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The inhibitory effect of *Azadirachta indica* extract on *Pseudomonas aeruginosa* biofilm, virulence factors, and quorum sensing

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ABSTRACT

Pseudomonas aeruginosa infections are versatile, persistent, and difficult to treat due to the ability to produce virulent factors, form biofilm, and resist antibiotics. This study has tested a novel protocol that targets *Pseudomonas aeruginosa* pathogenicity mechanisms and quorum sensing mechanisms to manage and control *P. aeruginosa* infections. The aim of this study is to assess the effectiveness of *Azadirachta indica* methanolic extracts in inhibiting *P. aeruginosa* biofilm, virulence factors, and quorum sensing. The findings showed that the *A. indica* extract has strong antibiofilm activity against *P. aeruginosa* at concentrations that are below what would normally stop the growth of the bacteria (1.56, 0.78, and 0.39 mg/mL). The results also showed that it has several antibiofilm effects that target swarming motility, aggregation, hydrophobicity, and the production of exopolysaccharide (EPS). Also, *A. indica* effectively stopped the production of several harmful substances, such as pyocyanin, rhamnolipids, and *LasA* protease. It's important to note that *A. indica* extract stops *P. aeruginosa* and *Chromobacterium violaceum* from making acyl-homoserine lactone and violacein, which shows that it can mess up quorum sensing mechanisms. The findings of this study suggest that *A. indica* extract is a promising choice for developing alternative therapeutic strategies aimed at controlling and treating biofilm-related infections in humans, animals, and plants.

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Introduction

The effectiveness of antibiotics has reduced greatly, as many bacteria can build biofilms which enable them to resist antibiotics. As a result, many antibiotics treatment protocols still used to combat bacterial infections have become ineffective (Bayramov & Neff 2017). Currently, the effects extend to cause serious health problems in humans and animals, In addition to plant growth and food production (Nazarov et al. 2020).

The challenge in treating infectious diseases has become the main focus for many researchers' efforts. Some of these efforts focus on finding new antibacterial agents from natural or synthetic sources. Additionally, there have been attempts to address this issue by altering the antibiotics currently in use. One of the innovative approaches focuses on finding agents that could interfere with the quorum sensing system, virulence factors, and biofilm formation of microbes. Antiquorum sensing agents are novel in that they reduce infection severity, improve treatment effectiveness, make infectious disease control and management possible, and reduce the possibility of antibiotic-resistant development (Naga et al. 2023).

Opportunistic gram-negative bacterium *Pseudomonas aeruginosa* features amongst the virulent nosocomial pathogens because it has a high capability of causing a spectrum of diseases ranging from very mild to life-threatening ones. These comprise both acute and chronic infections induced by *P. aeruginosa* in mammals, pets,

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and even infection of some crops (Mehmood et al. 2023). It is also an isolate commonly found in immunocompromised patients and those suffering from cystic fibrosis (Kreitmann et al. 2024). *P. aeruginosa* possesses intrinsic mechanisms for the production of virulence factors, biofilm formation, and the operation of its quorum-sensing system, making it one of the most virulent bacterial species (Jurado-Martín et al. 2021).

P. aeruginosa has four quorum sensing systems that function independently and dependently, namely, the *Las*, *Rhl*, and quinolone-based QS systems and the recently determined IQS-dependent system (Vetrivel et al. 2021; Ma'aitah 2024). Quorum-sensing is a process of cell to cell communication that relies on the production and release of extracellular signaling molecules termed auto-inducers, whose concentration increases as a function of cell density. When a critical threshold concentration of autoinducers is reached, quorum sensing bacteria respond as a group to alter the gene expression. Autoinducers can freely diffuse through the bacterial and enables population-mediated control of gene expression. Ultimately, this gene expression results in various phenotypes including the production of virulence factors and biofilm formation (Vetrivel et al. 2021). Targeting elements that regulate QS and biofilm formation might be a promising approach to combat antibiotic-resistant biofilm-related bacterial infections (Wang et al. 2020).

Azadirachta indica (Neem tree) is a tropical and subtropical plant that grows quickly, is hardy, and is evergreen. It belongs to the Meliaceae family. Every part of the neem tree has been traditionally used. It has been used to treat colds, influenza, skin infections, meningitis, and malaria infections (Bhatia et al. 2015). Reports showed that the neem (*Azadirachta indica*) extracts showed variable biological activities such as antiplasmodial, antitrypanosomal, antioxidant, anticancer, antibacterial, antifungal, antiviral, nematicidal, antiulcer, spermicidal, anthelminthic, antidiabetic, anti-implantation as well as immunomodulating, contraceptive, molluscidal, insecticidal, antifeedant, insect repellant and toxicological effects (Bansal et al. 2019; Moin et al. 2021). Here we are screening a methanolic extract of leaves from *A. indica* for its anti-infective property. The current study describes the assessment of potential antibiofilm, antivirulence, and antiquorum sensing activity of methanolic leaf extracts from *A. indica*.

Materials and methods

A bacterial starter cultures were made by culturing one single colony in 3 mL Luria Bertani broth (LB). The pretreated culture was incubated at 37°C for 24h. Bacterial suspension from LB culture was prepared and adjusted to 0.5 McFarland solution ($1.5X10^8$ CFU/mL) for all experiments. The incubation conditions were performed at 37°C for 24h, except if otherwise mentioned. In all supernatant based experiments, centrifugation was performed at 10000 rpm for 10 min. In all experiments, the effects on *P. aeruginosa* biofilm mediated factors, virulence factors, and quorum sensing were performed on bacterial cultures treated with *A. indica* at concentrations of 1.56, 0.78, and 0.39 mg/mL, compared with cultures treated with dimethyl sulfoxide (DMSO), and untreated culture. 10% of DMSO was used to prepare stock solutions. Culture treated with DMSO was used as a negative control while untreated culture was used as a positive control. The percentage of production was calculated by subtracting the absorbance of the treated culture from the absorbance of the untreated culture and dividing the result by the absorbance of the untreated culture, and multiplying the result by 100. The percentage of inhibition was calculated by subtracting the percentage of production from 100. All tests were performed in triplicates.

Plant materials and extraction

A. indica was collected from Naour, Amman, Jordan, in the summer of 2022. The freshly collected plant was identified by Prof. Saleh Al Qur'an (Department of Biology, Mutah University). A voucher specimen (NO. MU2022-02) was deposited at the Department of Biology, Mutah University.

The plant materials were cleaned and left to dry in the shade. The leaves were collected and ground into a fine powder. A 250 g of the leaf material was subjected to extraction by soaking in 500 mL of methanol (99%). The suspension was incubated at room temperature for 24h. The solvent was collected, filtrated, and exposed to a rotary evaporator. The produced crude extract was stored at 4°C for further investigation.

Isolation of pathogenic bacteria

P. aeruginosa pathogenic strain was isolated from a urine sample of a patient diagnosed with urinary tract-infection (AlKarak Government Hospital, AlKarak, Jordan). It was isolated and identified using standard methods. The identification was confirmed using Biomérieux VITEK® 2 system. The resistant pattern for the isolated strain was characterized as Beta-Lactemase *P. aeruginosa* using Biomérieux VITEK® 2 system. The quorum-sensing biosensor strain, *Chromobacterium violaceum* ATCC 12472, was purchased from the American Type Culture Collection (ATCC).

Antibacterial activity

A. indica methanolic extract's preliminary antibacterial activity against *P. aeruginosa* was evaluated using the disc diffusion method (ALrawashdeh et al. 2019). In brief, 100 µL of a bacterial suspension containing 10^8 CFU/mL (adjusted to a 0.5 McFarland solution) was added to Muller-Hinton agar and spread out evenly across the agar surface. Then, the extract-containing disc (0.5 mg) was transferred to the surface of the agar along with the positive control disc (Cefotaxime, 30 µg) and the negative control disc (DMSO). After 24 hours of incubation at 37°C, the inhibition zone observed was measured in mm.

To determine the exact concentration that inhibits the visible growth of *P. aeruginosa*, the minimum inhibitory concentration (MIC) of *A. indica* was estimated (Khaled et al. 2023). The extract was two-fold diluted in a 96-well plate (Polystyrene) to produce concentrations equal to 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, and 0.1 mg/mL. Then, 10 µL of 10^8 CFU/mL bacterial suspension was transferred to each well. Untreated culture and culture treated with DMSO were prepared similarly. The plate was then incubated at 37°C for 24h and the concentration that inhibited the visible growth was reported as MIC.

Antibiofilm activity and cell viability assay

The potential of *A. indica* extract to suppress the biofilm formation ability of *P. aeruginosa* was assayed using a crystal violet assay, as conducted by (Qaralleh et al. 2020; Qaralleh 2023). Briefly, in a 96-well plate, different extract concentrations (as used in the MIC test) were prepared and inoculated with 10 µL of 10^4 CFU/mL bacterial suspension, then incubated for 24 hours at 37°C. Next, the well contents were removed and washed with tap water to eliminate non-adherent cells. The plate was then subjected to staining and decolorization processing. Crystal violet was added to the wells and incubated for 15 minutes. Afterward, the crystal violet contents were discarded, the wells were washed, and refilled with absolute ethanol. The ethanol containing crystal violet was measured after 15 minutes at 590 nm using an ELISA reader (MCL-2100C, China), and the percentage inhibition in biofilm formation was calculated.

To determine the effect of the extract on viable cells biofilm production, a 96-well plate was prepared and processed similarly as in a crystal violet methodology (Gordya et al. 2017). To the empty wells, 200 µL of Triphenyl tetrazolium chloride (TTC) solution (0.2% glucose + 50 µL of 5 mg/mL TTC solution) was added. The TTC prepared plate was then incubated at 37°C in a shaker incubator at 150 rpm shaking rate. After 6h, the absorbance of the TTC solution was measured at 405 nm and the percentage of inhibition in viable cells was calculated.

Biofilm visualization

Two sets of *A. indica* (1.56, 0.78, and 0.39 mg/mL)-treated cultures were prepared in 24 well plates containing sterile coverslips in their bottoms. Untreated and DMSO-treated cultures were prepared similarly. The cultures were incubated at 37°C for 48h and one set of these coverslips was removed, washed, and stained with crystal violet (1 min) and subjected to light microscope (NIKON, Japan) observation. The other set of coverslips was processed for SEM observation. They were washed, fixed using 5% glutaraldehyde for 24h, and dehydrated using different concentrations of ethanol (10, 30, 50, 70, and 100%). The dried samples were visualized using SEM (Thermo Scientific Phenom Desktop SEM, JU-24112022, Waltham, MA, USA).

Biofilm mediated factors

Swarming motility

The effect of *A. indica* on *P. aeruginosa* swarming was evaluated using swarming media (0.5% agar), according to (Sagar et al. 2022). Swarming agar was prepared, autoclaved, and poured into sterile petri dishes containing *A. indica* extract. Untreated and DMSO-treated cultures were prepared similarly. After solidification, the center of the plate surface was inoculated with 1 µL of 24h old *P. aeruginosa* culture. After 48h of incubation at 37°C, the swarming zone was measured in mm.

Aggregation

The aggregation inhibitory potential of *A. indica* extract was evaluated (Shanks et al. 2008). In brief, the initial absorption (abs1) of the 24h old, prepared culture was measured at 600 nm (Shimadzu UV-1601, Japan). This culture was vortexed for 1 min and the second absorption (abs2) was measured at 600 nm. The data were represented as a percentage of aggregation which was calculated using the formula:

Aggregation (%) = (abs2- abs1)/abs2 *100

Hydrophobicity

The initial absorption (abs1) of the 24h old culture (1 mL) was measured at 600 nm. To this culture, 1 mL of n-hexadecane was added, mixed, and then removed, while the second absorption (abs2) for the remained culture was measured again (Krishnan et al. 2012). The data were represented as a percentage of hydrophobicity which was calculated using the formula:

Hydrophobicity (%) = (abs2- abs1)/abs2 *100

Exopolysaccharides

Briefly, the extraction of the total exopolysaccharides was performed by mixing 3 mL of cold ethanol and 1 mL of the culture supernatant. The mixture was maintained at 4°C for 24h, then centrifuged (10000 rpm, 10 min), the supernatant was removed, and to the pellet, distilled water (3 mL), 5% cold phenol (1 mL), and 98% sulfuric acid (5 mL) were added. Next, the absorption at 490 nm was measured and the data were represented as a percentage of EPS production (Razack et al. 2011).

Virulence factors

Pyocyanin

In this test, chloroform was used to extract the secreted pyocyanin from the culture supernatant. This was made by mixing 1 mL of chloroform (99%) with 1 mL of culture supernatant. The mixture was fractionated into two phases, and to the pyocyanin containing phase (chloroform), 1.5 mL of HCl (0.2N) was added. Next, the absorption at 520 nm was measured and the data were represented as a percentage of inhibition in pyocyanin production (Hossain et al. 2017).

Rhamnolipids

In this test, diethyl ether was used to extract the secreted rhamnolipids from the culture supernatant. This was made by mixing 3 mL of diethyl ether (99%) with 1 mL of culture supernatant. The mixture was fractionated into two phases, the rhamnolipids containing phase (diethyl ether) was collected and supplemented with 200 µL of water and 900 µL of 0.18% orcinol (w/v) in 53% (v/v) H_2SO_4. The mixture was heated at 24°C for 0.5h. Next, the absorption at 421 nm was measured and the data were represented as a percentage of inhibition in rhamnolipids production (Luo et al. 2017).

LasA protease

The effect of *A. indica* on *P. aeruginosa LasA* protease was evaluated using azocasein as a substrate and trichloroacetic acid (TCA) as a reaction terminator (Andrejko et al. 2013). The reaction was prepared using 1 mL of culture supernatant supplemented with 3 mL of 50 mM phosphate buffer, and 0.1 mL of azocasein. The reaction was activated by incubating the prepared solution at 37°C and 150 rpm, while it was terminated after 24h by adding 0.5 mL of TCA. Then, it was centrifuged (10000 rpm, 10 min) and the absorption at 366 nm was measured and the data were represented as a percentage of inhibition in *LasA* protease activity.

Quorum sensing

Violacein

The antiquorum sensing potential of *A. indica* was performed using *C. violaceum* according to (Gómez-Gómez et al. 2019) with some modifications. It is a biosensor strain that operates a quorum sensing system to produce a purple pigment known as violacein. Agents, that inhibit the production of this pigment, are described as antiquorum sensing agents. In this test, insoluble violacein pigment in the prepared culture was precipitated by centrifugation (13000 rpm for 15 min). To the collected pellet, 1 mL of DMSO was added to extract the violacein pigment. The extraction process was maintained at room temperature for 30 min, then it was centrifuged (10000 rpm, 10 min). The absorption of the violacein containing DMSO was measured at 575 nm and the data were represented as a percentage of inhibition in violacein production.

Acyl Homoserein Lactone (AHL)

AHL was extracted from the culture supernatant and qualitatively measured according to (Lee et al. 2017). AHL of 2 mL of supernatant was submitted to extraction using 3 mL of ethyl acetate (99%) and incubation at 40°C for 24h. Then, 40 µL of the AHL containing ethyl acetate was added to a mixture of 2 M hydroxyl amine and 3.5 M NaOH, and 90 µL from a solution that was prepared by mixing equal volumes of 10% ferric chloride in 4% HCL and 95% ethanol. The absorption at 520 nm was measured and the data were represented as a percentage of inhibition in AHL production.

Statistical analysis

The significant difference between the treatment groups and the untreated standard group was assessed using one-way ANOVA and expressed as stars (*, **, or ***) corresponding to the P-values of $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

Antibacterial activity

The bacteriostatic effect of *A. indica* leaf methanolic extract against *P. aeruginosa* was assigned based on the size of the inhibition zone and the value of the MIC (Table 1). The results showed that the inhibition zone of *A. indica* extract is 6.8 mm, and the MIC is 12.5 mg/mL. This may indicate that it possesses weak antibacterial activity. DMSO (10%) and cefotaxime were also evaluated and showed inhibition zones of 0.0 and 17.5 mm, respectively.

Antibiofilm activity

The ability of *A. indica* extract to prevent the formation of *P. aeruginosa* biofilm was evaluated using a crystal violet assay. In this test, the extract was evaluated

at a broad range of concentrations, including 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, and 0.1 mg/mL. The findings (Figure 1A) showed that the extract completely (≥90%) prevented the formation of biofilm at concentrations of 25, 12.5, 6.25, 3.13, and 1.56 mg/mL. At concentrations lower than 1.56 mg/mL, the inhibitory ability of the extract gradually decreased as the concentration of the extract decreased. In this context, the maximum percentage of inhibition in biofilm formation was 75.9% at 0.78 mg/mL, while the lowest percentage was 9.2% at 0.1 mg/mL. However, the minimum biofilm inhibitory concentration (MBIC50), the concentration that reduces the ability of *P. aeruginosa* to form biofilm by 50%, is near 0.39 mg/mL. The negative control (DMSO) showed no effect on the ability of *P. aeruginosa* to form biofilm.

Table 1. Inhibition zone (mm) and MIC (mg/mL) of *A. indica* leaf methanolic extract

Compound Concentrations	Inhibition zone (MIC) ±SD
A. indica leaf methanolic extract (0.5 mg/disc)	6.8±0.3 (12.5 mg/mL)
Cefotaxime (30 µg/disc)	17.5±0.5
DMSO (10%)	0.0±0.0

SD: Standard Deviation

Biofilm cell viability

The ability of *A. indica* extract to inhibit biofilm formation might be due to the antibiofilm activity or bacteriostatic effect. Therefore, the effect of the extract on biofilm viable cells was observed using the TTC assay (Figure 1B). The results showed that the ability of the extract to inhibit biofilm formation at concentrations greater than 1.56 mg/mL is due to its bacteriostatic action. At these concentrations, a significant reduction in *P. aeruginosa* viable cells was observed. At concentrations lower than 3.13 mg/mL, there was no significant reduction in cell viability, and the ability of *A. indica* extract to prevent biofilm formation is due to the antibiofilm activity of the extract. Therefore, the treatment concentrations of 1.56, 0.78, and 0.39 mg/mL have been selected in this study.

Light microscope and Scanning electron microscope (SEM)

Light and scanning electron microscopes have been used to observe the effect of *A. indica* extract on *P. aeruginosa* biofilm (Figures 2 and 3). *P. aeruginosa* cells clumped and arranged in 3D in the untreated culture (Figures 2A and 3A), demonstrating their ability to form biofilm. However, treating *P. aeruginosa* with 1.56, 0.78, and 0.39 mg/mL of *A. indica* extract stopped it from clumping together (Figures 2B-D and 3B-D). This effect was strongest when the highest concentration (1.56 mg/mL) was used. The signs, such as the presence of scattered, non-aggregated cells and the absence of multilayered 3D forms, were observed, indicating the prevention of biofilm formation.

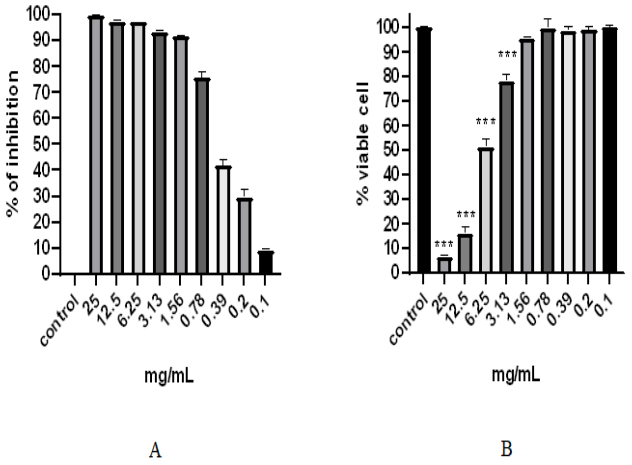

Fig. 1: The effect of A. indica methanolic extract on the biofilm (A) and biofilm cell viability (B) of *P. aeruginosa*.

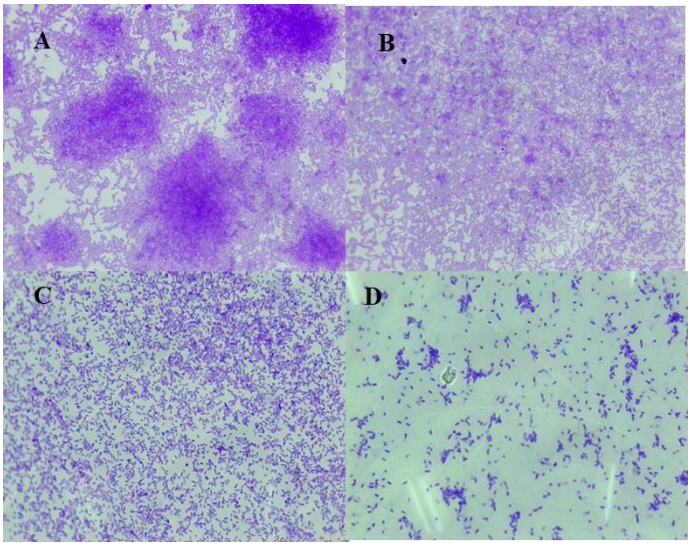

Fig. 2. Images of light microscope (40X) of *P. aeruginosa* untreated culture (A) and culture treated with A. indica extract at 0.39 (B), 0.78 (C), and 1.56 mg/mL (D).

Effects of A. indica extract on factor-mediated biofilm formation

Swarming motility

The effect on the swarming motility of *P. aeruginosa* was evaluated using swarming agar (Figure 4A). *A. indica* extract exhibited a dose-dependent effect

on *P. aeruginosa* swarming motility. Treatment with 1.56, 0.78, and 0.39 mg/mL of the extract significantly reduced the swarming motility from 55.0 mm to 16.0, 28.2, and 33.5 mm, respectively.

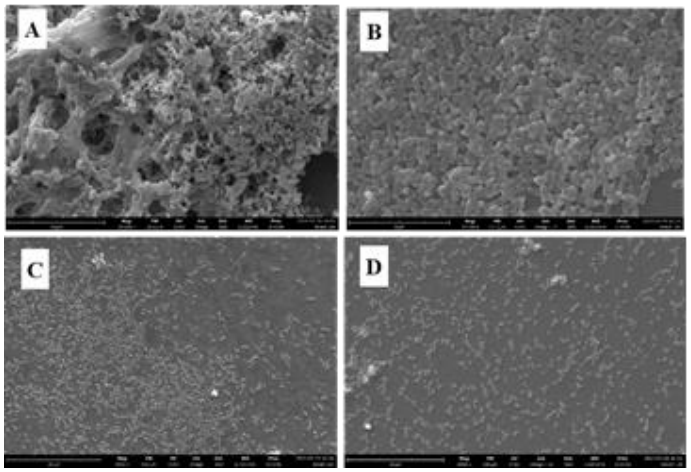

Fig. 3. Images of SEM of *P. aeruginosa* untreated culture (A) and culture treated with *A. indica* extract at 0.39 (B), 0.78 (C), and 1.56 mg/mL (D).

Aggregation

Dose-dependent inhibition of *P. aeruginosa* aggregation ability was observed. The Figure 4B illustrates the significant and mostly powerful potential of *A. indica* extract to suppress *P. aeruginosa'* aggregation ability at 1.56 and 0.78 mg/mL. These concentrations reduced the percentage of aggregation from 65.3% to 4.96 and 25.6%, respectively, and further reduced it to 51.7% at 0.39 mg/mL.

Hydrophobicity

The surface hydrophobicity of *P. aeruginosa* was evaluated using the n-hexadecane method. *A. indica* extract exhibited a dose-dependent inhibitory effect on hydrophobicity (Figure 4C). A significant reduction in the percentage of hydrophobicity from 67.6% to 33.3 and 44.0% was observed when the treatment was performed using 1.56 and 0.78 mg/mL, respectively.

EPS production

The effect of *A. indica* extract on the production of EPS was evaluated based on the extraction and quantification of the total polysaccharides in the culture medium. The results (Figure 4D) showed that *A. indica* extract exhibited a dose-dependent inhibitory effect on EPS production. The maximum reduction in EPS production was observed at 1.56 mg/mL, which resulted in a reduction in the percentage of EPS production from 100 to 51.7%. At 0.78 mg/mL, the treatment resulted in a significant reduction in EPS production to 74.9%, whereas at 0.39 mg/mL, it was not significant.

Effect of A. indica on P. aeruginosa virulence factors

Pyocyanin

The effect of *A. indica* on the production of pyocyanin was evaluated (Figure 5A). The results showed that *A. indica* possesses a dose-dependent inhibitory effect on pyocyanin production. However, the reduction was only significant at a treatment concentration of 1.56 mg/mL, resulting in a 26.8% inhibition of pyocyanin production. At lower concentrations, there was no significant reduction in the percentage of pyocyanin inhibition.

Rhamnolipids

The effect of *A. indica* extract on the production of rhamnolipids was evaluated using the orcinol method (Figure 5B). A dose-dependent inhibitory effect was observed. A significant reduction in rhamnolipid production was observed at 1.56 and 0.78 mg/mL. The percentage inhibitions in rhamnolipid production were 22.2 and 14.7%, respectively.

LasA protease

As shown in Figure 5C, *A. indica* extract exhibited a dose-dependent inhibitory effect on *P. aeruginosa* LasA protease activity. When 1.56 mg/mL and 0.78 mg/mL of *A. indica* extract were added, there was a big drop in LasA protease activity, as shown by inhibition percentages of 55.0 and 27.7%, respectively. There was no significant reduction at 0.39 mg/mL.

Effects of A. indica extract on quorum sensing

Violacein

The effect of *A. indica* methanolic extract on the quorum sensing system was evaluated using *C. violaceum* as a biosensor strain. It operates a quorum sensing system to produce a purple pigment known as violacein. Agents, that inhibit the production of this pigment, are described as antiquorum sensing agents. As shown in Figure 6A, *A. indica* extract exhibited a dose-dependent inhibitory effect on the production of violacein pigment by *C. violaceum*. At 1.56 and 0.78 mg/mL, a significant reduction in violacein pigment was observed. At these concentrations, a percentage inhibition of 35.5 and 18.1%, respectively, was reported.

Acyl-homoserine lactone (AHL)

The effect of *A. indica* on the production of AHL as an autoinducer was evaluated (Figure 6B). The results demonstrated that *A. indica* significantly reduced AHL p

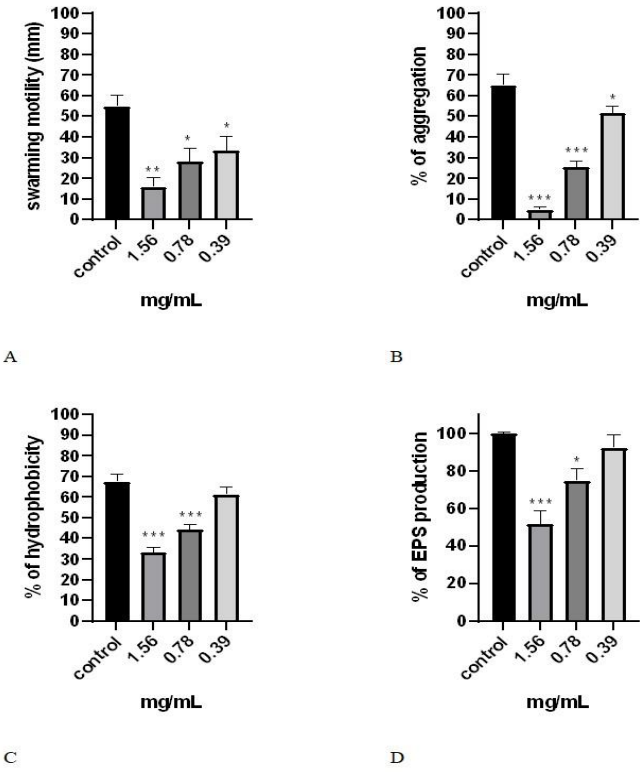

Fig. 4. Effects of *A. indica* extract on factor-mediated biofilm formation, including (A) swarming motility, (B) aggregation, (C) hydrophobicity, and (D) EPS production of *P. aeruginosa*.

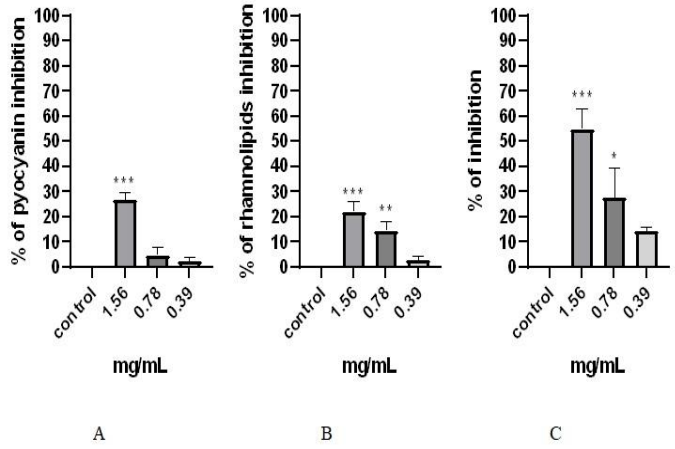

Fig. 5. Effects of *A. indica* extract on *P. aeruginosa* virulence factors, including (A) pyocyanin, (B) rhamnolipids, and (C) *LasA* protease.

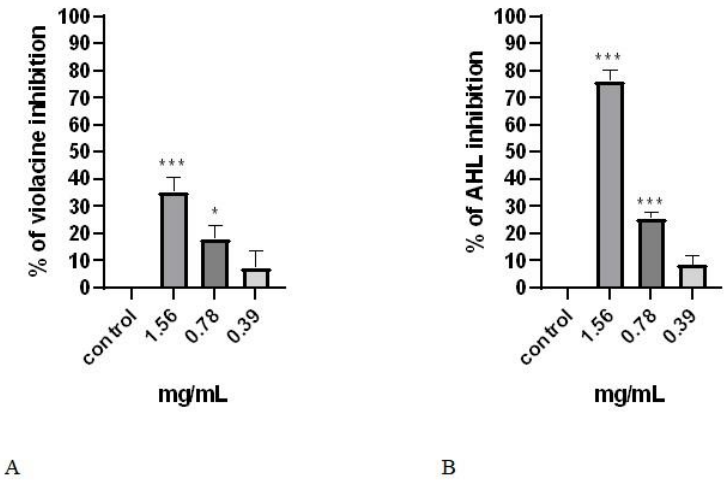

Fig. 6. Effects of *A. indica* extract on the quorum sensing of (A) *C. violaceum* as indicated by the percentage inhibition in violacein production, and (B) *P. aeruginosa* as indicated by the percentage inhibition of AHL production

production in a dose-dependent manner. The highest tested concentration (1.56 mg/mL) demonstrated a significant reduction in AHL production, as evidenced by the percentage inhibition of 76.4%. At 0.78 mg/mL, the percentage inhibition was lower (25.8%), but significant. There was no significant inhibition at 0.39 mg/mL.

Discussion

Natural products continue to be a valuable source of biologically active compounds, and they can be a major source for anti-quorum sensing agents. Plant extracts and their active ingredients have been found effective against antibiofilm, anti-virulence, and antiquorum sensing pathogenicity (Fernandes et al. 2023; Qaralleh et al. 2024a; Qaralleh et al. 2024b). In drug discovery programs, natural product based drugs are preferable due to their multiple effective modes of action (Ren et al. 2023). Furthermore, its frequent use in folk medicine provides strong evidence about its benefits and safety (Singh & Gohil 2023). Therefore, the traditionally used and broad biologically active *A. indica* (neem) was selected in this study.

In this study, the inhibition zone observed, and the value of MIC suggests strong evidence regards the mild antibacterial effect of *A. indica* methanolic extract against *P. aeruginosa*. This result is in agreement with other study results that found *A. indica* petroleum ether and methanolic leaf extract have no activity against *P. aeruginosa* (Fasher Bory et al. 2022). In another study conducted by Faujdar et al. (2020), the MIC value of *A.*

indica hydroethanolic extract against ESBL *P. aeruginosa* was found to be 6.25 mg/mL (Faujdar et al. 2020).

At sub-MIC, *A. indica* extract exhibited a significant antibiofilm activity as observed by crystal violet assay, light microscope, and SEM. Among them, the 1.56, 0.78, and 0.39 mg/mL treatment concentrations showed a significant biofilm inhibition without displaying any significant cell viability reduction. This may be indicative that the antibiofilm activity is due to the extract's antibiofilm effects rather than its bacteriostatic action. Previously, extracts from *A. indica* have been reported to suppress biofilm formation in *P. aeruginosa* and PAO1 strains (Harjai et al. 2013), *Streptococcus Sanguis* (Pai et al. 2004), *Streptococcus mutans* (Dinesh et al. 2023), and *Candida albicans* (Polaquini et al. 2006) effectively. *A. indica* at 6.25 mg/mL exhibited 54% and 57% inhibitions in biofilm formation of *P. aeruginosa* ATCC 27853 and clinically isolated *P. aeruginosa*, respectively (Mehrishi et al. 2022).

The factors that mediate biofilm formation play significant roles in the development of biofilm (Lebeloane et al. 2024). These factors, including bacterial motility, aggregation, hydrophobicity, and EPS production, were significantly suppressed when *A. indica* extract was applied. These factors are activated sequentially, and inhibiting one of these factors could result in co-suppression of the other factors. Possibly, the extract may display an inhibitory effect on one of these factors and suppress the others in a cascade manner, or it may exert multiple effects targeting more than one of these biofilm-mediated factors. For instance, agents that suppress motility or EPS production might suppress adhesion and aggregation, thus preventing the initiation of biofilm formation (Shrout et al. 2006).

Extracts from plants often contain a mixture of complex compounds that may exert multiple and potentially novel mechanisms of action. As bacterial motility contributes effectively to the formation of biofilms, substances that inhibit motility prevent the formation of biofilms. In addition, studies have shown that EPS plays an important role in stimulating adhesion, and inhibition of EPS production could result in preventing adhesion. It is possible that some of the components of the extract have bound to adhesion receptors and thus prevented bacterial aggregation and adhesion (Kassinger & van Hoek 2020).

Biofilm-forming bacteria are more resistant to antibiotics and immune responses. The cells move towards surfaces where they adhere, aggregate, and communicate with each other through the quorum sensing system to build a 3D structural community called biofilm (Høiby et al. 2010). Furthermore, *P. aeruginosa* possesses a multitude of quorum sensing-mediated virulence factors. The most important secreted virulence factors are proteases and elastases, which play an important role in adhesion and colonization. They also facilitate tissue invasion, necrosis, and the spread of pathogens. Pyocyanin, an oxidizing agent, is produced and act as a chelating agent to extract iron from proteins (Andrejević et al. 2023). In biofilms, pyocyanin complexes with eDNA increase surface hydrophobicity and aggregation, protecting the integrity of extracellular polymeric substances and the stability of the biofilm (Abdelaziz et al. 2023). Rhamnolipids are biosurfactant virulence factors that promote bacterial motility. Bacteria are sessile without rhamnolipids, but their motility will be restored if supplemented (O'May & Tufenkji 2011).

A. indica inhibited virulence factor production through elimination of the quorum sensing system as evidenced by the considerable reduction of AHL in response to *A. indica* treatment. The decrease in the production of the AHL leads to a decrease in the production of the virulence factors, hence reducing the pathogenicity of the microbes (Ahmed et al. 2019). Of the four cell systems in *P. aeruginosa* (Soukarieh et al. 2018), Pqs system genes encode for rhamnolipid production, pyocyanin, lectin A and B, whereas the Las system encodes for alkaline proteases. The *LasA* system is connected with the Pqs system to control the synthesis of Psl, Pel, elastase A, and elastase B, while the Pqs system is connected with the Rhl system to regulate the synthesis of rhamnolipids (Ueda & Wood 2009) (Muimhneacháin et al. 2018; Yan & Wu 2019).

Because of its intricate structure, extract activity often results from the combined effects of its active components or the action of a single ingredient. β-sitosterol, a metabolite of *A. indica*, has been previously documented to suppress biofilm formation and the production of extracellular polymeric substances (EPS). (Rasamiravaka et al. 2017). The isolated fatty acid, linoleic acid, was found effective in inhibiting quorum sensing, biofilm, and protease in *P. mirabilis* and *S. marcescens* (Marathe et al. 2018). Catechin exhibited superior antibiofilm and antiquorum sensing activities against biofilm-forming *Alcaligenes faecalis* and *Pseudomonas gingivalis* to quercetin, nimbolide, nimbin, and azardirachtin (Lahiri et al. 2021). Also, the antibiofilm and anti-virulence activities of limonoids rich extracts have been observed against MRSA (Zhang et al. 2022).

Conclusion

A. indica extract showed novel antibiofilm, anti-virulence factor, and anti-quorum sensing activities. These broad activities make *A. indica* extract a promising candidate to

be developed as a therapeutic agent that could be used to manage, control, and prevent chronic and persistent bacterial infections. Further investigations to isolate the active compounds, elucidate the mode of action, and evaluate the cytotoxicity are required.

References

Abdelaziz AA, Kamer AMA, Al-Monofy KB, Al-Madboly LA. (2023). Pseudomonas aeruginosa's greenish-blue pigment pyocyanin: its production and biological activities. Microb Cell Fact 22: 110.

Ahmed SAKS, Rudden M, Smyth TJ, Dooley JSG, Marchant R, Banat IM. (2019). Natural quorum sensing inhibitors effectively downregulate gene expression of Pseudomonas aeruginosa virulence factors. Appl Microbiol Biotechnol 103: 3521–3535.

ALrawashdeh I, Qaralleh H, Al-limoun M, Khleifat K. (2019). Antibactrial Activity of Asteriscus graveolens Methanolic Extract: Synergistic Effect with Fungal Mediated Nanoparticles against Some Enteric Bacterial Human Pathogens. J Basic Appl Res Biomed 5: 89–98.

Andrejević T, Ašanin D, Pantović B, Stevanović N, Marković V, Djuran M, Glisic B. (2023). Metal complexes with valuable biomolecules produced by Pseudomonas aeruginosa: a review of coordination properties of pyocyanin, pyochelin and pyoverdines. Dalt Trans. 52: 4276-4289

Andrejko M, Zdybicka-Barabas A, Janczarek M, Cytryńska M. (2013). Three Pseudomonas aeruginosa strains with different protease profiles. Acta Biochim Pol 60(1): 83-90

Bansal V, Gupta M, Bhaduri T, Shaikh SA, Sayed FR, Bansal V, Agrawal A. (2019) Assessment of Antimicrobial Effectiveness of Neem and Clove Extract Against Streptococcus mutans and Candida albicans: An: In vitro: Study. Niger Med J 60: 285–289.

Bayramov D, Neff J. (2017) Beyond conventional antibiotics—New directions for combination products to combat biofilm. Adv Drug Deliv Rev 112: 48–60.

Bhatia N, Sharma R, Kaur G, Kaur N, Singh G, Dhawan R. (2015) Beneficial effects of Morus alba and Azadirachta indica leaf extract based combination cream in scalding type burn injury in rats. Int J Basic Clin Pharmacol 4: 720.

Dinesh D, Imran K, Vijayaraghavalu S, Khan MS, Nikesh VV. (2023). In vitro evaluation of Antibiofilm activity of Methanolic leaf Extract of Azadirachta indica on cariogenic Streptococcus Mutans. J Nat Remedies 23: 276–281.

Fasher Bory W, Hammad M, Idris A. (2022). Evaluation of in vitro antimicrobial effects of Azadirachta indica (Neem) leaves extracts against selected pathogens. Microbes Infect Dis 3: 744–750.

Faujdar SS, Bisht D, Sharma A. (2020). Antibacterial potential of neem (Azadirachta indica) against uropathogens producing beta-lactamase enzymes: A clue to future antibacterial agent? Biomed Biotechnol Res J 4: 232–238.

Fernandes ES, da Silva Figueiredo IF, Monteiro CRAV, Monteiro-Neto V. (2023). Antimicrobial and Anti-Infective Activity of Natural Products—Gaining Knowledge from Novel Studies. Antibiotics 12: 1051.

Gómez-Gómez B, Arregui L, Serrano S, Santos A, Pérez-Corona T, Madrid Y. (2019). Selenium and tellurium-based nanoparticles as interfering factors in quorum sensing-regulated processes: Violacein production and bacterial biofilm formation. Metallomics 11: 1104–1114.

Gordya N, Yakovlev A, Kruglikova A, Tulin D, Potolitsina E, Suborova T, et al. (2017) Natural antimicrobial peptide complexes in the fighting of antibiotic resistant biofilms: Calliphora vicina medicinal maggots. PLoS One 12: e0173559.

Harjai K, Bala A, Gupta RK, Sharma R. (2013). Leaf extract of Azadirachta indica (neem): a potential antibiofilm agent for Pseudomonas aeruginosa. Pathog Dis 69: 62–65.

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. (2010). Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35: 322–332.

Hossain MA, Lee SJ, Park NH, Mechesso AF, Birhanu BT, Kang J. et al. (2017). Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. Sci Rep 7: 10618.

Jurado-Martín I, Sainz-Mejías M, McClean S. (2021). Pseudomonas aeruginosa: an audacious pathogen with an adaptable arsenal of virulence factors. Int J Mol Sci 22: 3128.

Kassinger SJ, van Hoek ML. (2020). Biofilm architecture: An emerging synthetic biology target. Synth Syst Biotechnol 5: 1–10.

Khaled FM, Haitham Q, Dmour S, Moath A, Yassin, A. (2023) Azadirachta indica (Neem) Leaf: chemical composition and antibacterial activity as determined by different polarity solvents. Res J Biotechnol 18: 2.

Kreitmann L, Helms J, Martin-Loeches I, Salluh J, Poulakou G, Pène F, Nseir S. (2024). ICU-acquired infections in immunocompromised patients. Intensive Care Med 50: 332–349.

Krishnan T, Yin WF, Chan KG. (2012). Inhibition of quorum sensing-controlled virulence factor production in Pseudomonas aeruginosa PAO1 by Ayurveda spice clove (Syzygium aromaticum) bud extract. Sensors 12: 4016–4030.

Lahiri D, Nag M, Dutta B, Mukherjee I, Ghosh S, Dey A. et al. (2021). Catechin as the most efficient

bioactive compound from Azadirachta indica with antibiofilm and anti-quorum sensing activities against dental biofilm: An in vitro and in silico study. Appl Biochem Biotechnol 193: 1617–1630.

Lebeloane MM, Famuyide IM, Dzoyem JP, Adeyemo RO, Makhubu FN, Elgorashi EE. et al. (2024). Influence of selected plant extracts on bacterial motility, aggregation, hydrophobicity, exopolysaccharide production and quorum sensing during biofilm formation of enterohaemorrhagic Escherichia coli O157: H7. South African J Bot 167: 197–208.

Lee JH, Kim Y, Lee J. (2017). Carvacrol-rich oregano oil and thymol-rich thyme red oil inhibit biofilm formation and the virulence of uropathogenic Escherichia coli. J Appl Microbiol 123: 1420–1428.

Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, et al. (2017). 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: e0176883.

Ma'aitah S. (2024). Pseudomonas aeruginosa biofilm formation: Antibiofilm Strategies and conventional methods of evaluation. *J Basic Appl Res Biomed* **10**: 11–28.

Marathe K, Bundale S, Nashikkar N, Upadhyay A. (2018). Influence of linoleic acid on quorum sensing in Proteus mirabilis and Serratia marcescens. Biosci Biotechnol Res Asia 15: 661–670.

Mehmood N, Saeed M, Zafarullah S, Hyder S, Rizvi ZF, Gondal AS. et al. (2023). Multifaceted impacts of plant-beneficial pseudomonas spp. in managing various plant diseases and crop yield improvement. ACS omega 8: 22296–22315.

Mehrishi P, Agarwal P, Broor S, Sharma A. (2022). Antibacterial and antibiofilm properties of Azadirachta indica (Neem), Aloe vera (Aloe vera), and Mentha piperita (Peppermint) against multidrug-resistant clinical isolates. Biomed Biotechnol Res J 6: 98–104.

Moin MS, Siddiqui JI, Alam MA, Khatoon F, Khan S. Minhajuddin A. (2021) Ethnomedicinal potential of widely used plant Azadirachta indica A. Juss: A comprehensive review. J Phytopharm 10: 456–467.

Muimhneacháin EÓ, Reen FJ, O'Gara F, McGlacken GP. (2018). Analogues of Pseudomonas aeruginosa signalling molecules to tackle infections. Org Biomol Chem 16: 169–179.

Naga NG, El-Badan DE, Ghanem KM, Shaaban MI. (2023). It is the time for quorum sensing inhibition as alternative strategy of antimicrobial therapy. Cell Commun Signal 21: 1–14.

Nazarov PA, Baleev DN, Ivanova MI, Sokolova LM, Karakozova MV. (2020). Infectious plant diseases: etiology, current status, problems and prospects in plant protection. Acta Naturae 12: 46.

O'May C, Tufenkji N. (2011). The swarming motility of Pseudomonas aeruginosa is blocked by cranberry proanthocyanidins and other tannin-containing materials. Appl Environ Microbiol 77: 3061–3067.

Pai MR, Acharya LD, Udupa N. (2004). Evaluation of antiplaque activity of Azadirachta indica leaf extract gel—a 6-week clinical study. J Ethnopharmacol 90: 99–103.

Polaquini SRB, Svidzinski TIE, Kemmelmeier C, Gasparetto A. (2006). Effect of aqueous extract from Neem (Azadirachta indica A. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans. Arch Oral Biol 51: 482–490.

Qaralleh H. (2023). Chemical Composition and Quorum Sensing Inhibitory Effect of Nepeta curviflora Methanolic Extract against ESBL Pseudomonas aeruginosa J Pharmacopuncture 26: 307–318.

Qaralleh H, Al-Limoun M, Khlaifat A, Khleifat K, Al-Tawarah N, Hashem AH, Alsharafa K. (2020). Antibacterial and Antibiofilm Activities of a Traditional Herbal Formula against Respiratory Infection Causing Bacteria. Trop J Nat Prod Res 4: 527–534.

Qaralleh, H., Majali, I., Al-limoun, M., ALdehayyat, R., Khleifat, K., Dmour, S., & Al Khutaba'a, G. (2024). Cynomorium coccineum extract inhibits biofilm formation and virulence factors and attenuates quorum sensing in Pseudomonas aeruginosa. Journal of Biological Studies, 7(4): 184–202.

Qaralleh H, Saghir SAM, Al-Limoun MO, Dmor SM, Khleifat K, Al-Ahmad BEM. et al. (2024). Effect of Matricaria aurea Essential Oils on Biofilm Development, Virulence Factors and Quorum Sensing-Dependent Genes of Pseudomonas aeruginosa. Pharmaceuticals 17: 386.

Rasamiravaka T, Ngezahayo J, Pottier L, Oliveira Ribeiro S, Souard F, Hari L. et al. (2017). Terpenoids from Platostoma rotundifolium (Briq.) AJ Paton alter the expression of quorum sensing-related virulence factors and the formation of biofilm in Pseudomonas aeruginosa PAO1. Int J Mol Sci 18: 1270.

Razack SA, Vijayagopal V, Viruthagiri T. (2011). Production of Exopolysaccharides by P. aeruginosa, P. fluorescens and Bacillus sp. IUP J Biotechnol 5(4):.14-19

Ren J, Yang L, Qiu S, Zhang AH, Wang XJ. (2023). Efficacy evaluation, active ingredients, and multitarget exploration of herbal medicine. Trends Endocrinol Metab 34: 146–157.

Sagar PK, Sharma P, Singh R. (2022). Inhibition of quorum sensing regulated virulence factors and biofilm formation by Eucalyptus globulus against multidrug-

resistant Pseudomonas aeruginosa. J Pharmacopuncture 25(1): 37-45

Shanks RMQ, Meehl MA, Brothers KM, Martinez RM, Donegan NP, Graber ML. et al. (2008). Genetic evidence for an alternative citrate-dependent biofilm formation pathway in Staphylococcus aureus that is dependent on fibronectin binding proteins and the GraRS two-component regulatory system. Infect Immun 76: 2469–2477.

Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. (2006). The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. Mol Microbiol 62: 1264–1277.

Singh MP, Gohil KJ. (2023). Therapeutic Efficacy and Cost Effectiveness of Herbal Drugs-A Reasonable Approach. Pharmacol Res Prod 2: 100009.

Soukarieh F, Williams P, Stocks MJ, Camara M. (2018). Pseudomonas aeruginosa quorum sensing systems as drug discovery targets: current position and future perspectives. J Med Chem 61: 10385–10402.

Ueda A, Wood TK. (2009). Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in Pseudomonas aeruginosa through tyrosine phosphatase TpbA (PA3885). PLoS Pathog 5: e1000483.

Vetrivel A, Ramasamy M, Vetrivel P, Natchimuthu S, Arunachalam S, Kim GS, Murugesan R. (2021). Pseudomonas aeruginosa biofilm formation and its control. Biologics 1: 312–336.

Wang CH, Hsieh YH, Powers ZM, Kao CY. (2020). Defeating antibiotic-resistant bacteria: exploring alternative therapies for a post-antibiotic era. Int J Mol Sci 21: 1061.

Yan S, Wu G. (2019). Can biofilm be reversed through quorum sensing in Pseudomonas aeruginosa? Front Microbiol 10: 1582.

Zhang L, Ismail MM, Rocchetti G, Fayek NM, Lucini L, Saber FR. (2022). The untargeted phytochemical profile of three meliaceae species related to in vitro cytotoxicity and anti-virulence activity against MRSA isolates. Molecules 27: 435.