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Estimation of the Anti-Biofilm Activity of Green-Synthesized Nanoparticles Against Clinical Isolates of Pseudomonas aeruginosa

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that causes serious infections and exhibits resistance to many antibiotics due to its ability to form biofilms and regulate virulence through quorum sensing (QS). This in vitro study aimed to evaluate the effect of green-synthesized silver nanoparticles (AgNPs) on biofilm formation by multidrug-resistant (MDR) clinical isolates of *P. aeruginosa* and on the expression of the QS-related lasI gene**.** Out of 150 clinical specimens, 23 MDR biofilm-producing *P. aeruginosa* isolates were identified. Silver nanoparticles were synthesized using clove (*Syzygium aromaticum*) extract as a green reducing and stabilizing agent. The antibacterial activity of AgNPs was evaluated using a resazurin-based broth microdilution assay, and the minimum inhibitory concentration (MIC) was determined as 3.125 mg/mL. Biofilm formation was assessed using the microtiter plate method. Statistical analysis was performed using one-way ANOVA and an independent-samples t-test, with $p < 0.05$ considered statistically significant**.** Using quantitative real-time polymerase chain reaction (qRT-PCR) revealed a significant decrease in lasI expression in AgNP-treated samples (0.0216-fold change; $p < 0.05$) compared with untreated controls. This downregulation indicates inhibition of QS signaling, which may contribute to biofilm suppression and reduced virulence. The findings suggest that green-synthesized AgNPs may have antibacterial and potential anti-virulence effects against MDR P. aeruginosa under *in vitro* conditions. However, further direct biofilm inhibition assays, in vivo experiments, and clinical studies are required to confirm their therapeutic potential.

Keywords: Pseudomonas aeruginosa, silver nanoparticles, biofilm inhibition, quorum sensing, lasI gene, multidrug resistance.

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen associated with a wide range of severe infections, including burns, wounds, urinary tract infections, and respiratory diseases, particularly in immunocompromised individuals [1]. This bacterium can adapt efficiently to host environments through multiple

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virulence mechanisms, including intrinsic antibiotic resistance, extracellular enzyme production, and biofilm formation [2].

Bacterial biofilm formation leads to enhanced resistance to antimicrobial agents, as well as to the persistence of chronic infections. Biofilm formation is regulated by quorum sensing (QS), a cell-to-cell communication system that coordinates bacterial behavior according to population density. The QS systems in Pseudomonas aeruginosa mainly include two regulatory circuits, Las and Rhl. The LasI/LasR **system** regulates the biosynthesis and detection of the autoinducer N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), while the RhlI/RhlR system regulates the biosynthesis of C4-HSL. The Las and Rhl systems are interconnected and function together to regulate the expression of virulence factors such as pyocyanin, rhamnolipids, elastase, and proteins associated with secretion [3,4], as illustrated in Figure 1.

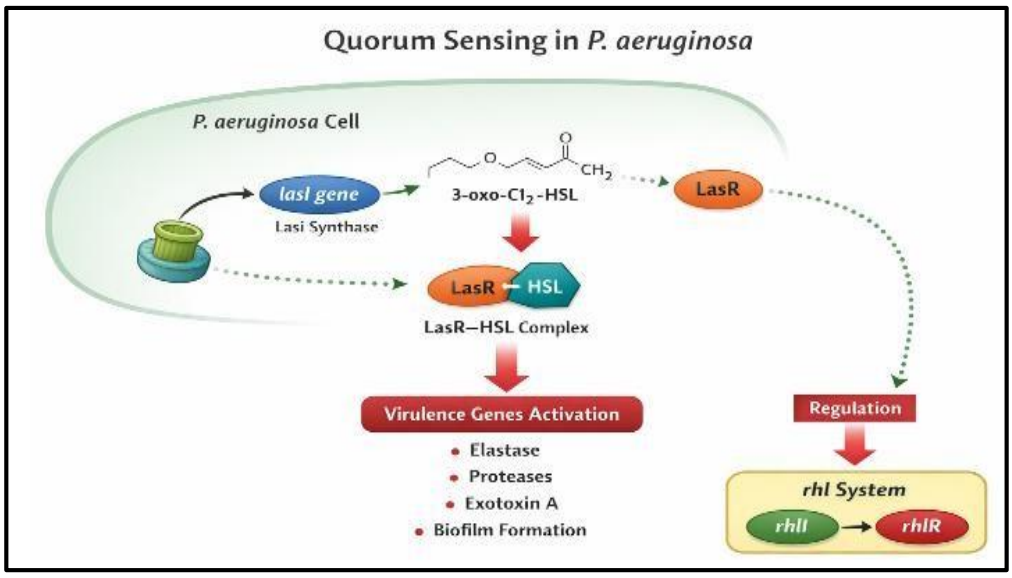

Figure 1. Schematic representation of the quorum sensing regulatory system in *Pseudomonas aeruginosa.* The lasI gene synthesizes the autoinducer molecule 3-oxo-C12-HSL, which binds to the LasR transcriptional regulator, activates virulence gene expression, and regulates the downstream Rhl quorum sensing system[5].

Quorum sensing also plays a crucial role in coordinating biofilm maturation, surface attachment, and resistance mechanisms, including efflux pump activation and stress response pathways [6]. Therefore, disruption of QS signaling has emerged as a promising anti-virulence strategy, as it attenuates pathogenicity without directly exerting bactericidal pressure, which may reduce the likelihood of resistance development [7].

Because of the increasing prevalence of MDR clinical isolates and the declining effectiveness of conventional antibiotics, alternative therapeutic strategies are needed. One alternative treatment of interest is silver nanoparticles (AgNPs) since they exhibit multiple antimicrobial activities, including reactive oxygen species (ROS) generation, disruption of bacterial cell membranes, and interference with bacterial metabolism [8].

Recent evidence suggests that AgNPs can also target bacterial communication systems by inhibiting quorum sensing pathways, thereby impairing biofilm formation and virulence regulation [9]. The lasI gene is an important component of QS regulation. It **encodes** the enzyme required to produce 3-oxo-C12-HSL, which is the key signaling molecule produced during QS. As the cell density increases in a population, the concentration of the

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signaling molecule 3-oxo-C12-HSL increases. When the abundance of 3-oxo-C12-HSL reaches a threshold concentration sufficient to activate gene expression, it binds to the LasR regulator. This results in the transcription of virulence-associated genes [10].

The las system is considered the major regulatory pathway within the QS network, controlling downstream systems such as Rhl. Therefore, changes in lasI gene expression can lead to profound effects on virulence factor production, biofilm development, and overall pathogenicity [11,12].

Thus, this study aimed to evaluate the antibacterial and anti-biofilm activity of green-synthesized (AgNPs) against MDR clinical isolates of *P. aeruginosa* and to investigate their effect on lasI gene expression as a marker of quorum sensing inhibition.

Materials and Methods

Clinical Specimens

From September to November 2025, a total of 150 clinical specimens were collected from patients admitted to burn units, surgical units, and outpatient clinics in several hospitals in Baghdad, Iraq. The specimens included burn wound swabs, surgical wound swabs, urine samples, and ear (otitis) swabs. All specimens were collected using sterile cotton swabs and transported immediately under aseptic conditions for bacteriological analysis.

Isolation and Identification of *Pseudomonas aeruginosa*

Each clinical specimen was inoculated onto Cetrimide agar, MacConkey agar, and Nutrient agar plates and incubated at 37 °C for 24 hours. Presumptive identification of *P. aeruginosa* was based on a combination of morphological, microscopic, and biochemical characteristics [13], as follows:

1. Microscopic examination: Gram-negative bacilli
2. Colony morphology: characteristic green pigmentation on Cetrimide agar and non-lactose fermenting pale colonies on MacConkey agar
3. Biochemical tests: oxidase-positive, catalase-positive, and non-fermentative metabolism
4. Automated identification: confirmation using the VITEK-2 system with GN identification cards, based on 41 biochemical reactions, providing automated identification within approximately 3 hrs.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of *P. aeruginosa* isolates was evaluated using both the Kirby–Bauer disk diffusion method and the VITEK-2 system (AST cards).

A bacterial suspension equivalent to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) was prepared in sterile 0.9% saline**.** Mueller Hinton agar plates were inoculated with a sterile cotton swab, and antibiotic disks were applied. The plates were then incubated at 37°C for 18 hrs. Inhibition zones were measured and interpreted according to CLSI guidelines (2023). Multiple antibiotic classes were represented, including:

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- Aminoglycosides
- Carbapenems (meropenem and doripenem)
- Ceftolozane/tazobactam
- Colistin
- Fluoroquinolones
- β-lactams

Isolates demonstrating resistance to multiple antibiotic classes were classified as multidrug-resistant (MDR) according to the standard definition proposed by Tzimotoudis [14], in which MDR refers to non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories. Accordingly, *P. aeruginosa* isolates showing resistance to at least one agent from three or more tested antibiotic classes were considered MDR and selected for subsequent nanoparticle-based experiments.

Biofilm Quantification Assay

Biofilm formation by *P. aeruginosa* isolates was quantitatively assessed using the standard microtiter plate assay, as previously described 14, with minor modifications. Bacterial cultures were incubated overnight, and their optical density (OD_{600}) was adjusted to 0.05 before adding 200 µL into sterile 96-well flat-bottom microtiter plates containing 1:100 diluted Luria–Bertani (LB) broth. The plates were then incubated at 37 °C for 24 hrs. under static conditions to allow biofilm formation on the surface of each well.

The non-adherent (planktonic) cells were carefully removed after incubation, and the wells were washed three times with sterile phosphate-buffered saline (PBS) to remove loosely attached bacteria. The adherent biofilms were then fixed **overnight** and stained for 15 min. using 0.1% (w/v) crystal violet. After staining, excess dye was discarded, and the wells were thoroughly rinsed with sterile distilled water and allowed to air dry. The bound crystal violet was then solubilized using 95% ethanol, and the biofilm biomass was quantified by measuring the absorbance at 570 nm using a microplate reader.

Preparation of Ag-NP Concentrations and Determination of Minimum Inhibitory Concentration (MIC)

A series of two-fold serial dilutions of the synthesized AgNPs was prepared in Mueller–Hinton broth (MHB) to obtain final concentrations ranging from 50 to 0.097 mg/mL.

The minimum inhibitory concentration (MIC) of AgNPs against *P. aeruginosa* was determined using the standard broth microdilution method in a 96-well microtiter plate, employing resazurin as a redox indicator of bacterial metabolic activity, with minor modifications [15].

An overnight bacterial culture was prepared in nutrient broth at 37°C. The culture was adjusted to 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL. The resulting bacterial suspension was diluted to a working inoculum of approximately 1×10^6 CFU/mL.

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Each individual well of a sterile 96-well plate was filled with 100 µL of Mueller–Hinton broth containing different concentrations of AgNPs prepared by serial dilution, followed by the addition of 10 µL of bacterial inoculum. The final volume in each well was 110 µL.

Appropriate controls were included in the experimental design, including:

- Sterility control (MHB only)
- Growth control (bacteria without AgNPs)

The plates were incubated aerobically at 37 °C for 18–24 hrs. After incubation, 5 µL of resazurin solution (6.75 mg/mL) was added to each well, followed by further incubation at 30 °C for 4 hrs.

A color change of resazurin from blue to pink indicated active bacterial metabolism (growth), whereas no color change indicated complete inhibition of bacterial growth.

The MIC was defined as the lowest concentration of AgNPs that prevented the color change of resazurin, indicating inhibition of bacterial viability.

RNA Extraction, cDNA Synthesis, and qRT-PCR Analysis of *lasI* Expression

To clarify the workflow of gene expression analysis, the strong biofilm-producing isolate PA-12 was selected for qRT-PCR analysis after AgNP treatment. The isolate was cultured in Luria–Bertani (LB) broth at 37°C for 24 hrs. under aerobic conditions. The bacterial culture was adjusted to OD600 = 0.05 and divided into two groups: an untreated control group and an AgNP-treated group. The treated group was exposed to AgNPs at the MIC concentration of 3.125 mg/mL, whereas the control group was maintained under the same conditions without AgNPs.

After treatment, bacterial cells were harvested by centrifugation at 8,000 rpm for 10 min. The supernatant was discarded, and the resulting pellets were used for total RNA extraction using a commercial RNA isolation kit under RNase-free conditions according to the manufacturer's instructions. RNA purity and concentration were assessed using a Nano-Drop spectrophotometer, and only RNA samples with acceptable A260/A280 ratios were used for downstream analysis.

Complementary DNA (cDNA) was synthesized from the extracted RNA using the GoScript™ Reverse Transcriptase Kit according to the manufacturer's protocol. The synthesized cDNA was then used as the template for qRT-PCR amplification of the lasI gene using SYBR Green chemistry on an ABI 7500 Real-Time PCR System. Each reaction was performed in a final volume of 20 µL and included SYBR Green qPCR master mix, forward and reverse primers, cDNA template, and nuclease-free water. All reactions were performed in triplicate to ensure reproducibility.

The pvdQ gene was used as the internal reference gene for normalization. It was selected because it encodes a ribosomal protein, is commonly used for bacterial gene expression normalization, and is not directly involved in the Las/Rhl quorum-sensing pathway. Therefore, it was considered suitable for comparing lasI expression between untreated and AgNP-treated samples.

Relative *lasI* expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The ΔCt value was calculated by

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subtracting the Ct value of the reference gene from the Ct value of lasI. The ΔΔCt value was then calculated by subtracting the ΔCt of the untreated control from the ΔCt of the AgNP-treated sample. A fold-change value below 1 indicated downregulation of *lasI* expression after AgNP treatment. Melt curve analysis was performed to confirm amplification specificity, are listed in Table (1).

Table (1): Oligonucleotide primers used in this study

Gene	Primer Sequence (5' → 3')	Tm (℃)	Amplicon size (bp)	Reference
lasI	F:GTGTTCAAGGAGCGCAAAG R:GAAACGGCTGAGTTCCCAGA	62	240	16
pvdQ	F:GGACTTCGTGCAGAACTCCA R:CCATCTCCTCGAGCGTCTTC	61		16

The thermal cycling conditions were applied as described in Table (2).

Table (2): qRT-PCR cycling conditions used for lasI and pvdQ amplification

Step	Temperature (°C)	Time (min)	Cycle
Initial denaturation	94	2	1
Denaturation	94	0.5	40
Annealing	58	0.5	
Extension	72	0.5	
Melting curve	65 – 95	0.5	1

The housekeeping gene pvdQ was used as an internal reference for normalization. The threshold cycle (Ct) was recorded during the exponential phase of amplification. Relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method [17], as follows:

- $\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$
- $\Delta\Delta Ct = \Delta Ct\ (treated) - \Delta Ct\ (control)$
- Fold change = $2^{-\Delta\Delta Ct}$

A fold change value <1 indicated downregulation of gene expression in treated samples compared to the control.

Statical analysis

Statistical analysis was performed using SPSS software, Version 31.0.2.0 (IBM Corp., USA). All experiments were performed in triplicate to ensure reproducibility, and data were expressed as mean ± standard deviation (SD). Data normality was assessed before applying parametric tests. Differences in biofilm formation and minimum inhibitory concentration (MIC) assays between treated and untreated groups were analyzed using one-way ANOVA, followed by appropriate post hoc comparisons when required. For gene expression analysis, relative lasI expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and

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differences between control and treated groups were analyzed using an independent-samples t-test. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Bacterial Isolation and Identification

A total of 150 clinical specimens from burn wounds, surgical wounds, urine, and ear infections were examined for the presence of *P. aeruginosa* based on colony morphology, pigmentation, microscopic examination, and biochemical characteristics. Identification was confirmed using the VITEK-2 system (BioMérieux, France). Among 27 presumptive isolates, 23 isolates were confirmed as *P. aeruginosa*, representing an overall prevalence rate of 15.3%.

The overall prevalence rate (15.3%) observed in this study falls within the range reported in previous studies (12.8%–29.3%), indicating consistency with global epidemiological trends [18,19]. However, variation in prevalence rates may be attributed to differences in infection control practices, patient populations, and healthcare settings. Notably, higher prevalence rates have been reported in burn-related infections, ranging from 19.6% to 44.4%, depending on clinical and environmental factors [20].

Most confirmed *P. aeruginosa* isolates were recovered from burn samples (52%), followed by wound samples (30%), urine samples (13%), and ear swabs (5%). This distribution supports the strong association of *P. aeruginosa* with burn and wound infections. The disruption of the skin barrier, local immunosuppression, and moist nutrient-rich conditions may facilitate bacterial colonization and persistence at these infection sites [21].

Previous studies have shown that *P. aeruginosa* accounts for approximately 27–36% of burn-related infections, making it one of the most common pathogens in burn units. The lower frequency of *P. aeruginosa* in urinary and ear infections may be related to differences in infection routes, host immune responses, and microbial competition at these anatomical sites.

These findings confirm the clinical importance of *P. aeruginosa* as an opportunistic and hospital-acquired pathogen, particularly in burn-associated infections.

Antibiotic Susceptibility Patterns

In the present study, all *P. aeruginosa* isolates showed MDR characteristics and resistance to several classes of antibiotics. High rates of resistance were recorded against amoxicillin (100%), cefazolin (96%), and tetracycline (88%), whereas resistance to piperacillin/sulbactam was (42%). Moderate resistance rates were observed for levofloxacin (33%), ciprofloxacin (28%), and tobramycin (25%). In contrast, lower resistance rates were found for meropenem (18%) and colistin (12%), as shown in Figure 2.

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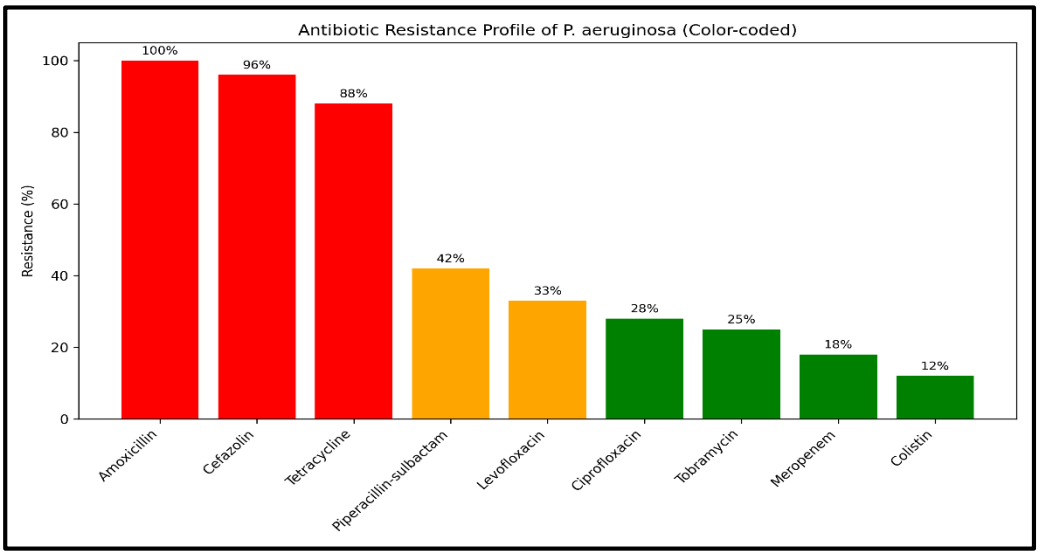

Figure 2. Antibiotic resistance profile of *Pseudomonas aeruginosa* isolates. The bar chart shows resistance percentages among the tested isolates

Previously reported rates of resistance to β-lactam antibiotics (penicillins and cephalosporins) in clinical isolates of *P. aeruginosa* have been significantly high, and this data supports the fact that resistance rates exceed 80% [22,23]. This resistance may be explained by multiple mechanisms, including β-lactamase production, reduced outer membrane permeability, and overexpression of efflux pump systems [24].

The high tetracycline resistance observed in this study is consistent with previous reports showing resistance rates of 70%–90%, suggesting limited therapeutic value of tetracycline against *P. aeruginosa* [23,25]. Similarly, the moderate resistance to fluoroquinolones and aminoglycosides agrees with studies reporting variable resistance rates of approximately 20–40% for these antibiotic classes [18,26].

In contrast, the relatively low resistance rates to meropenem (18%) and colistin (12%) indicate that these agents remain among the most effective therapeutic options for treating MDR *P. aeruginosa* infections, as supported by recent studies reporting resistance rates generally below 25% [26,27]. However, the emerging resistance to these last-line antibiotics represents a growing clinical concern, particularly due to the limited availability of alternative treatment options [27,28].

Overall, the resistance profile observed in this study highlights the reduced effectiveness of several conventional antibiotics against clinical *P. aeruginosa* isolates. These findings support the need for alternative approaches, including anti-virulence and nanoparticle-based strategies, alongside strict antimicrobial stewardship programs.

Biofilm Formation Ability

Biofilm formation by *P. aeruginosa* isolates was evaluated using the microtiter plate (MTP) assay. Among the 23 tested MDR isolates, 19 (82.6%) were weak biofilm producers, 3 (13.0%) were moderate biofilm producers, and 1 (4.4%) was a strong biofilm producer. Isolate PA-12 showed the highest biofilm-forming ability (OD_{570} = 1.88) and was therefore selected for nanoparticle-based experiments, Figure 3.

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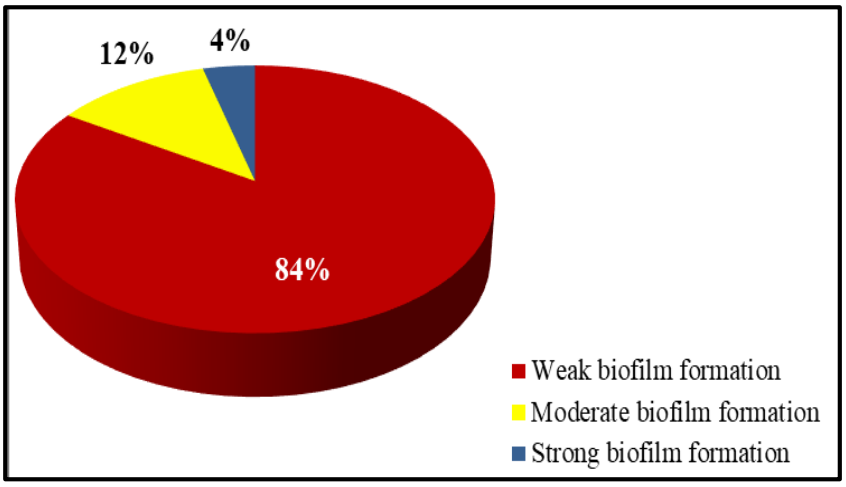

Figure (3): Biofilm-forming ability of *Pseudomonas aeruginosa* isolates determined by the microtiter plate assay

The results showed that *P. aeruginosa* isolates were capable of forming biofilms on abiotic surfaces, supporting their role as clinically important pathogens. This finding agrees with previous studies reporting that approximately 90%–100% of clinical *P. aeruginosa* isolates can produce biofilms [29,30].

Interestingly, the predominance of weak biofilm producers (84%) in the present study differs from some reports in which moderate to strong biofilm formation was more frequently observed (30%–60%) [31.] This variation may be attributed to differences in environmental conditions, genetic diversity among isolates, and methodological variations in biofilm quantification techniques.

Although most isolates were weak biofilm producers, this phenotype remains clinically relevant. Even low-level biofilm formation can enhance bacterial persistence, reduce antibiotic penetration, and protect cells from host immune responses[32]. Biofilms act as a protective matrix that limits antibiotic penetration and promotes horizontal gene transfer, thereby accelerating the development of multidrug resistance [33].

The identification of isolate PA-12 as a strong biofilm producer highlights the heterogeneity among *P. aeruginosa* strains. The elevated biofilm biomass (OD_{570} = 1.88) suggests an increased capacity for virulence and resistance, as strong biofilm formation is often associated with chronic, persistent, and difficult-to-treat infections [31,34].

Biofilm development is closely linked to quorum sensing (QS), particularly the LasI/LasR regulatory pathway, which controls the expression of key virulence factors. Therefore, targeting QS signaling may represent an effective anti-virulence strategy for reducing biofilm formation in MDR P. aeruginosa infections.

So, biofilm production contributes significantly to the pathogenicity of P. aeruginosa. These findings support the need for therapeutic strategies that disrupt both biofilm structure and quorum sensing activity, rather than relying only on conventional antibacterial agents.

Antibacterial Activity of AgNPs and Potential Anti-Biofilm Implications

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The antibacterial activity of green-synthesized AgNPs against *P. aeruginosa* was evaluated using a resazurin based microplate assay. The results showed concentration-dependent inhibition, effect, as wells containing lower AgNP concentrations showed a color shift from blue/purple to pink, reflecting active bacterial metabolism. In contrast, wells exposed to higher AgNP concentrations retained the blue color of resazurin, indicating suppression of bacterial metabolic activity.

The minimum inhibitory concentration (MIC) of AgNPs was determined to be 3.125 mg/mL, as shown in Figure 4. At this concentration, no visible color change was observed, confirming effective inhibition of bacterial viability. These findings indicate that green-synthesized AgNPs exert a strong antibacterial effect against *P. aeruginosa*. Since bacterial growth and metabolic activity are essential for biofilm establishment and maintenance, the observed inhibitory effect may contribute to limiting biofilm-associated bacterial persistence. However, direct quantification of biofilm biomass after AgNP treatment should be considered in future work to further confirm the anti-biofilm effect.

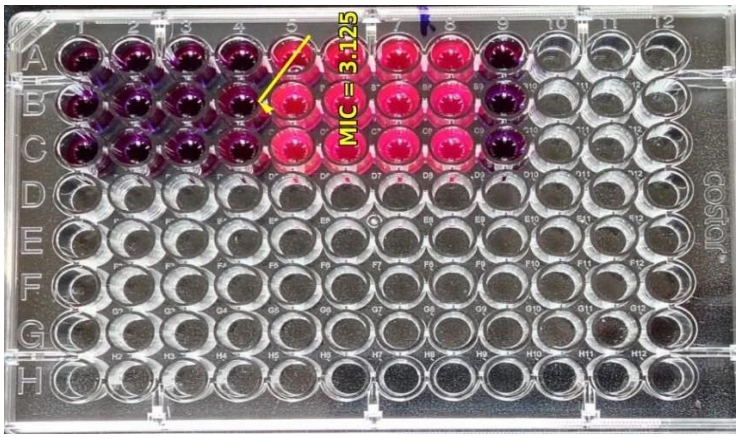

Figure (4): Determination of the minimum inhibitory concentration of synthesized AgNPs against *Pseudomonas aeruginosa* using a resazurin-based microplate assay

The concentration-dependent antibacterial activity observed in this study is consistent with previous reports showing that AgNPs exhibit broad-spectrum antimicrobial activity against *P. aeruginosa*. Reported MIC values commonly range from 1 to 10 mg/mL, depending on nanoparticle size, synthesis method, and experimental conditions [35,36]. Therefore, the MIC value obtained in the present study supports the effectiveness of the green synthesis approach.

The antimicrobial activity of AgNPs may be attributed to several mechanisms, including disruption of the bacterial cell membrane, generation of reactive oxygen species (ROS), and interference with intracellular components such as proteins and DNA [36]. In addition, AgNPs may affect biofilm structure and stability, which increases their activity against biofilm-associated infections [37].

The resazurin assay provides a useful indicator of bacterial viability because metabolically active cells reduce resazurin to resorufin. Therefore, the absence of color change at the MIC indicates suppression of bacterial metabolic activity and growth. These findings agree with previous studies reporting a dose-dependent inhibitory effect of AgNPs on bacterial metabolism [36,38].

Overall, the results indicate that green-synthesized AgNPs exert antibacterial activity against *P.*

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aeruginosa under *in vitro* conditions. Their effect on bacterial viability, together with the observed changes in quorum-sensing-related gene expression, suggests a possible role in limiting biofilm-associated mechanisms; however, direct quantitative biofilm inhibition after AgNP treatment requires further confirmation [39].

Effect of Nanoparticles on Quorum Sensing Gene Expression

The effect of green-synthesized AgNPs on the expression of the quorum sensing gene *lasI* in *P. aeruginosa* was evaluated using quantitative real-time PCR (qRT-PCR), with pvdQ used as the internal reference gene.

The results revealed a significant downregulation of *lasI* expression in AgNP-treated samples compared to the untreated control. The calculated ΔCt values were 3.114 for the control and 8.648 for the treated samples, resulting in a ΔΔCt of 5.533. Based on the $2^{-}\Delta\Delta Ct$ method, the relative expression level of *lasI* was determined to be 0.0216 ($p < 0.05$), corresponding to approximately 2.1% of the control level (Figure 5). This indicates strong suppression of *lasI* expression after AgNP treatment.

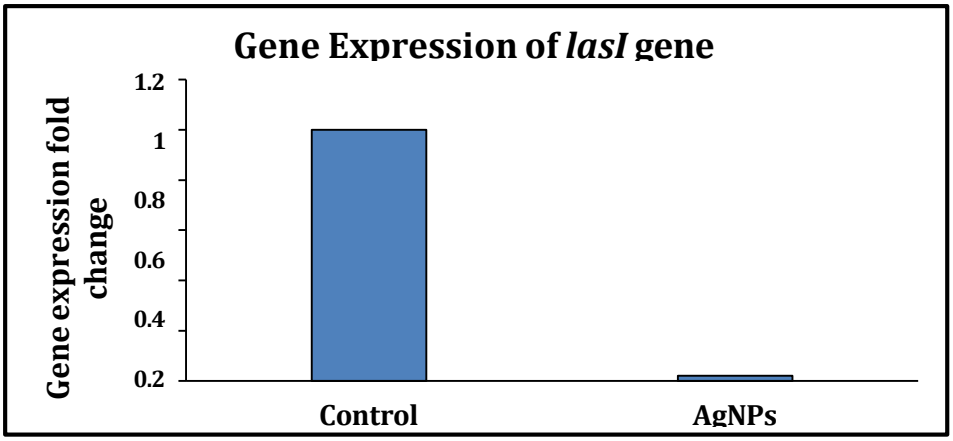

Figure (5): Relative expression of *lasI* in *Pseudomonas aeruginosa* treated with AgNPs compared with the untreated control, determined by qRT-PCR using the $2^{\wedge-\Delta\Delta Ct}$ method

The amplification plots further supported these findings, as treated samples exhibited delayed Ct values, reflecting reduced initial transcript abundance (Figure 6). In addition, melt curve analysis showed a single sharp peak, confirming the specificity and reliability of amplification (Figure 7).

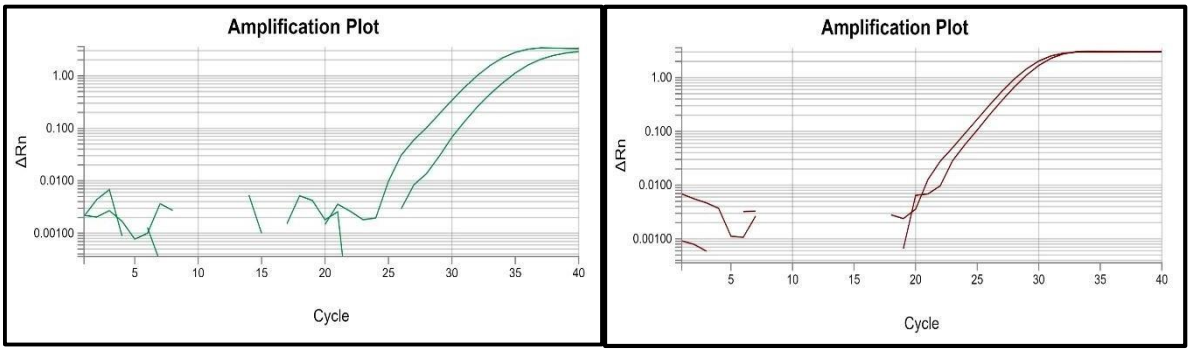

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Figure (6): Amplification plots of the *lasI* obtained by qRT-PCR. AgNP-treated samples showed delayed amplification compared with control.

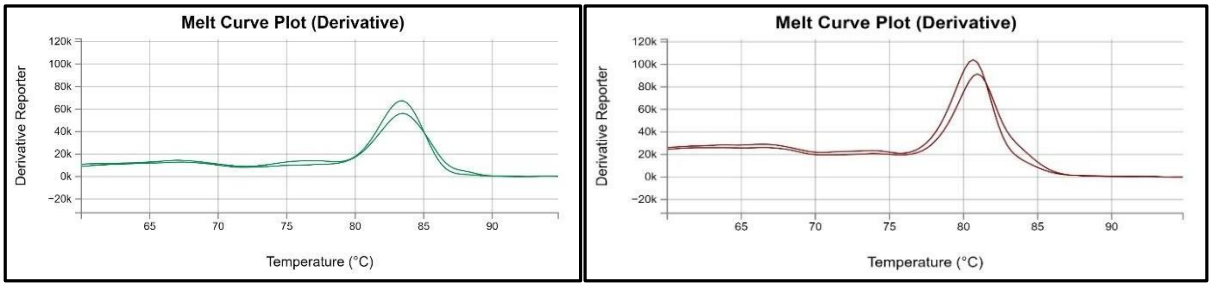

Figure (7): Melt curve analysis of *lasI* gene amplification showing a single sharp peak, confirming amplification specificity.

The observed marked downregulation of *lasI* is consistent with previous studies demonstrating that nanoparticles, particularly AgNPs, can interfere with quorum sensing (QS) regulatory systems. Reported reductions in QS-related gene expression typically range from 20% to 90%, depending on nanoparticle properties and experimental conditions [39,40]. However, the extremely low expression level observed in this study (2.1%) suggests a strong inhibitory effect of the synthesized AgNPs on QS signaling.

The *lasI* gene plays a critical role in QS regulation by controlling the synthesis of N-acyl homoserine lactone (AHL) signaling molecules, which coordinate the expression of virulence factors and biofilm formation [41]. Therefore, suppression of *lasI* may disrupt bacterial cell-to-cell communication, leading to reduced virulence and impaired biofilm development.

This mechanism represents an anti-virulence strategy, as it targets bacterial pathogenicity rather than only bacterial survival. Such an approach may reduce selective pressure and limit the likelihood of resistance development [42].

The inhibitory effect of AgNPs on QS gene expression may be attributed to multiple mechanisms, including interaction with regulatory proteins, inhibition of signaling molecule synthesis, and induction of oxidative stress (ROS production), all of which can interfere with gene transcription and cellular signaling pathways
[35]. Previous studies have also shown that AgNPs may downregulate several QS-related genes, including *lasI*, *lasR*, and *rhlI*, resulting in decreased virulence factor production and biofilm formation [39,43].

Notably, the strong suppression of *lasI* expression observed in this study provides a mechanistic explanation for the reduced biofilm formation and antibacterial activity reported earlier, highlighting the multi-target mode of action of AgNPs [44].

So, these findings indicate that AgNPs exert a significant inhibitory effect on QS-related gene expression, by reducing *lasI* expression to minimal levels. These findings suggest that AgNPs may act as potential anti-virulence agents by suppressing quorum-sensing-related gene expression in vitro. However, their role in controlling MDR *P. aeruginosa* infections, particularly biofilm-associated infections, requires further validation using direct biofilm inhibition assays and *in vivo* models.

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Conclusions

The present in vitro study showed that clinical isolates of Pseudomonas aeruginosa exhibited multidrug resistance and variable biofilm-forming ability. Green-synthesized AgNPs demonstrated antibacterial activity against *P. aeruginosa*, with a minimum inhibitory concentration (MIC) of 3.125 mg/mL. In addition, AgNP treatment was associated with significant downregulation of the quorum-sensing-related lasI gene, reducing its relative expression to 2.1% of the untreated control. These findings suggest that AgNPs may interfere with quorum sensing and could contribute to limiting biofilm-associated virulence. However, because this study was conducted under in vitro conditions, further direct biofilm inhibition assays, *in vivo* experiments, and clinical investigations are required to confirm the anti-biofilm and therapeutic potential of green-synthesized AgNPs against MDR *P. aeruginosa*.

Acknowledgments

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We also extend our deep appreciation to all researchers who contributed to the successful completion of this scientific work.

Author's Declaration

The authors declare that all figures and tables included in this manuscript are original and have been created by the authors, and have not been previously published or submitted elsewhere.
Ethical approval for this study was obtained from the local ethical committee at Al-Nahrain University/College of Biotechnology, ensuring full compliance with established ethical standards and the protection of participants' rights and well-being.

Author's Contribution Statement

- First Author: Conceived and designed the study, performed the experimental work, analyzed the data, and drafted the initial manuscript.
- Second Author: Contributed to conducting experiments and data collection, participated in the literature review, interpreted the results, and contributed to manuscript writing.
- Third Author: Supervised the overall research process, critically reviewed the manuscript for scientific and linguistic accuracy, and provided essential guidance to ensure the quality and integrity of the study.

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