

Antioxidant and Antibacterial Activities of Green Biosynthesized Silver Nanoparticles Using Freshwater Isolate of *Escherichia coli*

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Abstract: The use of microorganisms in the synthesis of nanoparticles has emerged as a clean, biocompatible, environmentally friendly method. The current study focuses on the synthesis of silver nanoparticles (AgNPs) by reducing aqueous Ag⁺ ions with the culture supernatants of freshwater-isolated *E. coli*. The color change in the bacterial supernatant initially observed during AgNP biosynthesis was further confirmed by UV spectroscopy and characterized by TEM, EDAX, X-ray diffraction, and FTIR. The UV-visible spectrum analysis of the aqueous solution containing silver nanoparticles showed a characteristic peak at 420 nm. The TEM micrograph testing revealed the formation of spherical silver nanoparticles, with a size range of 6–28 nm. Bio-synthesized AgNPs by the studied isolate were also used to explore their antibacterial and antifungal potential against pathogens such as *Salmonella typhi*, *Salmonella paratyphi*, *Vibrio cholerae*, *Staphylococcus aureus*, and *Candida albicans*, revealing a broad-spectrum biocidal activity and scavenging activity against DPPH.

Keywords: biosynthesized nanoparticles; silver; green synthesis; bactericidal.

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1. Introduction

Antimicrobial resistance has been identified as one of the alarming health concerns due to the indiscriminate and extensive use of commercially available antibiotics. The widespread multidrug resistance among pathogens was mainly due to mobile genetic elements, horizontal gene transfer, transformation, and transduction [1]. Nanotechnologies are involved in developing new, inexpensive, rapid, and safe solutions for the synthesis of nanoparticles, especially silver and gold nanoparticles [2], which are efficient against multidrug-resistant microorganisms that cause significant economic losses in agriculture, the food industry, and human health, among others [3-5]. Microorganisms as nanofactories have great potential as environmentally friendly, inexpensive, and non-toxic tools that require less energy for AgNP biosynthesis than currently used physicochemical methods. Employing plant extracts [6,7], algae [8,9], bacteria [10,11], fungi, and yeast [12,13] has been extensively studied as an environment-friendly, low-cost, and simple alternative to potentially toxic chemicals. Thus, while physical and chemical routes require several highly toxic chemicals and often generate

hazardous byproducts for both humans and the environment [14], biosynthesis of metal nanoparticles using a green plant approach can contribute to sustainable development goals [15]. Among the different nanoparticles studied to date, silver nanoparticles have attracted much more attention due to their diverse applications in new technologies across chemistry, electronics, medicine, and biotechnology [16,17]. The biosynthesis of AgNPs using prokaryotic bacteria has attracted significant attention in recent decades. This process offers cost-efficient, environmentally friendly, and scalable fabrication of nanoparticles without the use of toxic chemicals [18]. Different methods of bacterial-mediated synthesis of AgNPs are well established now. Studies involving the use of *Bacillus subtilis* [17], *Pseudomonas aeruginosa* [18], *Bacillus* sp. [19], *Bacillus stearothermophilus* [20], and *Bacillus licheniformis* [21-23] have been conducted for the production of AgNPs. It has been observed that nanoparticles synthesized by biological methods can be capped with biomolecules such as alkaloids, phenolic compounds, terpenoids, enzymes, coenzymes, proteins, polysaccharides, and lipids [24]. Due to the above-mentioned reasons, the present work aimed to employ *E. coli* cell-free extract for the extracellular biosynthesis of AgNPs from silver nitrates and to investigate its antioxidant properties and antibacterial activity against human pathogenic bacteria such as *S. aureus*, *Salmonella typhi*, *P. aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus aureus*, and *Vibrio cholerae*.

2. Materials and Methods

2.1. Culture media and antibiotics.

Culture media and antibiotics were purchased from Oxoid Ltd., England. Silver nitrate (AgNO_3) 99.9%, Methanol (CH_3OH), Ethanol ($\text{C}_2\text{H}_5\text{OH}$), ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemicals, USA. The pathogenic bacterial strains were obtained from the National Institute of Oceanography and Fisheries Culture Collection.

2.2. Isolation of metal-resistant bacteria.

Freshwater samples were aseptically collected from Al-Bahr El-Pherony, Menoufyia Governorate, Egypt, in sterile brown bottles, transported to the laboratory, and stored at 4°C until bacteriological analysis was completed. The membrane filter technique was applied using a filtration system (APHA, 2005). M-coli blue medium was used in the quantification of *E. coli* and incubated at 37°C for 24 h. The colonies obtained were further inoculated on nutrient agar supplemented with 1-20 mM concentrations of filter-sterilized AgNO_3 and incubated at 37°C for 24 h for the screening of Ag-resistant bacterial strains. The resistant colonies were subcultured, and one, named EC3, was randomly selected for nanoparticle synthesis studies. Morphological and biochemical studies characterized the isolates microbiologically using routine methods described in Bergey's Manual of Determinative Bacteriology (1984).

2.3. Screening for extracellular biosynthesis of silver nanoparticles.

For nanoparticle biosynthesis studies, the obtained bacterial isolate was inoculated into LB broth and incubated in a rotating shaker at 37°C and 120 rpm for 24 h in a dark room to avoid silver nitrate photoactivation. After incubation, the biomass and supernatant were obtained by centrifugation at 12000 rpm for 10 min, and the cell-free extract was used for the

extracellular biosynthesis of silver nanoparticles. Then, supernatants were mixed with filter-sterilized 5 mM AgNO₃ solution. The heat-inactivated supernatant incubated with silver nitrate and silver nitrate solution alone was also maintained as a control. The bio-reduction of Ag⁺ ions was initially monitored by visual observation of color changes. Also, the optical characteristics of synthesized silver nanoparticles were measured using a UV-visible spectrophotometer (Hitachi U5100) over the 200-800 nm range, with a control as the reference.

2.4. Optimization of AgNPs bio-manufacturing using EC3.

Microbial bio-reduction of AgNPs has been investigated under various conditions to maximize AgNP production. The effect of the silver nitrate concentration was determined by varying the AgNO₃ concentration (0.5, 1.0, 3.0, or 5.0 mM). The effect of pH was studied by adjusting the pH of the reaction mixtures to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, or 10 using 0.2 M. To study the effect of temperature on nanoparticle synthesis, the reaction mixtures were incubated at 28, 30, 37, and 50°C for 720 min. The effect of reaction time was evaluated by incubating the reaction mixtures with the optimum composition for 60, 180, 360, and 720 min. Biosynthesis of AgNPs was monitored by visual observation of a color change from clear to brown with a yellowish tint and by measuring the absorbance maximum at 420 nm in UV-Visible spectra.

2.5. Characterization of silver nanoparticles.

The reduction of silver ions was confirmed by measuring the reaction medium at 200-800 nm with a resolution of 1 nm using a UV-Vis spectrophotometer (Beckman DU-40). Further characterizations of AgNPs were carried out through X-ray diffraction (XRD) measurement, Fourier-Transform Infrared (FT-IR) Chemical Analysis, Transmission Electron Microscopy (TEM), and Energy Dispersive X-ray Analysis.

2.6. In vitro assay of antibacterial activity.

The biosynthesized AgNPs were tested for their antibacterial activity against pathogenic bacteria, including *E. coli*, *Salmonella sp.*, *Vibrio cholerae*, *P. aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, using the standard disc diffusion method on Mueller-Hinton Agar (MHA) plates. Wells were made on the surface of the Müller Hinton Agar plate, and then 100 µL of the biosynthesized AgNPs solution was poured into the corresponding well. As a control, 100 µL of 5 mM AgNO₃ solution was poured into the control well. After incubation at 37°C for 24 hours, the diameter of the zone of inhibition (mm) around each well was recorded.

2.7. Antioxidant Activity of AgNPs using DPPH.

In vitro, the DPPH assay reaction consisted of a mixture of freshly prepared 1 mL DPPH (0.1 mM) solution in methanol with different concentrations (5, 10, 25, 50, 75, and 100 µg/mL) of AgNPs, in a final volume of 0.1 mL in distilled water. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. After that, the absorbance was measured at 517 nm using a UV-visible 1800 spectrophotometer (Shimadzu). The percentage of DPPH scavenging activity was calculated using the formula:

$$\text{Inhibition\%} = (A_0 - A_1) / A_0 \times 100 \quad (1)$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the test. The results were compared with ascorbic acid as a standard.

3. Results and Discussion

3.1. Isolation and characterization of the potential isolate.

The potential isolate was identified following the routine morphological and biochemical examinations. Results showed that the isolated strain resembles and relates to *E. coli*. The isolated strain (EC3) was found to be a Gram-negative bacillus. Based on biochemical characterization, the isolate was identified as *E. coli* (Table 1). *E. coli* was then isolated, identified, and cultured to produce biomass. The *E. coli* isolate was inoculated into LB broth as the production culture and incubated at 37°C in an orbital shaker at 121 rpm.

Table 1. Biochemical test results of the EC3 isolate.

Characteristics of the potential strain	Results
Gram staining	Negative
Morphology	Bacilli
Methyl red	Positive
Voges Proskauer	Negative
Indole	Positive
Citrate	Positive
Urease	Negative

The newly isolated *E. coli* was employed for silver nanoparticle synthesis. The silver nanoparticles were characterized by UV–Visible spectroscopy. This technique has proved to be very useful for the analysis of metal nanoparticles. A characteristic broad peak of silver nanoparticles was observed in the UV-visible spectra at 420 nm (Figure 1). The characteristic absorption peak attributed to the surface plasmon resonance (SPR) of AgNPs induced by the collective oscillation of their free electrons in resonance with the light wave [25].

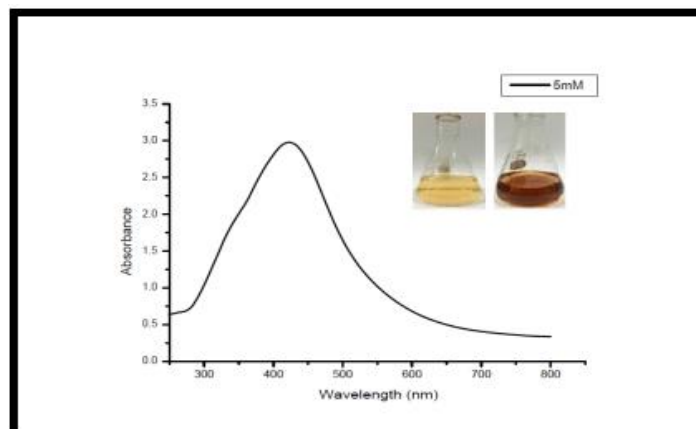


Figure 1. UV-visible spectra of silver nanoparticles synthesized using *E. coli* cell-free extract.

Microbial culture filtrate contains biomolecules that not only reduce Ag^+ ions but also act as natural capping agents. These biological extracts contain a combination of biomolecules, such as enzymes, proteins, amino acids, and carbohydrates, that help reduce and cap Ag^+ ions, thereby stabilizing AgNPs [17]. Among these enzymes, “Nitrate reductase” is the widely

accepted enzyme responsible for nanoparticle biosynthesis. Nitrate reductase is an enzyme that is responsible for the conversion of nitrate to nitrite and, during this process, catalyzes an electron shuttle to the incoming silver ions [26].

3.2. Extracellular synthesis of AgNPs.

Initially, the extracellular synthesis of silver nanoparticles was confirmed by visual observation of a color change in the reaction medium from yellow to deep brown within 24 h, and the color intensity increased with incubation time. A control group without bacteria showed no color change under the same conditions. The color change from pale yellow to dark brown was attributed to the culture media and antibiotics. Other authors made a similar observation [27, 6]. Although the exact mechanism is not fully understood, it is believed that bacteria can trap silver ions, either on their surfaces or within microbial cells, which are then reduced to nanoparticles by microbial enzymes [28].

By evaluating different pH values for AgNPs biosynthesis, it was found that pH 8 showed the highest absorbance; absorbance increased from pH 5 to 8 and decreased with increasing pH (Table 2). This shows that an alkaline environment favors the synthesis of silver nanoparticles. This result was corroborated by Othman et al. [29], who showed that maximum AgNPs production was obtained at pH 10 using the *Aspergillus fumigatus* DSM819; earlier, Gurunathan et al. [30] and Sanghi and Verma [31] reported that the reduction of metallic ions was sensitive to hydrogen ion concentrations and the morphology of the product. The proteins involved in synthesis may bind with silver at the thiol regions (–SH), forming a –S–Ag bond, a clear indication of what aids the conversion of Ag^+ to Ag^0 . In addition, an alkaline ion (–OH) is required to reduce metal ions.

Additionally, in alkaline conditions, the enzymatic activity has a high affinity for the reduction of metal ions [31]. We identified a suitable temperature for maximum AgNPs production at 37°C, which also yielded the highest absorbance. When the temperature increased from 2 to 5°C, the absorbance of the reaction mixture increased (Table 2). The results suggested that an increase in temperature above 37°C reduced the reduction process. This could be due to the inactivation or degradation of the biomolecules responsible for silver reduction. The extract at pH 8 showed the highest optical density (2.23) compared with other pH values, temperatures, AgNO_3 concentrations, and reaction times. While evaluating the effect of different concentrations of Ag^+ ions in the reaction mixture, it was found that 3.0mM Ag^+ ions showed the highest optical density (Table 2). The maximum absorbance was recorded at 720 min at 420 nm, corresponding to the highest AgNPs production (Table 2). With increasing incubation time, the production increased.

Table 2. The absorbance of different reaction times of produced AgNPs by *E. coli* with an aqueous solution of AgNO_3 at four different temperatures, pH, and five different extract volume concentrations at 420 nm.

Parameters	Temp (°C)				pH				AgNO ₃ conc (mM)				Reaction time (min)			
	28	30	37	50	5	7	8	10	0.5	1	3	5	60	180	360	720
OD at 420 nm	0.48	0.53	0.67	0.01	0.88	1.5	2.23	0.17	0.17	0.8	2.3	0.29	0.54	0.9	1.5	1.9

3.3. Characterization of AgNPs.

3.3.1. UV–vis spectroscopy.

The reduction of pure Ag^+ ions to Ag^0 was monitored using the UV–vis spectroscopy. The presence of a characteristic surface plasmon resonance (SPR) band at 420 nm confirmed the formation of silver nanoparticles (Figure 1). This absorption band is a well-known characteristic of noble-metal nanoparticles [22].

3.3.2. X-ray diffraction (XRD).

The XRD pattern of the silver nitrate-treated sample (Figure 2) corresponds to that of silver nanoparticles. The XRD pattern shows four intense peaks across the entire 2θ range from 30 to 80. It is important to determine the exact nature of the silver particles, which can be inferred from the sample's XRD spectrum. The Joint Committee has published XRD spectra of pure crystalline silver structures and pure silver nitrate on Powder Diffraction Standards (file no. 04-0783 and 84-0713). A comparison of our XRD spectrum with the standard sample confirmed that the silver nanoparticles had been formed in the form of nanocrystals, as was evidenced by the peaks at 2θ values of 38.25° , 46.37° , 64.60° , and 67.62° corresponding to 111, 200, 220, and 311 planes for silver, respectively. These sharp Bragg peaks may be attributed to the capping agent's role in stabilizing the nanoparticles. These intense Bragg reflections further suggest that the capping agents form strong X-ray scattering centers within the crystalline structure [32].

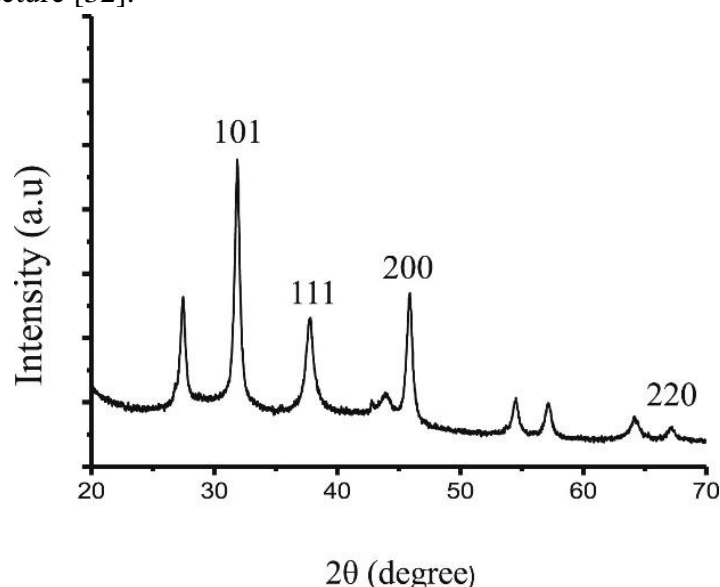


Figure 2. X-ray diffraction pattern of silver nanoparticles.

3.3.3. Fourier Transform Infrared spectroscopy (FT-IR) analysis.

Using Fourier transform infrared (FTIR) spectroscopic analysis to identify the potential functional groups of biomolecules in the extract that acted as reducing and capping agents for bio-reduced AgNPs. The spectrum showed several characteristic peaks, indicating the presence of various bond stretches associated with these functional groups (Figure 3).

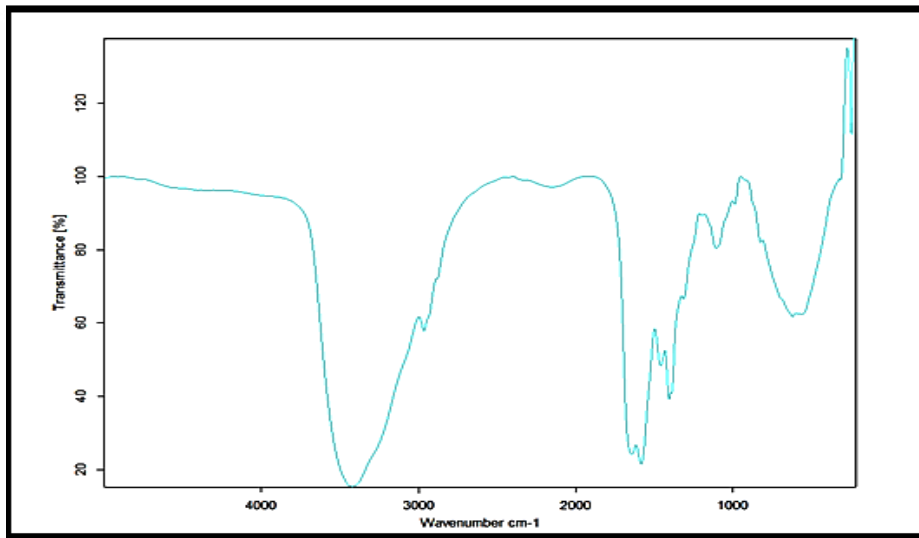


Figure 3. FT-IR spectroscopy of silver nanoparticles.

3.3.4. Transmission Electron Microscopy (TEM) analysis of AgNPs.

TEM is often used to provide further insight into the morphology and particle-size distribution of AgNPs. Transmission electron microscopy (TEM) confirmed the biosynthesis of spherical, polydispersed silver nanoparticles (AgNPs), ranging from 6 to 28 nm, using the cell-free *E. coli* extract (Figure 4). The small size and lack of significant agglomeration result in a large surface area, enhancing their reactivity and catalytic properties in various applications [30]. This is consistent with other studies reporting a wide range of AgNP sizes (5–100 nm) and morphologies produced using bacterial or plant extracts [27, 33].

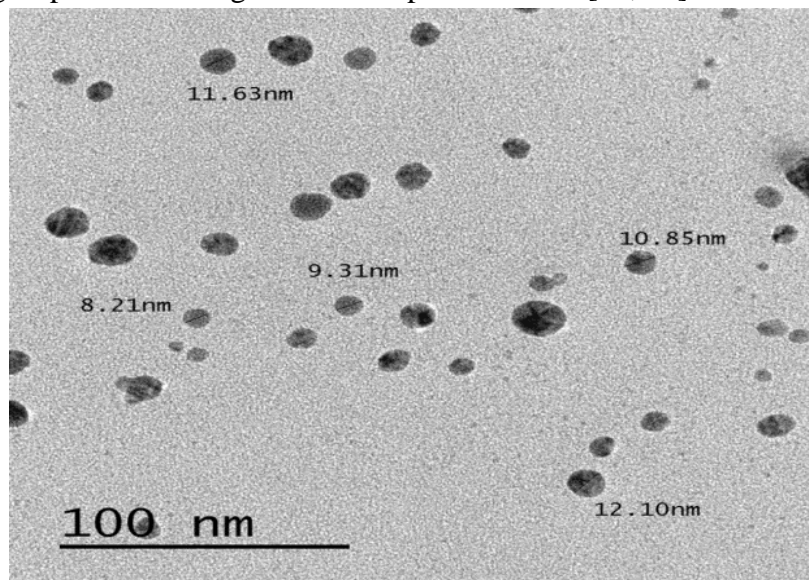


Figure 4. Microphotograph of the silver nanoparticles synthesized from *E. coli* supernatant.

3.3.5. EDAX.

The elemental composition of the nanoparticles by EDX (Figure 5) indicates a sharp peak at 2.9-3.0 keV, which shows the presence of silver as the base and dominant element. Additional peaks at 8.0-9.0 keV are due to copper, which is the grid's base material.

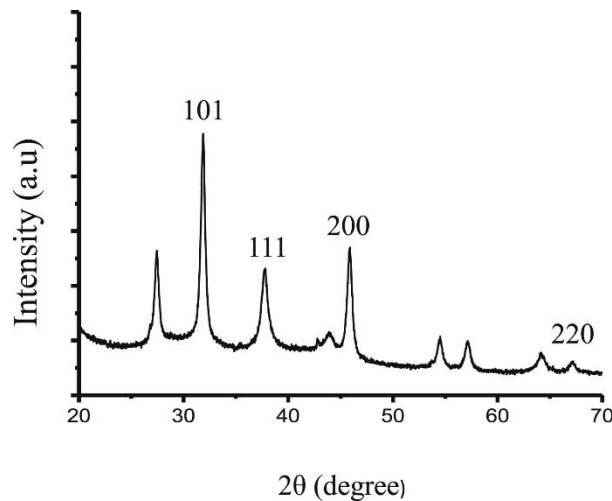


Figure 5. EDAX spectroscopy of the synthesized nanoparticles depicts silver as the base element.

3.4. Antimicrobial activity.

Due to the emergence of antibiotic resistance among pathogenic bacteria and fungi over the past decade, silver nanoparticles have attracted increased attention for their antimicrobial properties [34-38]. This study investigated the effectiveness of biosynthesized AgNPs against clinical pathogens, namely *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* 6633, *Escherichia coli*, and *Salmonella typhi*, representing both Gram-positive and Gram-negative bacteria. Biosynthesized AgNPs exhibited considerable antimicrobial effects against the tested pathogens, as evidenced by the average zone of inhibition shown in Table 3. This indicates that these AgNPs could have potential future applications in the medical field by effectively inhibiting the growth of pathogens. Although the exact mechanism of their antimicrobial action is still under investigation, two mechanisms are commonly accepted. One involves the interaction between the positively charged silver ions (Ag^+) and the negatively charged biomacromolecules (such as proteins and nucleic acids) found on or within bacterial cells. This interaction causes structural damage to the cell wall and membrane, ultimately disrupting metabolic functions and causing cell death. A second proposed mechanism suggests that free radicals produced on the nanoparticle surface increase the permeability of the bacterial membrane, also leading to cell death [39,40].

Table 3. Antimicrobial activity of the AgNPs synthesized from *E. coli*.

Tested bacteria	Zone of inhibition \pm SD
<i>Bacillus cereus</i>	10.5 \pm 0.5
<i>Staphylococcus aureus</i>	8.0 \pm 0.22
<i>Pseudomonas aeruginosa</i>	14.5 \pm 0.32
<i>Bacillus subtilis</i> 6633	11.2 \pm 0.2
<i>Escherichia coli</i>	13.0 \pm 0.6
<i>Salmonella typhi</i>	13.4 \pm 0.5
<i>Candida albicans</i>	7.8 \pm 0.02

3.5. Antioxidant activity of AgNPs.

DPPH is widely used to assess the preliminary radical-scavenging activity of a compound or plant extract. In the present study, the synthesized silver nanoparticles showed potential free radical-scavenging activity. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. The results of DPPH reduction are shown in Table 4 and Figure 6. The biosynthesized silver nanoparticles exhibited a strong capacity to scavenge the DPPH free radical. Our results are in accordance with previously reported studies [41,42]. The free radical-scavenging capacity increased steadily with the continuous increase in concentration. The antioxidant activities of the individual compounds in the extract may depend on structural features, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl groups, keto groups, free carboxylic groups, and other structural features.

Table 4. DPPH free radical scavenging activity of silver nanoparticles produced by *E. coli* cell-free supernatant.

Concentration (µl/ml)	Percentage of scavenging (%)	Cell-free extract	Ascorbic acid
25	68.22 ± 0.012	24±0.02	52±0.04
50	69.18 ± 0.03	30±0.011	58±0.02
75	70.1 ± 0.02	43±0.03	60.2±0.022
100	75.16 ± 0.04	47±0.014	63.6±0.01

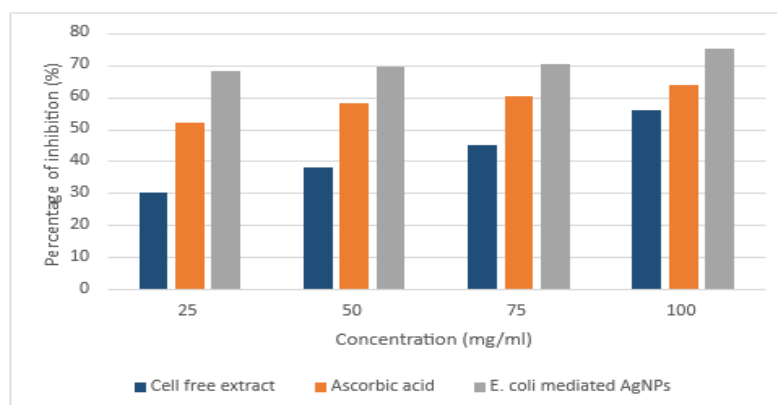


Figure 6. Percentage of antioxidant (DPPH) scavenging activity of bio-AgNPs and ascorbic acid (control).

4. Conclusion

A rapid one-step green approach was employed for nano-sized silver biosynthesis. Freshwater *E. coli* isolates acted mainly as reducing and capping agents during AgNPs biosynthesis. The fabricated silver nanoparticles exhibited broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria. Results also proved the antioxidant potency of silver nanoparticles against ascorbic acid.

Author Contributions

Investigation, H.B., W.T.H.; methodology, H.B., W.T.H.; software, H.B., W.T.H.; writing—original draft preparation, H.B., W.T.H.; writing—review and editing, H.B.. All authors contributed to writing and reviewing the article and approved the submitted version.

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Data Availability Statement

Data supporting the conclusion of this article are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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