Heliyon 11 (2025) e42899

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Biosynthesized silver nanoparticles at subinhibitory concentrations as inhibitors of quorum sensing, pathogenicity, and biofilm formation in *Pseudomonas aeruginosa* PAO1

Fatemeh Aflakian [1], Gholamreza Hashemitabar [*,1]

Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

ARTICLE INFO

Keywords:
Anti-quorum sensing
Anti-virulence
Anti-biofilm
Synthesized silver nanoparticles
Pseudomonas aeruginosa

ABSTRACT

Pseudomonas aeruginosa infections associated with biofilm are a significant clinical challenge due to the limited efficacy of traditional antibiotics or combination therapies. Hence, exploring novel strategies and assessing different compounds for their anti-biofilm or anti-quorum sensing (QS) properties is imperative. One of the various applications of silver nanoparticles (AgNPs) is to use them as an antimicrobial agent to target bacteria resistant to common antibiotics. This study evaluates the anti-biofilm and anti-virulence effect of biosynthesized AgNPs against *P. aeruginosa* PAO1 at subinhibitory concentration levels.

Minimum inhibitory concentrations (MICs) and biofilm formation capacity were evaluated by the microdilution method and crystal violet method, respectively. Motility assay and virulence factors were investigated in the presence of AgNPs.

It was observed that green-synthesized AgNPs at sub-MIC (50 μg/mL) suppressed *P. aeruginosa* biofilm formation by 78 %. Increased dose-dependent inhibitory effects on virulence phenotypes (LasB elastase, LasA protease, pyocyanin, and motility) regulated by QS were observed. In addition, the relative expression levels of biofilm-related genes including *algC, pslA*, and *pelA* were analyzed using RT-qPCR. The expression level of QS-regulated biofilm genes after AgNPs treatment sub-MIC led to a decrease in the expression of *algC, pslA,* and *pelA* by 77 %, 83 %, and 68 %, respectively.

The findings of this study demonstrated how green AgNPs can effectively inhibit QS at sub-MIC concentrations, indicating their potential as antivirulence agents to deal with challenges related to biofilm formation and antimicrobial resistance in *P. aeruginosa.* This presents a promising alternative to traditional antibiotics in antimicrobial therapy.

1. Introduction

Antibiotics and antimicrobial drugs have been utilized for a long time to restrain or kill bacteria. Nevertheless, resistance to antimicrobial drugs has extended on an incredibly massive scale over time, enormously decreasing their effectiveness, and becoming a worldwide challenge, so much so that the World Health Organization (WHO) has ranked antimicrobial resistance as the third global health threat [1]. In 2013, WHO announced that the world is heading towards a post-antibiotic period, where bacterial infections

* Corresponding author.
E-mail addresses: Fatemeh_aflakian@yahoo.com (F. Aflakian), hashemita@gmail.com (G. Hashemitabar).
[1] Contributing authors.

https://doi.org/10.1016/j.heliyon.2025.e42899
Received 1 December 2024; Received in revised form 19 February 2025; Accepted 20 February 2025
Available online 20 February 2025

cannot be treated with traditional antibiotics and can often lead to death [2]. Bacterial drug resistance has several negative consequences for medicine and public health. Drug-resistant bacterial diseases result in higher doses of drugs, the addition of remedies with more toxicity, increased treatment costs, longer treatment periods, and increased mortality [3].

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that can cause long-term and persistent human infections, especially in immunocompromised and hospitalized individuals [4]. The World Health Organization (WHO) has introduced multidrug-resistant (MDR) *P. aeruginosa* as a serious menace to public health that requires extensive research and development of novel antimicrobial drugs [4]. The presence of highly complex regulatory networks and the ability to produce biofilm in this bacterium has led to widespread antibiotic resistance and treatment failure [5].

Bacterial population density is regulated by a highly organized cell-to-cell signaling system called quorum sensing (QS), in which signaling molecules regulate the expression and production of virulence factors, including motility, and biofilm development [5]. In *P. aeruginosa*, there are three QS systems: rhlI/rhlR, lasI/lasR, and pqs/mvfR systems. In the las system, the activation of the transcription factor lasR is initiated by the autoinducer C12-HSL. This activation then leads to the expression of lasI and subsequently triggers the production of exotoxin A, LasB elastase, and LasA protease. Similarly, in the rhl system, the autoinducer C4-HSL enhances rhlI expression through its binding and interaction with rhlR. This induction causes the production of LasB elastase, rhamnolipids, and pyocyanin [6,7].

Therefore, interference with QS signalling systems can lead to reduced bacterial virulence and is a promising alternative strategy to combat multidrug-resistant *P. aeruginosa* and treat bacterial infections [2].

One of the most promising strategies for overcoming microbial resistance is the use of nanoparticles (NPs) [1]. The NPs offer the definitive solution to combat antibiotic-resistant bacteria, as they are not only used as antimicrobial and natural antibiotic carriers but also combat the bacteria themselves [8]. In addition, the exceptional physiochemical properties of NPs mainly due to their large surface area to volume proportion make them potential candidates to be utilized for better therapeutic effectiveness against resistant microbes [9]. Also, the multiple concurrent mechanisms of action of nanoparticles against bacteria have made it difficult for bacterial cells to evolve resistance to NPs [8].

In the medical field, silver nanoparticles (AgNPs) are particularly important and popular due to their antimicrobial properties [10]. For years, AgNPs have been recognized as an antimicrobial agent with a broad spectrum of activity and have been used in various industrial and medical applications against bacteria, viruses, and other microorganisms [11,12]. It is believed that green nanoparticles have a relative advantage over AgNPs that are produced through physical and chemical methods due to their natural coating and biocompatibility [13].

In our previous research, AgNPs were synthesized using the medicinal plant *Teucrium polium* and characterized by different analyses [14]. The antibacterial activity of green synthesized AgNPs led to the potential inhibition of six pathogens including *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus*, *Salmonella typhimurium*, *Klebsiella pneumonia,* and *Acinetobacter baumannii*. The increase in antibiotic resistance of *P. aeruginosa* and the placement of this bacterium at the top of the priority pathogens list of the WHO [15] have intensified the need to develop new treatment strategies against this bacterium.

Since the development of anti-biofilm treatments in the field of biomedicine is a challenge and a necessary need, the present study aimed to investigate the effect of sub-inhibitory concentrations of green synthesized AgNPs on QS system-related virulence factors and biofilm production of *Pseudomonas aeruginosa* PAO1. This study provides valuable insight into the anti-biofilm and anti-QS activity of synthesized AgNPs at sub-inhibitory concentrations against *Pseudomonas aeruginosa*, which can be very important for the effective management of infections and the overcoming of antimicrobial resistance, especially considering the global challenge of antimicrobial resistance.

2. Materials and methods

2.1. Green synthesis of silver nanoparticles using Teucrium polium leaf/flower

The green synthesis of AgNPs was carried out according to Hashemitabar et al., previous research [14]. To synthesize AgNPs, the aqueous extract of *Teucrium polium* at a concentration of 100 mg/mL was mixed with an aqueous solution of silver nitrate ($AgNO_3$, Merck, Germany) with a concentration of 5 mM at pH 10 and 60 °C temperature for 24 h. To separate and purify the synthesized AgNPs, the mixture was centrifuged at 10,000 rpm for 10 min. After centrifugation, the supernatant solution was discarded and the precipitate of nanoparticles was washed three times with sterile double distilled water (ddH_2O). The obtained nanoparticle sediment was subsequently dried at room temperature and kept in a dark location to assess the antibacterial properties.

2.2. Microorganisms

The study utilized two strains of *P. aeruginosa*: the quality control strain *P. aeruginosa* ATCC 27853 and the wild-type strain *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 is commonly employed as a model strain in biofilm and QS system research. Both these strains were obtained from the microbiology laboratory collection of Ferdowsi University of Mashhad, Iran. For further experiments, *P. aeruginosa* strains were grown on Luria-Bertani (LB, Merck, Germany) agar at 37 °C for 24 h.

2.3. Antibacterial activity of AgNPs

The antimicrobial activity of AgNP against *P. aeruginosa* was evaluated using the Nature protocol with slight modifications [16]. 95

μg/mL of Müller– Hinton broth (MHB, Sigma-Aldrich, St. Louis, MO, USA) was poured into each well of a 96-well plate (Sigma-Aldrich, Germany). The synthesized AgNPs powder was dissolved in ddH_2O and ultrasonicated at 400 Hz for 30 min to make it completely uniform. Two-fold serial dilutions of silver nanoparticles (concentration 400, 200, 100 to 6.25 μg/mL) were prepared using MHB medium and added to wells of the plate. Finally, the bacterial suspension with $OD_{600} = 0.01$ was added to each well to obtain a final 5×10^5 CFU/mL concentration. A column of wells containing sterile medium with AgNPs and a column containing a mixture of sterile medium with bacterial suspension were considered negative control and positive control, respectively. The plates were placed overnight in a shaker incubator at 37 °C. After that, the OD values were read by a microplate reader (Epoch2-microplate reader, BioTek Company, USA) at a wavelength of 600 nm to determine the quantitative growth of bacteria in each concentration. To determine the minimum bactericidal concentration (MBC), which is the lowest concentration that shows a specified reduction (e.g., 99.9 %) in CFU/mL compared to the MIC dilution, the dilution corresponding to the MIC and at least two of the more concentrated test product dilutions were inoculated on Nutrient agar.

2.4. Biofilm inhibition assay

The anti-biofilm effect of silver nanoparticles on *P. aeruginosa* was investigated using the microtiter plate method and crystal violet staining [17]. AgNPs in concentrations of 1/2 × MIC to 1/16 × MIC (equivalent to 50 to 6.25 μg/mL, respectively) were prepared with tryptic soy broth (TSB, Merck) and added to 96-well U-bottom polypropylene plates (Sigma-Aldrich, Germany) at a volume of 200 μL per well. The positive control comprised a sterile medium with a bacterial suspension (biofilm formation control), while the negative control consisted of a sterile medium with AgNPs. After incubation for 24 h at 37 °C, the OD_{600} was recorded to determine the quantitative growth of bacteria in different concentrations. After this step, to remove non-adherent cells, the wells were rinsed three times with sterile phosphate-buffered saline (PBS) of pH 7.2. After drying at room temperature, 210 μL of 0.1 % (w/v) crystal violet staining solution was added to each well and stained for 20 min at room temperature. Subsequently, the staining solution was eliminated, and the wells were rinsed three times with sterile distilled water. Finally, 220 μL of 70 % ethanol was added to each well and incubated for 20 min with gentle shaking. The OD values were read by a microplate reader (Epoch2-microplate reader, BioTek Company, USA) at a wavelength of 595 nm.

2.5. Biofilm eradication assay

The efficacy of AgNPs in eradicating mature biofilms of *P. aeruginosa* was assessed as previously described [18]. A diluted seed culture (200 μL with OD600 = 0.01) of the bacterial cells was added to a 96-well microtiter plate and incubated at 37 °C for 24 h. After this incubation, the planktonic cells were discarded, and the surface-attached biofilm cells were washed with sterile TSB. 200 μg/mL of various concentrations of AgNPs (100–6.25 μg/mL) were prepared in sterile TSB and added to the bacterial cells. The microplate was subsequently incubated at 37 °C for 24 h. Plankton cells were then removed, and biofilm cells were stained using the same method employed in the biofilm inhibition experiment.

2.6. Motility assay

To investigate the effect of AgNPs on virulence factors, the motility of *P. aeruginosa* was measured in the presence of synthesized nanoparticles [5,19]. *P. aeruginosa* strains were grown on LB agar at 37 °C for 24 h and then colonies were suspended in LB broth with a final concentration of 5×10^5 CFU/mL ($OD_{600} = 0.01$). The synthesized AgNPs were added to this suspension at a sub-minimum inhibitory concentration (sub-MIC, 50 μg/mL) and kept overnight at 37 °C. Next, 5 μL of the suspension was placed in the center of plates containing LB medium supplemented with agar at concentrations of 0.3 %, 0.5 %, and 1 %. Plates with concentrations of 0.3 %, 0.5 %, and 1 % agar were used to measure swimming, swarming, and twitching, respectively. After overnight incubation, the diameters of the zones for twitching, swarming and swimming were measured. All experiments were repeated three times.

2.7. SEM analysis

The inhibitory effect of silver nanoparticles on biofilm formation was analyzed using a scanning electron microscope (SEM) [20]. A polyvinyl chloride film as a biofilm carrier was cut to a diameter of 1 cm and exposed to ultraviolet radiation for 24 h in 75 % alcohol and then washed with sterile 0.9 % normal saline. The biofilm carrier was immersed in a bacterial solution with a concentration of 5×10^5 CFU/mL and silver nanoparticles at a concentration of 1/2 × MIC. In parallel, a biofilm carrier was immersed in a bacterial solution without AgNPs as a control. The samples were incubated at 37 °C for 24 h. Biofilm carriers were collected and gently washed three times with sterile PBS to remove plankton bacteria on the carrier surface. Then, the samples were fixed in 3 % (w/v) glutaraldehyde solution (Wuhan Saiweier Biotechnology Company, China) at 4 °C for 2 h. The biofilm carriers were washed three times using sterile PBS. Then dehydration was done with different concentrations of ethanol (30, 50, 70, 80, 90, and 100 %) for 10 min per step. After drying, the biofilms were observed by SEM (ZEISS LEO-1450 VP, Carl Zeiss AG, Jena, Germany).

2.8. Anti-quorum sensing activity assay

In this study, the effect of synthesized AgNPs on inhibiting the production of pathogenic factors of *P. aeruginosa* under the control of the QS system i.e., pyocyanin, LasB elastase and LasA protease was investigated.

Pyocyanin assay: An overnight culture of *P. aeruginosa* PAO1 was prepared and adjusted to OD_{600} = 0.01 in LB medium, then incubated with different concentrations of AgNPs from 1/2 × MIC to 1/16 × MIC (equivalent to 50 to 6.25 μg/mL, respectively) for 24 h. A group containing bacterial suspension without nanoparticles was considered as a control. The samples were centrifuged at 10,000 rpm for 20 min. 3 mL of the supernatant was collected and mixed with chloroform at a ratio of 3:2, the chloroform supernatant was extracted with 1 mL of 0.2 M HCl and vortexed. The absorbance of the upper pink layer in OD_{520} was measured [21].

LasA protease assay: The azocasein assay was used to assess the production of LasA protease [6]. Bacterial suspensions of *P. aeruginosa* PAO1 (with an optical density of 0.01 at 600 nm) were incubated at 37 °C for 24 h in the presence or absence of AgNPs at concentrations ranging from 1/2 × MIC to 1/16 × MIC at 37 °C for 24 h. To eliminate bacterial cells, the suspension was filtered using a 0.22 μm syringe filter (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany). Then, 150 μL of this filtered suspension was mixed with 250 μL of a solution containing 2 % azocasein (Sigma-Aldrich, St. Louis, MO, USA), prepared in 50 mM Tris-HCl. After incubating for 4 h at 4 °C, trichloroacetic acid (TCA) at a concentration of 10 % (1.2 mL) was added to precipitate the undigested substrate. The mixture was then centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was combined with 1.4 mL of a solution containing 1 M NaOH. The relative protease activity was determined by measuring the optical density at a wavelength of 440 nm.

LasB elastase assay: The production of LasB elastase was assessed using elastin-Congo red (ECR) assay [6]. Bacterial suspensions of

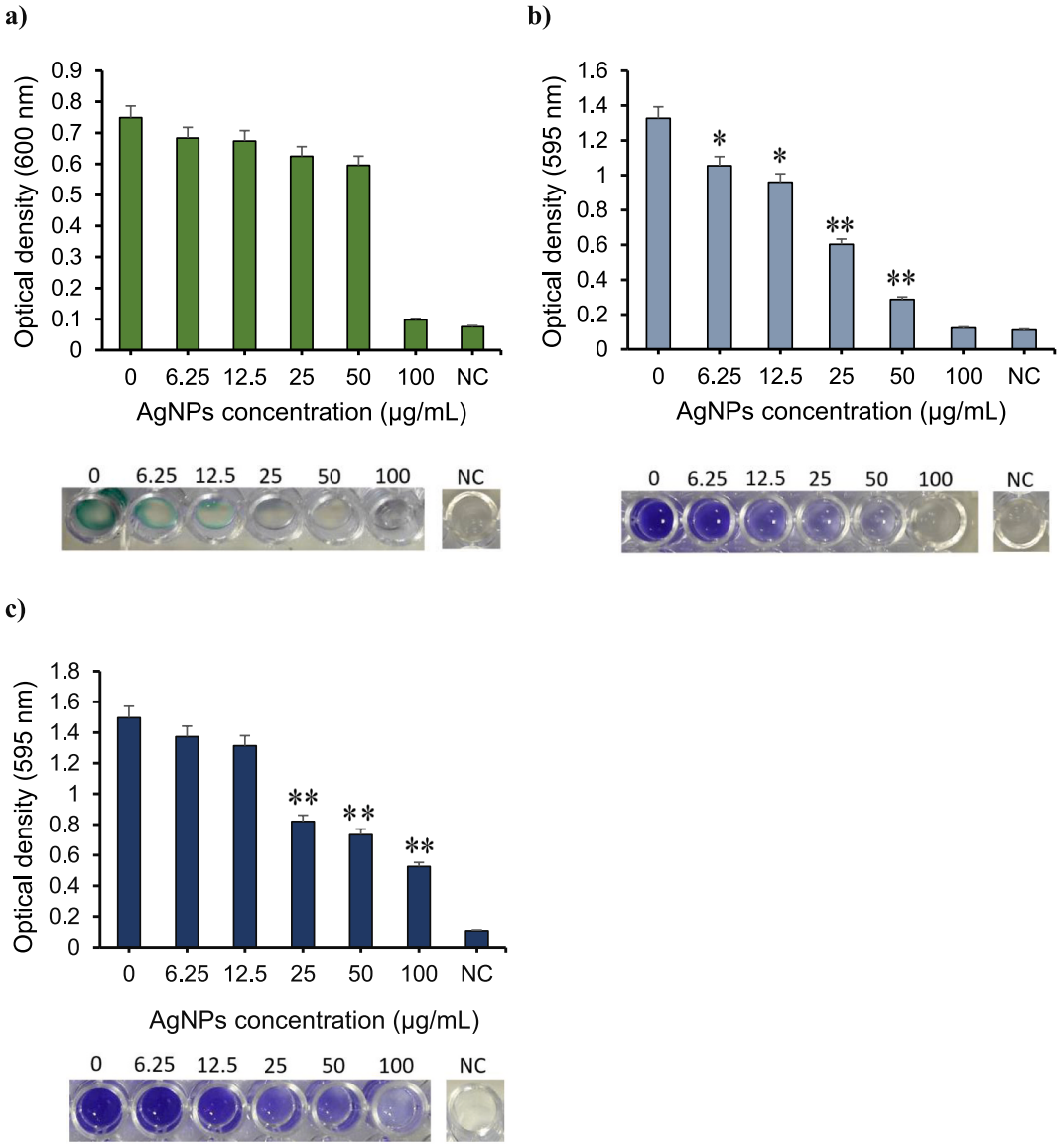

Fig. 1. The impact of different concentrations of AgNPs on the planktonic growth (a) and biofilm formation (b) the mature biofilm eradication (c) of *P. aeruginosa* PAO1. The values represent the mean of three independent replicates ± SD. "*" shows a significant difference (*$p \leq 0.05$, **$p \leq 0.01$) between AgNPs and control (0 μg/mL). NC: negative control.

P. aeruginosa PAO1 (OD600 = 0.01) cells were incubated in the presence or absence of AgNPs at concentrations from 1/2 × MIC to 1/16 × MIC for 24 h at 37 °C. The suspension was centrifuged at 10,000 rpm for 20 min, and the supernatant was collected. The volume of 100 μL from the supernatant was combined with 900 μL of ECR buffer (100 mM Tris-HCl, 1 mM CaCl2, pH 7.5, 10 mg ECR) (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 20 h with shaking. The reaction was stopped by introducing 100 mL of EDTA (0.12 M) solution. The reaction mixture was centrifuged to eliminate the insoluble ECR, and the absorbance of the supernatant was measured at OD_{495} to determine the activity level of elastase.

2.9. Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was used [22] to evaluate the effects of sub-MIC AgNPs on the expression levels of biofilm production genes (*algC*, *pslA*, and *pelA*) under the control of the QS system according to Aflakian et al., [5]. *P. aeruginosa* PAO1 (10^6 cells/mL) was incubated in LB medium supplemented with AgNPs at a concentration of 1/2 × MIC for 20 h at 37 °C. In parallel, bacterial suspension with no AgNP treatment was prepared as a control. Total bacterial RNA was extracted using a total RNA isolation kit (Denazist, ASIA). The complementary DNA (cDNA) was synthesized using an Easy cDNA synthesis kit (Parstous, Iran) following the manufacturer's guidelines.

Quantitative real-time PCR was performed using a BIO-RAD Real-Time PCR system (Rotorgen, USA) with SYBR Green qPCR RealQ Plus (Ampliqon, Denmark). The reaction was set up in a final volume of 20 μL, consisting of 10 μL 2 × Master Mix Green, 1 μL of 10 pM forward primer, 1 μL of 10 pM reverse primer and 1 μL cDNA template topped up with ddH_2O to 20 μL. The cycling program included an initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min. The *16S rRNA* gene was used as a reference gene for normalization, and fold change was determined using the ΔΔCT method ($2^{-\Delta\Delta CT}$ formula was used to analyze the data).

2.10. Statistical analysis

The statistical analysis was conducted through the use of Student's t-test of variance (ANOVA) using SPSS software version 26. All experiments were carried out three times, and the results are shown as means ± standard errors of the mean (SEM) and standard deviation (SD). Statistical significance was considered at $p \leq 0.05$ and $p \leq 0.01$.

3. Results

3.1. Characterization of green synthesized AgNPs

The green synthesis method of AgNPs which was determined in the previous study [14] showed that the aqueous extract of *T. polium* as a harmless reducing agent compatible with the environment can cause the synthesis of AgNPs. The synthesis of nanoparticles was confirmed by scanning the wavelength in the range of 400–450 nm (specifically at 420 nm). Additionally, scanning electron microscopy, energy-dispersive X-ray spectroscopy, and X-ray diffraction analysis confirmed the synthesis of AgNPs. Furthermore, analysis through dynamic light scattering, zeta potential, and transmission electron microscopy indicated that the nanoparticles exhibit a spherical shape with sizes ranging from 8 to 28 nm, and demonstrate high stability in the reaction mixture.

3.2. Antibacterial and antibiofilm activity of AgNPs

The antibacterial activity of synthesized AgNPs was evaluated using the broth microdilution method by determining MIC and MBC against *P. aeruginosa*. The obtained results showed a significant antibacterial effect at a concentration of 100 μg/mL (Fig. 1a). The viability of *P. aeruginosa* at concentrations below the MICs of AgNPs was investigated to determine whether there is a significant bacteriostatic effect at these concentrations. As shown in Fig. 1a, AgNPs at sub-MIC concentrations (50, 25, 12.5, 6.25 μg/mL) did not show significant inhibition in the growth of planktonic cells of *P. aeruginosa* PAO1 compared to the control group (0 μg/mL) ($P > 0.05$). The MBC of the synthesized AgNPs was 200 μg/mL, and no growth was observed at this concentration. Hence, concentrations below 100 μg/mL were selected for evaluation in future anti-biofilm and anti-QS assays.

As shown in Fig. 1b, AgNPs prevented the biofilm formation of *P. aeruginosa* at concentrations sub-MIC. Significant inhibition of biofilm formation at sub-MIC levels of about 78 % and 54 % was observed at 1/2 × MIC and 1/4 × MIC (50 μg/mL and 25 μg/mL), respectively. Student's t-test was performed to compare biofilm formation at sub-MIC levels of AgNPs to the control, which had 100 % biofilm.

The established mature biofilm was significantly disrupted by AgNPs (Fig. 1c). Maximum eradication of the mature biofilm occurred at the MIC (100 μg/mL) of AgNPs. Specifically, the eradication rate of the mature biofilm of *P. aeruginosa* was 64.8 % at its MIC value and 50.9 % at 1/2 × MIC. These results indicate that the highest level of biofilm eradication was achieved at the MIC of the AgNPs.

3.3. Effect of AgNPs on P. aeruginosa motility

The ability to move is an important factor in the spread and pathogenesis of *P. aeruginosa* infections. The sub-MIC effects of AgNPS on three types of motility (twitching, swimming and swarming) were assessed by inoculating overnight cultures of *P. aeruginosa* PAO1

on motility plates. As shown in Fig. 2b, the zone of swarming, swimming and twitching of *P. aeruginosa* PAO1 in the absence of AgNPs was 12 ± 1.2 mm, 17 ± 1.3 and 10 ± 2.1 mm, respectively. After treatment with AgNPs, the zone of swarming, swimming and twitching decreased to 6 ± 1.4 mm, 10 ± 1.3 mm and 5 ± 1.1 mm, respectively. In general, *P. aeruginosa* PAO1 exhibited a significant decrease ($p < 0.05$) in motility after AgNP treatment (Fig. 2a).

3.4. Effect of AgNPs on biofilm formation by scanning electron microscopy

SEM analysis was performed to reveal the inhibitory effects and the morphological changes of the treated cells. In the untreated group, *P. aeruginosa* grew extensively and the cells in the secreted matrix were cohesively attached to the polyvinyl chloride film and formed the primary biofilm (Fig. 3b), no biofilm was detected in the control group containing LB medium (Fig. 3a). In the treatment groups, the density of bacteria and the amount of adhesion to the biofilm carrier decreased with increasing concentrations of AgNPs. The absence of biofilm matrix around cells treated with concentrations of 50 μg/mL and 25 μg/mL indicates the inhibition of biofilm formation by nanoparticles at levels below MIC (Fig. 3c–d). The size of some bacteria decreased due to the effect of nanoparticles. Also, the damage of nanoparticles was caused by binding to the membrane and cell lysis. In comparison, control cells showed intact cell structure within the biofilms.

3.5. Anti-virulence effect of AgNPs on QS-controlled factors

Pyocyanin, LasA protease and LasB elastase production: Investigating the effect of synthesized AgNPs on the production of

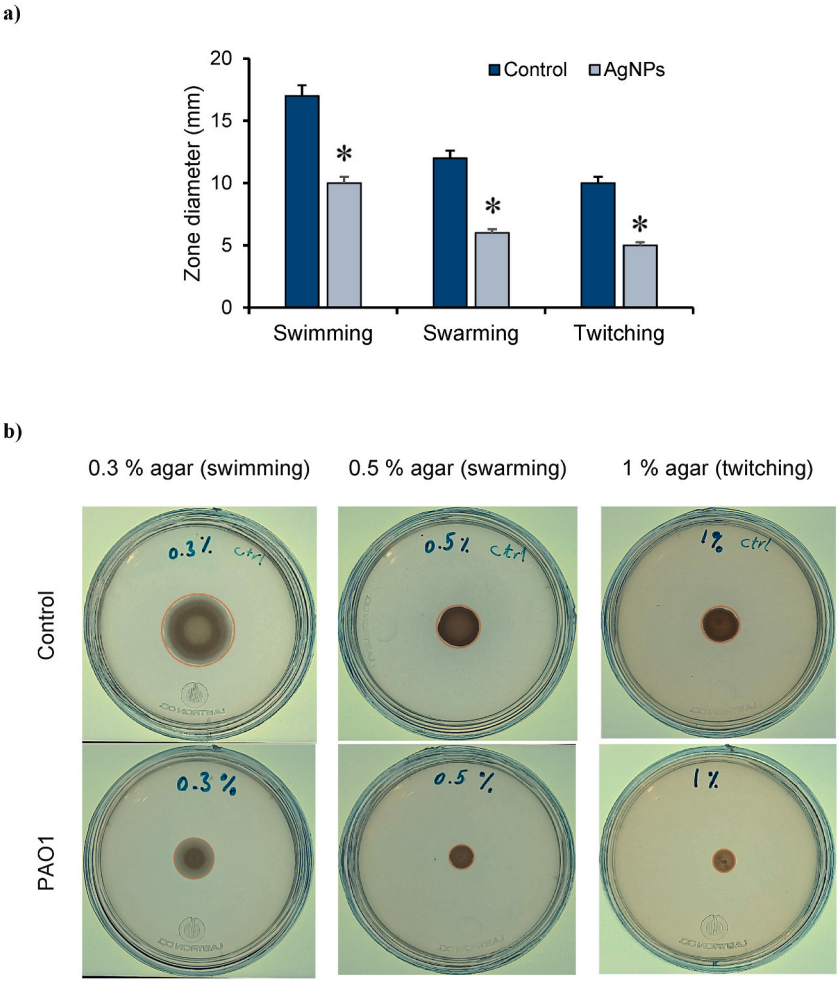

Fig. 2. Effect of synthesized AgNPs at 1/2 × MIC (50 μg/mL) on the swimming, swarming and twitching motility of *P. aeruginosa* PAO1. (a) The results indicate the means ± SEM of motility. Control samples were plates inoculated with bacteria in the absence of AgNPs. Statistical significance was determined by the student's t-test. "*" shows a significant difference ($*p < 0.05$) of AgNPs compared with the control. (b) Representative images of swimming, swarming and twitching of *P. aeruginosa* in the presence of AgNPs compared with the control. The brown circle line indicates the zone diameter of bacterial growth.

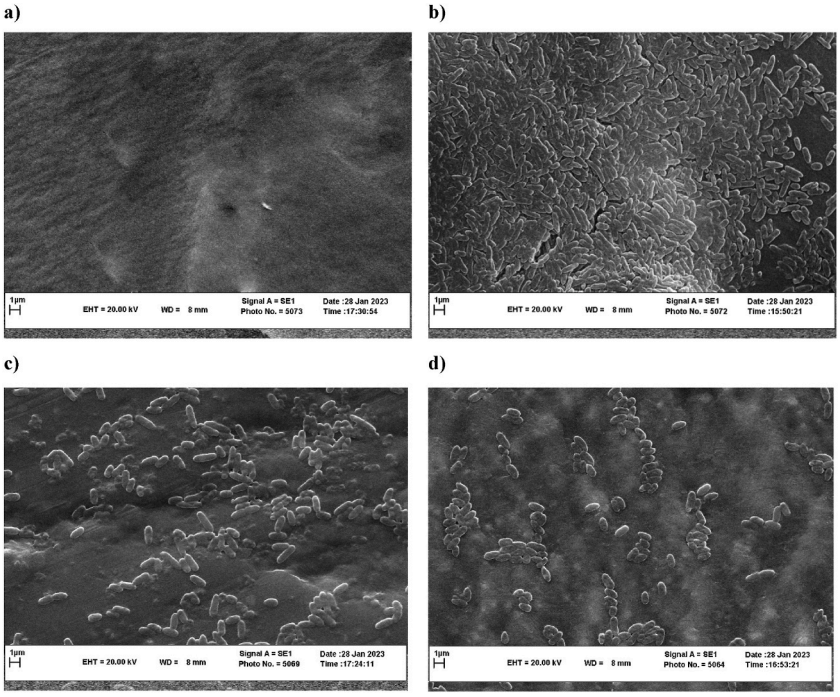

Fig. 3. The electron microscopy analysis of biofilm formation of *P. aeruginosa* PAO1 before and after treatment with AgNPs. (a) LB medium as negative control (no bacteria). (b) untreated group. Aggregation of *P. aeruginosa* cells and secretion of biofilm matrices after 24 h. (c) AgNPs treated group with the final concentration of 1/4 × MIC. (d) AgNPs treated group with the final concentration of 1/2 × MIC. Biofilm formation decreased with the increase in nanoparticle concentration.

pyocyanin, as a *P. aeruginosa* siderophore that facilitates pathogenic growth at the site of infection, showed a significant decrease in the amount of pyocyanin at a concentration of 1/2 × MIC - 1/4 × MIC (about 70 %–62 %), compared to the control sample (Fig. 4a). In parallel, the production of two other pathogenic factors associated with QS after exposure to AgNPs at sub-MIC (1/2 × MIC - 1/4 × MIC) was significantly reduced in a dose-dependent manner: 85 %–72 % for LasA protease inhibition, and 87 %–75 % for LasB elastase inhibition (Fig. 4b–c). These results confirmed the anti-virulent activity of synthesized AgNPs.

3.6. Biofilm genes expression

The *P. aeruginosa* biofilm matrix consists of three polysaccharides: Pel, Psl and alginate. Psl and Pel polysaccharides play a role in adhesion and the initial development of the biofilm. The *algC* produces alginate precursors for a mucosal phenotype that protects against antimicrobial agents and the host defence system. The gene expression levels related to biofilm formation (quorum sensing genes) when exposed to synthesized AgNPs showed a significant decrease at sub-MIC levels. According to the results in Fig. 5, the transcription levels of *algC, pslA* and *pelA* genes using RT-qPCR at the 1/2 × MIC)50 µg/mL) of AgNPs in the treated group decreased by approximately 77.5 %, 83 %, and 68 %, respectively compared to the untreated group ($P < 0.05$). AgNPs at 1/4 × MIC (25 µg/mL) repressed the expression levels of *algC, pslA* and *pelA* by 70.5 %, 75.3 % and 58.2 %, respectively. These inhibitory effects on biofilm genes are correlated with the results of biofilm formation inhibition and SEM analysis.

4. Discussion

Increasing multidrug resistance to traditional antimicrobials has prompted researchers to search for novel approaches to modulate bacterial virulence and control the spread of multidrug-resistant bacteria [5,19,23].

The unique properties of AgNPs, especially the antimicrobial properties against a wide range of bacteria, have made these to be used in the medical, drug delivery, food and wastewater treatment industries, and are considered next-generation antimicrobials [1, 24]. Also, studies have shown that resistance to antimicrobial treatment is difficult for AgNPs, as it requires three separate mutations in three separate bacterial systems in one generation of bacteria [25,26]. Since the synthesis method is one of the influential parameters in the size and antibacterial properties of nanoparticles. Also, due to environmental concerns, the synthesis of metal nanoparticles using plant extracts was used as the most economical and compatible method to reduce the involvement of toxic chemicals and has been the subject of many studies in recent years [27].

The antibacterial activity of Bio-AgNPs against gram-positive and gram-negative bacteria has been described by many studies. However, few studies have investigated the sub-inhibitory effects of silver nanoparticles on microorganisms [28,29]. The present study

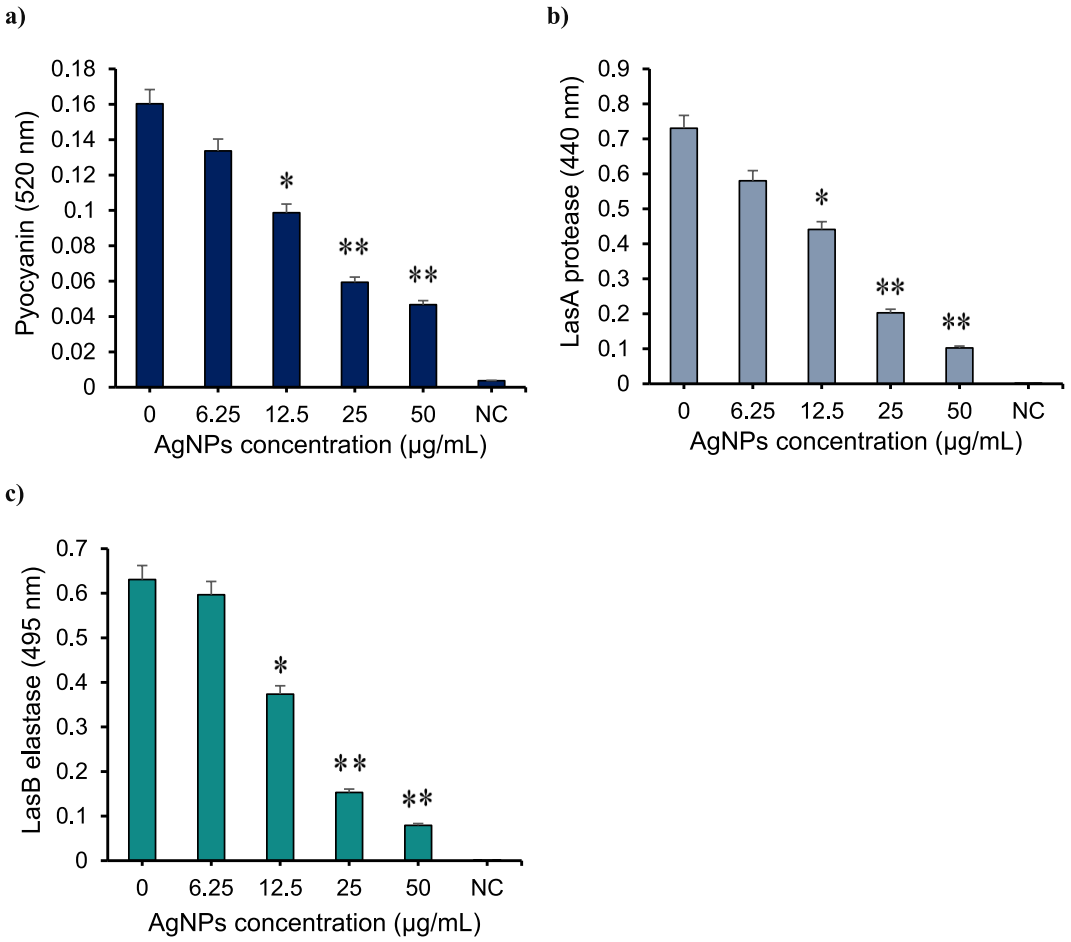

Fig. 4. Effect of different concentrations of AgNPs on the production of Pyocyanin (a), LasA protease (b) and LasB elastase (c) in *P. aeruginosa* PAO1, compared to the cells not treated with the AgNPs (0 µg/mL). The values are the mean ± SD of three independent experiments. Statistical significance was determined using the student's t-test. *$p \leq 0.05$ and **$p \leq 0.01$ compared with the control group. NC: negative control.

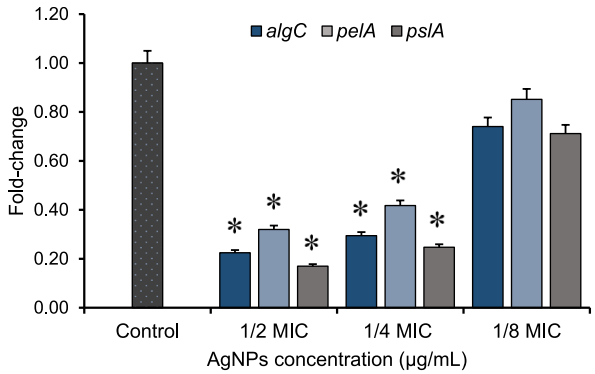

Fig. 5. The levels of relative expression of QS-regulated biofilm genes (*algC, pslA,* and *pelA*) in the presence of sub-MICs of AgNPs (50, 25, and 12.5 µg/mL). *$P < 0.05$ indicates a significant difference compared to the control group (untreated). Error bars represent the standard deviation of three parallel measurements.

was conducted to investigate the effect of sub-inhibitory concentrations of green AgNPs (16 nm) synthesized by Hashemitabar et al. [14], on the inhibition of virulence factors, motility and biofilm formation regulated by QS system of *P. aeruginosa*.

Biofilm-forming bacterial pathogens are resistant to drugs, environmental stressors, and the host immune system, and they produce virulence factors that contribute to chronic infections. The results of investigating the effect of synthesized AgNPs on inhibiting biofilm

formation showed that nanoparticles were able to inhibit biofilm formation in a concentration-dependent manner. It was found that at a concentration of 1/2 × MIC (50 μg/mL) of AgNPs, it inhibits the biofilm formation of *P. aeruginosa* by 78 %. In examining the impact of nanoparticles on the dispersion of mature biofilms, we found that the highest eradication rate of mature biofilms was 64.8 % at the MIC of AgNPs. Notably, at 1/2 × MIC, the eradication rate for mature *P. aeruginosa* biofilms was 50.9 %. Based on these findings, reducing the biofilm formation and elimination of mature biofilms, as well as neutralizing the virulence properties of *P. aeruginosa*, could be a potential approach to managing infections associated with this bacterium.

Loo et al., investigated the effectiveness of AgNPs against *P. aeruginosa* PAO1 and showed that EPS matrix degradation resulted in a 95 % reduction in Pseudomonas biofilm production [30].

Our findings were phenotypically similar to the findings of Singh et al., [31]. They showed a decrease in *P. aeruginosa* PAO1 biofilm formation ability using green synthesized AgNPs. The results of this study showed a partial lethal effect of silver nanoparticles at the sub-MIC level, which led to the inhibition of bacterial growth and adhesion. Therefore, the inhibition of biofilm formation at sub-minimum inhibitory concentrations (MIC) could be attributed to non-lethal damage or the suppressive effect on the expression of related genes.

One of the important factors in bacterial pathogenicity is motility because it plays a role in host cell adhesion, colonization, and biofilm formation [32]. In the present study, *P. aeruginosa* PAO1 showed a significant reduction in swarming, swimming and twitching motility when exposed to AgNPs. The study of de la Fuente-Nunez et al., showed well that the reduction of swimming and twitching motility, and the suppression of the expression of various genes involved in motility directly affect the biofilm formation [33].

In accordance with these results, Hussain et al. showed that AgNPs at sub-MIC (1/16, 1/8, 1/4 and 1/2 MIC), caused a 42–81 % reduction in swarming motility and 79-22 % reduction in biofilm formation of *P. aeruginosa* PAO1 [34].

In the research by Khan et al., sub-MIC concentrations of gold nanoparticles led to a significant decrease in swimming, swarming, twitching, and biofilm formation [35].

Contrary to our results, Saeki et al., in investigating the effect of synthesized silver nanoparticles (75 nm) using *Fusarium oxysporum*, showed that sub-inhibitory concentrations of biological nanoparticles (1/2 MIC, 7.81–31.25 μM) significantly ($p < 0.05$) increased swarming, swimming, twitching and biofilm formation capacity [19]. This difference could be associated with the size, shape, surface charge and biological corona of nanoparticles. Liao et al., confirmed that smaller nanoparticles have more antibacterial activity than larger nanoparticles, so the size of the nanoparticles can determine the regulation of the virulence factor [36]. Grun et al., found that the treatment of pre-grown freshwater biofilms with 30–70 nm AgNPs increased biofilm formation and led to a change in the biofilm community [37].

Considering that the adhesion of bacteria to different surfaces, especially host tissues, is considered the initial stage of the formation and spread of bacterial biofilms, in this study the inhibitory effect of silver nanoparticles against bacterial adhesion on polyvinyl chloride film was evaluated by SEM. The biofilm matrix was present in the untreated cells, which caused the cells to adhere to one another and the surface. The results of the SEM analysis were in agreement with the results of other experiments in inhibiting biofilm formation in the presence of different concentrations of AgNPs. Similar results of biofilm inhibition using SEM were shown by Singh et al., [38].

At sub-MIC levels, one of the possibilities is the absorption of nanoparticles by *P. aeruginosa* due to the very small size of the NPs, and spherical shape, which allows the endocytosis or ingestion of nanoparticles by the treated cells. When AgNPs are introduced, various mechanisms have been reported for nanoparticles to cell damage, including the production of reactive oxygen species (ROS), DNA damage, disruption of protein and ribosome function, and interference with the QS system [1,26]. Also, AgNPs release silver ions (Ag+) from their surface when exposed to aerobic conditions. These released silver ions show a strong antimicrobial effect by interacting with the cell walls and membranes of bacteria. This mechanism is the main cause of the toxicity of AgNPs [39].

Since pathogenicity, biofilm formation capacity and the most virulence factors of Pseudomonas are controlled by the QS system, this study, in addition to the phenotypic investigation of biofilm inhibition investigated the expression of genes related to the biofilm polysaccharide matrix (Psl, Pel, and alginate). The lasI/lasR system in the QS system directly controls the *psl* gene expression. The lasR protein influences the expression of *pel* and *alg* genes by activating the rhlI/rhlR system, which is the second QS system [5]. In this research, the transcriptional expression of *algC*, *pslA*, and *pelA* genes was significantly decreased when exposed to 1/2 × MIC - 1/4 × MIC of AgNPs. Specifically, *algC* exhibited a reduction of 77 %–70 %, *pslA* of 83 %–75 %, and *pelA* of 68 %–58 %.

In QS system, lasR and rhlR are involved in coding transcription activators and LasI and RhlI in the synthesis of autoinducers [40]. AgNPs may interfere with the las and rhl pathways and prevent the production of signalling molecules or the transcription of genes under the control of this system [41].

Al-Momani et al., showed that biosynthesized AgNPs (11 nm) at sub-inhibitory concentrations have significant inhibitory effects on growth, biomass metabolism and biofilm formation. Also, the expression of seven genes encoding biofilm and QS in clinical isolates of *P. aeruginosa* (*lasR, lasI, lssB, rhIR, rhII, pqsA* and *pqsR*) decreased significantly upon exposure to the nanoparticles [41].

Pyocyanin, a pigment produced by *P. aeruginosa* regulated by QS, plays a key role in the pathogenesis of *P. aeruginosa* infections by causing oxidative stress, damaging host cells, promoting tissue destruction, and Interference with the host immune response. Additionally, *P. aeruginosa* produces pyocyanin, which aids in iron acquisition, bacterial growth, and immune response stimulation. The bacterium also secretes enzymes LasA protease and LasB elastase, contributing to tissue damage and colonization in host infections [42]. The results of investigating the effect of synthesized AgNPs on the production of three virulence factors (pyocyanin, LasA protease, and LasB elastase) in this study showed a significant decrease in the levels of pyocyanin, LasA protease, and LasB elastase (70 %, 85 %, and 87 %, respectively) at a concentration of 1/2 × MIC.

In a similar study, Singh et al., showed that synthesized bio-AgNPs (28 nm) prevented the formation of biofilm and several virulence factors such as LasA protease (86 %), LasB elastase (86 %), pyocyanin (96 %) and reduced the production of C12-AHL and

C4-AHL of PAO1. Further gene quantification analyses revealed that AgNPs significantly decreased the expression of QS-regulated genes, especially those responsible for producing virulence factors [38].

Rather and Mandal investigated the effects of plant-synthesized silver nanoparticles on QS-dependent virulence factors and biofilm of *P. aeruginosa*. AgNPs showed significant reduction of QS-dependent virulence factors and biofilm at Sub-MICs. Significant inhibition of 88 %, 79 %, 73 % and 61 % of biofilm formation, QS, pyocyanin and LasB elastase were reported, respectively [43].

In the study of Awadelkareem et al., biologically synthesized AgNPs were tested on Gram-negative bacteria to assess their impact on biofilm formation and virulence factors controlled by QS. In this study, a dose-dependent decrease in virulence factors of *P. aeruginosa* (pyocyanin, pyoverdin, LasB elastase and LasA protease) was observed. Biofilm development was reduced by 72.56 % at the highest sub-MIC. In addition, a significant decrease in biofilm formation on glass surfaces, swimming motility and exopolysaccharides (EPS) was also observed [42].

5. Conclusions

There is a growing interest in the development and synthesis of environmentally friendly and non-toxic metal nanoparticles [14] for medical or clinical purposes. This study evaluated the anti-bacterial and anti-virulence effects of AgNPs synthesized from the medicinal plant *Teucrium polium* extract on *P. aeruginosa*. Biosynthesized AgNPs showed a significant reduction in multiple QS-regulated functions in *P. aeruginosa*. *P. aeruginosa* showed a reduction in its ability to form biofilm at sub-MIC levels, with a suppression rate of 78 % in a dose-dependent manner. This reduction in biofilm formation is crucial, as biofilms contribute to the persistence and resistance of bacterial infections, particularly in chronic conditions.

In addition, the inhibition of virulence factors, including pyocyanin, LasB elastase, and LasA protease, at a concentration of 1/2 × MIC was 70 %, 87 %, and 85 %, respectively. These factors are integral to the pathogenicity of *P. aeruginosa*, and their suppression could lead to diminished virulence and enhanced susceptibility to host defences.

There was a notable reduction in three types of motility (swarming, swimming and twitching), further indicating that AgNPs disrupt the motility and colonization capabilities of *P. aeruginosa*. This is particularly significant as motility is a key factor in the establishment of infections and biofilm development.

After treatment with AgNPs, the relative expression of biofilm-related genes controlled by QS, including *algC, pslA,* and *pelA*, decreased by 77 %, 83 %, and 68 %, respectively. The reductions in gene expression emphasize the ability of AgNPs to interfere with the QS signalling pathways that govern biofilm development and maintenance.

These results indicate the promising future for AgNPs as potential alternative antimicrobial agents or in combination with antibiotics. Due to the significant increase in drug absorption in the presence of nanoparticles, AgNPs could serve as an effective drug delivery system. However, further in-vivo studies are necessary to assess the therapeutic effectiveness of AgNPs against infections caused by pathogens resistant to conventional antibiotics.

CRediT authorship contribution statement

Fatemeh Aflakian: Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Gholamreza Hashemitabar:** Writing – review & editing, Supervision, Project administration, Investigation.

Ethics approval

Not applicable. The research did not involve human or animal participants, and their biological data or materials.

Data availability

All data generated or analyzed during this study are included in this published article. No supplementary materials are required.

Code availability

Not applicable.

Funding

The authors would like to acknowledge the Ferdowsi University of Mashhad, Iran for financing this research (FUM grant: 75,492).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

References

[1] F. Aflakian, F. Mirzavi, H.T. Aiyelabegan, A. Soleimani, J.G. Navashenaq, I. Karimi-Sani, A.R. Zomorodi, R. Vakili-Ghartavol, Nanoparticles-based therapeutics for the management of bacterial infections: a special emphasis on FDA approved products and clinical trials, Eur. J. Pharmaceut. Sci. (2023) 106515, https://doi.org/10.1016/j.ejps.2023.106515.
[2] D. Hu, L. Zou, Y. Gao, Q. Jin, J. Ji, Emerging nanobiomaterials against bacterial infections in postantibiotic era, View 1 (3) (2020) 20200014, https://doi.org/10.1002/VIW.20200014.
[3] A.J. Huh, Y.J. Kwon, "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era, J. Contr. Release 156 (2) (2011) 128–145, https://doi.org/10.1016/j.jconrel.2011.07.002.
[4] D.R.A. Sanya, D. Onésime, G. Vizzarro, N. Jacquier, Recent advances in therapeutic targets identification and development of treatment strategies towards *Pseudomonas aeruginosa* infections, BMC Microbiol. 23 (1) (2023) 86, https://doi.org/10.1186/s12866-023-02832-x.
[5] F. Aflakian, M. Rad, G. Hashemitabar, M. Lagzian, M. Ramezani, Design and assessment of novel synthetic peptides to inhibit quorum sensing-dependent biofilm formation in *Pseudomonas aeruginosa*, Biofouling 38 (2) (2022) 131–146, https://doi.org/10.1080/08927014.2022.2028280.
[6] D. Shang, X. Han, W. Du, Z. Kou, F. Jiang, Trp-containing antibacterial peptides impair quorum sensing and biofilm development in multidrug-resistant *Pseudomonas aeruginosa* and exhibit synergistic effects with antibiotics, Front. Microbiol. 12 (2021) 611009, https://doi.org/10.3389/fmicb.2021.611009.
[7] S.S. Pattnaik, S. Ranganathan, D.R. Ampasala, A. Syed, F. Ameen, S. Busi, Attenuation of quorum sensing regulated virulence and biofilm development in *Pseudomonas aeruginosa* PAO1 by Diaporthe phaseolorum SSP12, Microb. Pathog. 118 (2018) 177–189, https://doi.org/10.1016/j.micpath.2018.03.031.
[8] L. Wang, C. Hu, L. Shao, The antimicrobial activity of nanoparticles: present situation and prospects for the future, International journal of nanomedicine (2017) 1227–1249, https://doi.org/10.2147/IJN.S121956.
[9] A. Naskar, K.-s. Kim, Nanomaterials as delivery vehicles and components of new strategies to combat bacterial infections: advantages and limitations, Microorganisms 7 (9) (2019) 356, https://doi.org/10.3390/microorganisms7090356.
[10] P. Singh, I. Mijakovic, Antibacterial effect of silver nanoparticles is stronger if the production host and the targeted pathogen are closely related, Biomedicines 10 (3) (2022) 628, https://doi.org/10.3390/biomedicines10030628.
[11] S. Kumar, I.B. Basumatary, H.P. Sudhani, V.K. Bajpai, L. Chen, S. Shukla, A. Mukherjee, Plant extract mediated silver nanoparticles and their applications as antimicrobials and in sustainable food packaging: a state-of-the-art review, Trends Food Sci. Technol. 112 (2021) 651–666, https://doi.org/10.1016/j.tifs.2021.04.031.
[12] P. Singh, S. Pandit, C. Jers, A.S. Joshi, J. Garnæs, I. Mijakovic, Silver nanoparticles produced from Cedecea sp. exhibit antibiofilm activity and remarkable stability, Sci. Rep. 11 (1) (2021) 12619, https://doi.org/10.1038/s41598-021-92006-4.
[13] X. Liu, J.-L. Chen, W.-Y. Yang, Y.-C. Qian, J.-Y. Pan, C.-N. Zhu, L. Liu, W.-B. Ou, H.-X. Zhao, D.-P. Zhang, Biosynthesis of silver nanoparticles with antimicrobial and anticancer properties using two novel yeasts, Sci. Rep. 11 (1) (2021) 15795, https://doi.org/10.1038/s41598-021-95262-6.
[14] G. Hashemitabar, F. Aflakian, A.H. Sabzevar, Assessment of antibacterial, antioxidant, and anticancer effects of biosynthesized silver nanoparticles using *Teucrium polium* extract, J. Mol. Struct. 1291 (2023) 136076, https://doi.org/10.1016/j.molstruc.2023.136076.
[15] E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D.L. Monnet, C. Pulcini, G. Kahlmeter, J. Kluytmans, Y. Carmeli, Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis, Lancet Infect. Dis. 18 (3) (2018) 318–327, https://doi.org/10.1016/S1473-3099(17)30753-3.
[16] C.R. Belanger, R.E. Hancock, Testing physiologically relevant conditions in minimal inhibitory concentration assays, Nat. Protoc. 16 (8) (2021) 3761–3774, https://doi.org/10.1038/s41596-021-00572-8.
[17] E.F. Haney, M.J. Trimble, R.E. Hancock, Microtiter plate assays to assess antibiofilm activity against bacteria, Nat. Protoc. 16 (5) (2021) 2615–2632, https://doi.org/10.1038/s41596-021-00515-3.
[18] N. Tabassum, F. Khan, G.-J. Jeong, D.-M. Jo, Y.-M. Kim, Silver nanoparticles synthesized from *Pseudomonas aeruginosa* pyoverdine: antibiofilm and antivirulence agents, Biofilm 7 (2024) 100192, https://doi.org/10.1016/j.bioflm.2024.100192.
[19] E.K. Saeki, A.Y. Yamada, L.A. De Araujo, L. Anversa, D.d.O. Garcia, R.L.B. De Souza, H.M. Martins, R.K.T. Kobayashi, G. Nakazato, Subinhibitory concentrations of biogenic silver nanoparticles affect motility and biofilm formation in *Pseudomonas aeruginosa*, Front. Cell. Infect. Microbiol. 11 (2021) 656984, https://doi.org/10.3389/fcimb.2021.656984.
[20] Z. Ye, L. Ye, D. Li, S. Lin, W. Deng, L. Zhang, J. Liang, J. Li, Q. Wei, K. Wang, Effects of daphnetin on biofilm formation and motility of *Pseudomonas aeruginosa*, Front. Cell. Infect. Microbiol. 12 (2022) 1033540, https://doi.org/10.3389/fcimb.2022.1033540.
[21] J. Luo, B. Dong, K. Wang, S. Cai, T. Liu, X. Cheng, D. Lei, Y. Chen, Y. Li, J. Kong, Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances *Pseudomonas aeruginosa* clearance in a mouse peritoneal implant infection model, PLoS One 12 (4) (2017) e0176883, https://doi.org/10.1371/journal.pone.0176883.
[22] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, Nat. Protoc. 3 (6) (2008) 1101–1108, https://doi.org/10.1038/nprot.2008.73.
[23] P. Piewngam, J. Chiou, P. Chatterjee, M. Otto, Alternative approaches to treat bacterial infections: targeting quorum-sensing, Expert Review of Anti-Infective Therapy 18 (6) (2020) 499–510, https://doi.org/10.1080/14787210.2020.1750951.
[24] S. Shah, S. Gaikwad, S. Nagar, S. Kulshrestha, V. Vaidya, N. Nawani, S. Pawar, Biofilm inhibition and anti-quorum sensing activity of phytosynthesized silver nanoparticles against the nosocomial pathogen *Pseudomonas aeruginosa*, Biofouling 35 (1) (2019) 34–49, https://doi.org/10.1080/08927014.2018.1563686.
[25] S. Silver, L.T. Phung, G. Silver, Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds, Journal of industrial microbiology and biotechnology 33 (7) (2006) 627–634, https://doi.org/10.1007/s10295-006-0139-7.
[26] X. Xin, C. Qi, L. Xu, Q. Gao, X. Liu, Green synthesis of silver nanoparticles and their antibacterial effects, Frontiers in Chemical Engineering 4 (2022) 941240, https://doi.org/10.3389/fceng.2022.941240.
[27] C. Vanlalveni, S. Lallianrawna, A. Biswas, M. Selvaraj, B. Changmai, S.L. Rokhum, Green synthesis of silver nanoparticles using plant extracts and their antimicrobial activities: a review of recent literature, RSC advances 11 (5) (2021) 2804–2837, https://doi.org/10.1039/d0ra09941d.
[28] E.P. Figueiredo, J.M. Ribeiro, E.K. Nishio, S. Scandorieiro, A.F. Costa, V.F. Cardozo, A.G.d. Oliveira, N. Durán, L. Panagio, R. Kobayashi, New approach for simvastatin as an antibacterial: synergistic effect with bio-synthesized silver nanoparticles against multidrug-resistant bacteria, International journal of nanomedicine (2019) 7975–7985, https://doi.org/10.2147/IJN.S211756.
[29] S. Saeed, A. Iqbal, M.A. Ashraf, Bacterial-mediated synthesis of silver nanoparticles and their significant effect against pathogens, Environ. Sci. Pollut. Control Ser. 27 (30) (2020) 37347–37356, https://doi.org/10.1007/s11356-020-07610-0.
[30] C.-Y. Loo, P.M. Young, R. Cavaliere, C.B. Whitchurch, W.-H. Lee, R. Rohanizadeh, Silver nanoparticles enhance *Pseudomonas aeruginosa* PAO1 biofilm detachment, Drug development and industrial pharmacy 40 (6) (2014) 719–729, https://doi.org/10.3109/03639045.2013.780182.
[31] P. Singh, S. Pandit, M. Beshay, V. Mokkapati, J. Garnaes, M.E. Olsson, A. Sultan, A. Mackevica, R.V. Mateiu, H. Lütken, Anti-biofilm effects of gold and silver nanoparticles synthesized by the Rhodiola rosea rhizome extracts, Artificial cells, nanomedicine, and biotechnology 46 (sup3) (2018) 886–899, https://doi.org/10.1080/21691401.2018.1518909.
[32] E. Sun, S. Liu, R.E. Hancock, Surfing motility: a conserved yet diverse adaptation among motile bacteria, J. Bacteriol. 200 (23) (2018), https://doi.org/10.1128/JB.00394-18, 10.1128/jb.00394-18.

[33] C. De La Fuente-Núñez, V. Korolik, M. Bains, U. Nguyen, E.B. Breidenstein, S. Horsman, S. Lewenza, L. Burrows, R.E. Hancock, Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide, Antimicrobial agents and chemotherapy 56 (5) (2012) 2696–2704, https://doi.org/10.1128/AAC.00064-12.

[34] A. Hussain, M.F. Alajmi, M.A. Khan, S.A. Pervez, F. Ahmed, S. Amir, F.M. Husain, M.S. Khan, G.M. Shaik, I. Hassan, Biosynthesized silver nanoparticle (AgNP) from Pandanus odorifer leaf extract exhibits anti-metastasis and anti-biofilm potentials, Front. Microbiol. 10 (2019) 411648, https://doi.org/10.3389/fmicb.2019.00008.

[35] F. Khan, P. Manivasagan, J.-W. Lee, D.T.N. Pham, J. Oh, Y.-M. Kim, Fucoidan-stabilized gold nanoparticle-mediated biofilm inhibition, attenuation of virulence and motility properties in *Pseudomonas aeruginosa* PAO1, Mar. Drugs 17 (4) (2019) 208, https://doi.org/10.3390/md17040208.

[36] S. Liao, Y. Zhang, X. Pan, F. Zhu, C. Jiang, Q. Liu, Z. Cheng, G. Dai, G. Wu, L. Wang, Antibacterial activity and mechanism of silver nanoparticles against multidrug-resistant *Pseudomonas aeruginosa*, International journal of nanomedicine (2019) 1469–1487, https://doi.org/10.2147/IJN.S191340.

[37] A.Y. Grün, C.B. App, A. Breidenbach, J. Meier, G. Metreveli, G.E. Schaumann, W. Manz, Effects of low dose silver nanoparticle treatment on the structure and community composition of bacterial freshwater biofilms, PLoS One 13 (6) (2018) e0199132, https://doi.org/10.1371/journal.pone.0199132.

[38] B.R. Singh, B.N. Singh, A. Singh, W. Khan, A.H. Naqvi, H.B. Singh, Mycofabricated biosilver nanoparticles interrupt *Pseudomonas aeruginosa* quorum sensing systems, Sci. Rep. 5 (1) (2015) 13719, https://doi.org/10.1038/srep13719.

[39] A.I. Mekkawy, M.A. El-Mokhtar, S.M. El-Shanawany, E.H. Ibrahim, Silver nanoparticles-loaded hydrogels, a potential treatment for resistant bacterial infection and wound healing: a review, J. Pharm. Res. Int 14 (2016) 1–19, https://doi.org/10.9734/BJPR/2016/30525.

[40] K. Brindhadevi, F. LewisOscar, E. Mylonakis, S. Shanmugam, T.N. Verma, A. Pugazhendhi, Biofilm and Quorum sensing mediated pathogenicity in *Pseudomonas aeruginosa*, Process Biochemistry 96 (2020) 49–57, https://doi.org/10.1016/j.procbio.2020.06.001.

[41] H. Al-Momani, M. Almasri, D.A. Al Balawi, S. Hamed, B.A. Albiss, N. Aldabaibeh, L. Ibrahim, H. Albalawi, S. Al Haj Mahmoud, A.I. Khasawneh, The efficacy of biosynthesized silver nanoparticles against *Pseudomonas aeruginosa* isolates from cystic fibrosis patients, Sci. Rep. 13 (1) (2023) 8876, https://doi.org/10.1038/s41598-023-35919-6.

[42] A.M. Awadelkareem, A.J. Siddiqui, E. Noumi, S.A. Ashraf, S. Hadi, M. Snoussi, R. Badraoui, F. Bardakci, M.S. Ashraf, C. Danciu, Biosynthesized silver nanoparticles derived from probiotic Lactobacillus rhamnosus (AgNPs-LR) targeting biofilm formation and quorum sensing-mediated virulence factors, Antibiotics 12 (6) (2023) 986, https://doi.org/10.3390/antibiotics12060986.

[43] M.A. Rather, M. Mandal, Attenuation of biofilm and quorum sensing regulated virulence factors of an opportunistic pathogen *Pseudomonas aeruginosa* by phytofabricated silver nanoparticles, Microb. Pathog. 185 (2023) 106433, https://doi.org/10.1016/j.micpath.2023.106433.