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Hibiscus tiliaceus ethanolic leaf extract modulates quorum sensing-facilitated virulence factors

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Abstract. *Husnah M, Suhartono S, Ismail YS. 2023.* Hibiscus tiliaceus *ethanolic leaf extract modulates quorum sensing-facilitated virulence factors. Biodiversitas 24: 5962-5971.* Bacterial pathogenicity and infections are often associated with the presence of virulence factors generated mostly through quorum-sensing (QS) mechanisms. Therefore, QS suppression is considered an effective strategy for combatting pathogens. Many studies to search for new anti-QS agents, particularly those of plant origin, have been intensively conducted. *Hibiscus tiliaceus* L. is one of the medicinal plants exhibiting various pharmacological activities including antibacterial potential; however, the study regarding its effects on bacterial QS systems has not been reported. Therefore, this study aimed to investigate the potency of *Hibiscus tiliaceus* ethanolic leaf extract (HELE) as a modulating agent against *Chromobacterium violaceum* ATCC122472, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* PA01. The extract was obtained through maceration and phytochemical screening was carried out by GC-MS. To evaluate antibacterial and anti-QS potentials of HELE, minimum inhibitory concentration (MIC); minimum bactericidal concentration (MBC); growth inhibition rate; disc diffusion; and inhibition of violacein, pyocyanin, swarming motility, and biofilm formation were assayed. Statistical analysis was performed using SPPS software and Analysis of Varian (ANOVA) was employed to determine significant differences between the control and treatment groups. Phytochemical screening revealed the presence of 16 different compounds in HELE, represented predominantly by diterpenoids (phytol), fatty acids (palmitic acid and oleic acid), and p-Tolualdehyde. MIC was found at 10 mg/mL against both *C. violaceum* ATCC122472 and *S. aureus* ATCC 29213, and 160 mg/mL against *P. aeruginosa* PA01. The MBC for the first two bacteria was 20 mg/mL, whereas that against *P. aeruginosa* PA01 remains to be determined. HELE was able to suppress the production of violacein, pyocyanin, and swarming motility at sub-MIC concentrations, highlighting the anti-QS potential of the extract. HELE at sub-MICs was found to stimulate biofilm formation, whereas at 2 x MIC exhibited an inhibitory effect. These findings justified that HELE might serve as a promising candidate not only as a future QS-inhibitory but also as a concentration-dependent QS-modulating agent.

Keywords: Biofilm, biofilm induction, HELE, pyocyanin, swarming motility, violacein

INTRODUCTION

Bacterial infection, pathogenicity, and resistance to various available antibiotics continue to increase over time and have become major health concerns worldwide (Kothari et al. 2017; Azam and Khan 2019; Onyancha et al. 2020). High incidences of morbidity and mortality (60-70%) due to bacterial resistance toward existing antibiotics have been reported in developed countries (Walters et al. 2015; Azam and Khan 2019). The resistance observed in bacteria is frequently linked to their capacity to generate a diverse array of virulence factors, which are primarily regulated by quorum sensing (QS) mechanisms. QS enables bacterial cell-to-cell communication through the utilization of small signaling molecules known as autoinducers (AIs) (Azimi et al. 2020; Wang et al. 2020; Boo et al. 2021). This process results in the expression of specific genes encoding virulence factors, such as proteins, pigments, cell surface-associated properties, motility-associated factors, and biofilm formation, once a certain population density threshold is reached (Mukherjee and Bassler 2019; Acet et al. 2021; Ruhal and Kataria 2021). Biofilm, in particular, contributes to an increase in bacterial resistance and pathogenicity as they protect bacteria from host immune responses and reduces the efficacy of antibiotics (Rasamiravaka et al. 2015; Munita and Arias 2016) and is responsible for approximately 17.9-100% of hospital-acquired infection worldwide and 80% of chronic infection in human (Elhabibi and Ramzy 2017; Preda and Săndulescu 2019; Assefa and Amare 2022). This dramatic emergence of antibiotic-resistant bacterial strains due to their ability to develop a defense against available antibiotics has encouraged the search for novel molecules to be developed into new antibacterial (Llor and Bjerrum 2014).

A significant number of bacterial pathogens depend on quorum sensing (QS) mechanisms to regulate the expression of their virulence factors. Consequently, interfering with QS mechanisms has emerged as a promising approach to combat bacterial infections and prevent the development of bacterial resistance (Alasil et al. 2015). The anti-QS substances have been reported to effectively interfere with bacterial QS activity (Soheili et al. 2019). These molecules are widely available in nature,

including molecules of plant origin (Ćirić et al. 2019; Asif 2020; Bouyahya et al. 2022). Unlike animals endowed with specialized immune systems to fight against biotic threats, plants do not possess specialized immune cells to combat invading pathogens. Nonetheless, plants have evolved a complex defense mechanism, where each plant cell is shifting into battle mode by producing various secondary metabolites whenever attacked (Koh et al. 2013; Subramanian et al. 2022). Phytochemical compounds have been reported to exhibit antimicrobial effects, including anti-QS activities, to conquer QS pathogens. This situation has made plant-based bioactive constituents a potential and preferable option to overcome bacterial infection (Asif 2020; Bouyahya et al. 2022; Subramanian et al. 2022).

One of the plants widely known to possess various bioactive constituents is *Hibiscus tiliaceus* L. (Andriani et al. 2017; Samsudin et al. 2019; Wong and Chan 2022). *H. tiliaceus*, a plant native to the tropical coastlines of the Pacific and Indian Oceans, has since become prevalent in subtropical and tropical regions globally. The plant exhibits distinctive heart-shaped leaves and bell-shaped flowers, which possess maroon-colored centers and stigmas. Notably, the flowers undergo a color transformation, appearing yellow in the morning, reddish-orange in the evening, and mauve the following morning. The plant has diverse applications, with various components utilized for different purposes, such as food and beverages, decorative ornaments, and traditional medicinal remedies. In particular, Indonesian and Malaysian indigenous medicine employs the leaves, primarily in the form of a decoction, to address ailments such as fever, diarrhea, cough, bronchitis, and other respiratory disorders (Lim 2014; Chan et al. 2016; Wong and Chan 2022). Previous studies suggested that *H. tiliaceus* contains bioactive substances exhibiting antimicrobial (Hossain et al. 2015; Abdul-Awal et al. 2016; Sumardi et al. 2018; Samsudin et al. 2019; Andriani et al. 2023) and antibiofilm activities (Andriani et al. 2017; Suhartono et al. 2019). However, the study regarding *H. tiliaceus* effects on quorum sensing, in particular, has not been reported. Therefore, the objective of this investigation was to assess the anti-quorum sensing (anti-QS) properties of *H. tiliaceus* ethanolic leaf extract (HELE) and to characterize its phytochemical constituents. To the best of authors' knowledge, this represents the inaugural study examining the modulatory influence of *H. tiliaceus* on QS signaling pathways.

MATERIALS AND METHODS

Samples collection and extraction

The leaves of *H. tiliaceus* were collected from rice-farming areas in Aceh Besar, Indonesia. The leaves were thoroughly washed, minced into small pieces, and air-dried for two weeks before grinding and sieving into a fine powder. Extraction was performed using the maceration method at a 1:7.5 ratio. Therefore, 1,500 g of simplicial was placed into a maceration container containing 11.25 L of 96% ethanol, homogenized, sealed, and incubated for five days with occasional stirring. Subsequently, the concoction underwent filtration employing Whatman filter paper, yielding macerate and lees fractions. The macerate fraction was meticulously preserved at ambient conditions, shielded from light exposure, while the lees fraction underwent a repeat maceration process for an additional five-day period utilizing 3.75 L of ethanol as the solvent. The complete filtrate comprising the macerate fraction was subjected to controlled evaporation below 50°C, resulting in the attainment of a concentrated formulation of 100%. Ultimately, the resultant extract was quantified, precisely weighed, and subsequently stored at a temperature of 4°C until it was ready for utilization.

Phytochemical analysis

HELE was analyzed through a comprehensive phytochemical screening to ascertain the existence of specific phytochemical constituents. This examination was conducted utilizing a Gas Chromatography-Mass Spectrometry (GC-MS) apparatus (Shimadzu GCMS-QP 2010 Ultra) equipped with a 30 m x 0.25 mm fused silica capillary column. Five microliter of prepared sample (1 g of HELE in ethanol with a ratio of 1:2) was injected into the GC-MS system using a splitless mode. The injection temperature was 250°C and helium gas was utilized as carrier with a flow rate of 0.72 mL/minute and pressure of 37.1 kPa. The column temperature was adjusted from 70-270°C (raised from 70 to 230°C by 10°C/min, held for 3 min, and raised to the final temperature of 270°C). Ion was generated by a 70 kV electron impact, with ion source temperature of 230°C. Identification of metabolites was performed by comparing their mass spectra with fragmentation pattern stored in the National Institute of Standards and technology (NIST) MS database (Suhendar and Sogandi 2019).

Bacterial strains and culture condition

In this study, the antibacterial and anti-quorum sensing (anti-QS) efficacies of HELE were assessed using three bacterial strains: *Chromobacterium violaceum* ATCC 122472, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* PA01. These bacterial strains were cultured and maintained under specific growth conditions. The culture media employed for bacterial propagation included Luria Bertani broth (LBB) (Difco, USA), Muller Hinton broth (MHB) (Difco, USA), and tryptic soy broth (TSB) (Oxoid, UK). Incubation temperatures were optimized for each bacterial strain: 30°C for *C. violaceum*, 37°C for *P. aeruginosa*, and 28°C for *S. aureus*.

Antibacterial activity

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the HELE were ascertained using a modified protocol as described earlier (Hossain et al. 2015). Briefly, a range of extract concentrations (0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40, 80, 100, and 160 mg/mL) were prepared and dispensed into a 96-well microtiter plate. Tetracycline, at a concentration of 16

mg/mL, served as the positive control. Subsequently, bacterial inocula, adjusted to a final density of 5×10^5 CFU/mL, were introduced into the wells. A well containing bacterial inoculum devoid of the extract functioned as the growth control. The MIC was identified as the lowest concentration of the extract at which no visible bacterial growth was observed (evidenced by a clear well) following a 24-h incubation period. Aliquots of 20 µL from wells exhibiting concentrations at or above the MIC were subcultured onto Mueller Hinton Agar (MHA) plates and incubated for an additional 24 h at ambient temperature for the determination of MBC. The MBC was defined as the lowest concentration at which complete bacterial growth inhibition was observed on the MHA plates.

Bacterial inhibition rate

The bacterial inhibition rate was assessed using previously described methods (Hossain et al. 2015; Ušjak et al. 2019). In a succinct manner, bacterial strains *C. violaceum* and *S. aureus* were inoculated into Erlenmeyer flasks containing 10 mL of sterile MHB supplemented with varying concentrations of HELE: 1/2× MIC (5 mg/mL), 1 × MIC (10 mg/mL), and 2 × MIC (20 mg/mL). On the other hand, the extract concentrations were adjusted to 1/21 × MIC (7.5 mg/mL), 1/11 × MIC (15 mg/mL), and 1/5 × MIC (30 mg/mL) in different Erlenmeyer flasks for *P. aeruginosa*. The final bacterial density in each flask was standardized to 5×10^5 CFU/mL prior to incubation at 150 rpm for 24 h at their respective temperatures. A growth control comprising MHB and bacterial inoculum, devoid of the extract, was incubated under identical conditions. Aliquots were extracted from each Erlenmeyer flask at time intervals of 0, 2, 4, 8, and 24 h post-incubation and were subsequently subjected to serial ten-fold dilutions, ranging from 10^{-2} to 10^{-6}. A volume of 20 µL from each dilution was then plated onto tryptic soy agar (TSA) plates and incubated for an additional 24 h at the respective temperatures to enumerate the colony-forming units (CFU).

Quorum sensing (QS) inhibition

To evaluate the quorum sensing (QS) inhibitory activities of the HELE, a series of assays were conducted, encompassing the inhibition of violacein and pyocyanin production, swarming motility, and biofilm formation. In this study, extract concentrations of 1/2 × MIC (5 mg/mL), 1 × MIC (10 mg/mL), and 2 × MIC (20 mg/mL) were employed against *C. violaceum* and *S. aureus*. Notably, for *P. aeruginosa*, the experimentally determined MIC of 160 mg/mL was deemed prohibitively high for QS inhibitory analysis. Consequently, the study adopted a benchmark concentration of 15 mg/mL as suggested in a former study (Andriani et al. 2017), resulting in the utilization of concentrations of 7.15 mg/mL (1/21 × MIC), 15 mg/mL (1/11 × MIC), and 30 mg/mL (1/5 × MIC) for this bacterial strain. These concentrations were also employed in the growth inhibition assays against the respective bacterial species.

Inhibition of violacein

The inhibitory effect of HELE on violacein production was evaluated by the disk-diffusion and flask incubation methods for qualitative and quantitative analyses, respectively. Molten Luria Bertani agar (LBA) was inoculated with *C. violaceum* to make a $5x10^6$ CFU/mL inoculum. Various extract concentrations (0.31, 0.63, 1.25, and 2.5 mg/disk), 3% hydrogen peroxide (H_2O_2) (20 µL/disk), tetracycline (16 mg/disk), and ethanol 96% (60 µL/disk) were injected into the sterile paper disks (6 mm in diameter). After adequate drying, the paper disks were plated on LB agar and incubated at 30°C for 24 h. The detected colorless ring, yet viable cells around the disks, verified anti-QS activity, whereas the clear zone (viable cell-free area) around the disk indicated antibacterial activity (Pratiwi 2015; Hossain et al. 2015). A calibrated digital slide caliper was used to measure the diameter of the pigment and bacterial growth inhibition. H_2O_2, tetracycline, and ethanol were positive controls for QS inhibition, antibacterial activity, and diluent control, respectively.

Violacein quantification was performed by inoculating *C. violaceum* culture (5×10^5 CFU/mL) into different flasks containing LBB supplemented beforehand with ½ × MIC, 1 × MIC, and 2 × MIC of the extract, and 3% H_2O_2 (positive control). A bacterial culture in drug-free LB broth was also prepared as a negative control (Hossain et al. 2015). After incubation at 30°C for 24 h in a shaking incubator (150 rpm), pigment production was quantified as previously described (Choo et al. 2006). Briefly, 1 mL of the mixture from each flask was centrifuged at 9,700 rpm for 10 minutes to obtain a pellet. Next, the pellet was dissolved in 1 mL DMSO and centrifuged at 9,700 rpm for another 10 minutes to obtain the supernatant. Violacein concentration was determined by measuring the absorbance of the supernatant using a microplate reader (Bio-Rad) at 585 nm, and the percentage of violacein inhibition was calculated.

Inhibition of pyocyanin

Pyocyanin inhibition was assessed as previously reported (Hossain et al. 2015). *P. aeruginosa* PA01 was cultured overnight at 37°C in 5 mL of LBB, supplemented with varying concentrations of the extract: 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC. H_2O_2 at a concentration of 3% and LBB devoid of any additives were employed as positive and growth (negative) controls, respectively. Subsequently, the mixture was subjected to centrifugation at 10,000 rpm for a duration of 10 minutes to facilitate the collection of the supernatant. To this supernatant, 3 mL of chloroform was added. Following the removal of the aqueous phase, the residual chloroform layer was combined with 1 mL of 0.2 M hydrochloric acid (HCl), vortexed, and centrifuged to yield a pink layer. Next, the optical density of the resultant pink layer was quantitatively assessed at a wavelength of 520 nm. Each recorded absorbance value was then multiplied by the predetermined extinction coefficient of 17.072 to calculate the concentration of pyocyanin present in the supernatant.

Inhibition of swarming motility

To assess the inhibitory effects of the HELE on bacterial motility, swarming activity of *P. aeruginosa* PA01 was evaluated. Specifically, the inhibition of swarming motility was investigated by inoculating *P. aeruginosa* PA01 onto the center of semi-solid LBA plates. These plates were formulated with 0.5% agar and 0.5% glucose and were pre-supplemented with varying concentrations of the extract: 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC. Plates devoid of the extract served as negative controls. The diameter of the resultant swarm zones was quantitatively measured employing a digital slide caliper, as described in previous studies (Pratiwi 2015; Ušjak et al. 2019). The assay was conducted in triplicate to ensure statistical robustness.

Inhibition of biofilm formation

The impact of the HELE on biofilm formation by *P. aeruginosa* and *S. aureus* was assessed employing a microtiter plate biofilm assay, as delineated in prior studies (Andriani et al. 2017; Suhartono et al. 2019). Specifically, 100 µL of LBB supplemented with 0.5% glucose and varying concentrations of HELE (1/21 × MIC, 1/11 × MIC, and 1/5 × MIC for *P. aeruginosa*; 1/2 × MIC, 1 × MIC, and 2 × MIC for *S. aureus*), was dispensed into each well of a 96-well microplate. This was followed by the addition of 100 µL of the respective bacterial suspensions, standardized to a final density of 5×10^5 CFU/mL. Control wells containing LBB with 0.5% glucose and 3% H_2O_2 served as the positive control, while untreated medium functioned as the negative control. Following a 24-h incubation period at 27°C, the microplate was subjected to three washes with sterile phosphate-buffered saline (PBS). Thereafter, 200 µL of 95% ethanol was added to each well and allowed to stand for 15 minutes prior to draining and air-drying. Staining was subsequently conducted using 0.1% crystal violet (200 µL) for a 15 minutes duration, followed by rinsing with sterile distilled water. After air-drying, each well was filled with 200 µL of 30% glacial acetic acid and incubated for an additional 15 min. The absorbance, indicative of the extent of biofilm formation, was quantitatively measured at a wavelength of 570 nm utilizing a microtiter plate reader (Bio-Rad, CA, USA).

Data analysis

All experiments were conducted by comparing the HELE-treatment groups with the control (untreated samples). Descriptive statistics, including the standard deviation (SD) and arithmetic mean, were computed for each dataset. To evaluate the statistical significance of the observed differences between the treated and control groups, initial data normality was assessed using the Shapiro-Wilk test. Subsequently, a parametric one-way Analysis of Variance (ANOVA) was employed, followed by a post-hoc Tukey test for multiple comparisons. All statistical computations were conducted using IBM SPSS Statistics software, and a *p*-value threshold of ≤0.05 was established as the criterion for statistical significance.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical analysis of HELE was carried out using the GC-MS procedure, and the results revealed the presence of 16 eluting peaks within the first 3 to 23 minutes of the retention time, suggesting the presence of 16 different compounds in HELE (Figure 1 and Table 1). 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,(CAS) Phytol, Hexadecanoic acid (CAS) Palmitic acid, benzaldehyde, 4-methyl- (CAS) p-Tolualdehyde, and 9-Octadecenoic acid (Z)- (CAS) Oleic acid were the four top abundant constituents found in the extract, with percent area of 42.72, 17.46, 10.64, and 5.78% respectively.

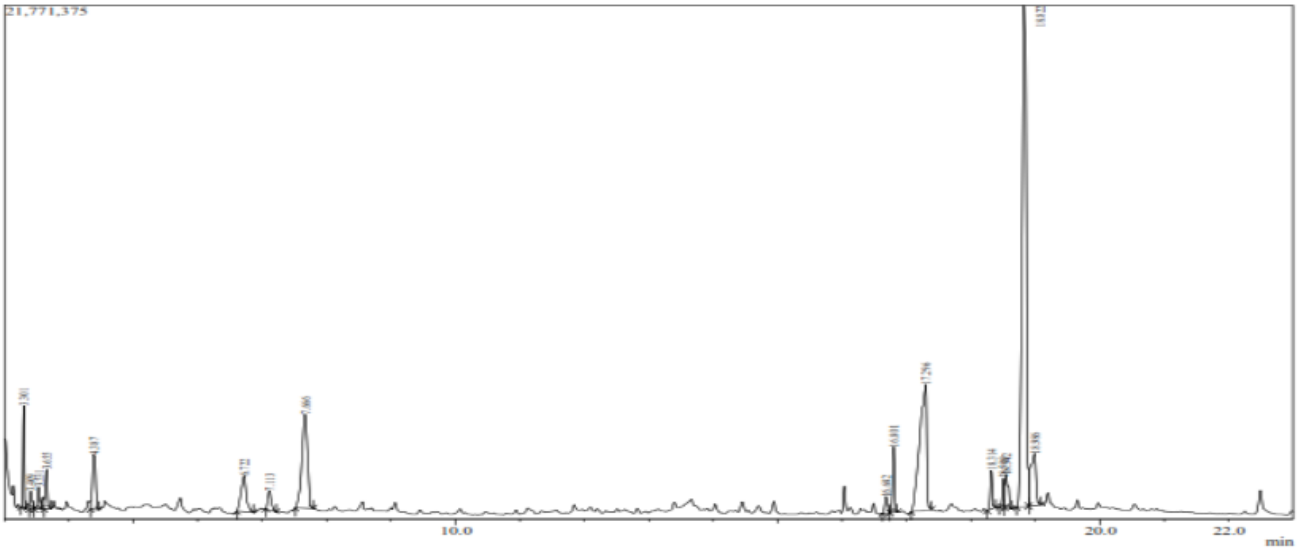


Figure 1. Chromatogram of the GC-MS analysis of the *Hibiscus tiliaceus* ethanolic leaf extract

Table 1. The result of GC-MS analysis of *Hibiscus tiliaceus* leaf extract

Peak	Retention time (minute)	Area (%)	Compound	Formula	Molecular weight (g/mol)
1	3.301	2.68	(Diethoxy) Methyl Ester of Acetic Acid	$C_7H_{14}O_4$	162.0
2	3.409	0.76	2H-Pyran-2-one (CAS) 2-Pyrone	$C_5H_4O_2$	96.1
3	3.531	0.89	2-Furanmethanol (CAS) Furfuryl alcohol	$C_5H_6O_2$	98.0
4	3.655	1.63	Propane, 1,1-diethoxy-2-methyl	$C_8H_{18}O_2$	146.0
5	4.387	3.71	Butane, 1,1-diethoxy-3-methyl-	$C_9H_{20}O_2$	160.0
6	6.722	3.78	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144.0
7	7.113	1.35	1,4,4-Trimethyl-8-oxa-bicyclo[3.2.1]oct-6-en-one	$C_{10}H_{14}O_2$	166.0
8	7.666	10.64	Benzaldehyde, 4-methyl- (CAS) p-Tolualdehyde	C_8H_8O	120.0
9	16.682	0.96	Octadecanoic acid	$C_{18}H_{36}O_2$	284.0
10	16.801	2.64	Pentadecanoic acid, 14-methyl-, methyl ester (CAS)	$C_{17}H_{34}O_2$	270.0
11	17.296	17.46	Hexadecanoic acid (CAS) Palmitic acid	$C_{16}H_{32}O_2$	256.0
12	18.314	1.65	Cyclohexanol, 5-methyl-2-(1-methylethyl)-	$C_{17}H_{26}O$	246.4
13	18.500	1.25	11,14-Eicosadienoic acid, methyl ester (CAS)	$C_{21}H_{38}O_2$	322.0
14	18.542	2.09	9,12,15-Octadecatrienoic acid, methyl ester,	$C_{19}H_{32}O_2$	292.0
15	18.822	42.72	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,(CAS) Phytol	$C_{20}H_{40}O_2$	296.0
16	18.986	5.78	9-Octadecenoic acid (Z)- (CAS) Oleic acid	$C_{18}H_{34}O_2$	282.0

Bacterial inhibition

HELE inhibited the growth of the tested bacteria at the MIC of 10.00 mg/mL against *C. violaceum* and *S. aureus* and 160 mg/mL against *P. aeruginosa*. The MBC was twice as high as the MIC (20 mg/mL) against *C. violaceum* and *S. aureus*, whereas that against *P. aeruginosa* remained to be determined (Table 2). In the presence of ½ × the MIC of HELE, *C. violaceum* was unable to grow rapidly within the first 4 h. However, the bacterial count increased sharply after that time point and reached the untreated control level at the end of the incubation period. When treated with 1 × MIC, *C. violaceum* failed to grow during the first 8 h and was eliminated afterward completely. No increase in CFU was observed in 2 x MIC-treated cultures for the first 2 h, followed by total elimination after that time point (Figure 2A). Similarly, *S. aureus* could not grow during the first 2 h when incubated with ½ x MIC of the extract; however, the bacterial population consistently increased afterward, reaching the growth level of untreated control over 24 h of the incubation. About one log reduction in CFU was noted for *S. aureus* at 1 x MIC and 2 x MIC within the first 4 h of the incubation; however, the bacterial growth consistently elevated by approximately two logs in 1 x MIC-treated culture and eliminated completely in 2 x MIC-treated culture after that time point (Figure 2B). *Pseudomonas aeruginosa* was notably unable to grow within the first 2 h of incubation in the presence of all tested HELE concentrations; however, the CFU number then increased by two logs at 1/21 x MIC and by one log at both 1/11 x MIC and 1/5 x MIC over 4 h of the incubation; after that time, the bacterial population consistently escalated and reached the untreated control level at the end of the incubation period (Figure 2C).

Quorum sensing inhibition

The results of disk-diffusion assay exhibited a significant dose-dependent inhibitory activity of HELE on violacein production by *C. violaceum* (Figure 3A). The respective turbid zones formed around the disk loaded with 0.31, 0.63, 1.25, and 2.50 mg/mL of the extract were 5.13 ± 0.12 mm, 11.43 ± 0.12 mm, 12.37 ± 0.12 mm, and 14.70 ± 0.10 mm, which were significantly higher than that of H_2O_2 (3.70 ± 0.00 mm). Furthermore, the result of the quantitative study revealed 57.5, 64.8, and 74.1% violacein inhibitions in ½ x MIC, 1 x MIC, and 2 x MIC of HELE-treated cultures, respectively, as compared to the untreated control, whereas H_2O_2 inhibited the pigment production by 52.9% (Figure 3B). These findings suggest a significant effect of HELE on violacein production, which seems to have potential as a source of a novel anti-QS agent.

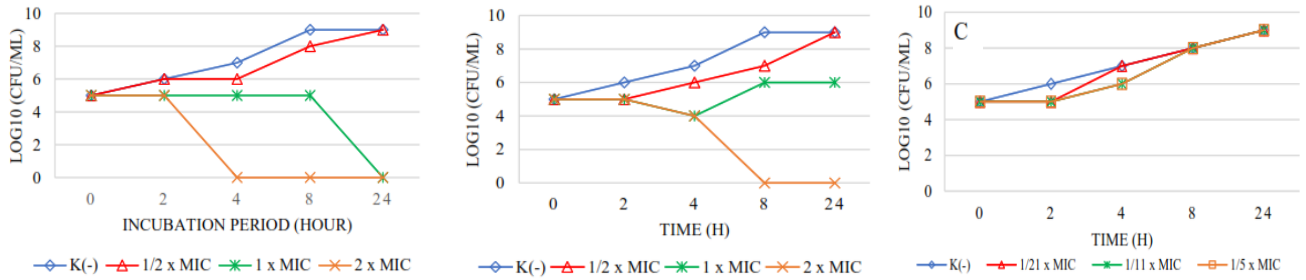
In terms of its effect on pyocyanin, HELE significantly reduced pyocyanin production by approximately 24.08, 23.74, and 59.23% in cultures supplemented with 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC of HELE, respectively, whereas H_2O_2 exhibited a lower inhibitory effect (12.23%) (Figure 4). This justifies the better anti-QS potential of HELE compared to the available conventional quorum quenching agent. Reduced swarming motility of *P. aeruginosa* PA01 was observed in the presence of HELE. The average diameter of the swarming zone over the extract-free semi-solid agar was 21.37 ± 2.10 mm. In contrast, those on 1/21 x MIC, 1/11 x MIC, and 1/5 x MIC HELE-supplemented medium were 7.70 ± 0.26 mm, 5.83 ± 1.04 mm, and 5.13 ± 0.06 mm, respectively, indicating approximately 63.97, 72.72, and 75.92% swarming activity inhibition at the respective HELE concentrations as compared with the untreated control (Figure 5).

Table 2. MIC and MBC values of HELE against target bacteria

Bacterial strains	MIC (mg/mL)	MBC (mg/mL)
Chromobacterium violaceum ATCC122472	10.00	20.00
Staphylococcus aureus ATCC 29213	10.00	20.00
Pseudomonas aeruginosa PA01	160.00	ND

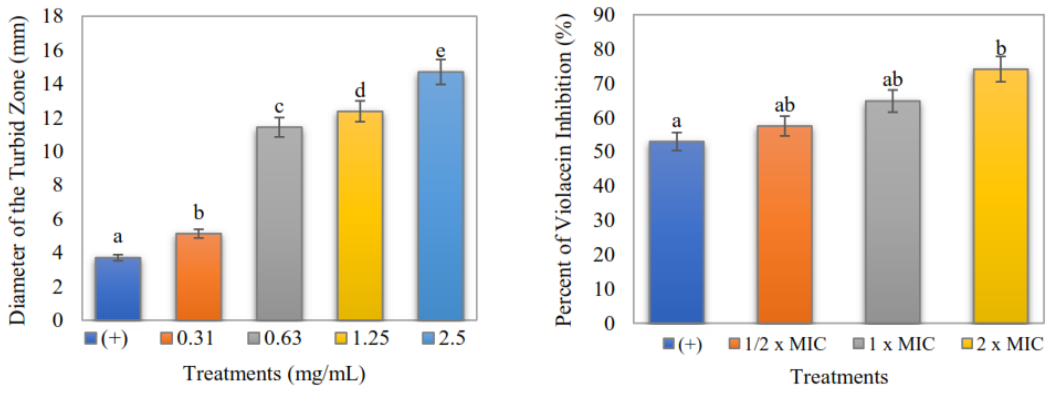
Note: ND: Not detected

The results of biofilm inhibition assay revealed that HELE at all sub-MIC concentrations (7.5, 15, and 30 mg/mL) could not interfere with biofilm formation by *P. aeruginosa*. The addition of 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC of HELE triggered *P. aeruginosa* biofilm formation by 3-, 7-, and 8-fold, respectively, compared to the untreated control, suggesting a significant induction of biofilm formation by HELE in a dose-dependent manner. H_2O_2, on the contrary, exhibited inhibitory activities toward *P. aeruginosa* biofilm formation by 81.9% (Figure 6A). Similarly, HELE at ½ × MIC and 1 × MIC stimulated *S. aureus* biofilm formation by 5- and 1.5-fold, respectively, compared to the extract-free control. The inhibitory effect of the extract against *S. aureus* biofilm formation was observed at 2 × MIC (54.3%), whereas H_2O_2 inhibited biofilm formation by 70.7% (Figure 6B).



A **B** **C**

Figure 2. Time-dependent inhibitory activities of HELE towards: A. *C. violaceum*; B. *S. aureus* in the presence of ½ x MIC (5 mg/mL), 1 x MIC (10 mg/mL), and 2 x MIC (20 mg/mL), and C. *P. aeruginosa* in the presence of 1/21 x MIC (7.5 mg/mL), 1/11 x MIC (15 mg/mL), and 1/5 x MIC (30 mg/mL). (-): growth control (LBB without the addition of HELE)



A **B**

Figure 3. The inhibition of violacein production of *C. violaceum*: A. Diameter of turbid zones in disk-diffusion assay, indicating anti-QS activities of HELE at various concentrations; B. The percentage of violacein inhibition (quantitative study) in the presence of ½ x MIC, 1 x MIC, and 2 x MIC of the extract. (+): positive control (3% H_2O_2). Different superscripts above each column indicated statistically significant differences

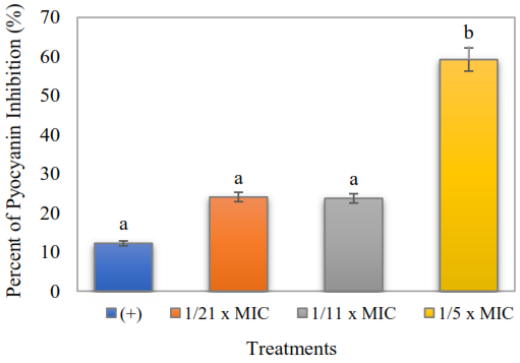


Figure 4. Pyocyanin inhibition of *P. aeruginosa* PA01 in the presence of 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC of HELE. (+) Positive control (3% H_2O_2). Different superscripts above each column indicated statistically significant differences

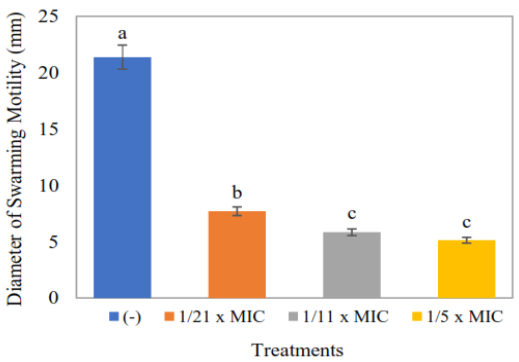


Figure 5. Swarming motility inhibition of *P. aeruginosa* PA01 in the presence of 1/21 × MIC, 1/11 × MIC, and 1/5 × MIC. (-): negative control (extract-free semi-solid LBA + bacteria). Different superscripts above each column indicated statistically significant differences

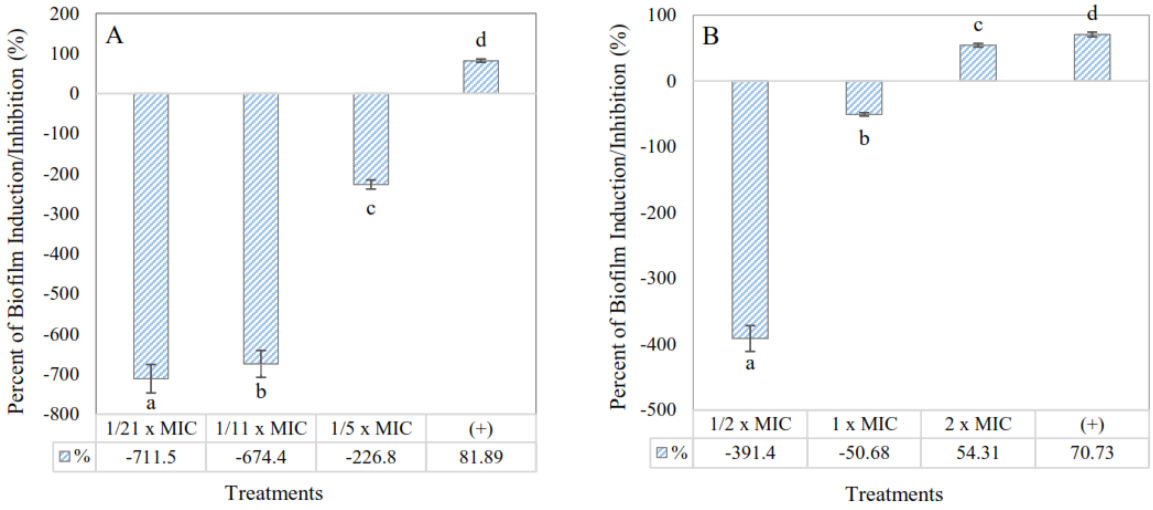


Figure 6. Biofilm formation modulation against (A) *P. aeruginosa* PA01 in the presence of 1/21 × MIC, 1/11 × MC, and 1/5 × MIC of HELE; and (B) *S. aureus* ATCC 29213 in the presence of ½ × MIC, 1 × MIC, and 2 × MIC of the extract. (+) Positive control (3% H_2O_2). The different superscripts in each column indicate statistically significant differences

Discussion

Overcoming bacterial infections is becoming increasingly challenging owing to their increased resistance and virulence (Kothari et al. 2017; Azam and Khan 2019). Therefore, searching for novel antimicrobial agents and developing effective therapeutic strategies, such as targeting QS regulatory mechanisms, continue to be an urgent need in human battles against pathogens. Plant-derived secondary metabolites with widely known antimicrobial properties may become potential sources for new anti-QS agents. *Hibiscus tiliaceus* is one of the plants reportedly rich in bioactive compounds possessing antimicrobial (Samsudin et al. 2019) and antibiofilm properties (Suhartono et al. 2019a), leading to an assumption that *H. tiliaceus* might possess quorum quenching potentials. In this study, we investigated the ability of HELE to interfere with or modulate QS-facilitated virulence factors.

Phytochemical screening revealed the presence of diterpenoids (phytol), aromatic aldehydes (benzaldehyde and 4-methyl- (CAS) p-Tolualdehyde), fatty acids (palmitic and oleic acids), and alcohols (Table 1), which were similar to that reported in a previous study (Nandagopalan et al. 2015). The presence of terpenoids in the ethanolic and methanolic extracts of *H. tiliaceus* leaves has also been reported previously (Andriani et al. 2017; Suhartono et al. 2019). Phytol is the most abundant diterpenoid component detected in HELE, which has been reported to induce ROS accumulation in *P. aeruginosa*, leading to oxidative stress-associated DNA damage and cell death (Lee et al. 2016). Phytol was also reported to be capable of inhibiting pyocyanin and biofilm formation of *P. aeruginosa* PA01 at concentrations below the MIC (Pejin et al. 2015) and reducing the swarming motility of *Serratia marcescens* through the inhibition of QS-regulated gene expression (Srinivasan et al. 2017).

In the current study, HELE exhibited antibacterial activity against Gram-positive and Gram-negative bacteria at the lowest concentration of 10 mg/mL against *S. aureus* and *C. violaceum*, and 160 mg/mL against *P. aeruginosa* PA01 (Table 2). These MICs were slightly higher than the maximum effective concentration (≤8 mg/mL) recommended for crude extracts (Ncube et al. 2012) and were also higher than the MICs (0.25-2.5 mg/mL) reported in previous investigations for *H. tiliaceus* methanol extracts against various Gram-positive and Gram-negative bacteria (Hemaiswarya et al. 2009; Wong et al. 2010), suggesting a slightly lower antibacterial potency of HELE in the current study. A lower MIC value (20 mg/mL) was also reported for *H. tiliaceus* leaf water fraction against *P. aeruginosa* (Andriani et al. 2023). Several factors, including the polarity of extraction solvents, extraction methods, target bacteria used in each study, and types of phytochemical constituents contained in *H. tiliaceus* plant parts, might have been associated with this heterogeneity in the MIC values of *H. tiliaceus* among studies (Mostafa et al. 2018). However, similar MICs (10, 8-10, and 150 mg/mL) as those obtained in the present study have been reported in several previous investigations for ethanol leaf extract of *Moringa oleifera* against *C. violaceum* (Suhartono et al. 2019), ethanol extracts of various plant species against *S. aureus* (Bussmann et al. 2010), and ethanol and chloroform leaf and stem extracts of *Viscum album* L. against various strains of *P. aeruginosa* (Erdönmez et al. 2018), respectively.

Regarding the inhibitory rates of HELE on bacterial growth, HELE at sub-MICs showed different inhibitory levels on the growth of all the tested bacteria; however, the inhibitory effect did not persist until the end of the incubation period (Figure 2A-C). Fluctuating inhibitory rates throughout the incubation period were manifested at 1 × MIC against *C. violaceum* and *S. aureus*, whereas 2 × MIC exhibited a complete killing effect on both bacteria

over different hours of incubation (Figure 7 A-B). This demonstrates the dose-dependent bacterial inhibitory activity of HELE against the tested bacteria.

The QS inhibitory effect of HELE on *C. violaceum* was determined by evaluating violacein pigment production. The presence of turbid zones around the disks in the qualitative assay indicated the anti-QS activity of the extract. HELE inhibited violacein production at a very low extract concentration (0.31 mg/mL or 1/32 × MIC) without affecting bacterial growth, confirming the quorum-quenching potential of the extract (Figure 3A). Furthermore, the results of the quantitative study revealed that HELE suppressed violacein production by more than 57% at a concentration of ½ × MIC, confirming the potency of HELE to interfere with the bacterial QS mechanism. In contrast to previously reported significant dose-dependent violacein inhibitory patterns of *M. oleifera* (Suhartono et al. 2019) and *Nymphaea tetragona* (Hossain et al. 2015), the results of the present study indicated insignificant concentration dependence. This variety of findings might be attributed to the differences in the bioactive components contained in each plant extract. QS-regulated violacein production involves the activation of *the vio*ABCDE operon consisting of five genes (*vio*A, *vio*B, *vio*C, *vio*D, and *vio*E), transcription regulators (CviI/CviR), and AHL signaling (Kothari et al. 2017). Thus, bioactive constituents in HELE are assumed to affect the regulation of this operon system by either interfering with signal molecule synthesis, mimicking the structure or function of this signal, or suppressing the expression of related genes. As previously reported, suppressing the expression of *the cvi*I gene to inhibit AHL synthesis (Champalal et al. 2018), repressing the expression of the *vio*A gene (Devescovi et al. 2017), or the presence of anti-QS molecules resembling the structure and function of AHL can inhibit violacein production without restricting bacterial growth (Vasavi et al. 2013; Subramanian et al. 2022).

Pyocyanin production and swarming motility in *P. aeruginosa* are among QS-based biological processes. Pyocyanin is a toxic blue compound of the phenazine group that causes damage to the host cells during *P. aeruginosa* infection (Hall et al. 2016; Azam and Khan 2019), whereas swarming motility is multicellular rapid and coordinated locomotion of flagellated bacteria over a semi-solid surface (Rütschlin and Böttcher 2020), contributing to higher bacterial resistance to antibiotic (Finkelshtein et al. 2015; Rütschlin and Böttcher 2020). In the presence of ½ × MIC of HELE, pyocyanin production and swarming motility were significantly reduced. This led to an assumption that the extract might possess *rhl*-inhibitory properties, hampering the expression of QS-associated virulence factors. As elucidated in previous investigations, QS mechanisms in *P. aeruginosa* are modulated through the *rhl* system, which regulates the expression of the phenazine operon (*phz*1 and *phz*2) for the production of pyocyanin (Azam and Khan 2019) and activation of *the rhl*AB operon for the expression of rhamnolipid and 3-hydroxyalconics acid (HAA) required for swarming activity (Pollitt and Diggle 2017). Besides affecting the synthesis of biosurfactants, HELE inhibitory effects on the swarming motility of *P. aeruginosa* might also be associated with the disruption of flagella-related processes. Furthermore, both pyocyanin and swarming inhibition in the present study were dose-dependent compared to the previous study's findings (Hossain et al. 2015), underlining the promising anti-QS potential of HELE.

The effect of HELE on biofilm formation against both Gram-negative and Gram-positive bacteria was also assessed in this study, and unlike its inhibitory effect on the production of violacein, pyocyanin, and swarming motility, HELE at sub-MICs demonstrated a significant stimulating effect on biofilm formation by *P. aeruginosa* and *S. aureus*. A triggering effect was also observed at 1 × MIC against *S. aureus*; however, at a higher concentration (2 × MIC), the extract exhibited biofilm inhibitory activity against this Gram-positive bacterium. Such phenomena have been reported previously in several investigations, describing that at sub-inhibitory concentrations, plant-derived secondary metabolites and antibiotics could trigger biofilm formation of various Gram-negative and Gram-positive bacteria, including *P. aeruginosa* and *S. aureus* (Vacheva et al. 2011; Plyuta et al. 2013; Elken et al. 2022) as their response to stress (Kumar and Ting 2013; Ranieri et al. 2018; Alves de Lima e Silva et al. 2021). Subinhibitory antibiotics have been proposed to induce phage elements in *P. aeruginosa* and *S. aureus* and regulates programmed-cell death owing to environmental stress, leading to explosive release of eDNA and cellular contents as components of biofilm matrix, and thereby seeds biofilm formation. Another suggested mechanism is by triggering the production of cyclic dimeric guanosine monophosphate (c-di-GMP) that regulates gene expression, including those encoding cell surface properties. This may lead to an alteration and upregulation of adhesion-associated proteins, causing increased bacterial adherence that serves as a prominent initial step in biofilm formation (Ranieri et al. 2018). It is assumed that HELE might contain bioactive constituents, which, at a low concentration, would trigger biofilm formation by the respective bacteria through one or more scenarios mentioned above. In addition, the fact that HELE did not stimulate, indeed, attenuated the swarming motility of *P. aeruginosa* at sub-MICs, stimulation of biofilm formation in the present study was presumably not associated with bacterial swarming activity.

In conclusion, HELE in the present study exhibited slightly low antibacterial potency against all tested bacteria, particularly *P. aeruginosa*. However, the extract effectively inhibited QS-controlled virulence factors, including the production of violacein, pyocyanin, and swarming motility, highlighting promising HELE potential as a novel quorum-quenching compound. Furthermore, the stimulating effects of the extract on biofilm formation by *P. aeruginosa* and *S. aureus* at sub-inhibitory concentrations justify the potency of HELE not only as an anti-QS but also as a QS-modulating agent, rendering a new insight into its application in combatting bacterial resistance and pathogenicity. Further investigations should be carried out to identify the specific bioactive constituents of the extract

responsible for QS interference and their molecular mechanisms of action in bacterial QS suppression.

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