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RESEARCH PAPER

Green Synthesis of Silver Nanoparticles Utilization of Honey: Characterization and Assessment of Antibacterial and Antibiofilm Activities

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ABSTRACT

In this study, silver nanoparticles (AgNps) were successful synthesized using honey as antural, ecofriendly, cost effective and stabilizing agent. The formation of AgNPs was confirmed using several characterization techniques included (UV)visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), and energy dispersive X-ray spectroscopy (EDS). The results indicated that the synthesized AgNps were stable, solid and uniformly distributed, these finding suggest that honey can be effectively used as a green alternative for synthesis silvers nanoparticles. The antibacterial efficacy of the biosynthesized AgNPs was examined against *Staphylococcus aureus* and *Pseudomonas aeruginosa* utilizing the agar well diffusion method and minimum inhibitory concentration (MIC) assay. The findings demonstrated significant antibacterial efficacy against both bacterial types. The antibiofilm efficacy of AgNPs was assessed via the crystal violet microtiter plate assay, revealing a substantial decrease in biofilm development in a concentration dependent manner. The synergistic antibacterial and antibiofilm results demonstrate that honey-mediated AgNPs possess significant antimicrobial efficacy. The results indicate that biosynthesized AgNPs may be viable candidates for biomedical applications, especially in the prevention of biofilm-associated illnesses and the creation of antimicrobial coatings.

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INTRODUCTION

one of the most critical problems facing modern medicine is the rising incidence of antibiotic resistant microorganisms [1]. Treatment of the infections is becoming more challenging due to the development of resistance in many bacteria that were formerly easily managed with medicines [2]. wound infections represent a significant clinical challenge, particularly when pathogens such as *Pseudomonas aeruginosa* and

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Staphylococcus aureus which can cause chronic infections that are resistant to standard therapies [3]. One of the major factors contributing to there resistant is their ability to form biofilms which protect the bacterial cells from antimicrobial agents and the host immune response [4]. Silver nanoparticles (AgNPs) have gained increasing attention as promising alternatives to traditional antibiotics, mainly because of their broad-spectrum antimicrobial activity and their effectiveness against biofilm-forming pathogens [5]. Unlike conventional antibiotics, which usually target specific bacterial processes, AgNPs act through multiple mechanisms. This multi-target action reduces the chances of bacteria developing resistance [6]. These mechanisms consist of compromising the bacterial cell membrane, obstructing metabolic processes, and preventing DNA replication [7]. AgNPs can also get through biofilm structures and kill bacteria that are stuck in them. This makes them especially useful for treating chronic wound infections [8]. However, widely used chemical approaches for making AgNPs often use toxic reducing agents and harsh reaction conditions. This makes people worry about how safe it is for the environment and how well it works with living things [9]. Additionally, these methods can lead to synthesis nanoparticles with varying sizes and shapes, potentially impacting their biological activity significantly [10]. Therefore, green synthesis techniques using natural substance have gained increasing attention as eco-friendly alternatives, Honey has proven to be excellent candidates due its rich content of reducing sugars, enzymes and bioactive phytochemicals that facilitate both reduction and stabilization processes [11]. In mild conditions, the natural compounds in honey may efficiently reduce silver ions and stabilize the resultant nanoparticles, creating friendly nanoparticles without the use of hazardous chemicals [12]. This study aims to synthesize silver nanoparticles using an eco-friendly green method based on Sidr honey, followed by characterization of the produced nanoparticles using appropriate analytical techniques. It also considers first time locally to investigate the antibacterial activity of biosynthesis silver nanoparticles from honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, in addition to evaluating their effectiveness in inhibiting biofilm formation.

MATERIALS AND METHODS

Chemicals and Samples

Silver nitrate ($AgNO_3$) was used as the silver ion precursor [7]. Sidr honey was sourced from a reliable commercial supplier. clinical strains of *Staphylococcus aureus and Pseudomonas aeruginosa* were used for the microbiological assays which isolated from wounds infections at Medical City Hospitals in Baghdad from the period from February 2024 to April 2024.

Green Synthesis of Silver Nanoparticles (AgNPs)

The synthesis of AgNPs was carried out by adding 3 % honey extract which served as the bioreductor to 0.1 M silver nitrate ($AgNO_3$) as the precursor in the ratio of 1:1 (v/v). The reaction mixture, under stirring using a magnetic stirrer (200–300 rpm) at room temperature (27–30 °C) for 10 min, was well mixed. The solution was, therefore, exposed to a halogen lamp for about 30 min. This was the period that the formation of silver nanoparticles, as evidenced by the color change of the solution to brown, occurred [7,8].

Characterization of Silver Nanoparticles

UV–Vis spectroscopy

The formation of silver nanoparticles was confirmed using UV–visible spectroscopy by scanning the absorbance spectra in the range of 300–700 nm. The characteristic surface plasmon resonance (SPR) peak of AgNPs was recorded.

Fourier transform infra-red

FTIR analysis was performed to identify the functional groups involved in the reduction and stabilization of AgNPs. The spectra were recorded in the range of 400–4000 cm^{-1}.

X-ray diffraction

The crystalline structure of the synthesized AgNPs was analyzed using X-ray diffraction (XRD) with Cu-Kα radiation (λ = 1.5406 Å). Diffraction patterns were recorded over a 2θ range of 20–80°.

Scanning electron microscopy (SEM)

The surface morphology and particle size of AgNPs were examined using FESEM after appropriate sample preparation.

Energy-Dispersive X-ray Spectroscopy

Elemental composition and confirmation

of silver presence were determined using EDS coupled with SEM.

Antibacterial activity test

Agar well diffusion method

The antibacterial activity of AgNPs synthesized using Sidr honey was evaluated against S. aureus (Gram-positive) and P. aeruginosa (Gram-negative) using the agar well diffusion method. Muller Hinton agar was prepared by dissolving 20 g/L in distilled water, followed by boiling. The medium and associated equipment were sterilized via autoclaving at 121 ◦C and 15 psi for 15 min. After sterilization, the agar was cooled to 45 ◦C. Bacterial suspensions were standardized to a McFarland 0.5 turbidity equivalent in 0.9 % NaCl.Approximately 15 mL of agar was poured into sterile Petri dishes, and 100 µL of bacterial suspension was evenly spread across the solidified medium. Wells with a diameter of 6 mm were created in the agar, and 20 µL of AgNP sample prepared with honey was introduced into the wells. Plates were incubated at 37 ◦C for 24 h. Antibacterial activity was determined by measuring the diameter of the inhibition zones in horizontal, vertical, and diagonal orientations using calipers. experiment was performed in triplicate to ensure reproducibly [13].

MIC Determination

MIC Determination (Colorimetric Microdilution Using Resazurin, 96-well Plate)

The minimum inhibitory concentration (MIC) of the biosynthesized silver nanoparticles from honey was determined using a colorimetric microdilution assay in sterile 96-well microtiter plates. Two-fold serial dilutions of the nanoparticles were prepared in BHI broth to obtain concentrations ranging from 5 mg/ml to 0.156 mg/ml. In each well, 100 µl of the nanoparticle dilution was mixed with 100 µl of a standardized bacterial suspension adjusted to 1×10^8 CFU/ml (0.5 McFarland standard), yielding a final volume of 200 µl per well.

Controls consisted of a positive control (broth inoculated without nanoparticles) and a negative control (broth only). The plates were incubated at 37 °C for 24 h after the addition of 20 µl of 0.01% resazurin. Blue wells indicated growth inhibition, while a colour shift to pink indicated bacterial growth [14].

Ant biofilm Activity Assay

Ant biofilm activity was evaluated using the crystal violet assay [15]. Biofilms were formed

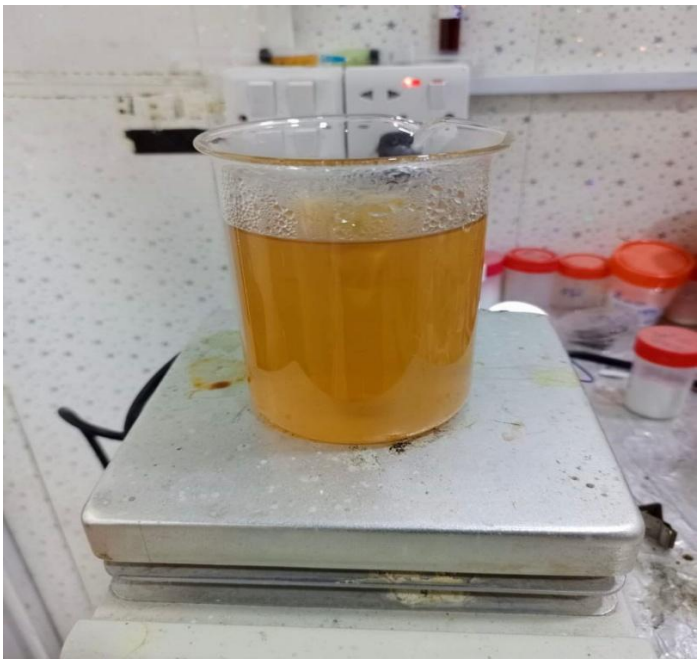

Fig. 1. Successful formation l of AgNPs.

in 96-well plates using bacterial suspensions adjusted to 0.5 McFarland and incubated at 37 °C for 24 h. After washing with PBS, biofilms were treated with different concentrations of AgNPs and re-incubated for 24 h. Wells were fixed, stained with 0.1% crystal violet, and the dye was solubilized for absorbance measurement at 570 nm. Biofilm inhibition (%) was calculated relative to untreated controls. Experiments were performed in triplicate.

Statistical Analysis

The Statistical Packages of Social Sciences -SPSS (2019) program was used to detect the effect of difference concentration in study parameters. Least significant difference-LSD was used to significant compare between means in this study.

RESULTS AND DISCUSSION

Bacterial isolation

clinical samples were collected from patients with suspected wound infections. All bacterial isolates were diagnosis using (selective and differential culture media, microscopic examination, biochemical tests) vitek compact 2 system and 16srRNA were done for conformation. Staphylococcus aureus was the most frequently isolated organism, accounting for 60% of all isolates. *Pseudomonas aeruginosa* became recognized as the second most common isolated bacteria, which represented 40% of the bacterial species obtained from wound samples. The significant prevalence of both *S. aureus* and *P. aeruginosa* was the second most prevalent isolated bacteria with 40% of the bacterial species obtained from wound samples. The high prevalence of *S. aureus* and *P. aeruginosa* in wound infections is of great clinical importance. These pathogens are often co-isolated in polymicrobial infections, where their coexistence can promote synergistic interactions that increase virulence and lead to delayed wound healing.

Green Synthesis of Silver Nanoparticles (AgNPs)

The addition of honey to the silver nitrate solution resulted in a slow change of colour from clear to dark Amber within about 30 minutes, which indicate the formation of AgNps (Fig. 1). This colour change is due to the excitation of surface plasmon resonance (SPR).

Characterization of AgNPs

The characterization analyses confirmed successful green synthesis of stable silver nanoparticles (AgNPs) using honey as a reducing and stabilizing agent. UV–Vis spectroscopy

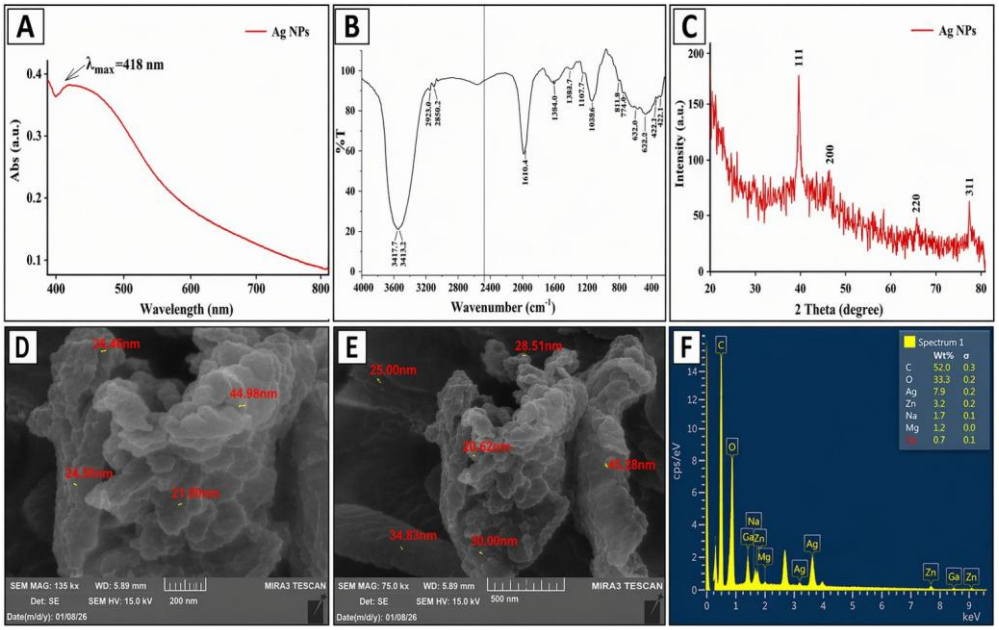

Fig. 2. Characterization of AgNPs used in this study. (A)UV-visible adsorption spectrum (B) FTIR spectrum (C) X-ray diffraction pattern (D and E) SEM analysis and size distribution (F) EDX spectrum.

revealed a well-defined surface Plasmon resonance (SPR) peak at 418 nm, indicating the formation of nanoparticles with a relatively uniform size distribution (Fig. 2A).FTIR analysis identified key functional groups, including O–H, C–H, C=O, and C–O, suggesting the involvement of honey derived biomolecules in both the reduction of silver ions and the stabilization of the synthesized nanoparticles (Fig. 2B).Furthermore, X-ray diffraction (XRD) patterns exhibited distinct peaks at 2θ values of 38.2°, 44.2°, 64.5°, and 77.5°, which correspond to the (111), (200), (220), and (311) crystallographic planes of face-centered cubic (fcc) silver. The calculated crystallite size ranged from 18 to 54 nm, with an average size of approximately 30 nm, confirming the crystalline nature of the synthesized AgNPs (Fig. 2C).FESEM images showed mainly quasi-spherical particles with slight aggregation and sizes between 20–45 nm (Fig. 2D and E), while EDX analysis confirmed elemental silver through a strong peak around 3 keV, along with carbon and oxygen from honey organic compounds (Fig. 2F). The present findings demonstrate that honey can serve as an efficient natural reducing and stabilizing agent for the green synthesis of silver nanoparticles with desirable physicochemical properties. The formation of crystalline, predominantly spherical AgNPs with nanoscale size range and good stability suggests their suitability for biomedical and antimicrobial

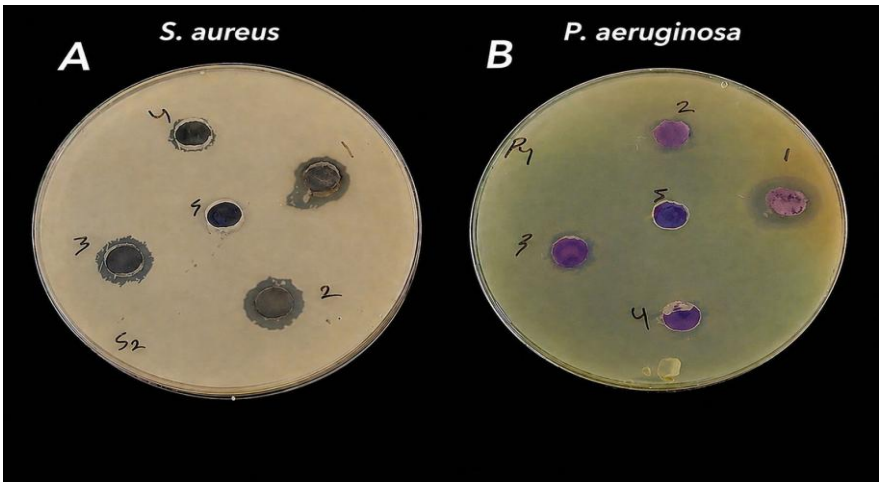

Fig. 3. Detection of Antibacterial activity of biosynthesized AgNPs from honey against Bacteria. (A) *S.aureus* and (B) *p.aeruginosa* Wells: (1) 2.5, (2) 1.25, (3) 0.625, (4) 0.312 mg/mL, (5) control.

Table 1. Antibacterial activity of biosynthesized silver nanoparticles against *S.aureus* using agar well diffusion method.

	Concentration					L.S.D.	P-value
Isolate	Control	2.5	1.25	0.6	0.312		
1	0 d	22 a	19 a	12 b	7 c	4.91 **	0.0001
2	0 c	18 a	14 ab	10 b	4 c	4.07 **	0.0001
3	0 d	21 a	18 ab	13 bc	8 c	5.18 **	0.0001
4	0 d	23 a	17 ab	12 b	6 c	5.47 **	0.0001
5	0 d	20 a	16 a	11 b	5 c	4.31 **	0.0001
6	0 d	18 a	12 b	7 c	4 cd	4.27 **	0.0001
7	0 d	15 a	10 b	9 b	5 c	3.92 **	0.0001
8	0 e	19 a	14 b	10 cd	7 d	4.57 **	0.0001

** (P≤0.01).

applications. These results are consistent with previous studies that reported the successful biosynthesis of AgNPs using natural products rich in sugars, proteins, and phenolic compounds as reducing and capping agents [15,16]. Slight particle aggregation observed in some analyses may be attributed to intermolecular interactions of surface-bound biomolecules, which is commonly reported in biologically synthesized nanoparticles [17]. Overall, the eco-friendly, cost-effective, and simple synthesis approach used in this study highlights the potential of honey-mediated AgNPs as promising candidates for future pharmaceutical, medical, and antimicrobial applications [18].

Antibacterial activity

Agar well diffusion method

The antibacterial activity of the biosynthesized silver nanoparticles (AgNPs) was evaluated against Staphylococcus aureus and Pseudomonas aeruginosa using the agar well diffusion method. The results showed that AgNPs exhibited clear antibacterial activity against both bacterial strains, as evidenced by the formation of well-defined zones of inhibition (Tables 1 and 2).

The diameters of the inhibition zones produced by AgNPs against S. aureus and P. aeruginosa were nearly comparable, indicating a similar level of susceptibility of both bacterial species to the synthesized nanoparticles. Furthermore, the inhibition zones increased with increasing AgNP concentration, demonstrating a concentration dependent antibacterial effect (Fig. 3).

The antibacterial activity observed in both bacterial strains can be attributed to the ability of AgNPs to interact with the bacterial cell membrane, leading to membrane damage, increased permeability, and subsequent leakage of intracellular components. Also, AgNPs generate reactive oxygen species (ROS) and also change the function of important cellular enzymes and DNA, leading to the inhibition of bacterial growth. The similar antibacterial effects of biosynthesised AgNPs against Gram-positive and Gram-negative bacteria have been reported in recent studies [19-21]. The results suggest that biosynthesised AgNPs have good antibacterial activity against both Staphylococcus aureus and Pseudomonas aeruginosa, indicating their potential to be used as antimicrobial agents for biomedical applications.

The minimum inhibitory concentration (MIC) Method

The (MIC) assay was conducted to determine the lowest concentration of AgNPs required to inhibit the visible growth of Staphylococcus aureus and Pseudomonas aeruginosa. The results demonstrated that AgNPs effectively inhibited the growth of both bacterial strains at low concentrations.

The MIC value was found to be 0.15 mg/mL, as indicated by the lowest concentration showing no color change (purple), confirming inhibition of bacterial growth.

The MIC values obtained for S. aureus and P. aeruginosa were found to be comparable, further supporting the agar diffusion results and indicating that both bacterial species exhibit similar sensitivity to the biosynthesized AgNPs (Table 3). Beyond their physical disruption of bacterial cells, AgNPs also have another trick up their sleeve, they trigger the generation of reactive oxygen species (ROS), which essentially overwhelm the bacteria from the inside. On top of that, they interfere with

Table 2. Antibacterial activity of biosynthesized silver nanoparticles against *p.aeruginosa* using agar well diffusion method.

Isolate	Control	Concentration				L.S.D.	P-value
		2.5	1.25	0.6	0.3		
1	0 d	20 a	17 a	8 bc	5 cd	5.02 **	0.0001
2	0 d	22 a	16 b	10 c	7 c	4.69 **	0.0001
3	0 c	19 a	15 a	9 b	4 bc	5.17 **	0.0001
4	0 d	21 a	19 ab	14 b	8 c	5.33 **	0.0001
5	0 c	23 a	18 a	11 b	6 b	5.82 **	0.0001
6	0 c	20 a	16 a	10 b	7 b	4.55 **	0.0001
7	0 c	22 a	17 a	9 b	6 b	5.61 **	0.0001
8	0 d	21 a	15 b	8 c	5 cd	5.97 **	0.0001

** (P≤0.01).

critical enzymes and attack DNA directly, leaving bacteria with little chance of survival or continued growth. And this isn't an isolated finding several recent studies have reported strikingly similar results, with biosynthesized AgNPs proving effective against both Gram-positive and Gram-negative bacteria alike [19-21].

Putting it all together, the evidence here is fairly compelling. The AgNPs produced in this study demonstrated real, meaningful antibacterial activity against both Staphylococcus aureus and Pseudomonas aeruginosa two of the most problematic pathogens in wound infections. That alone makes them worth taking seriously as potential antimicrobial agents, particularly for biomedical applications where safer, more natural alternatives to conventional treatments are increasingly needed.

Antibiofilm Activity

The antibiofilm activity of the biosynthesized (AgNPs) was estimated against both bacterial that used in the study by using the crystal violet microtiter plate assay (MTP). The results explained that AgNPs effectively inhibited biofilm formation in both bacterial species Treatment with AgNPs resulted in a significant reduction in biofilm biomass compared to untreated controls. Increasing concentrations of AgNPs led to a gradual decrease in biofilm formation, indicating that the antibiofilm effect is dose dependent. The percentages of biofilm inhibition observed for S. aureus and P. aeruginosa were nearly comparable (Tables 4 and 5]), suggesting that the synthesized AgNPs were equally effective against biofilms formed by both bacterial species. The antibiofilm activity of AgNPs can be attributed to

Table 3. The MIC, Sub-MIC and MBC used in this study against isolated bacteria.

Bacteria	Isolate	MIC	Sub-MIC	MBC
S.aureus	1	0.15 b	0.075 b	0.30 a
	2	0.60 a	0.30 a	0.30 a
	3	0.15 b	0.075 b	0.30 a
	4	0.07 b	0.035 b	0.15 b
	5	0.07 b	0.035 b	0.15 b
	6	0.07 b	0.035 b	0.15 b
	7	0.07 b	0.035 b	0.15 b
	8	0.07 b	0.035 b	0.15 b
P.aeruginosa	1	0.07 b	0.035 b	0.15 b
	2	0.15 b	0.075 b	0.30 a
	3	0.15 b	0.075 b	0.30 a
	4	0.15 b	0.075 b	0.30 a
	5	0.07 b	0.035 b	0.15 b
	6	0.07 b	0.035 b	0.15 b
	7	0.07 b	0.035 b	0.15 b
	8	0.07 b	0.035 b	0.15 b
L.S.D. (0.0001)		0.187 ** (0.0001)	0.0551 ** (0.0001)	0.128 ** (0.0084)

** (P≤0.01).

Table 4. Detection of Biofilm Inhibition by AgNPs against *S.aureus* isolates.

Concentration (mg/ml)	Mean of OD	SD	Inhibition (%)
5	0.064	±0.02	91.00 a
2.5	0.066	±0.04	91.00 a
1.25	0.116	±0.03	84.00 a
0.6	0.071	±0.02	90.00 a
0.3	0.109	±0.07	85.00 a
0.15	0.210	±0.13	72.00 b
0.07	0.269	±0.14	64.00 c
0.03	0.299	±0.14	60.00 cd
0.01	0.355	±0.15	53.00 d
0.008	0.336	±0.05	55.00 d
C+ (Control)	0.750	±0.06	0.00 e
C- (Negative)	0.035	±0.02	0.00 e
L.S.D.	---	---	7.802 **
(P-value)			(0.0001)
** (P≤0.01).			

Table 5. Detection of Biofilm Inhibition of AgNPs against *Pseudomonas aeruginosa* isolates.

Concentration (mg/ml)	Mean OD	SD (±)	Inhibition %
5	0.080	±0.02	85% a
2.5	0.098	±0.03	82% a
1.25	0.078	±0.02	86% a
0.6	0.083	±0.04	85% a
0.3	0.146	±0.09	73% b
0.15	0.081	±0.01	84% a
0.07	0.077	±0.02	85% a
0.03	0.115	±0.04	79% ab
0.01	0.336	±0.03	40% c
0.008	0.560	±0.05	0% d
C+ (Control)	0.560	±0.05	0% d
C- (Negative)	0.030	±0.01	0% d
L.S.D.	---	---	8.219 **
(P-value)			(0.0001)
** (P≤0.01).			

their ability to interfere with the early stages of biofilm development, including bacterial adhesion and extracellular polymeric substance (EPS) production. Silver nanoparticles are known to penetrate the biofilm matrix, disrupt cell-to-cell communication, and impair the structural integrity of mature biofilms. In addition, the generation of reactive oxygen species (ROS) and the interaction of silver ions with key cellular components further contribute to biofilm disruption and inhibition. [23,24] have reported similar antibiofilm effects of biosynthesized AgNPs against S. aureus and P. aeruginosa, highlighting their potential to overcome biofilm-associated antimicrobial resistance The comparable antibiofilm activity observed in both bacterial strains indicates that the effectiveness of AgNPs is not significantly influenced by differences in cell wall structure between Gram-positive and Gram-negative bacteria. Overall, these findings confirm that the biosynthesized AgNPs possess strong antibiofilm activity against both Staphylococcus aureus and Pseudomonas aeruginosa. This property, combined with their antibacterial effectiveness, supports their potential application in preventing biofilm-related infections, particularly in biomedical applications.

CONCLUSION

The green synthesis of silver nanoparticles using honey is a simple, cost-effective, and environmentally friendly approach. The

synthesized AgNPs are stable and exhibit excellent antibacterial and antibiofilm properties. These findings support the potential use of honey-mediated AgNPs as alternative antimicrobial agents in treating infectious diseases and preventing biofilm-related complications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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