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Inhibitory effects of *Tilia cordata* aqueous and ethanolic extracts on *Pseudomonas aeruginosa* biofilm, virulence factors and quorum sensing: phenotypic and gene expression analysis

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Abstract

Aim: This study was designed to evaluate the anti-quorum sensing (QS), anti-biofilm, and anti-virulence properties of *Tilia cordata* (*T. cordata*) leaf extracts against *Pseudomonas aeruginosa*.

Methods: Ethanol (EtOH) and aqueous (H_2O) extracts were prepared from *T. cordata* leaves. Minimum inhibitory concentrations (MICs) were determined via broth microdilution. Sub-MIC effects on biofilm development, bacterial viability inside the biofilm matrix [using the 2,3,5-triphenyltetrazolium chloride (TTC) assay], initial adherence phenotypes [swarming, aggregation, hydrophobicity, exopolysaccharide (EPS)], and virulence factor secretion (pyocyanin, rhamnolipids, LasA protease, chitinase) were quantified. Relative expression of core QS circuit genes (*lasI*, *lasR*, *rhlI*, *rhlR*) was evaluated by RT-qPCR.

Results: Both extracts exhibited an identical MIC of 4.8 mg/mL. At sub-MIC levels (1.2–1.4 mg/mL), both extracts achieved 95–100% biofilm inhibition [minimum biofilm inhibitory concentration ($MBIC_{50}$) of 0.15 mg/mL for H_2O and 0.3 mg/mL for EtOH] with minimal viability loss (9.7%). Sub-MIC treatments significantly suppressed pyocyanin (up to 57.1% inhibition) and rhamnolipids (up to 66.1% inhibition), while inhibiting LasA protease by up to 83.3% and chitinase in a dose-dependent manner. Swarming motility, aggregation (reduced from 31.5% to 9.1%), surface hydrophobicity, EPS, and *N*-acyl-homoserine lactone (AHL) synthesis were significantly suppressed. RT-qPCR revealed that the EtOH extract (0.6 mg/mL) induced significant transcriptional downregulation of *lasI* (0.02-fold), *lasR* (0.16-fold), *rhlI* (0.29-fold), and *rhlR* (0.12-fold) ($p < 0.01$).

Conclusions: Although both extracts exhibit robust phenotypic antibiofilm and anti-virulence efficacy, their underlying modes of action diverge: the ethanolic extract functions through the transcriptional repression of core QS regulatory genes (*lasI*, *lasR*, *rhlI*, and *rhlR*), whereas the H_2O extract appears to mediate its effects primarily via post-transcriptional or non-transcriptional pathways. These findings highlight the potential of *T. cordata* as a therapeutic adjuvant, natural food preservative, and anti-biofilm sanitizing agent in food processing environments.

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Keywords

quorum sensing, *Pseudomonas aeruginosa*, *Tilia cordata*, biofilm, virulence factors, exopolysaccharide, acyl homoserine lactone

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a major causative agent of many fatal nosocomial infections [1]. The bacterium is responsible for several human diseases, such as urinary tract and burn-related infections. Infections caused by *P. aeruginosa* are known to be persistent, a direct result of their resistance to antibiotics and ability to form biofilm [2]. Furthermore, the pathogenicity of *P. aeruginosa* is attributed to the presence of numerous virulence factors, the production of which relies on a set of quorum sensing (QS) systems, including Las, Rhl, and pseudomonas quinolone signal (Pqs). Virulence factors such as pyocyanin, elastase B, exotoxin A, proteases, and hydrogen cyanide are considered the major contributors to *P. aeruginosa* pathogenicity [3]. In QS systems, activation of the Las pathway leads to the production of the homoserine lactone autoinducer. This autoinducer, in turn, activates the LasR quorum-sensing circuit, which stimulates the expression of three genes, namely *lasB*, *apr*, and *toxA*. Initiation of Las regulon positively activates the Rhl system; the latter's role is amplified through the production of various autoinducers, which in turn activate a set of genes responsible for producing virulence factors called rhamnolipids and pyocyanin. The final QS system—Pqs system—is responsible for producing quinolones; these, in turn, act as signaling molecules that maintain regulatory links between the other components of the QS system in *P. aeruginosa* [4]. While the in-depth study and investigation of the aforementioned systems have yielded promising results for the development of innovative antibacterial therapies, the challenge of antibiotic resistance in *P. aeruginosa* complicates the management of infections caused by this bacterium. Consequently, researchers are rapidly pursuing innovative approaches to identify antimicrobial agents capable of overcoming this resistance with minimal host toxicity. The use of effective QS inhibitors reduces bacterial pathogenicity and the capacity to cause disease, thereby enabling the host's immune system to combat the infection efficiently. In general, the management of bacterial infections should include the prescription of anti-inflammatory agents, as the progression of the disease depends on both bacterial pathogenicity and the body's immune response. The importance of prescribing anti-inflammatory medicines lies in the fact that an excessive immune response in the body can lead to edema, organ failure, and systemic hyperthermia. Using Anti-inflammatory medications can mitigate the compounded effects of bacterial infection and the body's immune response, thereby breaking the cycle linking the two [5].

Vast arrays of plant-based remedies have been utilized for centuries in order to combat bacterial infections and minimize inflammation. There is a prevailing belief that these substances possess a low level of toxicity, hence rendering them suitable for therapeutic utilization [6]. In addition to their novel immunosuppressive activity, natural product compounds possess the capability to disrupt the QS system and, ultimately, attenuate the mechanism of bacterial pathogenicity without influencing the viable bacterial population. *T. cordata*, often known as the lime tree or Zezafoon, is a member of the Malvaceae family of trees. Usually, the leaves are brewed into an herbal tea to treat catarrh, coughing, and common cold symptoms. The extracts of *T. cordata* have a variety of secondary metabolites such as *p*-coumaric, caffeic, chlorogenic acids, quercetin, isoquercitrin, kaempferol, myricetin, hyperoside, citral, eugenol, citronellol, menthone, limonene, alpha-pinene, and nerol [7, 8]. Additionally, numerous pharmacological studies have shown that the isolated chemicals and extracts from *T. cordata* exhibit a wide range of biological activity, supporting their extensive and varied traditional usage. According to pharmacological research, *T. cordata* has anti-fungal, anti-inflammatory, anti-viral, and antioxidant properties [9, 10]. While our previous report indicated that *T. cordata* possesses the capability to prevent the formation of *P. aeruginosa* biofilm [11], the underlying mechanisms remained largely uncharacterized. Specifically, the precise molecular mechanisms, the impact on key extracellular virulence phenotypes (such as pyocyanin secretion and swarming motility), and the direct regulatory effects on the core QS circuit genes (*lasR*/*I*, *rhlR*/*I*, and *pqsA*) remained completely

unexplored. The present study was therefore designed to systematically investigate these phenotypic and transcriptional targets to establish the anti-virulence and quorum-quenching mode of action of *T. cordata* polar extracts.

Materials and methods

Plant materials and extraction

Tilia cordata (*T. cordata*) leaves were obtained from a local herbalist market located in the city of Mu'tah, AlKarak, Jordan. A sample of the plant's leaves has been officially deposited as a voucher sample under the identification code M121B at the Department of Biological Sciences, Mutah University, Jordan. The outer surfaces of the plant leaves were cleaned, then dried in the shade, and subsequently ground into a fine powder. To prepare the ethanolic extract, 250 g of the plant leaf powder was soaked in 600 mL of 95% ethanol (EtOH); meanwhile, to prepare the aqueous (H_2O) extract, the same amount of the plant powder was soaked in 600 mL of distilled water. Both mixtures were incubated at ambient temperature for 24 hours. Both extracts were filtered to remove the insoluble leaf powder residue; the ethanolic and H_2O *T. cordata* leaf extract filtrates were collected and subjected to evaporation using a rotary evaporator. The resulting crude residues from both extracts were stored at 4°C [12]. A total crude yield of 11.4% was obtained from the ethanolic leaf extract, and 6.8% from the H_2O extract, based on the initial dry weight of the plant leaf powder used.

Bacterial strains

The *P. aeruginosa* and *Staphylococcus aureus* (*S. aureus*) strains used in this study were obtained from AlKarak Government Hospital, Jordan. These isolates were recovered from clinical urine samples and identified using the Biomérieux VITEK® 2 system. *Chromobacterium violaceum* (*C. violaceum*) ATCC 12472 was obtained from the American Type Culture Collection (ATCC).

Effect of *T. cordata* ethanolic and H_2O extracts on the growth of P. aeruginosa planktonic cells

Disc diffusion method

This assay was conducted to evaluate the antibacterial efficacy of *T. cordata* extracts. Briefly, a 0.5 McFarland suspension was used to prepare bacterial cultures with 1×10^8 CFU/mL. Using a sterile swab, approximately 100 µL of the bacterial suspension was spread onto the surface of Petri dishes containing Mueller-Hinton agar. Discs containing 1 mg of the extracts were placed on the inoculated agar surfaces using sterile forceps under aseptic conditions. The diameter of the inhibition zone was measured after incubating the plates for 24 hours at 37°C. All experiments were performed in triplicate for each extract.

Minimum inhibitory concentration (MIC)

The steps of this experiment were performed as described by Jaafreh et al. [13], with the microdilution method modified to estimate the MIC. Using 96-well plates, two fold serial dilutions of the leaf extracts (10 mg/mL) were performed to obtain concentrations of 4.8, 2.4, 1.2, 0.6, 0.3, 0.15, 0.08, 0.04, and 0.02 mg/mL. Following this, 10 µL of bacterial suspension containing 5×10^4 CFU/wells was cultured in the plate wells for analysis. The organic solvent DMSO (10%) was diluted using the same method as the plant extracts and served as a negative control. After incubating the plates with shaking for 24 hours at 37°C, absorbance readings of the cultured wells in the 96-well plates were taken using an ELISA reader at a wavelength of 600 nm. Subsequently, the net growth was quantified, and the MIC was determined as the concentration of the tested extracts that exhibited a 95% inhibition of bacterial growth.

Screening for antibiofilm and anti-QS activity

Antibiofilm activity

The crystal violet method was used to evaluate the anti-biofilm activity of the prepared extracts. The extracts were tested at sub-MIC concentrations (0.04–1.2 mg/mL) using the same procedure previously employed to determine the MIC in 96-well plates inoculated with bacteria. Wells containing culture

medium and the test extracts at various concentrations-but lacking the bacterial suspension-served as blanks. After 24 hours of incubation at 37°C, the contents of the wells were discarded, and the wells were rinsed three consecutive times with tap water. Subsequently, 150 µL of crystal violet was added to the wells; they were incubated for 15 minutes, after which the crystal violet was discarded. This step is followed by introducing 200 µL of 95% EtOH into the wells to dissolve the crystal violet remaining after the liquid was discarded; this incubation lasts for 15 minutes. After the 15 minutes, 150 µL of the EtOH from the wells is transferred to a clean 96-well plate, and the absorbance is measured at 590 nm.

Minimum biofilm inhibitory concentration ($MBIC_{50}$) values were reported as a percentage of biofilm formation inhibition as follows:

MBIC inhibition (%) = $[(OD_{590,\ control} - OD_{590,\ extracts})/OD_{590,\ control}] \times 100$

$MBIC_{50}$ was determined to be the lowest concentration of the studied extracts needed to prevent producing 50% of the biofilm.

Effect on the production of violacein

The well-diffusion method was employed to determine the efficacy of *T. cordata* leaf extracts in inhibiting violacein production by *C. violaceum* [14]. Briefly, the method involved using a bacterial suspension adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/mL). A 100 µL aliquot of this suspension was mixed with 30 mL of molten Mueller-Hinton agar and poured into Petri dishes. Once the agar solidified, 6 mm-diameter wells were created and filled with 100 µL of Mueller-Hinton broth containing 1 mg of *T. cordata* extracts. Following a 24-hour incubation period at 37°C, the diameters of the zones of inhibition produced by the extracts were measured. Testing was performed in triplicate for each extract.

To quantitatively assess anti-QS activity, violacein pigment production was evaluated using *C. violaceum* according to Cheng et al. [15]. Following exposure to the extracts, bacterial cells were harvested by centrifugation, and the supernatant was discarded. The pellet was resuspended in 1 mL of DMSO and incubated for 3 h at ambient temperature to extract cell-bound violacein. After re-centrifugation, the absorbance of the violacein-enriched DMSO fraction was recorded at 575 nm. The relative inhibition of violacein synthesis was then computed against untreated controls to quantify QS disruption.

Effect on the viable cells in *P. aeruginosa* biofilm matrix

The extracts were tested at sub-MIC concentrations (0.15–1.2 mg/mL) using the same procedure previously employed to determine the anti-biofilm activity in 96-well plates inoculated with bacteria [16]. Wells containing culture medium and the test extracts at various concentrations-but lacking the bacterial suspension-served as control experiments. After 24 hours of incubation at 37°C, the contents of the wells were discarded, and the wells were rinsed three consecutive times with tap water to flush un-adhered cells. Subsequently, 200 µL of Mueller-Hinton broth medium containing 0.2% (w/v) glucose and 50 µL of 2,3,5-triphenyltetrazolium chloride (TTC) solution (5 mg/mL) were carefully put into each individual well; the 96-well plates were incubated for 6 hours at 37°C and 150 rpm, after which the absorbance was measured at 405 nm. The percentage of cell viability reduction was calculated as follows:

Cell viability reduction (%) = $[(OD_{405,\ control} - OD_{405,\ extract})/OD_{405,\ control}] \times 100$

Effect of *T. cordata* ethanolic and H_2O extracts on the *P. aeruginosa* virulence factors

Pyocyanin assay

This experiment was conducted following the steps of the method described by Hossain et al. [17], with some specific modifications. Briefly, 4.5 mL of chloroform was added to 7.5 mL of the supernatant portion of *P. aeruginosa* cultures after centrifugation and treatment with various concentrations of *T. cordata* leaf extracts. After the formation of a blue-green layer on the surface of the mixture and its transfer to a clean test tube, 1.5 mL of hydrochloric acid (0.2 M) was added to a 3 mL fraction of the resulting blue-green mixture. As a result of the preceding chemical treatment, a pink solution appeared. The pyocyanin content

in this pink solution was quantified by measuring the absorbance at 520 nm. The percentage of pyocyanin production was reported as follows:

Pyocyanin production (%) = $[(OD_{520, extract})/OD_{520, control}] \times 100$

Supernatant of *P. aeruginosa* culture without treatment with *T. cordata* leaf extracts served as the control experiment.

Rhamnolipids assay

The orcinol technique was adopted to assess the amount of rhamnolipids content produced in the culture medium [18]. Briefly, 3 mL of diethyl ether was added to 1 mL of *P. aeruginosa* cell free supernatant treated with different concentrations of ethanolic and H_2O extracts of *T. cordata* leaves. Following the addition, centrifugation was performed, and the diethyl ether layer that formed on top of the mixture was collected in a clean tube. The diethyl ether solvent was evaporated using a rotary evaporator, and the remaining material was dissolved in 200 µL of distilled water. In a subsequent step, 900 µL of orcinol solution prepared at a concentration of 0.18% (w/v) in 53% (v/v) sulfuric acid (H_2SO_4) was introduced, and the resulting mixture was subjected to boiling. Following a 30-minute incubation period at ambient temperature, the optical density at a wavelength of 421 nm was measured and used to quantify the percentage of rhamnolipid production. The percentage of rhamnolipid production was calculated as follows:

Rhamnolipids production (%) = $(OD_{421, extract}/OD_{421, control}) \times 100$

Supernatant of *P. aeruginosa* culture without treatment with *T. cordata* leaf extracts served as the control experiment.

LasA staphylolytic assay

LasA protease activity was assessed using the LasA staphylolytic assay [19]. A culture of *S. aureus* was prepared in 50 mL of Mueller-Hinton liquid medium. After 24 hours of incubation, the bacterial culture was boiled for 10 minutes, followed by centrifugation. The precipitate was then collected and suspended in Na_2HPO_4 (10 mmol). In parallel with the aforementioned experiment, *P. aeruginosa* was cultured in Mueller-Hinton medium with and without (control) treatment with different concentrations of *T. cordata* leaf extracts. At the end of the incubation period, the cultures were centrifuged. After collecting the supernatant obtained from centrifugation, 100 µL of the *P. aeruginosa* culture supernatant was added to 900 µL of the prepared *S. aureus* solution. The mixture of both was then incubated for 60 minutes, followed by absorbance measurement at 600 nm, and the LasA staphylolytic inhibition percentage was calculated as follows:

LasA staphylolytic inhibition (%) = $[(OD_{600, control} - OD_{600, extract})/OD_{600, control}] \times 100$

Evaluation of LasA protease activity

In this section, the total nonspecific activity of the LasA protease enzyme was calculated [20]. One milliliter of cell-free supernatant obtained from *P. aeruginosa* culture treated with various doses of *T. cordata* extracts was combined with 5 mL of 0.65% (w/v) casein solution prepared in 50 mM Tris-HCl buffer (pH 7.8). Following the mixing process, the mixture was incubated at 37°C for 30 minutes. Then, 5 mL of pre-cooled 10% (w/v) trichloroacetic acid solution was added, and the reaction was halted by maintaining the temperature at 35°C for 30 minutes. After centrifugation for 5 minutes at 10,000 rpm, 5 mL of 0.5 M sodium carbonate solution and 1 mL of Folin's reagent were added to the resulting supernatant. After the 30-minute incubation period, absorbance readings were taken at 660 nm, and the following equation was used to calculate the LasA protease inhibition percentage:

LasA protease inhibition (%) = $[(OD_{660, control} - OD_{660, extracts})/OD_{660, control}] \times 100$

Supernatant of *P. aeruginosa* culture without treatment with *T. cordata* leaf extracts served as the control experiment.

Evaluation of chitinase activity

The substrate chitin azure was used to evaluate the activity of the chitinase enzyme in this experiment [21]. Chitin azure substrate solution was prepared by dissolving 1.3 mg of the compound in 130 mL of a sodium phosphate buffer (200 mM) at pH 7.0. The mixture was incubated at 37°C and 150 rpm for 7 days with continuous shaking. To measure the activity of the chitinase enzyme, *P. aeruginosa* cell free supernatant treated with different concentrations of *T. cordata* leaf extracts was used. Supernatant of *P. aeruginosa* culture without treatment with *T. cordata* leaf extracts served as the control experiment. 0.5 mL of *P. aeruginosa* supernatant was introduced into a 4.5 mL solution of chitin azure and the resulting mixture was then incubated at 37°C for 24 hours. Following the incubation, the assay mixture was centrifuged at 16,000 rpm for 10 minutes and the OD measurements were recorded at 570 nm. Chitinase inhibition percentage was calculated using the following equation:

$$\text{Chitinase inhibition (\%)} = [(OD_{570,\text{ control}} - OD_{570,\text{ extract}})/OD_{570,\text{ control}}] \times 100$$

Effect of *T. cordata* H_2O and EtOH extracts on biofilm formation

Swarming motility

The effect of *T. cordata* leaf extracts on *P. aeruginosa* swarming motility was determined using swarming agar prepared according to the method described by Krishnan et al. [22]. The test extracts were introduced into the liquefied agar medium (0.5% agar), and upon solidification, *P. aeruginosa* was injected at the central region of the Petri dish. The swarming motility was quantified in millimeters after a 24-hour incubation period at 37°C.

Evaluation of aggregation ability

The aggregation test was performed following the method described by Shanks et al. [23], with minor modifications. After incubating *P. aeruginosa* cultures with various concentrations of *T. cordata* leaf extracts for 24 hours at 37°C, the absorbance of the cultures was measured at 600 nm and recorded as the pre-vortex reading. After taking the pre-vortex reading, the cultures were vortexed for 1 minute, and their OD was measured again at 600 nm and recorded as the post-vortex reading. The percentage of aggregation was calculated according to the following formula:

$$\text{Aggregation (\%)} = [(OD_{600,\text{ post-vortex}} - OD_{600,\text{ pre-vortex}})/OD_{600,\text{ post-vortex}}] \times 100$$

Evaluation of hydrophobicity

Relative *P. aeruginosa* cell surface hydrophobicity, expressed as hydrocarbon separation ratio (FPc), was evaluated as previously documented [24]. The surface hydrophobicity assay for *P. aeruginosa* was performed after cultivation of the bacterial strain in the presence of varying treatments of leaf extracts derived from *T. cordata* for 24 hours at 37°C. The resulting cultures were then further incubated statically at 37°C for 2 h. The OD at 600 nm was measured and reported as initial adherence (Ai). After that, a portion of 1.5 mL of the treated culture was mixed with n-hexadecane and incubated for 15 min. Then the OD at 600 nm was measured and reported as final adherence (Af). The FPc was calculated using the following formula:

$$\text{Hydrophobicity index (FPc \%)} = [(Ai - Af)/Ai] \times 100$$

Evaluation of exopolysaccharides (EPSs) production

To determine the amount of EPS produced by *P. aeruginosa* after treatment with various concentrations of *T. cordata* leaf extracts and by the control experiment (without treatment), the phenol-sulfuric acid (PSA) assay was used [25]. In summary, part of the free cell supernatant was combined with 3-fold volume cold EtOH and incubated at 4ºC for 24 hours. Following centrifugation (10,000 rpm, 15 minutes), the solid pellet was subsequently suspended in deionized water. One milliliter portion from the extracted EPS was introduced into 1 mL of chilled phenol (5% w/v) and 5 mL of concentrated H_2SO_4. The OD at 490 nm was measured, and EPS inhibition % was calculated using the following formula:

$$\text{EPS inhibition \%} = [(OD_{490,\text{control}} - OD_{490,\text{extract}})/OD_{490,\text{control}}] \times 100$$

Evaluation of *N*-acyl-homoserine lactone (AHL) activity

To calculate the activity of the auto inducer called AHL, the previously published method was followed [26]. Cell free supernatant obtained from *P. aeruginosa* cultures, which were treated with various quantities of leaf extracts from *T. cordata*, was combined with ethyl acetate and subjected to agitation for 10 minutes. The AHL layer that was obtained was thereafter gathered and concentrated using a rotary evaporator. A volume of 40 µL of the extracted AHL was combined with 50 µL of a hydroxylamine solution (2 M) in a 1:1 ratio with NaOH (3.5 M). Following this, 90 µL of a 1:1 mixture of ferric chloride (10% in 4 M HCl) and 95% EtOH was introduced. The OD at 520 nm was measured and used in the following formula to calculate AHL production inhibition%.

$$\text{AHL inhibition (\%)} = [(OD_{520,\text{control}} - OD_{520,\text{extract}})/OD_{520,\text{control}}] \times 100$$

QS genes expression

The impact of *T. cordata* extracts (0.6 mg/mL) on QS gene expression (*rhlR*, *rhlI*, *lasR*, and *lasI*) in *P. aeruginosa* was investigated using the Gentier 48E RT-PCR system (Xi'an Tianlong Science and Technology, China). Total RNA extraction was performed using the Easy Pure RNA (TransGen Biotech Co., Ltd, China). RNA concentrations were measured with a UV/VIS Nano Spectrophotometer (MicroDigital, Korea). To synthesize first-strand cDNA, Easy Script First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd, China) was used. RT-PCR was subsequently conducted using SYBR Green PCR master mix and gene-specific primers listed in Table 1. Each experiment was independently repeated three times. Gene expression data were normalized to the housekeeping gene *rpoD*, which served as an internal control, and the relative gene expression changes were calculated using the ΔΔCt method. Primer efficiencies (> 90%, R^2 > 0.98) and single-peak amplicon specificities were validated via serial cDNA dilutions and melt curves prior to $2^{-\Delta\Delta Ct}$ quantification. These fold changes were compared with those observed in untreated control samples [27].

Table 1. List of primers used in this study.

Gene	Type	Primer sequence
lasI	Forward	5'-TTGCTCGCCGCACATCTG-3'
	Reverse	5'-ATCATCTTCTCCACGCCTAC-3'
lasR	Forward	5'-ACCGTTTCATAGAGTCGGTC-3'
	Reverse	5'-ACCACTGCAACACTTCCTTC-3'
rhlI	Forward	5'-TCTTCATCGAGAAGCTGGGC-3'
	Reverse	5'-ACGATGTAGCGGGTTTGCG-3'
rhlR	Forward	5'-TGATGTCCAACCCGGTCTG-3'
	Reverse	5'-TCGAACTTCTTCTGGATGTTC-3'
rpoD	Forward	5'-ACGAGGAAGAAGAAAGCG-3'
	Reverse	5'-ACCAGCTTGATCGGCATG-3'

Statistical analysis

Data are presented as mean ± standard deviation (SD) and figures were generated utilizing GraphPad Prism (8.0). Data were subjected to normality and homoscedasticity testing prior to parametric analysis. The one-way ANOVA followed by Tukey's honestly significant difference (HSD) post-hoc test was used. For two-group comparisons, Student's two-tailed t-test was applied. Differences were considered statistically significant at $p < 0.05$, with significance levels designated as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Effect of *T. cordata* ethanolic and H_2O extracts on the growth of *P. aeruginosa* planktonic cells

The results of antibacterial effects of *T. cordata* leaf extracts on *P. aeruginosa* cell growth using disc diffusion and MIC assays are shown in Table 2. The results showed weak antibacterial activity for both the H_2O and ethanolic extracts of *T. cordata* leaves. The inhibition zone diameter results were recorded for both extracts as 7 mm, which is consistent with the weak MIC value of 4.8 mg/mL for both extracts.

Table 2. Antibacterial activity of *T. cordata* ethanolic and aqueous extracts against *P. aeruginosa*.

Extracts	Inhibition zone (mm)	MIC (mg/mL)
EtOH	7.0 ± 1.0	4.8 ± 0.0
H_2O	7.0 ± 0.5	4.8 ± 0.0

Inhibition zone diameters (mm) represent the total clear zone diameter inclusive of the filter paper disc (6.0 mm). Values are expressed as mean ± SD of three independent biological replicates performed in triplicate. EtOH: ethanol; H_2O: aqueous; MIC: minimum inhibitory concentration; SD: standard deviation.

Screening for antibiofilm and anti-QS activity of *T. cordata* extracts against *P. aeruginosa*

Antibiofilm activity

The ability of different concentrations at sub-MIC values (0.04–2.4 mg/mL) of H_2O and ethanolic extracts of the *T. cordata* leaves to inhibit biofilm formation by vegetative *P. aeruginosa* cells was evaluated, as shown in Figure 1. The percentage of biofilm inhibition was calculated and compared to that of untreated cells. The control experiment showed no activity in inhibiting biofilm formation by the cells, confirming that the components of the culture medium do not interfere with the crystal-violet staining. The results of bacterial cells treated with extracts indicate that the biofilm formation ability of *P. aeruginosa* was significantly hindered, and that the inhibition was concentration-dependent. The results show a similarity in effect between the ethanolic and H_2O extracts, as both exhibited a 95–100% inhibition of biofilm formation at the tested sub-MIC concentrations of 2.4 and 1.2 mg/mL. The 50% inhibition of biofilm production by cells, recorded at 0.15 and 0.3 mg/mL concentrations of the H_2O and ethanolic extracts, respectively, indicates that both concentrations represent the $MBIC_{50}$. It is worth noting that the results also showed that DMSO (10%), as a negative control, exhibited no inhibitory activity.

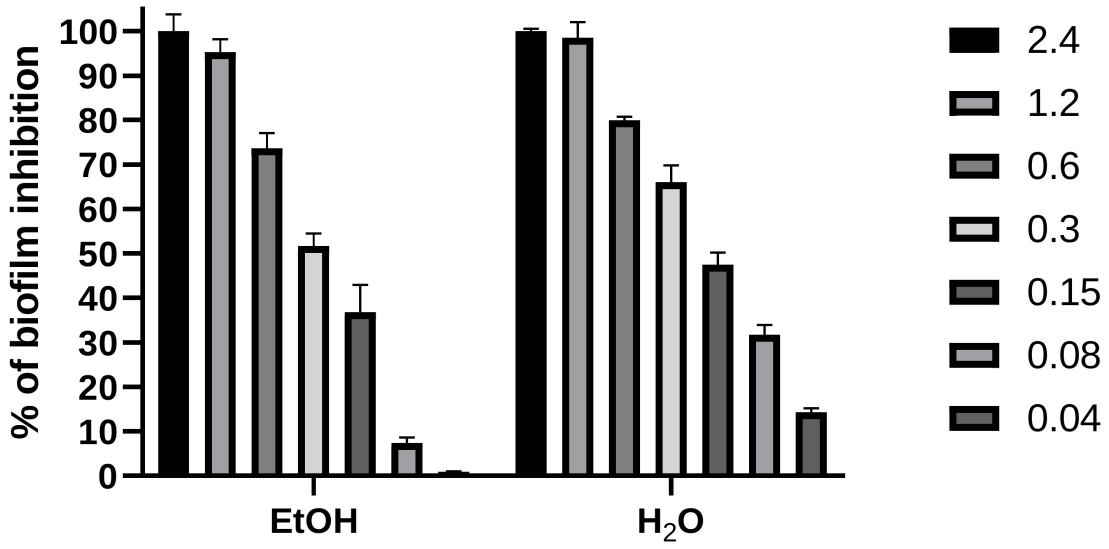


Figure 1. Antibiofilm activity of *T. cordata* extracts (0.04–2.4 mg/mL) against *P. aeruginosa*. The percentage of Biofilm inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Effect of EtOH and H_2O extracts of *T. cordata* on the production of violacein

The results obtained from examining the effect of using 1 mg of leaf extract on violacein production by *C. violaceum*, shown in Table 3 and Figure 2, indicate that both extracts exhibited the ability to inhibit violacein production using the well-diffusion method. The inhibition zone for the H_2O extract was recorded at 13.5 mm, while the zone for the ethanolic extract was 12.0 mm.

Table 3. Effect of *T. cordata* ethanol and H_2O extracts on the production of violacein using well diffusion method.

Extract	mm
EtOH	12.0 ± 0.1
H_2O	13.5 ± 0.3

Data represent as mean ± SD (n = 3). Each well contains 1 mg/well of the tested extract. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

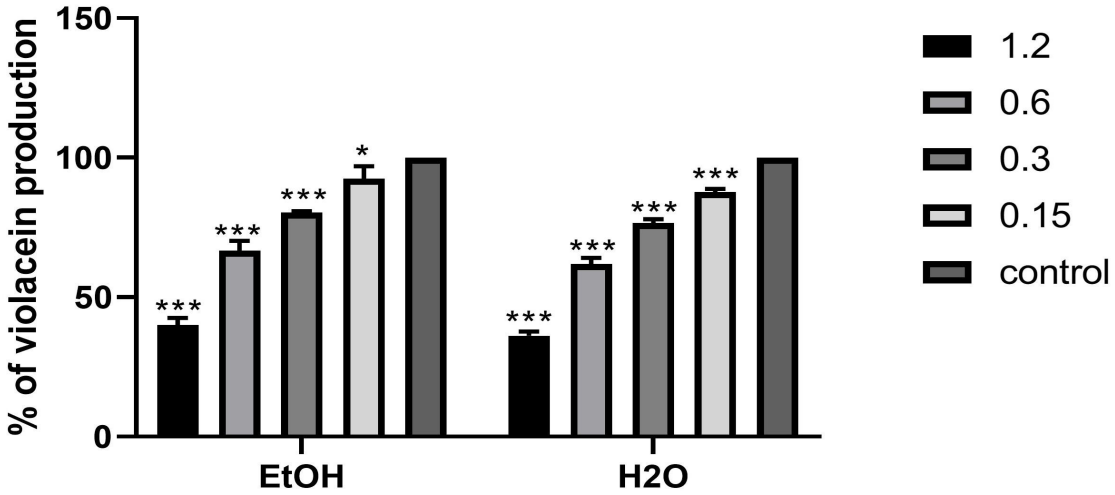


Figure 2. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on the production of violacein. The percentage of violacein inhibition of untreated *C. violaceum* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Quantitative analysis of violacein production inhibition was performed using spectrophotometry. Figure 2 shows a significant reduction in violacein production when *C. violaceum* was treated with *T. cordata* EtOH and H_2O extracts. In the case of the H_2O extract, the results showed a high and significant (p < 0.001) reduction in violacein production, reaching 36.2%, 61.9%, 76.5%, and 87.7%, respectively, when using concentrations of 1.2, 0.6, 0.3, and 0.15 mg/mL. Similarly, the ethanolic extract also had a significant (p < 0.001) and substantial effect, with inhibition percent of 40.1, 66.7, and 80.3 recorded at concentrations of 1.2, 0.6, and 0.3 mg/mL, respectively. Specifically, using a concentration of 0.15 mg/mL of the EtOH extract, the inhibition % was significantly high (p < 0.05), reaching 92.4%.

Effect of *T. cordata* extracts on the viable cells in *P. aeruginosa* biofilm matrix

This test was performed using TTC, which provides a direct association between the concentration of the intact viable cells within the biofilm matrix and the intensity of the red formazan. The wells of the 96-well plate containing the plant extract combined with the growth media exhibited no effect, confirming that the extracts do not chemically reduce TTC. The results disclosed that none of the *P. aeruginosa* cultures treated with different concentrations of *T. cordata* caused a significant reduction in viable cell numbers (Figure 3). The maximum decrease in viable cell number was at the highest concentration tested (1.2 mg/mL), which exerted a 9.7% reduction in viable cells. The minimum reduction of 8.9% was observed for the lowest concentration tested (0.15 mg/mL).

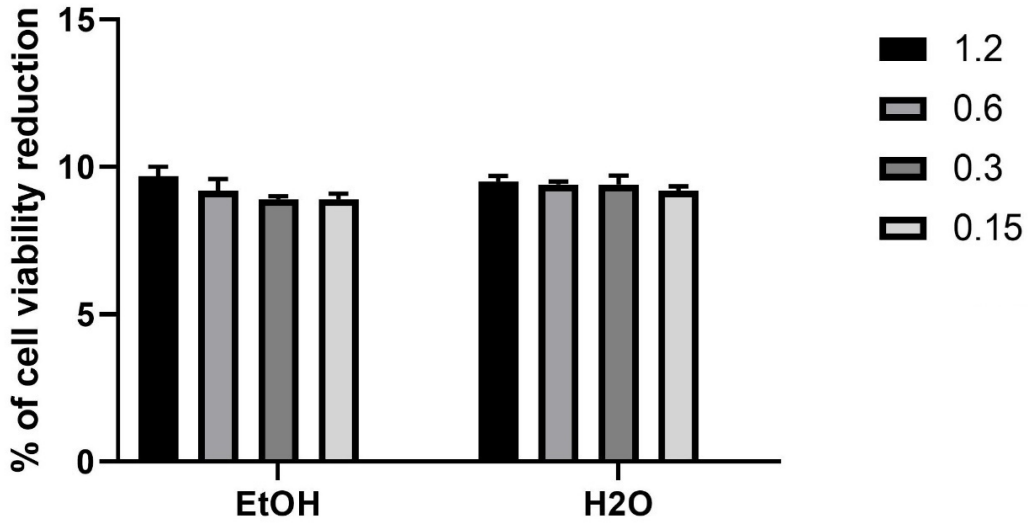


Figure 3. The effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on *P. aeruginosa* viable cells. The percentage of cell viability reduction of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Effect of *T. cordata* EtOH and H_2O extracts on the *P. aeruginosa* virulence factors

Pyocyanin production

This study investigated the effect of H_2O and ethanolic extracts of *T. cordata* leaves on the virulence factor pyocyanin, which is normally produced by *P. aeruginosa* as an extracellular green pigment secretion important for bacterial pathogenesis and virulence. As shown in Figure 4, the H_2O and EtOH extracts significantly reduce the production of pyocyanin in a dose-dependent manner. Significant ($p < 0.001$) reductions in pyocyanin production with the H_2O extract, reaching 42.9, 56.9, 78.9 and 88.1% ($p < 0.01$) were recorded when the treatment was done at 1.2, 0.6, 0.3 and 0.15 mg/mL, respectively. With the EtOH extract, the significant ($p < 0.001$) reduction in pyocyanin production of 49.9, 66.3% and 81.0% ($p < 0.01$) was observed at 1.2, 0.6, and 0.3 mg/mL, respectively. The reduction was insignificant at 0.15 mg/mL.

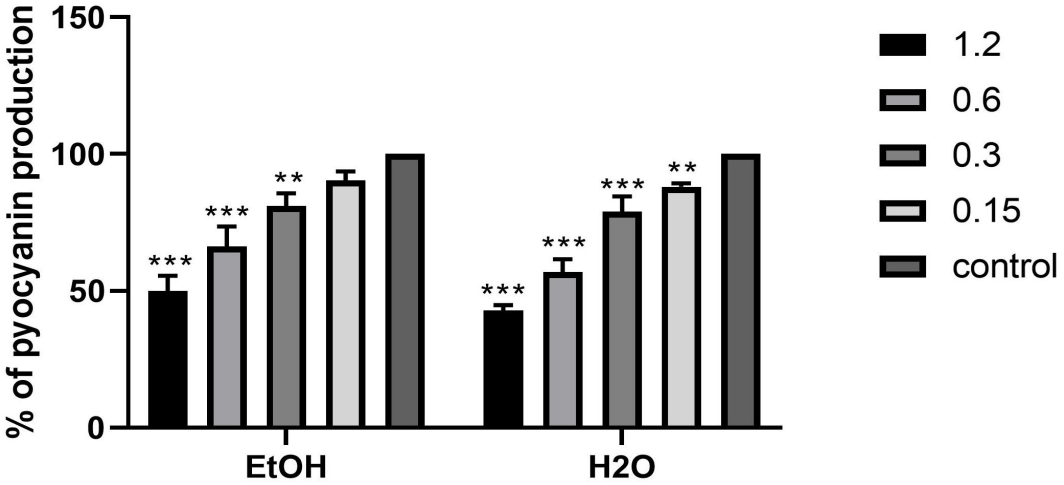


Figure 4. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on pyocyanin production. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Rhamnolipids production

As shown in Figure 5, the H_2O extract at concentrations of 1.2, 0.6, and 0.3 mg/mL caused a significant reduction in rhamnolipids production to 33.9%, 41.8% ($p < 0.001$) and 78.7% ($p < 0.01$), respectively. A similar pattern of results was observed with the ethanolic extract, where significant ($p < 0.001$) declines of

33.9%, 74.6%, and 78.8% were recorded, respectively. At a concentration of 0.15 mg/mL, neither the ethanolic nor the H_2O extract significantly reduced rhamnolipids production.

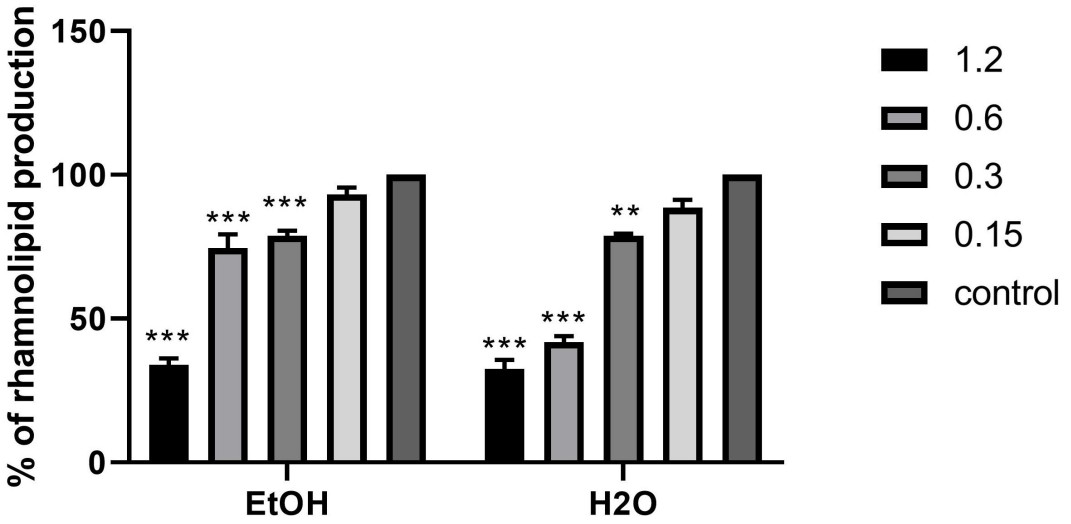


Figure 5. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on rhamnolipid production. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

LasA staphylolytic protease activity

LasA staphylolytic activity was assessed and compared with the results of the untreated group. The reported results of all treatments showed a significant decrease in LasA staphylolytic activity when applying both extracts (Figure 6). Highly significant (p < 0.001) reductions ranging from approximately 60 to 73% were observed for both extracts at 1.2 and 0.6 mg/mL, respectively. However, lower significant reductions were documented for both extracts at 0.3 mg/mL (p < 0.01) and 0.15 mg/mL (p < 0.05).

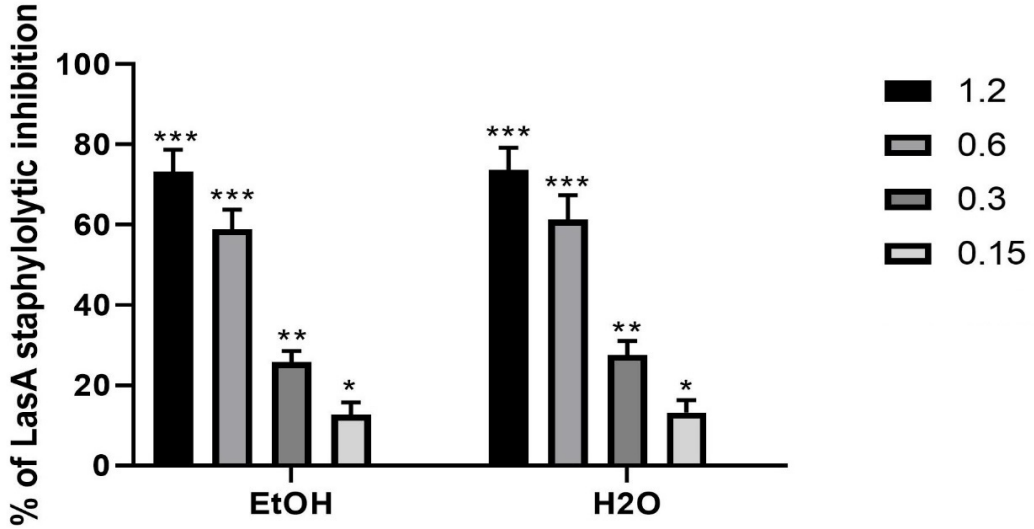


Figure 6. Percentage inhibition of LasA staphylolytic protease activity of *P. aeruginosa* treated with different concentrations of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL). The percentage of LasA staphylolytic inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. SD: standard deviation.

LasA protease activity

The overall pattern of results from this experiment, as shown in Figure 7, indicates a significant inhibition of LasA protease activity by both extracts at all concentrations used. The maximum reduction in enzyme

activity was recorded at a concentration of 1.2 mg/mL for both extracts, reaching 83.3% with the ethanolic extract and 75.8% with the H_2O extract.

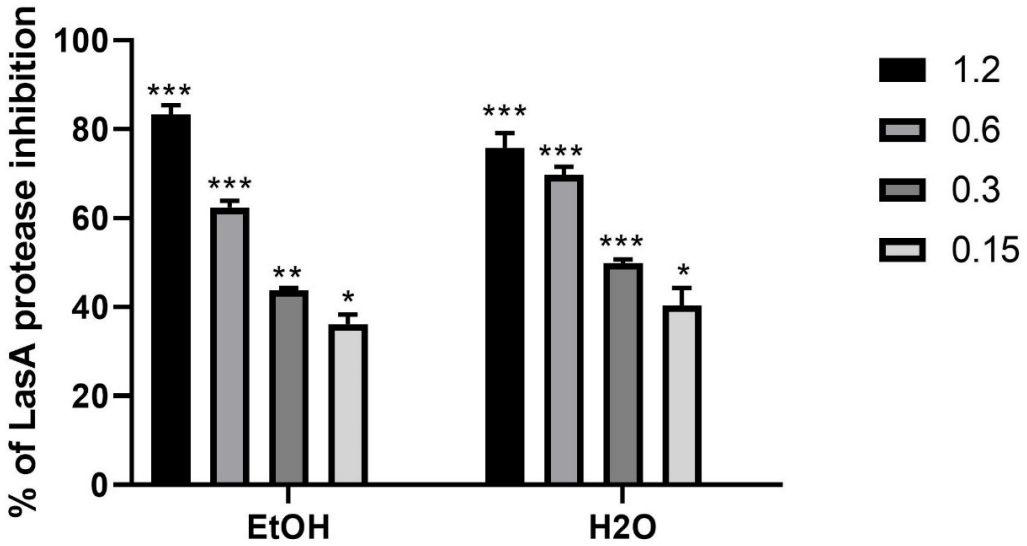


Figure 7. Percentage inhibition of LasA protease activity of *P. aeruginosa* treated with different concentrations of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL). The percentage of LasA protease inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Chitinase activity

The results regarding the ability of both the H_2O and ethanolic extracts to inhibit chitinase activity appear similar to those documented for the LasA protease enzyme, as indicated by the results in Figure 8. The results indicate that the maximum significant (p < 0.001) inhibitory effect for both extracts was observed at concentrations of 1.2 and 0.6 mg/mL, with the degree of inhibition ranging from 29% to 49% across these concentrations. At the lower concentrations-specifically 0.3 and 0.15-the inhibition rate was significant (p < 0.05–0.01), though lower than that observed with the higher concentrations.

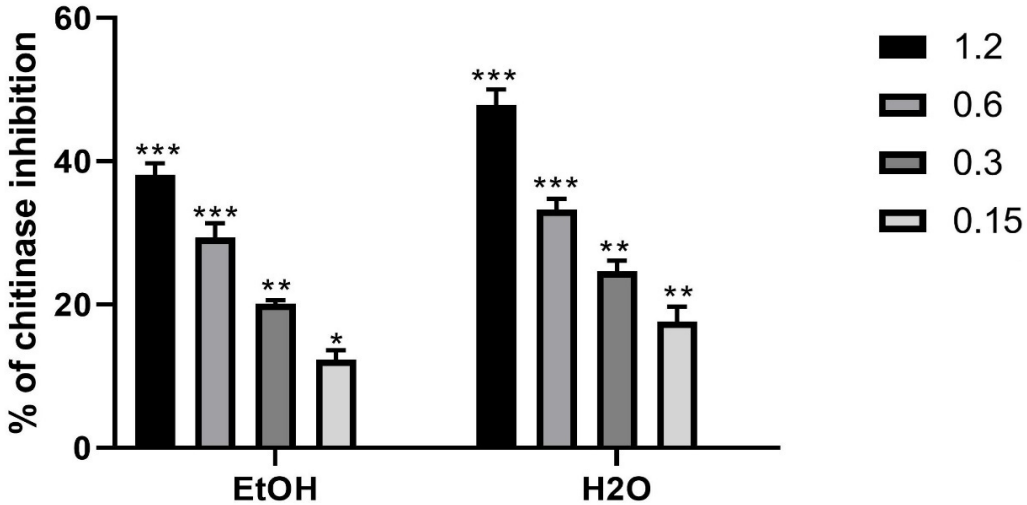


Figure 8. Percentage inhibition of Chitinase activity of *P. aeruginosa* treated with different concentrations of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL). The percentage of chitinase inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Effect of *T. cordata* H_2O and EtOH extracts on biofilm formation

Swarming motility

The effect of both H_2O and ethanolic extracts on the swarming motility of *P. aeruginosa* was investigated. Experimental results were evaluated by comparing the swarming zones observed on swarming plates for *P. aeruginosa* cultures treated with the extracts against untreated controls. Overall, the swarming zone values measured in the presence of either the H_2O or ethanolic extract, compared to their absence, as shown in Figure 9, indicated a statistically significant reduction in bacterial swarming motility. The results showed a significant reduction in swarming motility from 65.5 mm in the control to 46.0 mm, 39.0 mm, 30.5 mm, and 28.0 mm for the H_2O extract whereas it was 57.5 mm, 45.5 mm, 33.5 mm, and 23.0 mm for the ethanolic extract at concentrations of 0.15, 0.3, 0.6, and 1.2 mg/mL, respectively.

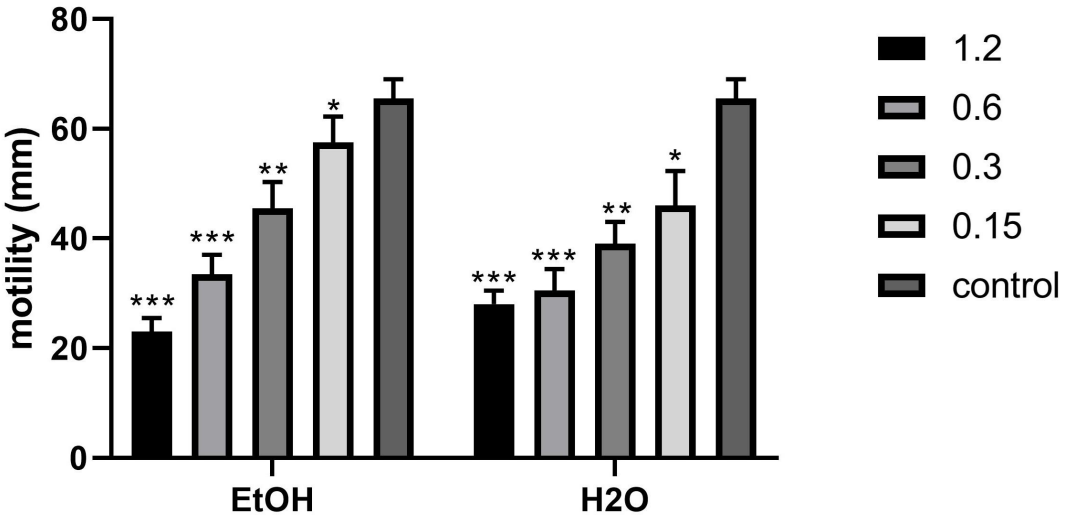


Figure 9. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on the swarming motility of *P. aeruginosa*. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H2O: aqueous; SD: standard deviation.

Aggregation ability

The effect of both the H_2O and ethanolic extracts on the aggregation of planktonic *P. aeruginosa* cells is shown in Figure 10. Overall, a significant reduction in the cells aggregation capacity was observed at concentrations of 1.2, 0.6, and 0.3 mg/mL for both extracts. The aggregation ability of the control group was 31.5%, whereas lower values were recorded with the H_2O extract, reaching a minimum of 9.1% at the highest concentration (1.2 mg/mL). At this same concentration (1.2 mg/mL) of the ethanolic extract, aggregation capacity of 17.21% was recorded. The effect of the H_2O extract at 0.15 mg/mL on aggregation ability was statistically significant, whereas for the same concentration of the ethanolic extract it was not.

Hydrophobicity

When examining the effect of applying different concentrations of the two extracts on reducing the hydrophobicity of *P. aeruginosa*, the general pattern of the results obtained was of inhibition, as shown in Figure 11. Significant inhibition was observed at concentrations of 1.2, 0.6, and 0.3 mg/mL for both extracts. The aggregation value recorded with the H_2O extract at 1.2 mg/mL was 38.8%, compared to 47.1% FPc for the control. It was 44.1% when using 1.2 mg/mL of the ethanolic extract, compared to 53.4% for the control. No significant reduction in hydrophobicity at 0.15 mg/mL with both extracts.

EPS production

The results shown in Figure 12 demonstrate a significant inhibitory effect of both extracts—at all concentrations tested—on the ability of bacterial cells to produce EPSs. The results indicate that the inhibition process is concentration-dependent, showing a clear direct relationship between the degree of

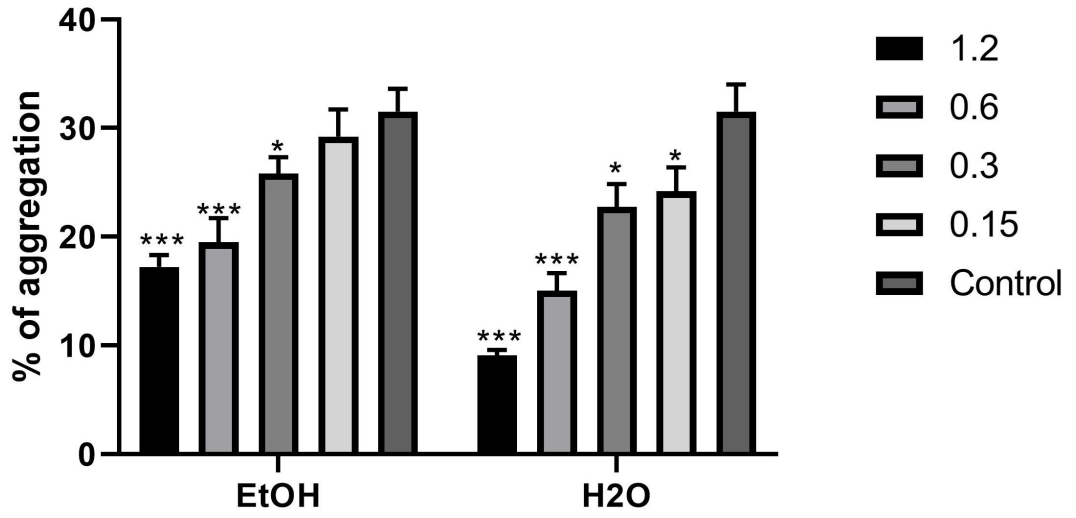


Figure 10. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on the aggregation ability of *P. aeruginosa*. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

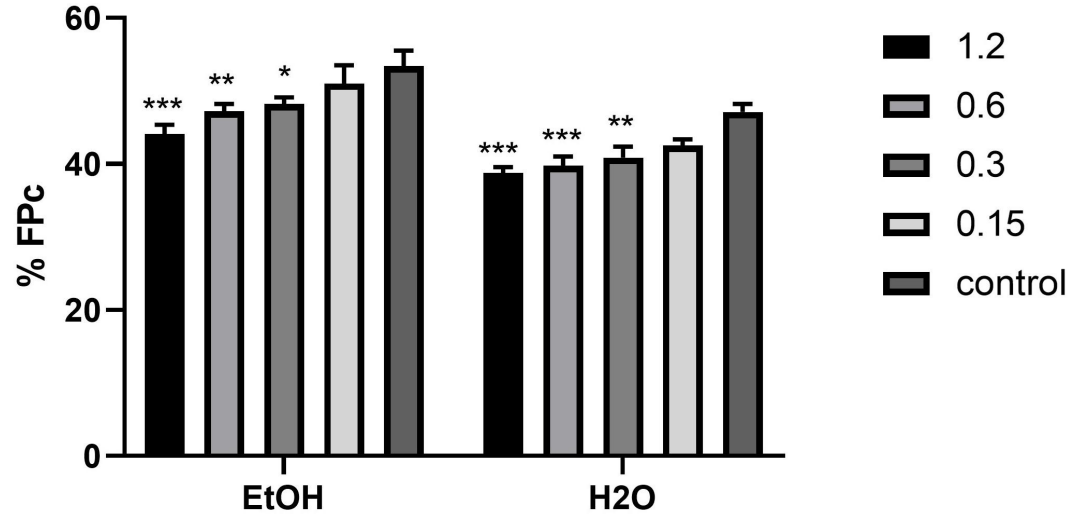


Figure 11. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on the cell surface hydrophobicity of *P. aeruginosa*. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; FPc: hydrocarbon separation ratio; H_2O: aqueous; SD: standard deviation.

inhibition and the concentration of the extract used. Consequently, the strongest inhibitory effect on EPS production was observed at the highest extract concentration (1.2 mg/mL), with inhibition percentages of 66.4 and 53.8 for the H_2O and ethanolic extracts, respectively. On the other hand, the weakest inhibitory effect was observed at 0.15 mg/mL, with inhibition percentages of 27.6 and 23.3 for the H_2O and ethanolic extracts, respectively.

AHL production

As shown in Figure 13, both the H_2O and ethanolic extracts of *T. cordata* leaves demonstrated comparable efficacy in reducing AHL production by *P. aeruginosa*. The general nature of the reduction depended on the concentration used, exhibiting a direct relationship between the concentration applied and the resulting reduction. The greatest reduction in AHL production was observed at a concentration of 1.2 mg/mL for both extracts, with reduction reaching 85.2% and 78.7% for the H_2O and ethanolic extracts, respectively. The lowest reduction was observed at 0.15 mg/mL for both extracts, with reduction reaching 14.1% and 11.1% for the H_2O and ethanolic extracts, respectively.

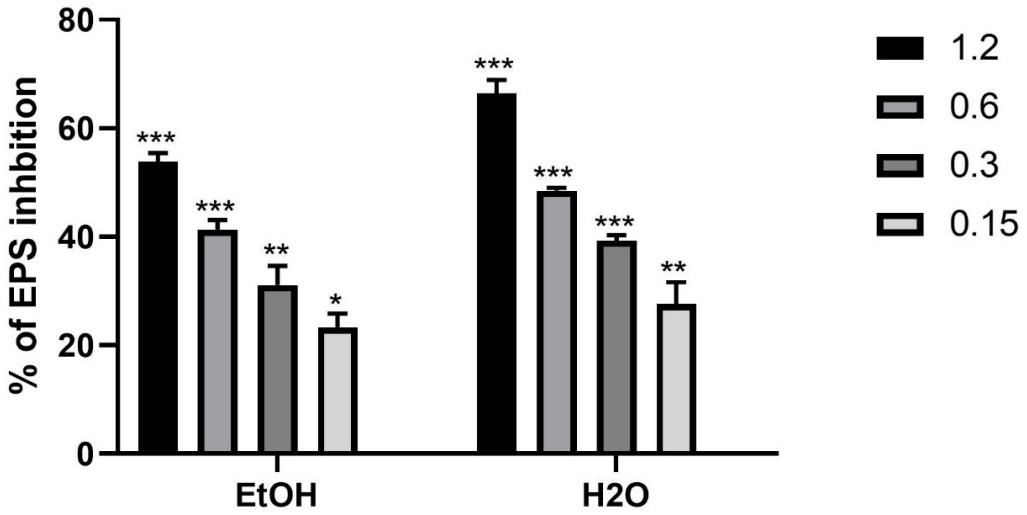


Figure 12. Effect of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL) on EPS production. The percentage of EPS inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EPS: exopolysaccharide; EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

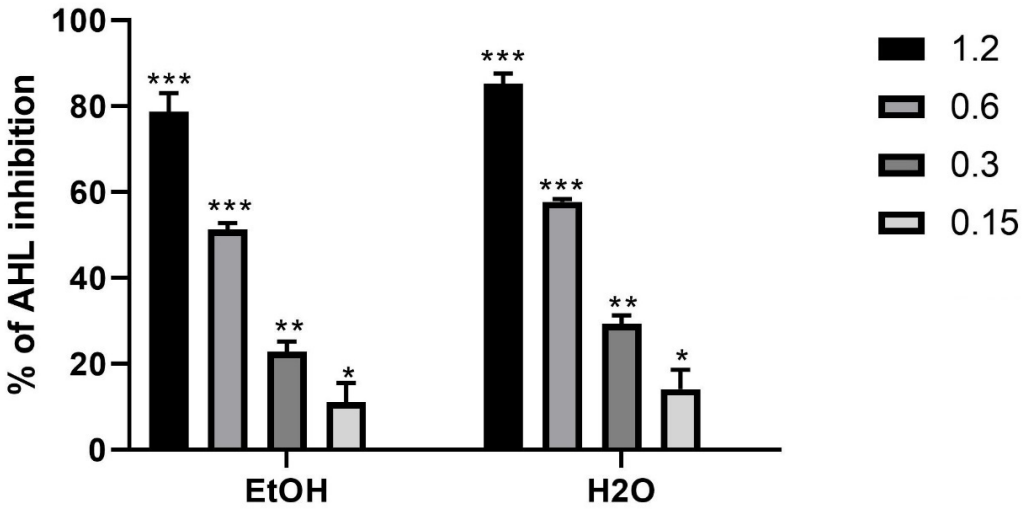


Figure 13. Percentage of inhibition of AHL production in *P. aeruginosa* treated with different concentrations of *T. cordata* ethanol and aqueous extracts (0.15–1.2 mg/mL). The percentage of AHL inhibition of untreated *P. aeruginosa* (control) was calculated as 0.0%. Data represent mean ± SD (n = 3). Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. AHL: *N*-acyl-homoserine lactone; EtOH: ethanol; H_2O: aqueous; SD: standard deviation.

Genes expression

The results regarding the effect of both extracts on the expression of certain critical QS genes (Figure 14), conclusively demonstrated that the H_2O extract had no significant impact on gene expression, whereas the ethanolic extract produced a significant effect. Treatment with the EtOH extract (0.6 mg/mL) significantly (p < 0.01) reduced the expression of critical QS genes namely *lasI*, *lasR*, *rhlI*, and *rhlR*, compared to the untreated control group. The treatment with the ethanolic extract induced a highly significant (p < 0.01) downregulation of *lasI* (0.02-fold relative to control), *lasR* (0.16-fold), *rhlI* (0.29-fold), and *rhlR* (0.12-fold). On the other hand, the H_2O extract at 0.6 mg/mL results in slight non-statistically significant reduction in gene expression levels. Melt-curve analysis confirmed single specific amplification products for each primer set, and relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Discussion

P. aeruginosa is considered a highly opportunistic bacterium that causes numerous medical complications, particularly in hospitalized and immunocompromised patients. It secretes various extracellular virulence

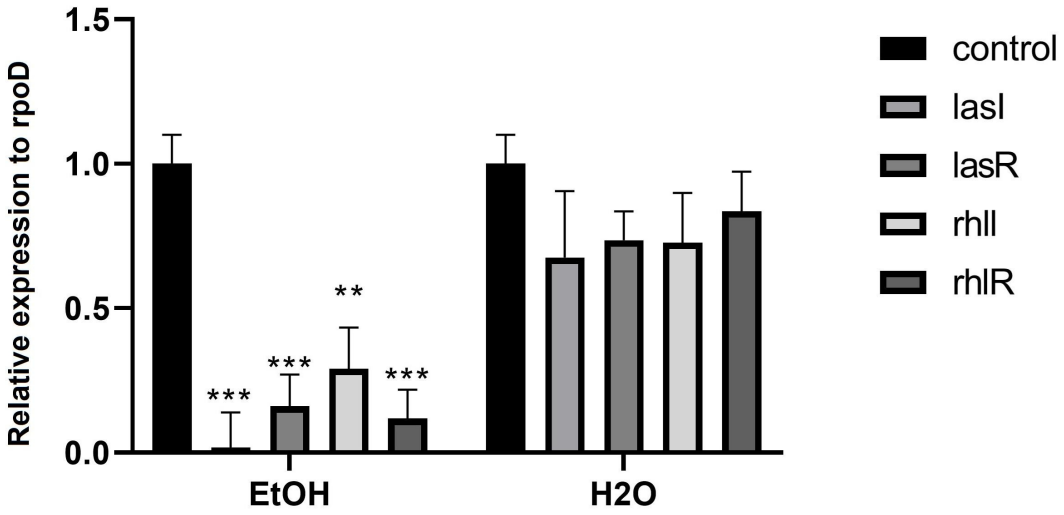


Figure 14. Relative expression of quorum sensing genes in *P. aeruginosa* treated with 0.6 mg/mL *T. cordata* H_2O and EtOH extracts. Asterisks denote statistical significance: *p < 0.05, **p < 0.01, and ***p < 0.001. EtOH: ethanol; H_2O: aqueous.

factors that determine its pathogenicity, the production of which is regulated by QS systems. Therefore, inhibiting QS is a novel strategy for combating *P. aeruginosa* infections [28].

Our findings of *T. cordata*'s antibacterial activities are consistent with earlier research. According to Abdel Gawad et al. [29], essential oil from *T. cordata* had no antibacterial effects. With less than 37% inhibitory activity reported, the hydroethanolic extract of *T. cordata* showed only moderate antibacterial activity at 50 µg/mL against MRSA and *P. aeruginosa* [29]. Qaralleh et al. [11] confirmed that the MIC values of *T. cordata* EtOH and H_2O extracts were 6.25 and 12.5 mg/mL against *P. aeruginosa*. Accordingly, sub-MIC concentrations of less than 2.4 mg/mL were chosen in the crystal violet assay. In this study, the crystal violet assay and the violacein inhibition assay have been used to estimate the potential of using *T. cordata* bioactive extracts as antibiofilm and anti-QS agents. The test extracts inhibited the development of biofilm and violacein production. The results indicated that *T. cordata* H_2O and EtOH extracts possess strong antibiofilm and anti-QS activities. Qaralleh et al. [11] reported an inhibition of *P. aeruginosa* biofilm development for *T. cordata*. The inhibition demonstrated by the ethanolic and H_2O extracts was at a concentration of 0.8 mg/mL, and the 50% inhibition ($MBIC_{50}$) was observed at a concentration equivalent to 0.4 mg/mL [11].

In this study, a cell viability assay was established to study the influence of varying extract concentrations on viable cells within the biofilm matrix. The findings showed that the maximum reduction in viable cells was 9.7% when the *P. aeruginosa* biofilm was treated with 1.2 mg/mL. This result indicates a very slight loss in viability, consistent with the weak bacteriostatic activity of the extracts.

The presence of extracellular virulence factors in *P. aeruginosa* exudate is indicative of the effective functioning of the QS system. These factors have been shown to significantly contribute to the organism's ability to invade host tissues. The regulation of elastase and protease secretions is governed by the Las system, which serves crucial roles in the colonization of the host tissues. The Rhl system regulates the synthesis of pyocyanin, a compound that enhances the virulence of this particular pathogen by sequestering iron from transferrin [18]. The surface mobility and initiation of biofilm formation in *P. aeruginosa* are reliant on the rhamnolipids regulated by the Rhl system, which serve as essential surfactants. The results obtained from the experiments in the current project, when using concentrations lower than the MIC of *T. cordata*, demonstrated a notable reduction in all the virulence components studied. This suggests that these extracts have the ability to effectively mitigate the virulence of *P. aeruginosa*, hence potentially reducing the risk of infection.

The creation of biofilms has been found to be associated with the development of antibiotic resistance, hence posing challenges in the treatment of such infections. Therefore, there is a prioritization of the quest for highly effective novel chemicals targeting the production of bacterial biofilms. The sequential steps of

biofilm production begin with the establishment of the conditioning biofilm, followed by cell migration to the surface, adhesion, and eventual replication to form a mature biofilm. Eventually, detachment of the biofilm takes place [30]. The findings of this investigation indicate that the extracts derived from *T. cordata* exhibited a notable reduction in bacterial hydrophobicity, competing with the initial attachment and surface adherence steps during biofilm initiation. The decrease in hydrophobicity observed, evidenced by the decline in FPc values, may be attributed to the presence of plant constituents that bind to the bacterial adhesion sites, hence diminishing their hydrophobic properties. In addition to the foregoing, the current results demonstrate the significant capacity of *T. cordata* extracts to reduce cell aggregation and swarming motility, processes considered fundamental steps in biofilm formation [31].

The QS system functions by detecting the signals emitted by the bacterial population. The present study examined the impact of sub-MICs of *T. cordata* extracts on swarming motility, EPS secretion, rhamnolipid and AHL production. The results indicate a dose-dependent relationship, implying that these extracts could potentially disrupt the QS system. The bioactive components of the extracts reduced swarming by targeting the flagellin protein associated with the flagellum. Reduced rhamnolipids production enhances suppression of swarming motility induced by the extracts. A reduction in EPS secretion weakens the architecture of biofilm, microcolony development, and antibiotic resistance, thereby promoting the exposure of biofilms to antimicrobials and facilitating their clearance [32–35]. A substantial decrease in AHL production was likely achieved by the attenuating action of leaf extracts on the QS systems, such as the Las and Rhl systems [36].

The noticeable decrease in QS gene expression following treatment with the *T. cordata* EtOH extract strongly implies that EtOH is particularly effective at pulling out active components that interfere with bacterial communication in *P. aeruginosa*. It's plausible this inhibitory effect originates from phenolic or flavonoid compounds, both recognized for their anti-QS properties [37]. This observation aligns well with earlier studies highlighting EtOH-based plant extracts as promising inhibitors of bacterial virulence by disrupting QS. Additionally, since both the Las and Rhl regulatory systems were suppressed, there's a good chance the EtOH extract reduces virulence factors and disrupts biofilm formation, underscoring its potential as a natural weapon against bacterial infections [38, 39]. Conversely, the lack of significant transcriptional downregulation by the H_2O extract suggests that its potent phenotypic anti-virulence activity operates primarily through non-transcriptional pathways. These include competitive inhibition of signal-receptor binding, direct inactivation/quenching of AHL signal molecules, or post-translational interference with virulence factor secretion. Importantly, none of the *P. aeruginosa* cultures treated with sub-inhibitory concentrations of *T. cordata* extracts exhibited a significant reduction in viable cell counts compared to untreated controls. This confirms that the observed inhibition of biofilm biomass, pyocyanin production, swarming motility, and QS gene expression occurs through specific anti-virulence and quorum-quenching regulatory mechanisms rather than non-specific bactericidal toxicity or metabolic growth arrest. Consequently, this mode of action exerts minimal selective pressure on bacterial viability, substantially reducing the risk of triggering rapid antimicrobial resistance development.

P. aeruginosa as a persistent biofilm-forming spoilage organism and opportunistic pathogen in food processing chains and water systems. The demonstrated quorum-quenching and anti-biofilm capabilities of *T. cordata* polar extracts highlight their practical utility as natural, plant-derived biopreservatives and food-contact surface sanitizers. *Tilia* spp. has a well-documented history of safe dietary consumption as herbal teas and botanical supplements. Therefore, these extracts represent viable candidates for incorporation into functional foods, dietary nutraceuticals, and active packaging materials aimed at mitigating bacterial biofilm formation without accelerating antimicrobial resistance.

This study confirms that both *T. cordata* extracts effectively reduce *P. aeruginosa* pathogenicity at sub-MIC levels. While both extracts demonstrate robust phenotypic antibiofilm and anti-virulence activities, the ethanolic extract specifically operates via the transcriptional downregulation of core QS regulatory genes (*lasI*, *lasR*, *rhlI*, and *rhlR*), whereas the H_2O extract acts primarily through non-transcriptional or post-transcriptional mechanisms. It is strongly advised to conduct additional research in order to isolate and

identify the active constituents. Investigating the impact of these extracts on the expression of QS genes, such as *las*, *rhl*, and *pqs*, may provide valuable insights into the underlying processes of their anti-QS properties.

Abbreviations

Af: final adherence

AHL: *N*-acyl-homoserine lactone

Ai: initial adherence

ATCC: American Type Culture Collection

EPSs: exopolysaccharides

EtOH: ethanol

FPc: hydrocarbon separation ratio

H_2O: aqueous

H_2SO_4: sulfuric acid

$MBIC_{50}$: minimum biofilm inhibitory concentration

MIC: minimum inhibitory concentration

Pqs: pseudomonas quinolone signal

QS: quorum sensing

TTC: 2,3,5-triphenyltetrazolium chloride

Declarations

Author contributions

RA: Investigation, Visualization, Writing—original draft. MOAI: Conceptualization, Formal analysis, Supervision, Validation, Visualization, Writing—review & editing. HQ: Conceptualization, Formal analysis, Supervision, Validation, Writing—review & editing. All authors read and approved the submitted version.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request

Funding

Not applicable.

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References

1. Palavutitotai N, Jitmuang A, Tongsai S, Kiratisin P, Angkasekwinai N. Epidemiology and risk factors of extensively drug-resistant Pseudomonas aeruginosa infections. PLOS ONE. 2018;13:e0193431. [DOI] [PubMed] [PMC]
2. Ciofu O, Tolker-Nielsen T. Tolerance and Resistance of Pseudomonas aeruginosa Biofilms to Antimicrobial Agents—How P. aeruginosa Can Escape Antibiotics. Front Microbiol. 2019;10:913. [DOI] [PubMed] [PMC]
3. Fong J, Yuan M, Jakobsen TH, Mortensen KT, Delos Santos MM, Chua SL, et al. Disulfide Bond-Containing Ajoene Analogues As Novel Quorum Sensing Inhibitors of Pseudomonas aeruginosa. J Med Chem. 2017;60:215–27. [DOI] [PubMed]
4. Rajkumari J, Borkotoky S, Murali A, Suchiang K, Mohanty SK, Busi S. Attenuation of quorum sensing controlled virulence factors and biofilm formation in Pseudomonas aeruginosa by pentacyclic triterpenes, betulin and betulinic acid. Microb Pathog. 2018;118:48–60. [DOI] [PubMed]
5. Lu H, Tu C, Zhou T, Zhang W, Zhan Y, Ding J, et al. A ROS-scavenging hydrogel loaded with bacterial quorum sensing inhibitor hyperbranched poly-L-lysine promotes the wound scar-free healing of infected skin in vivo. Chem Eng J. 2022;436:135130. [DOI]
6. Sadlon AE, Lamson DW. Immune-modifying and antimicrobial effects of Eucalyptus oil and simple inhalation devices. Altern Med Rev. 2010;15:33–47. [PubMed]
7. Linden flower. In: Blumenthal M, Goldberg A, Brinckmann J, editors. In: Herbal Medicine: Expanded Commission E Monographs. Newton: Integrative Medicine Communications; 2000. pp. 240–3.
8. Al-Essa MK, Mohammed FI, Shafagoj YA, Afifi FU. Studies on the Direct Effects of the Alcohol Extract of Tilia cordata. on Dispersed Intestinal Smooth Muscle Cells of Guinea Pig. Pharm Biol. 2008;45:246–50. [DOI]
9. Shah SMA, Akhtar N, Akram M, Shah PA, Saeed T, Ahmed K, et al. Pharmacological activity of Althaea officinalis L. J Med Plants Res. 2011;5:5662–6.
10. Murti Y, Sharma S. FLAVONOID: A PHARMACOLOGICALLY SIGNIFICANT SCAFFOLD. World J Pharm Pharm Sci. 2017;6:488–504. [DOI]
11. Qaralleh HA, Al-Limoun MO, Khlaifat A, Khleifat KM, Al-Tawarah N, Alsharafa KY, et al. Antibacterial and antibiofilm activities of a traditional herbal formula against respiratory infection causing bacteria. Trop J Nat Prod Res. 2020;4:527–34. [DOI]
12. Azwanida NN. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Med Aromat Plants. 2015;04:1000196. [DOI]
13. Jaafreh M, Khleifat K, Qaralleh H, Al-limoun M. Antibacterial and Antioxidant Activities of Centeurea damascena Methanolic Extract. J Basic Appl Res Biomed. 2019;5:55–63. [DOI]
14. Oliveira BD, Rodrigues AC, Cardoso BMI, Ramos ALCC, Bertoldi MC, Taylor JG, et al. Antioxidant, antimicrobial and anti-quorum sensing activities of Rubus rosaefolius phenolic extract. Ind Crops Prod. 2016;84:59–66. [DOI]
15. Cheng KC, Hsiao HC, Hou YC, Hsieh CW, Hsu HY, Chen HY, et al. Improvement in Violacein Production by Utilizing Formic Acid to Induce Quorum Sensing in Chromobacterium violaceum. Antioxidants. 2022;11:849. [DOI] [PubMed] [PMC]

16. Sabaeifard P, Abdi-Ali A, Soudi MR, Dinarvand R. Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. J Microbiol Methods. 2014;105: 134–40. [DOI] [PubMed]
17. Hossain MA, Lee SJ, Park NH, Mechesso AF, Birhanu BT, Kang J, et al. Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. Sci Rep. 2017;7:10618. [DOI] [PubMed] [PMC]
18. Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, et al. Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances Pseudomonas aeruginosa clearance in a mouse peritoneal implant infection model. PLOS ONE. 2017;12:e0176883. [DOI] [PubMed] [PMC]
19. Andrejko M, Zdybicka-Barabas A, Janczarek M, Cytryńska M. Three Pseudomonas aeruginosa strains with different protease profiles. Acta Biochim Pol. 2013;60:83–90. [DOI]
20. Cupp-Enyard C. Sigma's Non-specific Protease Activity Assay - Casein as a Substrate. J Vis Exp. 2008: 899. [DOI] [PubMed] [PMC]
21. Shen CR, Chen YS, Yang CJ, Chen JK, Liu CL. Colloid Chitin Azure Is a Dispersible, Low-Cost Substrate for Chitinase Measurements in a Sensitive, Fast, Reproducible Assay. SLAS Discov. 2010;15:213–7. [DOI] [PubMed]
22. Krishnan T, Yin WF, Chan KG. Inhibition of Quorum Sensing-Controlled Virulence Factor Production in Pseudomonas aeruginosa PAO1 by Ayurveda Spice Clove (Syzygium Aromaticum) Bud Extract. Sensors. 2012;12:4016–30. [DOI] [PubMed] [PMC]
23. Shanks RM, Meehl MA, Brothers KM, Martinez RM, Donegan NP, Graber ML, et al. 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. 2008;76:2469–77. [DOI] [PubMed] [PMC]
24. Rosenberg M, Gutnick D, Rosenberg E. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett. 1980;9:29–33. [DOI]
25. Packiavathy IA, Priya S, Pandian SK, Ravi AV. Inhibition of biofilm development of uropathogens by curcumin – An anti-quorum sensing agent from Curcuma longa. Food Chem. 2014;148:453–60. [DOI] [PubMed]
26. Lahiri D, Nag M, Dutta B, Dey S, Mukherjee D, Joshi SJ, et al. Antibiofilm and anti-quorum sensing activities of eugenol and linalool from *Ocimum tenuiflorum* against *Pseudomonas aeruginosa* biofilm. J Appl Microbiol. 2021;131:2821–37. [DOI] [PubMed]
27. Banerjee M, Moulick S, Bhattacharya KK, Parai D, Chattopadhyay S, Mukherjee SK. Attenuation of Pseudomonas aeruginosa quorum sensing, virulence and biofilm formation by extracts of Andrographis paniculata. Microb Pathog. 2017;113:85–93. [DOI] [PubMed]
28. Wahman S, Emara M, Shawky RM, El-Domany RA, Aboulwafa MM. Inhibition of quorum sensing-mediated biofilm formation in *Pseudomonas aeruginosa* by a locally isolated *Bacillus cereus*. J Basic Microbiol. 2015;55:1406–16. [DOI] [PubMed]
29. Abdel Gawad SM, Hetta MH, Ross SA, Badria FA. Antiprotozoal and antimicrobial activity of selected medicinal plants growing in Upper Egypt, Beni-Suef Region. World J Pharm Pharm Sci. 2015;4:1–21.
30. Renner LD, Weibel DB. Physicochemical regulation of biofilm formation. MRS Bull. 2011;36:347–55. [DOI] [PubMed] [PMC]
31. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol Microbiol. 2006;62:1264–77. [DOI] [PubMed]
32. Sauer K, Camper AK. Characterization of Phenotypic Changes in *Pseudomonas putida* in Response to Surface-Associated Growth. J Bacteriol. 2001;183:6579–89. [DOI] [PubMed] [PMC]
33. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol. 2005;13:34–40. [DOI]

34. Yildiz FH, Schoolnik GK. *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci. 1999;96:4028–33. [DOI] [PubMed] [PMC]
35. Brackman G, Defoirdt T, Miyamoto C, Bossier P, Van Calenbergh S, Nelis H, et al. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in Vibrio spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. BMC Microbiol. 2008;8:149. [DOI] [PubMed] [PMC]
36. Touati A, Ibrahim NA, Tighilt L, Idres T. Anti-QS Strategies Against Pseudomonas aeruginosa Infections. Microorganisms. 2025;13:1838. [DOI] [PubMed] [PMC]
37. Santos CA, Lima EMF, Franco BDGM, Pinto UM. Exploring Phenolic Compounds as Quorum Sensing Inhibitors in Foodborne Bacteria. Front Microbiol. 2021;12:735931. [DOI] [PubMed] [PMC]
38. Qaralleh H. Limonene as a Multi-Target Antibiofilm and Quorum Sensing Inhibitor Against Pseudomonas aeruginosa. J Basic Appl Res Biomed. 2024;10:80–8. [DOI]
39. Ma'aitah S. Pseudomonas aeruginosa biofilm formation: Antibiofilm Strategies and conventional methods of evaluation. J Basic Appl Res Biomed. 2024;10:11–28. [DOI]