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Myrtus communis leaf compounds as novel inhibitors of quorum sensing-regulated virulence factors and biofilm formation: In vitro and in silico investigations

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ABSTRACT

Antibiotic resistance of the Gram-negative bacterium *Pseudomonas aeruginosa* and its ability to form biofilm through the Quorum Sensing (QS) mechanism are important challenges in the control of infections caused by this pathogen. The extract of *Myrtus communis* (myrtle) showed strong anti-QS effect on *Chromobacterium. violaceum* 6267 by inhibiting 80 % of the production of violacein pigment at a sub-MIC concentration of 1/8 (31.25 μg/mL). In addition, the extract exhibited an inhibitory effect on virulence factors of *P. aeruginosa* PAO1 at half MIC (125 μg/mL), significantly reducing the formation of biofilms (72.02 %), the swarming activity (75 %), and the production of protease (61.83 %) and pyocyanin (97 %). The active fraction also downregulated the expression of selected regulatory genes involved in the biofilm formation and QS in the *P. aeruginosa* PAO1 strain. These genes included the autoinducer synthase genes (*lasI* and *rhlI*), the genes involved in the expression of their corresponding receptors (*lasR* and *rhlR*), and the *pqsA* genes. The analysis of the active fraction by HPLC/UV/MS and NMR allowed the identification of three phenolic compounds, 3,5-di-*O*-galloylquinic acid, myricetin 3-*O*-α-L-rhamnopyranoside (myricitrin), and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside. *In silico* studies showed that 3,5-di-*O*-galloylquinic acid, with an affinity score of −9.20 kcal/mol, had the highest affinity to the active site of the CviR protein (3QP8), a QS receptor from *C. violaceum*. Additionally, myricetin 3-*O*-α-L-rhamnopyranoside (myricitrin) and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside interact to a lesser extent with 3QP8. In conclusion, this study contributed significantly to the discovery of new QS inhibitors from *M. communis* leaves against resistant Gram-negative pathogens.

1. Introduction

In recent decades, there has been a significant increase in the prevalence of resistant human pathogenic bacteria, a trend that is closely related to high rates of morbidity and mortality in both clinical and community settings. One of the key mechanisms contributing to resistance to conventional drugs is biofilm formation, which is regulated by a bacterial cell-to-cell communication system known as quorum sensing (QS) [1]. This system, based on LuxI/LuxR homologues, governs a wide array of functions in bacteria including virulence, luminescence, motility, sporulation, pigment production, and the development of genetic competence [2,3]. Consequently, inhibiting QS emerges as a promising anti-infective strategy that could potentially prevent bacterial infections and circumvent the drug-resistance phenomenon. The opportunistic human pathogen *P. aeruginosa* is known to induce severe and persistent infections, particularly in immune-compromised patients, and it is responsible for approximately 57 % of nosocomial infections. *Pseudomonas. aeruginosa* secretes extracellular virulence factors that

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inflict significant damage to host tissues [4]. Interestingly, these virulence factors are regulated by the QS phenomenon.

Two dominating QS systems are identified in *P. aeruginosa*: the Las and Rhl systems [5]. Both QS systems are characterized by the presence of one transcriptional factor and one synthase enzyme that produces the signal molecule. In the case of Las system, LasR is the transcriptional activator while LasI is the homoserine lactone synthase (AHLs) which produces the signal molecule *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3OC12-HSL) [6]. On the other hand, Rhl system is composed by the transcriptional activator RhlR and the synthase enzyme RhlI, involved in the synthesis *N*-butanoyl-L-homoserine lactone (C4-HSL). As cell density increases, 3OC12-HSL diffuses into the bacterial environment and activates the LasR receptor, while C4-HSL interacts with its corresponding receptor, RhlR. Together, LasR and RhlR orchestrate the transcription of virulence genes [7]. A third QS system identified in *P. aeruginosa* is the *Pseudomonas* quinolone signal (*pqs*), which plays a crucial role in regulating the expression of virulence-related genes including *lasR*, *lasI*, *rhlR*, *rhlI*, and *pqsR* [8]. Consequently, it regulates the production of virulence factors such as elastase, pyocyanin and extracellular polymeric substrates (EPS) involved in biofilm formation [8,9]. Therefore, inhibiting bacterial QS emerges as a promising strategy to not only prevent *P. aeruginosa* resistance but also to mitigate the expression of virulence factors [10].

Medicinal plants serve as a rich reservoir of bioactive molecules, presenting a potential alternative to traditional drugs. These phytochemical substances, renowned for their broad spectrum of therapeutic effects, have demonstrated their capability to inhibit QS, combat antibiotic resistance and foster the development of more potent antimicrobials [11]. Several QS inhibitors have been identified, including hordenine, a natural plant phenylethylamine alkaloid [12], as well as other natural products such as luteolin, coumarin, clove oil, ginseng, clove oil, butein, and sappanol [13].

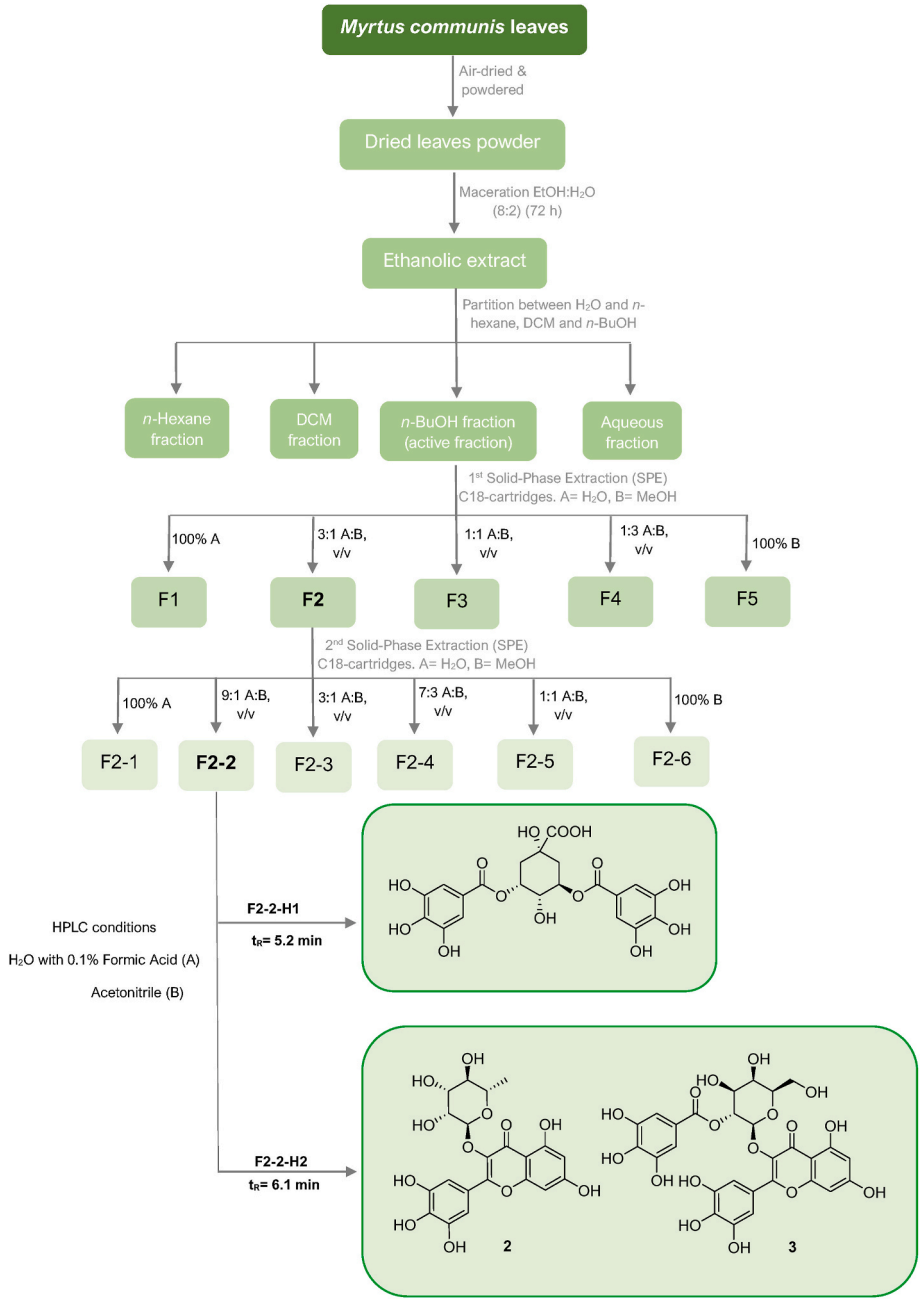

Fig. 1. Schematic representation of the extraction, partition, fractionation, and HPLC purification of *M. communis* leaves.

Continuing with our studies of medicinal plants from Tunisia [14, 15], we turned our attention to *Myrtus communis*, commonly known as myrtle, which is prevalent in the Mediterranean region and the Middle East [16]. Since antiquity, it has served as a spice, a crucial ingredient in food preparation, and for medicinal purposes, notably as an antiseptic agent [17]. Several studies have outlined the antimicrobial properties of *M. communis* extracts [18]. However, their effect on the QS system and biofilm formation in *P. aeruginosa* has not been well illustrated and documented [19].

Therefore, the main goal of this research was to assess the anti-QS activity of the *Myrtus communis* extract and its corresponding fractions against *Chromobacterium. violaceum* 6267 and to explore their inhibitory effect on the virulence factors and biofilm formation in *P. aeruginosa* PAO1. In addition, our study investigated the influence of the active fraction of *Myrtus communis* on the expression of quorum sensing regulatory genes in *P. aeruginosa* PAO1. *In silico* molecular docking analysis of the identified compounds from *M. communis* leaf ethanolic extract was also conducted.

2. Results

Leaves of *M. communis,* collected at Ain Sebaa, in Tabarka, from North-West region of Tunisia, were air-dried and powdered and then, they were extracted with a mixture of ethanol and water to give an ethanolic extract. The resulting crude extract was submitted to successive liquid-liquid extraction between water and *n*-hexane (*n*-Hex), dichloromethane (DCM), and *n*-butanol (*n*-BuOH) to yield, after evaporation, the corresponding fractions (Fig. 1).

2.1. Polyphenol content

Total phenolics, flavonoids, and tannins from the *M. communis* leaves ethanolic extract and its corresponding partitioned fractions (*n*-hexane, DCM, and *n*-BuOH) were determined and the results are displayed in Table 1. The *n*-BuOH fraction exhibited the highest amount of phenolic compounds (197.13 ± 1.22 mg GAE/gDE), being even higher than the ethanolic extract (188.10 ± 1.77 mg GAE/gDE). Conversely, the DCM fraction showed the lowest level with 46.22 ± 2.16 mg GAE/gDE. Moreover, the *n*-BuOH fraction was found to be the most effective solvent for extracting flavonoids and condensed tannins with 73.2 ± 2.2 and 47.1 ± 2.67 mg CE/g DE, respectively.

2.2. Antibacterial susceptibility testing and MIC determination

The antibacterial activity of the *M. communis* ethanolic extract and its partitioned fractions were determined against *P. aeruginosa* PAO1 (Fig. 2A, Table 2) and *C. violaceum* (Table 2). The *n*-BuOH fraction resulted to be the most active with MIC values of 250 and 500 μg/mL against *C. violaceum* and PAO1, respectively, being even more active than the ethanolic extract (Table 2). The *n*-hexane and dichloromethane fractions did not display antibacterial activity against PAO1, displaying only weak activity against *C. violaceum* with a MIC value of 1000 μg/mL.

The antibiotic imipenem, known for its effectiveness against *P. aeruginosa*, with a MIC value of 31.25 μg/mL against *P. aeruginosa* PAO1 showed higher potency than the *M. communis n*-BuOH fraction. Interestingly, the *M. communis n*-BuOH fraction displayed a similar antibacterial activity against *P. aeruginosa* PAO1 than the antibiotic vaamox®, both having a MIC value of 500 μg/mL. Cinnamaldehyde, recognized for its substantial anti-QS activity against Gram-negative bacteria, with MIC values of 31.25 μg/mL against *C. violaceum*, displayed higher activity than that of the *M. communis n*-BuOH fraction with MIC value 250 μg/mL.

Table 1
Phenolic content of the *M. communis* leaves extract and partitioned fractions.

Samples	Total phenolic content (mg GAE/g DE)	Total flavonoid content (mg CE/g DE)	Condensed tannin content (mg CE/g DE)
Ethanolic extract	188.10 ± 1.77	48.00 ± 0.5	35.7 ± 0.29
n-Hexane fraction	80.92 ± 1.57	45.0 ± 3.1	14.0 ± 0.59
Dichloromethane fraction	46.22 ± 2.16	43.5 ± 2.7	7.5 ± 1.48
n-Butanol fraction	197.13 ± 1.22	73.2 ± 2.2	47.1 ± 2.67

GAE: Gallic acid equivalent; CE: Catechin equivalent; DE: Dry extract.
Data reported as means ± SD (n = 3).

2.3. Anti-quorum sensing activity

The QS of *C. violaceum* was related to the production of the purple pigment violacein, and so, the loss of purple pigmentation, without any interference with bacterial growth of *C. violaceum*, is an indicator of QS inhibition [20].

For the qualitative assessment of QS inhibition, *M. communis* extracts and fractions were tested at their sub-inhibitory concentrations (Fig. 2B). The results indicated that the *n*-BuOH fraction exhibited significant anti-QS activity. It notably inhibited violacein production by 10.2–14.8 mm at sub-inhibitory concentrations starting from 125 μg/mL, without affecting cell growth (Fig. 2B).

For the quantitative assay, *M. communis* extracts and fractions showed varying levels of violacein pigment inhibition, and this inhibition was found to be concentration dependent (Fig. 3). The *n*-BuOH fraction displayed notable potency, with inhibition levels ranging from 93.55 % to 61.4 % at concentrations from ½ MIC (125 μg/mL) to 1/16 MIC (15.625 μg/mL). Notably, the *n*-BuOH fraction exhibited similar activity compared to the positive control cinnamaldehyde, which achieved 93.4 % and 87.8 % inhibitory effect at ½ and ¼ MIC values, respectively. Furthermore, the ethanolic extract showed a lower concentration of violacein pigment at ½ MIC corresponding to 250 μg/mL (68.72 %). In contrast, the *n*-hexane and DCM fractions exhibited a weak inhibitory effect at sub-inhibitory concentrations (Fig. 3).

2.4. Inhibition of QS-mediated virulence factors of P. aeruginosa PAO1

The effect of *n*-BuOH fraction at sub-MIC concentrations (15.62–250 μg/mL) on pycoyanin pigment production was assessed (Fig. 4). Notably, this fraction displayed a potent inhibition of pyocyanin production (97 % inhibition at ½ MIC). Interestingly, this inhibition was maintained even at 1/16 MIC, with 80.13 % of inhibition, and it was reduced at 1/32 MIC with only 25.05 % inhibition. Similarly, protease activity in the cell-free supernatant of the treated PAO1 culture was also reduced in a dose-dependent manner (Fig. 4). Treatment with the extract at ½ MIC resulted in a substantial reduction in total protease activity, by 61.83 ± 1.19 % compared to untreated samples and only 15 ± 2.88 % inhibition of protease activity was noted at ¼ MIC (125 μg/mL). Furthermore, the *n*-BuOH fraction was evaluated for swarming inhibition assay of *P. aeruginosa* PAO1 on agar medium. The results showed that the *n*-BuOH fraction significantly inhibited the swarming motility in a dose-dependent manner at sub-MIC concentrations (Fig. S1). It significantly inhibited the swarming motility by 75 % at 250 μg/mL compared to untreated control (Fig. 4). This inhibitory effect was also observed at 1/8 MIC sub-inhibitory concentration, resulting in 45 % inhibition. A lower inhibitory effect was recorded at reduced concentrations corresponding to 1/16 and 1/32 MICs (Fig. 4).

2.5. Antibiofilm activity

The *n*-BuOH fraction of *M. communis* was investigated for its antibiofilm effect against *P. aeruginosa* PAO1 strain (Fig. 5A). An inhibition percentage above 50 % was considered as a good anti-biofilm activity,

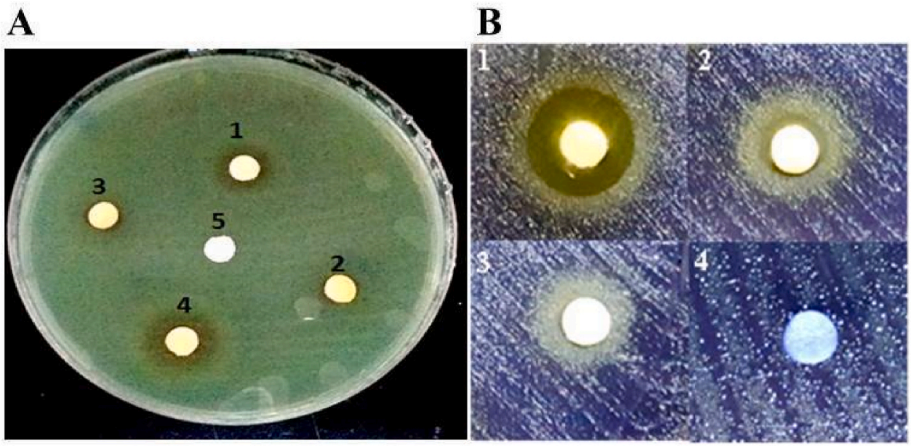

Fig. 2. **A**: Antibacterial activity of *M. communis* leaves extract and partitioned fractions against *P. aeruginosa* PAO1 strain: (1) ethanolic extract, (2) *n*-hexane fraction, (3) dichloromethane fraction, (4) *n*-butanol fraction at concentration of 1000 μg/mL (5) Negative control (pure ethanol). **B**: Anti-QS activities of the *n*-BuOH fraction using *C. violaceum* 6267 as the reference strain. The biosensor plates were spotted with different concentrations of the *n*-BuOH fraction at (1) 250 μg/mL and at sub-inhibitory concentrations: (2) 125 μg/mL and (3) 62.5 μg/mL (4) Negative control (pure ethanol).

Table 2
Antibacterial and anti-QS activities against *P. aerugionsa* PAO1 and *C. violaceum* of the ethanolic extract, partitioned and SPE fractions from the *M. communis* leaves along with two known antibiotics and an anti-QS active compound, used as standard controls.

Samples	MIC (μg/ml)		Anti-QS activity (μg/mL) and percentage of inhibition (%)
	P. aeruginosa PAO1	C. violaceum	
Ethanolic extract	2000	500	250 (68.72 ± 1.55 %)
n-Hexane	>2000	1000	>1000
Dichloromethane	>2000	1000	>1000
n-Butanol	**500**	**250**	**31.25 (82.64 ± 1.82 %)**
First C18-SPE			
F1	2000	2000	>2000
F2	**1000**	**500**	**31.25 (95.67 ± 1.13 %)**
F3	>2000	2000	>2000
F4	>2000	>2000	>2000
F5	>2000	>2000	>2000
Second C18-SPE			
F2-1	>2000	>2000	>2000
F2-2	**1000**	**500**	**31.25 (97.52 ± 0.79 %)**
F2-3	2000	1000	500 (76.42 ± 2.24 %)
F2-4	>2000	>2000	>2000
F2-5	>2000	>2000	>2000
F2-5	>2000	>2000	>2000
Vaamox®	500	n.d.	n.d.
Imipenem	31.25	n.d.	n.d.
Cinnamaldehyde	n.d.	31.25	7.81 (87.8 ± 2.32 %)

n.d.: Not determined.

while those with an inhibition percentage between 0 and 50 % were considered as weak anti-biofilm activity. Although values less than 0 were considered to lack antibiofilm activity and they were considered enhancers of biofilm formation [21]. Interestingly, the *n*-BuOH fraction inhibited bacterial biofilm formation by 72.02 % and 52.15 % at sub-inhibitory concentrations, corresponding to ½ (250 μg/mL) and ¼ MIC (125 μg/mL) concentrations, respectively. A reduced inhibitory effect was recorded at lower concentrations (Fig. 5A).

The effectiveness of the *n*-BuOH fraction in inhibiting PAO1 biofilm formation was further confirmed through microscopic visualization (Fig. 5B), demonstrating a significant reduction in biofilm formation compared to the untreated control. In contrast, the antibiotic agents (vaamox® and imipenem) exhibited lower antibiofilm effect than the *n*-BuOH fraction of *M. communis* (percentage of inhibition: 54.15 %, 47.24 % and 72 %, respectively) (Fig. 5A). Microscopic examination revealed that the antibiotic imipenem had less impact on reducing the biofilm formation of *P. aeruginosa* PAO1 compared to the *n*-BuOH fraction of *M. communis* (Fig. 5B).

2.6. Haemolytic effect of tested samples

The haemolytic activity of the *n*-BuOH fraction, vaamox®, and imipenem was evaluated against human erythrocytes (Table 3) and neither of them show cytotoxicity effect. Thus, the *M. communis* leaves ethanolic extract exhibited very low haemolytic effects (10.63 % ± 0.7 % and 15.87 % ± 2.4 % at 4 MIC and 8 MIC, respectively) toward human erythrocytes. Interestingly, the *n*-BuOH fraction was less cytotoxic than vaamox®, which resulted in 32.43 ± 1.72 % haemolysis at a concentration of 8 MIC. Imipenem did not induce haemolytic effect even at 8 MIC, with only 3.72 ± 2.51 % haemolysis at this concentration.

2.7. Chromatographic separation of the bioactive n-BuOH fraction and antibacterial evaluation of the resulting subfractions

The bioactive *n*-BuOH fraction was submitted to Solid Phase Extraction (SPE) on a Sep-Pak C-18 cartridge eluted with water-methanol as mobile phase to afford F1–F5 subfractions (Fig. 1). Among them, the F2 subfraction, eluted with a 3:2H_2O/MeOH mixture, resulted to be the most active fraction, showing inhibition of violacein production at 31.25 μg/mL (95.67 ± 1.13 % inhibition) and inhibition of *P. aeruginosa* PAO1 growth at 1000 μg/mL (Table 2). Subsequently, the F2 subfraction was subjected to a second SPE, using the same chromatographic conditions as before, to give other six subfractions, from F2-1 to F2-6. As indicated in Table 2, F2-2 subfraction, eluted with a 9:1 H_2O/MeOH mixture, was the most active one, inhibiting the growth of *P. aeruginosa* PAO1 at 1000 μg/mL and reducing violacein production at 31.25 μg/mL by 97.52 ± 0.79 %. F2-3 subfraction, eluted with a 3:2 H_2O/MeOH mixture, exhibited lower activity than F2-2, with a MIC value of 2000 μg/mL against *P. aeruginosa* PAO1 and inhibited violacein production by 76.42 ± 2.24 % at a concentration of 500 μg/mL. The remaining collected subfractions, F2-1, F2-4, F2-5, and F2-6, did not show antibacterial activity against *P. aeruginosa* PAO1 or inhibition of violacein production against *C. violaceum*.

2.8. Effect of the F2-2 subfraction on QS gene expression

The *pqsA*, *lasI*, *lasR*, *rhlI* and *rhlR* genes are key components of QS system in *P. aeruginosa* PAO1. The expression of these genes regulates other genes involved in virulence, biofilm formation, antibiotic resistance, and motility. Consequently, the relative expression level of these

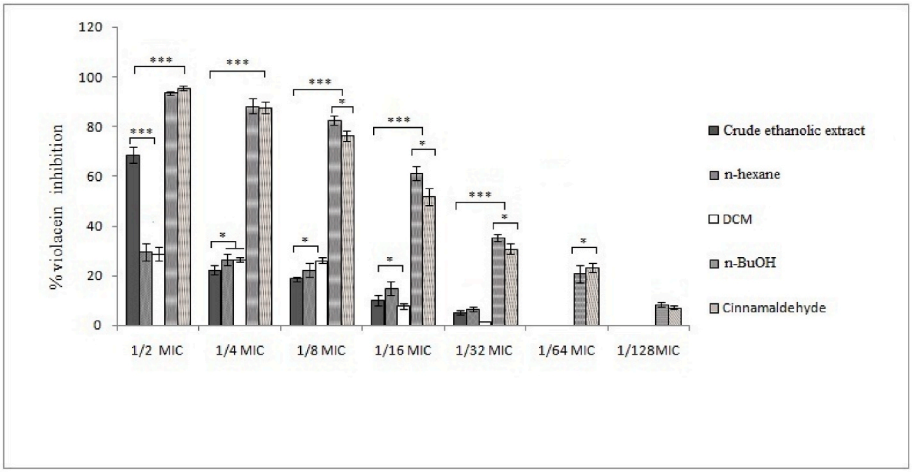

Fig. 3. Inhibition of violacein production by *Myrtus communis* ethanolic extract, *n*-hexane, dichloromethane and *n*-butanol fractions, and cinnamaldehyde at their sub-MICs concentrations. Data represent the mean ± SD of triplicate assays (n = 3). Statistically significant difference was analysed by calculating p-values: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001.

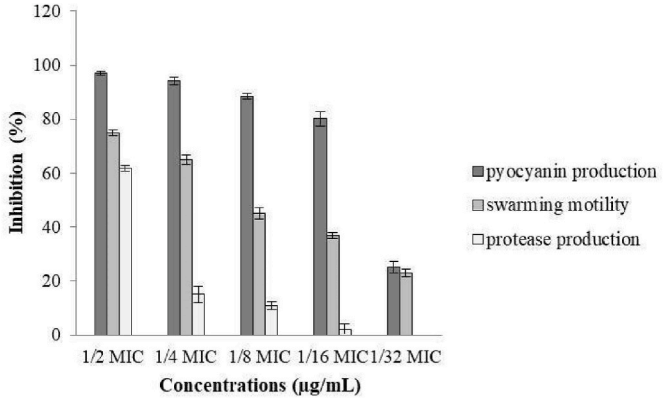

Fig. 4. Effect of the *n*-BuOH fraction from the *Myrtus communis* ethanolic extract on the virulence factors of *P. aeruginosa* PAO1: pyocyanin, and protease production, swarming motility at sub-inhibitory concentrations. Data represent the mean ± SD of triplicate assays (n = 3).

genes was assessed using RT-PCR after treating *P. aeruginosa* PAO1 cells with the most active subfraction, F2-2, at sub-lethal concentration. In this way, the F2-2 subfraction displayed a significant reduction in the expression of all tested genes when compared to untreated cells. Notably, 3 and 2-fold reduction was observed in the expression of *lasI* and *lasR* at ½ MIC (500 µg/mL). Furthermore, a significant reduction in the expression of *rhlI*, *rhlR*, and *pqsA* genes was recorded by 2-fold, 5.5-fold and 4.5-fold, respectively (Fig. 6).

2.9. Identification of the major compounds present in F2-2 subfraction

The active F2-2 subfraction was analysed by HPLC and two major chromatographic peaks was observed as shown in the chromatogram of Fig. S2, which were collected in two HPLC fractions, named F2-2-H1 and F2-2-H2 (Fig. 1). HPLC-UV-HRESIMS analysis, along with NMR studies, of both HPLC fractions allowed the identification of three major compounds **(1–3)** present in the active F2-2 subfraction (Fig. 1).

The HPLC fraction F2-2-H1 was eluted with a Rt of 5.2 min, showing λ max at 235 and 261 nm in its UV spectrum (Fig. S3). Its corresponding high resolution electrospray ionization mass spectrum in negative ion mode, (−)-HREIMS, showed an intense $[M - H]^-$ ion peak at *m/z* 495.0779 (Fig. 7) which was associated to the molecular formula for $C_{21}H_{19}O_{14}$ (calculated *m/z* 495.0780) that matched to that of 3,5-di-*O*-galloylquinic acid (**1**). Although the presence of 3,5-di-*O*-galloylquinic acid (**1**) could not be confirmed due to the lack of the appropriate standard, nevertheless, its presence was corroborated by the characteristic ion peaks observed in its (−)-HREIMS corresponding to the successive loss of one and two galloyl units at *m/z* 343.0667 (calculated for $C_{14}H_{15}O_{10}$ *m/z* 343.0665) and 191.0558 (calculated for $C_7H_{11}O_6$ *m/z* 191.0561), respectively [22].

The F2-2-H2 HPLC fraction was eluted with a Rt of 6.1 min, showing λ max at 229, 266, and 355 nm in its UV spectrum (Fig. S3). Its corresponding (−)-HREIMS showed two intense $[M - H]^-$ ion peaks. The first one at *m/z* 463.0879 was associated to the molecular formula for $C_{21}H_{19}O_{12}$ (calculated *m/z* 463.0882), matching to that of myricetin 3-*O*-α-L-rhamnopyranoside (**2**). The second one at *m/z* 631.0936 was correlated to the molecular formula for $C_{28}H_{23}O_{17}$ (calculated *m/z* 631.0941) that matched that of myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**). Again, although the presence of myricetin 3-*O*-α-L-rhamnopyranoside (**2**) and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**) could not be fully confirmed due to the lack of the appropriate standards, nevertheless, their presence was corroborated by the characteristic fragmentation in their (−)-HREIMS. Thus, a [M-rhamnosyl-H]$^-$ ion peak at *m/z* 316.022 (calculated for $C_{15}H_8O_8$ *m/z* 316.0225) was assigned to the loss of a rhamnosyl unit from myricetin 3-*O*-α−L-rhamnopyranoside (**2**) [18]. On the other hand, the [M-galloyl-H]$^-$ ion peak at *m/z* 479.0828 (calculated for $C_{21}H_{19}O_{13}$ *m/z* 479.0826) and [M-galloyl-gallactosyl-H]$^-$ ion peak at 316.0221 (calculated for $C_{15}H_8O_8$ *m/z* 316.0225) were assigned to the successive loss of galloyl and gallactosyl units from myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**), respectively (Fig. 8) [22].

The ^{1}H NMR, ^{13}C NMR spectra, and the HSQC experiment (CD_3OD, 300 MHz) of the F2-2- H1 (Fig. S4, S5 and S6) and F2-2-H2 (Figs. S7 and S8) HPLC fractions displayed all NMR proton and carbon chemical shift signals corresponding to **1** [23], **2** [24] and **3** [25]. Consequently, these data corroborated the presence of these compounds in the active F2-2 subfraction.

2.10. Molecular docking analysis of 1–3

Once the three compounds **1–3** were identified in the most active F2-2 subfraction, they were submitted to molecular docking analysis with the CviR protein which plays a significant role in *C. violaceum* QS. All of them showed an interesting affinity for that protein. The 3,5-di-*O*-galloylquinic acid (**1**) recorded the highest affinity score with a value of -9.20 kcal/mol (Table 4). The interaction of **1** with the CviR protein 3QP8 active site involved seventeen (17) conventional hydrogen bonds, two

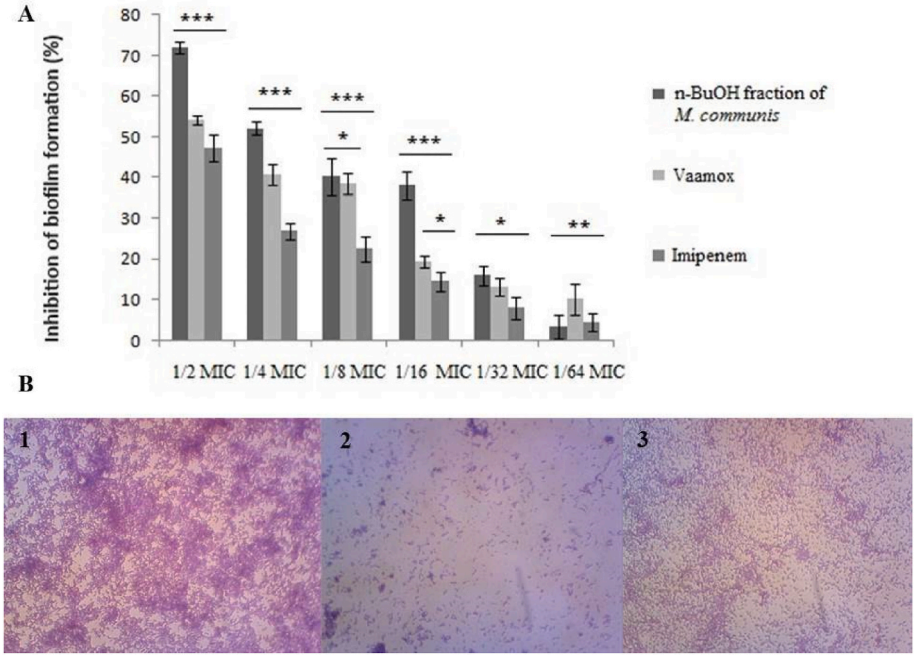

Fig. 5. A. Inhibition of *P. aeruginosa* PAO1 biofilm formation by *n*-BuOH fraction of *M. communis*, vaamox®, and imipenem Data represent the mean ± SD of triplicate assays (n = 3). The statistical significant difference was analysed by calculating p-values: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001. **B**. Microscopic observation of *P. aeruginosa* PAO1 biofilm, performed at 40× magnification after treatment with: (2) *n*-BuOH fraction of *M. communis*, (3) antibiotic imipenem. (1) PAO1 without treatment as negative control.

Table 3
Cytotoxicity effect of the *n*-BuOH fraction of *M. communis* leaves, vaamox®, and imipenem on human erythrocytes across a range of concentrations from 1 MIC to 8 MIC.

Concentrations (µg/mL)	Haemolysis (%)
***n*-BuOH fraction**	
4000	15.87 ± 2.4
2000	10.63 ± 0.7
1000	7.09 ± 1.55
500	3.01 ± 0.4
Vaamox	
4000	32.43 ± 1.72
2000	15.73 ± 3.18
1000	2.39 ± 0.53
500	0
Imipenem	
250	3.72 ± 2.51
125	2.41 ± 0.68
62.5	0.72 ± 0.4
31.25	0.42 ± 1.5

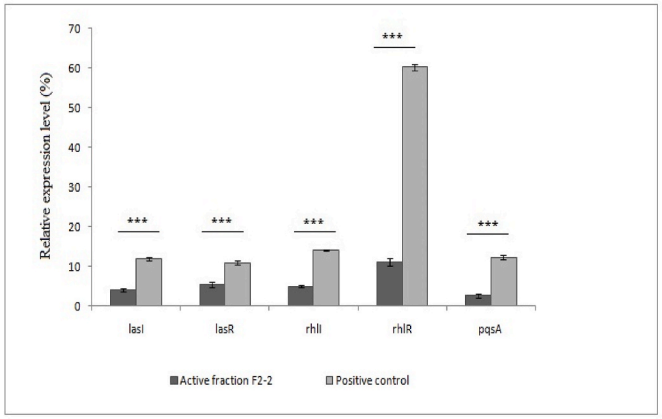

Fig. 6. Expression level of QS regulated genes after treatment with the active subfraction F2-2 at sub-inhibitory concentration corresponding to ½ MIC. Error bars represent standard deviation (n = 3). Statistical significance of the data was analysed by calculating p values with respect to control ***p ≤ 0.001.

(02) hydrophobic alkyl interactions and seven (07) van Der Waals bonds (Fig. 9A and B).

Myricetin 3-*O*-*α*-L-rhamnopyranoside (**2**) and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**) showed lower affinity scores with CviR 3QP8 protein, displaying values of −8.90 and −8.80 kcal/mol, respectively. The interaction myricetin 3-*O*-*α*-L-rhamnopyranoside (**2**)/ CviR protein 3DP8 involved two types of hydrogen bond interactions: fifteen (15) conventional hydrogen bonds and one (01) carbon-hydrogen bond, one (01) hydrophobic Pi-alkyl interaction, one (01) electrostatic interaction (attractive charge/Pi anions) and four (04) van Der Waals bonds (Fig. 9C and D). Similarly, the interaction of myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**) with the target protein involved two types of hydrogen bond interactions: fourteen (14) conventional hydrogen bonds and one (01) carbon-hydrogen bond, two (02) hydrophobic Pi-alkyl interactions, one (01) electrostatic interaction (attractive charge/Pi anions) and four (04) van Der Waals bonds (Fig. 9E and F). Noteworthy, 3,5-di-*O*-galloylquinic acid (**1**) exhibited higher binding affinity than the inducer *N*-(3-hydroxydecanoyl)-DL-homoserine lactone (3-OHC10-HSL) (−8.00 kcal/mol). This AHL is recognized as an inducer of violacein production by *C. violaceum* [26,27]. The interaction of the inducer with CviR 3QP8 protein involved two types of hydrogen bond interactions: nine (09) conventional hydrogen bonds and two (02) carbon-hydrogen bonds, three types of hydrophobic interactions as seven (07) alkyl, two (02) Pi-alkyl and one (01) Pi-sigma bonds and seven (07) van der Waals bonds (Fig. 9G and H). Noteworthy, 3, 5-di-*O*-galloylquinic acid (**1**) interacts with similar amino acids as the inducer C10-HSL with 3QP8 protein: A/ILE.57, A/LEU.85, A/TYR.88, A/SER89 and D/LEU.48 (Table 4).

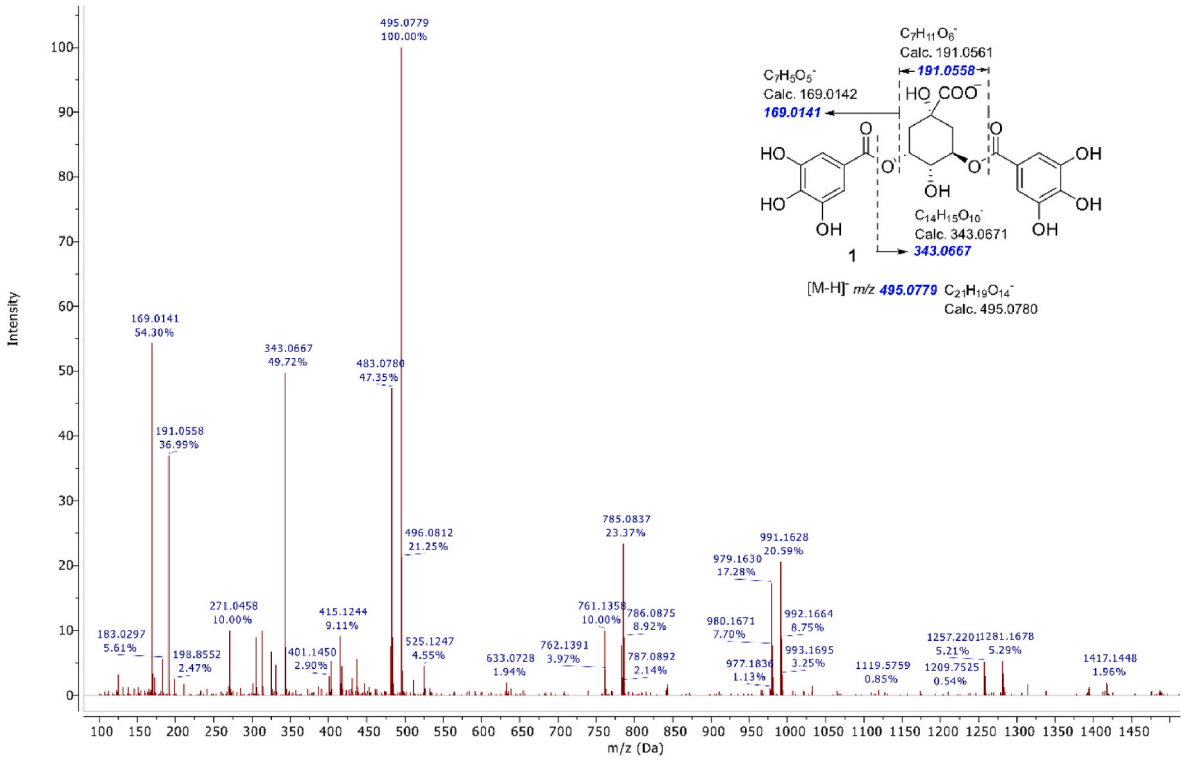

Fig. 7. (−)-HRESI-MS of the HPLC fraction F2-2-H1 (Rt. 5.2 min) showing the $[M - H]^-$ ion at *m/z* 495.0779 which matched to that 3,5-di-*O*-galloylquinic acid (**1**).

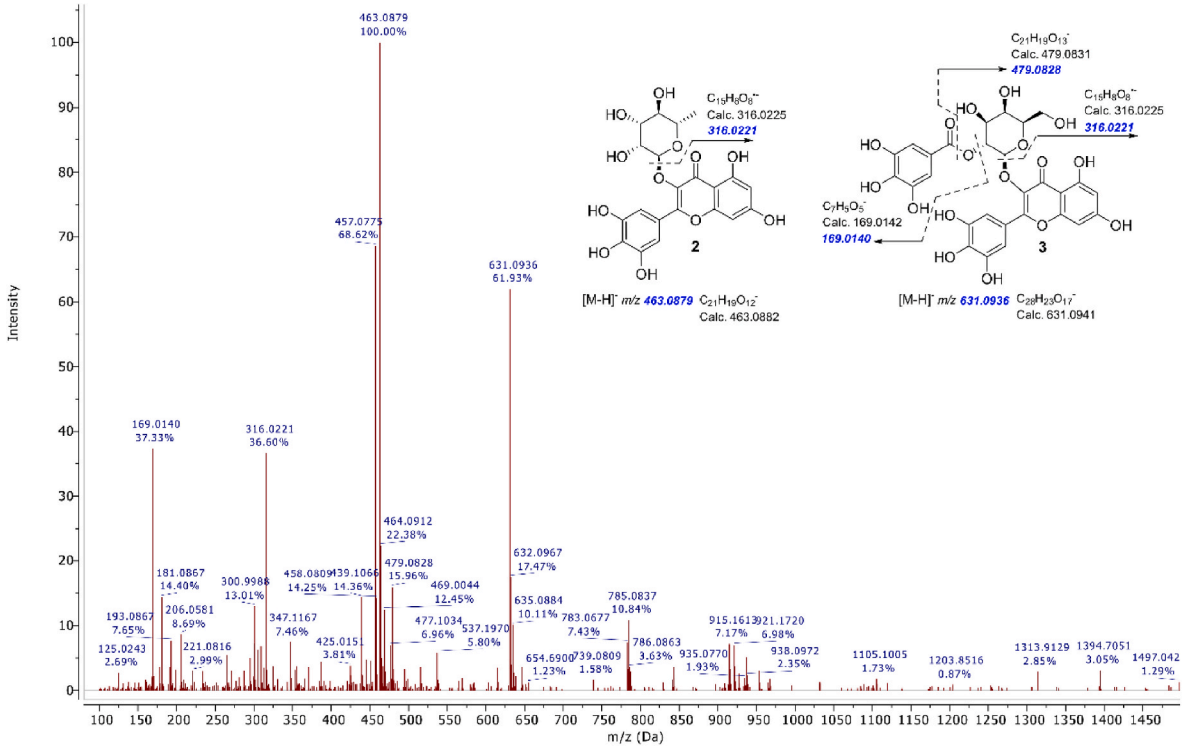

Fig. 8. (−)-HRESI-MS of the HPLC fraction F2-2-H2 (Rt. 6.1 min) and structures of myricetin 3-*O*-α-L-rhamnopyranoside (**2**) and myricetin-3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**).

Table 4
Molecular docking results of 1–3 compounds detected in F2-2 fraction from *M. communis* leaves extract and the inducer C10-HSL with CviR receptor active site.

Receptor	Ligands	Binding energy (Kcal/mol)	Active site aminoacids	Interaction type
CviR (*C. violaceum*)	C10-HSL	−8.00	A/VAL.59, A/LEU.76, A/TYR.80, A/TRP.84, A/ASP.97, A/SER.131, A/ILE.139, A/THR.140, A/SER.155	Hydrogen
			A/ILE.57, A/MET.72, A/VAL.75, A/LEU.85, A/TYR.80, A/TYR.88, D/PRO.52	Alkyl
			A/TRP.111, A/PHE.126	Pi-Alkyl
			A/TYR.80, A/ASP.97	Carbone-hydrogen
			A/TRP.111	Pi-Sigma
			A/SER.89, A/PHE.115, A/ALA.130, A/MET.135, A/ILE.153, D/LEU.48, D/SER.49	Van der Waals
	Quinic acid, 3, 5-di-*O*-gallate (**1**)	−9.20	A/LYS.41, A/LYS.74, A/ASN.77, A/SER.82, A/TYR.88, A/SER.89, A/GLN.90, A/ASN.92, A/PHE.93, A/ALA.94, D/ASP.45, D/ASN.46, D/SER.53, D/ASP.54, D/ARG.55, D/ILE.56, D/VAL.78	Hydrogen
			A/LEU.85, D/LEU.48	Alkyl
			A/GLU.37, A/ILE.57, A/LEU.76, A/ASN.86, D/LYS.41, D/ALA.42, D/SER.79	Van der Waals
	Myricetin 3-*O*-α-L-rhamnopyranoside (**2**)	−8.90	A/LYS.41, A/ASN.77, A/TYR.80, A/SER.82, A/TYR.88, A/ASN.92, A/PHE.93, A/ALA.94, D/LYS.41, D/ALA.51, D/SER.53, D/ASP.54, D/ARG.55, D/ILE.56, D/VAL.78	Hydrogen
			D/LEU.48	Pi-Alkyl
			D/SER.49	Carbon-hydrogen
			D/ASP.45	Attractive charge/Pi anions
			A/VAL.59, A/VAL.75, A/ASN.86, D/SER.79	Van der Waals
	Myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**)	−8.80	A/LEU.60, A/ARG.62, A/GLN.70, A/ARG.71, A/MET.72, A/VAL.75, A/TYR.88, A/ASN.92, A/ALA.94, A/GLN.95, D/ALA.51, D/SER.53, D/LYS.159, D/ASN.164	Hydrogen
			A/ALA.94, D/LYS.159	Pi-Alkyl
			A/GLN.70	Carbon-hydrogen
			D/ASP.54	Attractive charge/Pi anions
			D/PRO.52, D/GLY.134, D/GLU.160, D/ARG.163	Van der Waals

3. Material and methods

3.1. Plant collection and identification

Myrtus communis was collected at Ain Sebaa, in Tabarka, from North-West region of Tunisia (36°95′ 20.7′′N and 8° 92′ 70.7″). The identification of the plant was performed by Pr. A. Smaoui (Biotechnology Center of Borj Cedria, Tunisia) and a voucher specimen [Pls-BC 50-120] was deposited in the laboratory of Bioactive Substances.

3.2. Bacterial strains and culture conditions

The model organism *Chromobacterium violaceum* 6267 was used for the anti-QS tests. It was kindly provided by E.-B. Kerekes, Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Hungary. *Pseudomonas aeruginosa* PAO1 was also used in this study. Both strains were cultured in Luria broth (LB) medium containing 1 % peptone, 0.5 % yeast extract, and 0.5 % NaCl. These strains were stored in 20 % glycerol at −80 °C.

3.3. Chemicals

The following antibiotics were used as conventional antimicrobial drugs: vaamox® (amoxicilline and clavulanic acid) and imipenem (UNIMED laboratories). Cinnamaldehyde (Sigma-Aldrich, USA) was used as QS inhibitor.

3.4. Extraction of the leaves and partition of the corresponding crude extract

Air-dried leaves of *M. communis* were mechanically transformed in powder using an electrical commercial blender. This powdered material was then subjected to a maceration extraction process for a duration of 72 h, utilizing a solution of ethanol and water mixed in an 80:20 vol ratio. After filtration, the extract was evaporated and dried at 50 °C under reduced pressure using a rotary evaporator (Buchi, R200, Switzerland). The resulting ethanolic extract was suspended in deionized water and subjected to successive liquid-liquid extraction using *n*-hexane (*n*-Hex), dichloromethane (DCM), and *n*-butanol (*n*-BuOH). Both organic and aqueous fractions were evaporated under reduced pressure at 45 °C, dissolved in 80 % aqueous methanol, and stored at - 20 °C for further analysis (Fig. 1).

3.5. Total phenolic content (TPC)

Total phenolic content was determined using the Folin-Ciocalteu method as previously described with minor modifications [28]. Briefly, a volume of 0.125 mL of extracts was mixed with 0.5 mL distilled water and 0.125 mL Folin-Ciocalteu reagent. After 6 min in the dark, 1.25 mL of 7 % sodium carbonate (w/v) was added and the solution was adjusted to 3 mL with distilled water. After a 90 min incubation period in the dark at room temperature, the absorbance of the reaction was quantified at a wavelength of 760 nm using a UV/Vis spectrophotometer (Optizen 2120 UV plus, Mecasys, Korea). Gallic acid was used as a reference and results were expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g DE). Each sample was subjected to analysis in triplicate.

3.6. Total flavonoid content

The total flavonoid content was quantified using the method proposed by Dewanto and collaborators with minor modifications [28]. Briefly, 0.075 mL of 5 % $NaNO_2$ was added to 0.25 mL of extracts, followed by 0.15 mL of 10 % $AlCl_3$. The mixture was incubated for 5 min at room temperature and then 0.5 mL of NaOH (1 M) was added. The mixture was incubated at room temperature for 15 min and the absorbance was measured at 510 nm. Catechin was used as a standard for the calibration curve. All the samples were analysed in triplicates and the results were expressed as mg catechin equivalent per gram of dry extract (mg CE/g DE).

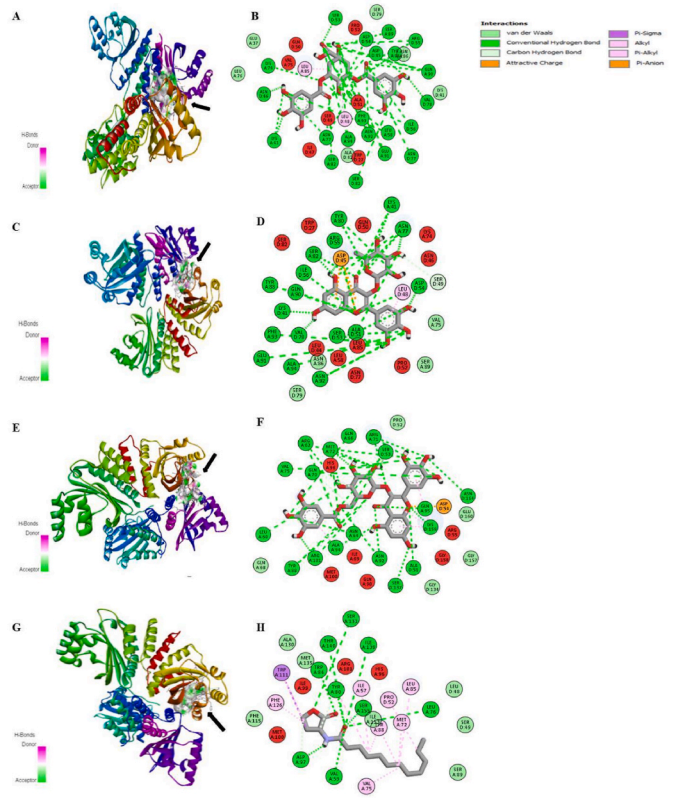

Fig. 9. Docking model of CviR 3QP8 protein binding of **1**–**3** active compounds and the inducer C10-HSL: (A, B) 3,5-di-*O*-galloylquinic acid (**1**), (C, D) myricetin 3-*O*-*α*-L-rhamnopyranoside (**2**), (E, F) myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**), and (G, H) the inducer C10-HSL. (A, C, E and G): backbone representations. (B, D, F and H): 2D interaction of the amino acids of 3QP8 protein with **1**–**3** and the inducer C10-HSL, respectively. Black arrows indicate binding sites.

3.7. Determination of tannin content

Tannin content was evaluated using the vanillin method as previously described [29]. An aliquot of 0.125 mL of tested extracts was added to 0.75 mL of vanillin and 0.375 mL of concentrated hydrochloric acid. The mixture was incubated in the dark at room temperature for 20 min and the absorbance was measured at 500 nm. The same conditions were used for the preparation of the standard calibration curve of catechin. All the samples were analysed in triplicates and the results were expressed as mg catechin equivalents per gram of dry extract (mg CE/g DE).

3.8. Determination of the antibacterial activity

The antibacterial activity of the crude ethanolic extract and fractions was assessed using the disc diffusion method as described previously with slight modification [30]. In brief, 25 µL of the suspension was impregnated into the paper disc with 5 mm diameter and placed on Muller Hinton plates inoculated with the tested bacteria. The plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibitory zone and the results were expressed as the average of three determinations.

3.9. Minimal inhibitory concentration "MIC" determination

The minimal inhibitory concentrations (MIC) of tested extracts against *P. aerugionsa* PAO1 and *C. violaceum* 6267 were determined in triplicate in sterile 96-well microlitre plates using the broth microdilution method as previously described [30]. Briefly, 10^6 CFU/mL of bacterial strains were treated with serial two-fold dilutions of the extracts. After overnight incubation at 37 °C, MIC was determined as the lowest concentration of the extract that inhibited the microorganism growth. Vaamox® and imipenem were used as positive controls [31].

3.10. Anti-quorum sensing activity

The anti-QS activity of *M. communis* extracts was initially evaluated by Agar disk-diffusion method at sub-MIC concentrations using *C. violaceum* 6267. Briefly, the overnight broth culture of *C. violaceum* was adjusted to 0.5 McFarland scale and was inoculated on Muller-Hinton (MH) culture medium. Sub-MIC concentrations were loaded over sterile paper disks and placed on the solidified agar plates. Plates were incubated at 25 °C under light exposure for 24 h. The QS inhibition was detected by a colourless viable halo on a purple background around the disks. Furthermore, quantitative evaluation of QS inhibition was carried out in 96 well plates as previously described with some modifications [32]. Briefly, serial dilutions of *M. communis* extracts (in LB medium) were incubated with 10^6 CFU/mL of bacterial suspension. Cinnamaldehyde was used as a known QS inhibitor [32] and ethanol 80 % was used as a negative control. After incubation at 30 °C under light exposure overnight, the microplate was centrifuged (3000 rpm, 20 min) to precipitate the insoluble violacein. The culture supernatants were removed and the pellet was suspended in 100 µl pure DMSO. The microplate was centrifuged again (3000 rpm, 20 min) and violacein was quantified spectrophotometrically at 585 nm. The percentage of violacein inhibition was calculated according to the following formula:

Violacein inhibition % = 100 - [(OD extract – OD negative control)/ (OD positive control – OD negative control)] x 100.

3.11. Inhibition of QS-mediated virulence factors in P. aeruginosa PAO1

3.11.1. Pyocyanin assay

The effect of tested samples on pyocyanin production was performed as previously described with some modifications [33]. Briefly, serial two-fold dilutions of the extract were incubated with 10^6 CFU/mL bacterial suspension at 37 °C for 24 h. Cells were separated from culture fluids by centrifugation at 3000 rpm for 20 min, and pyocyanin was extracted from 100 µL of culture supernatant by adding an equal volume of chloroform. The amount of pyocyanin was determined in the organic layer at 520 nm and the percentage of pyocyanin inhibition was calculated.

3.11.2. Swarming motility assay

Swarming motility, a factor associated with the antibiotic resistance of *Pseudomonas* strains, was assessed by inoculating *P. aeruginosa* colony on swarming agar medium with and without plant extracts according to Krishnan et al. (2012) [34]. The plant extract was added to the medium at sub-MICs concentrations and 2 µL of overnight cultures of *P. aeruginosa* PAO1 were inoculated on the agar surface at the middle of the plates. Plates were incubated at 37 °C for 24 h. Swarming assay was performed in triplicate and the mean of the diameter was assigned. A control plate containing the solvent was also performed.

3.11.3. Total proteolytic activity assay

The ability of the extract to inhibit protease production by *P. aeruginosa* PAO1 was determined according to Gharbi et *al.*, (2018) with slight modifications [35]. Briefly, *P. aeruginosa* PAO1 was cultured in LB broth supplemented with sub-MIC concentrations of the plant extract and was incubated at 37 °C for 24 h. The culture was centrifuged at 15.000 rpm for 20 min. Then, 25 µL of each culture supernatant fluid

was mixed with 75 μl of 5 % (w/v) azocasein solution (Sigma-Aldrich, France) prepared in 0.1 M Tris-HCl buffer pH 9. Samples were incubated for 1 h at 37 °C and the reaction was stopped by adding 600 μL of 10 % (w/v) trichloroacetic acid (TCA). After centrifugation (10.000 rpm for 5 min), 700 μL of each supernatant was added to 600 μL 1 N NaOH. The OD was measured at 440 nm. One unit of proteolytic activity was defined as the amount of enzyme that increases the absorbance by 0.01 per min under the assay conditions.

3.12. Inhibition of P. aeruginosa PAO1 biofilm formation

The inhibitory effect of the biofilm formation was carried out by the method of crystal violet staining as previously reported [36]. Serial dilutions of the tested extract (7.81–500 μg/mL), vaamox® (3.9–250 μg/mL), and imipenem (0.015–0.97 μg/mL) were incubated with 10^5 CFU/mL of PAO1 suspension in LB broth at 37 °C overnight. The biomass was quantified using the crystal violet staining method and biofilm inhibition was evaluated by measuring the absorbance at 600 nm using a microplate reader (Synergy HT, Bio-Teck). Microscopic evaluation of PAO1 cells in biofilm was performed using an inverted microscope (IVU6000, Labomed).

3.13. Haemolytic assay

The haemolytic activity was assayed on human erythrocytes as previously described [37]. Briefly, aliquots of human erythrocyte suspension (10^7 cells/mL in 0.9 % NaCl) were incubated with various concentrations of the tested extract (500–4000 μg/mL), vaamox® (250–2000 μg/mL), and imipenem (0.97–7.81 μg/mL) corresponding to MIC – 8 MIC values. After incubation at 37 °C for 30 min, the samples were centrifuged at 3500 rpm for 15 min and the supernatant was measured at 450 nm. Complete haemolysis was obtained by suspending erythrocytes in distilled water and the mixture was used as a positive control. Each experiment was performed in triplicate and the percentage of haemolysis was calculated.

3.14. Solid Phase Extraction (SPE)

The *n*-BuOH active fraction was fractioned as shown in Fig. 1. Briefly, it was submitted to Solid Phase Extraction (SPE) on a C18 Sep-Pak cartridge and was eluted with a gradient of water-methanol mixtures: 1:0; 3:1; 1:1; 1:3; 0:1 (v:v) to give five subfractions, named F1–F5, respectively. The F2 active fraction, eluted with a 3:1 mixture of water-methanol, was then submitted to a second SPE on a Sep-Pak C-18 cartridge eluted with a gradient of water-methanol mixtures: 1:0; 9:1; 8:2; 7:3; 1:1, and 0:1 (v:v) to give other six subfractions, named from F2-1 to F2-56, respectively. The collected active F2-2 subfraction, eluted with a 9:1 mixture of water-methanol, was analysed by LC/MS using a Thermo Scientific liquid chromatography system coupled with an LQT-Orbitrap Discovery mass spectrometer. An Atlantis C18 column (5 μm, 4.6 × 100 mm) was used. The mobile phase consisted of 0.1 % formic acid in water (Solvent A) and acetonitrile (Solvent B). A linear gradient with a flow rate of 0.5 mL/min was performed: 0–5 min, from 5 to 10 % (B); 5–10 min at 10 % (B); 10–15 min, from 10 to 100 % (B), and then 15–20 min at 100 % (B). MS analysis was run in negative ionization mode using an electrospray ionization (ESI) source. In this way, two HPLC fractions, named as F2-2-H1 and F2-2-H2, were collected (Fig. 1).

3.15. Identification of the active compounds

The identification of the compounds from the active fraction was based on the analysis of their corresponding HRESIMS and the comparison of their MS/MS fragmentation and NMR spectral data to those of the reported known compounds. MestReNova software version 14.1 was used for NMR data analysis. ^{1}H NMR [^{1}H (300 MHz)] and ^{13}C NMR [^{13}C (75 MHz)] analysis of both HPLC fractions was performed using a Bruker 300 MHz Neo Avance spectrometrer.

3.16. RNA extraction and cDNA synthesis

P. aeruginosa PAO1 was treated with the purified active fraction at sub-inhibitory concentration for an overnight incubation at 37 °C in 12-well plates. Untreated cells served as a positive control. Subsequently, total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA USA). The quantification of RNA was performed using a Nanodrop spectrophotometer. cDNA was then synthesized with oligo (dT) primers using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and was stored at −20 °C until use.

3.17. Quantitative real-time PCR analysis

SYBR Green qRT-PCR was used to monitor the expression of the QS-regulated genes of *P. aeruginosa* PAO1 treated with the active fraction at sub-inhibitory concentration corresponding to half MIC. Each PCR tube contained: 10 μl of SYBR Green PCR Master Mix, 0.2 μM of both forward and reverse primers of target genes (Table S1) [38], 3.4 μl of nuclease-free water, and 2.5 μL of cDNA. *rpoD* gene was used as an internal control (Table S1). The reaction was accomplished in a 7300 Real-Time PCR System (Applied Biosystems). Each PCR reaction was set up in triplicate and the relative expression of the target genes was estimated using $2^{-\Delta Ct}$ relative expression method.

3.18. Molecular docking study of CviR and identified compounds

Molecular docking studies were carried out with the CB-DOCK2 vina Tools (version 2021.09.15). The crystal structure of the target protein CviR from *Chromobacterium violaceum* (PDB ID: 3qp8) was downloaded from the protein databank (https://www.rcsb.org, access September 2023). In the crystal structure, missing residues were added and water molecules around the receptor were removed using the protein preparation wizard. The 3D structures of the auto-inducer C10-HSL ($C_{14}H_{25}NO_3$), the native ligand of 3QP8, 3,5-di-*O*-galloylquinic acid (**1**, $C_{21}H_{20}O_{14}$), myricetin 3-*O*-α-L-rhamnopyranoside (**2**, $C_{21}H_{20}O_{12}$), and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**, $C_{28}H_{24}O_{17}$) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov, access April 2024) and USCF Chimera 1.17. Docking analysis was performed to obtain a possible conformation and orientation of the ligand at the binding site. The cubical grid box that encloses the active sites of CviR had a dimension of 26 x 26 x 26 for x, y and z axis, respectively. The best conformation with the lowest binding energy was chosen [39]. The docked structures were visualized using PyMOL version 0.99. The interaction between target proteins and ligands was also determined by Biovia Discovery Studio v21.1.0.20298 (Biovia, D.S 2021).

3.19. Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean ± standard deviation. Data were considered statistically significant at minimum level of $p < 0.05$. Microsoft Excel 2010 statistical package was used for all analyses.

4. Discussion

The widespread misuse of antibiotics in both human and veterinary medicine has led to the emergence and proliferation of antimicrobial resistance among bacterial populations [38]. The QS system plays an important role not only in the regulation of virulence factors, such as biofilm, by triggering the activation of genes implicated in biofilm formation in Gram-negative bacteria [40], but also initiates the activation of genes involved in antibiotic resistance mechanisms, such as efflux pumps [41].

P. aeruginosa is well known to use QS auto-inducer molecules in order

to regulate a wide range of features, including virulence factors, biofilm formation, and antibiotic resistance [42]. In this sense, the disruption of the QS in *P. aeruginosa* bacterial strains could make them more susceptible to antibiotics by preventing the coordination and activation of resistance mechanisms [3,43,44].

Considering that medicinal plants constitute a rich natural source of QS inhibitors [45], we decided to perform the first study of the anti-QS activity of the ethanolic extract of *Myrtus communis* leaves, utilizing *C. violaceum* 6267 and *P. aeruginosa* PAO1 as bacterial models.

The phenolic analysis of *M. communis* leaves ethanolic extract displayed the contents of the total polyphenols (from 46.22 to 197.13 mg GAE/g DE) along with those of flavonoids (from 43.5 to 73.2 mg CE/g DE) and condensed tannins (from 7.5 to 47.1 mg CE/g DE). The highest level of polyphenols was found in the ethanolic extract and the *n*-BuOH fraction.

Previous studies have shown that the concentration of phenolic compounds in the *M. communis* extract depends on the solvent used in the extraction process. Thus, by increasing the polarity of the solvents, the yield of phenolic compounds in the organic extract increases [46]. In consequence, both water and ethanol extracts of *M. communis* have been found to contain significant amounts of phenolic content [47]. On the other hand, it has been observed that the leaves extract of myrtle contains a notably higher amount of total phenolic compounds compared to other organs of the plant [48]. For example, Hennia and coworkers outlined that *M. communis* leaves extracts are rich sources of galloyl, flavonols, and flavonol derivatives responsible for their antioxidant properties [49].

There are several reports on the antibacterial activity along with the phyto-constituents found in myrtle extracts from different locations [50]. For example, *M. communis* leaves extract collected in Jazan, a region in the Southwestern part of Saudi Arabia, showed inhibitory effects on the growth of numerous Gram-positive bacterial strains, being ineffective against both Gram-negative bacteria and various fungal strains [51].

In the present work, *M. communis* leaves ethanolic extract, collected in Tunisia, and its corresponding *n*-BuOH fraction inhibited the growth of *C. violaceum* 6267 and *P. aeruginosa* PAO1 Gram-negative bacterial strains with MIC values between 500 and 2000 μg/mL and 250–500 μg/mL, respectively.

Likewise, Amensour et *al.* [46] reported in 2010 the antibacterial activity against *P. aeruginosa* of a leaves extract from *M. communis* collected in Morocco, displaying a MIC value of 5 mg/mL which is lower in comparison to that of one collected in Tunisia in this work. Further observations revealed a dose-dependent reduction in bacterial viability of *P. aeruginosa* PAO1. Specifically, a methanolic extract from *M. communis* leaves displayed its highest bactericidal activity against *P. aeruginosa* at 2 MIC after an exposure period of 10 h [46]. In a separate study, Mansouri reported that *P. aeruginosa* PAO1 showed sensitivity to a leaves extract from *M. communis* collected in Iran, albeit at a higher MIC value exceeding 1000 μg/mL [52].

The antibacterial potential of *M. communis* essential oil against the Gram-negative bacterium *C. violaceum* was also reported [53], revealing a MIC value of 1500 μg/mL which was higher than that observed for the *M. communis* ethanolic extract (MIC value of 500 μg/mL) in the present study. In addition to the antibacterial potency, several phenolic compounds isolated from this plant have been reported to show inhibitory effects on acyl-homoserine lactone (AHL) dependent QS in *C. violaceum* [54]. The production of the violacein pigment by the bacterium is regulated by the QS system, which involves the production of signalling molecules *N*-acyl-homoserine lactones "AHLs" [26].

In this work, besides the antibacterial effect of *M. communis* leaves ethanolic extract and the *n*-BuOH fraction on *C. violaceum* 6267 with MIC values at 250 and 500 μg/mL, they also inhibited the production of violacein at sub-inhibitory concentrations. Noteworthy, the *n*-BuOH fraction showed the most potent inhibition in violacein production, ranging from 93.55 % to 61.42 % at concentrations between ½ MIC (125 μg/mL) and 1/16 MIC (15.62 μg/mL). The inhibitory effect of a methanolic leaves extract of *M. communis* collected in Saudi Arabia in violacein pigment production of *C. violaceum* was previously reported, displaying a significant decrease of about 65 % at 200 μg/mL of concentration [55].

Previous studies have highlighted the potent inhibition of QS regulated virulence factors produced by *C. violeceum* when exposed to several plant extracts, suggesting that these extracts could provide opportunities in therapeutic applications as anti-QS based antiseptic agents [56,57]. For example, *Syzygium aromaticum*, *Dionysia revoluta* Boiss, and *Eucalyptus camaldulensis* Dehnh extracts showed potent inhibition of QS regulated virulence factor of *C. violeceum* biosensor strain [57]. Another report outlined that the fruit ethyl acetate extract from *Passiflora edulis* inhibited violacein pigment production in a dose-dependent manner [58].

The QS system is involved in the regulation of *P. aeruginosa* virulence factors including biofilm formation, exopolysaccharide production (extracellular polymeric substances in biofilm), pyocyanin excretion, and swarming motility which are involved in early stage of biofilm formation [54,59]. Furthermore, the proteolytic activity of *P. aeruginosa* has crucial role in bacterial pathogenesis since it breaks down proteins and is responsible for cell damage to acquire nutrients and to colonize host tissue [42]. Additionally, pyocyanin is known to trigger oxidative stress, leading to an increase in the intracellular levels of reactive oxygen species (ROS) such as superoxide O_2.- and hydrogen peroxide H_2O_2, resulting in oxidative damage to cellular components [60].

Biofilm formation, a virulence factor involved in the *P. aeruginosa* infection process, is a complex process that involves the production of extracellular polymeric substances (EPS) that protect bacteria from the effects of phagocytic cells and antimicrobial agents. Notably, bacteria that form biofilms exhibit a form of resistance to antibiotic treatments, presenting a significant challenge in treatment strategies [61].

Swarming motility is another virulence factor involved in the *P. aeruginosa* infection process. It is essential for the bacterium to reach nutrient-rich regions and to form biofilm on various surfaces. A published study highlighted that the inhibition of virulence factors in *P. aeruginosa* could potentially attenuate the pathogen, facilitating the elimination of the bacteria by the host's immune system. This phenomenon was observed in mice, underscoring the promising prospects of targeting virulence factors to enhance immune response against the pathogen [62]. Interestingly, the *n*-BuOH fraction from *M. communis* leaves ethanolic extract inhibited *P. aeruginosa* PAO1 virulence factors including pyocyanin, biofilm formation, swarming motility, and proteolytic enzymes, even at sub-inhibitory concentrations.

Our findings are consistent with those reported by Alyousef and coworkers who observed a significant reduction in total protease production in *P. aeruginosa* after treatment with onion peel extracts. Furthermore, it was noticed that *M. communis* leaves ethanolic extract reduced the expression of virulence factors, including proteases and elastase, inhibition of pyocyanin production, and biofilm formation [55]. It should be noted they have found that linalool is the active compound.

The effect of plant extracts in reducing the expression of virulence factors, like pyocyanin in *P. aeruginosa* PAO1 and biofilm formation, has been reported previously [61,63,64]. Another study outlined the QS inhibitory effect of the active ingredient of *Agrimonia ilosa* on the production of virulence factors, including pyocyanin, elastase, proteolytic enzymes, motility, and biofilm formation [8].

In relation to the cytotoxicity of the *n*-BuOH fraction from *M. communis* noted in the present work, its biological evaluation displayed a weak cytotoxic effect on human erythrocytes at a concentration of 4 mg/mL (15.87 % ± 2.4). Noteworthy, this concentration surpasses the MIC against *P. aeruginosa* PAO1 cells (500 μg/mL) by eightfold and is 128 times greater than the MIC required for anti-QS activity which is 31.25 μg/mL. Interestingly, this concentration is 16 times greater than the one found effective in inhibiting all the virulence factors produced

by the *P. aeruginosa* PAO1 strain. These factors include biofilm formation, pyocyanin production, proteolytic enzymes production, and swarming motility, with the effective concentration being 250 μg/mL. Thus, it seems that the anti-QS activity and the inhibitory potential of QS-mediated virulence factors in *P. aeruginosa* PAO1 strain of *n*-BuOH fraction from *M. communis* are likely unrelated to the cytotoxic effect. A recent reported study also confirmed the absence of haemolysis caused by *M. communis* extract at concentrations higher than those with effective antibacterial effects. The aqueous extract exhibited a significantly low haemoglobin leakage rate compared to other tested plants, with only 20.14 ± 3.11 % of total haemolysis recorded at 200 mg/mL [65]. Additionally, it was noted that *M. communis* essential oil induced remarkable cytotoxic activity by apoptosis without affecting normal cell lines, suggesting a possible application of the bioactive compounds in *M. communis* as a natural anticancer drug [17].

For the phytochemical analysis of the *M. communis* leaves ethanolic extract in this work, the *n*-BuOH fraction was fractionated by two consecutive SPE processes and the resulting most active subfraction, F2-2, was submitted to RP-HPLC to give two HPLC fractions, F2-2-H1 and F2-2-H2. Chemical analysis of the HRESIMS, LC/MS, and NMR data of these two HPLC fractions allowed us to identify three phenolic compounds as their major components: 3,5-di-*O*-galloylquinic acid (**1**), myricetin 3-*O*-*α*-L- rhamnopyranoside (myricitrin) (**2**), and myricetin 3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**). These findings agree with previous studies that reported the presence of these phenolic compounds from *M. communis*. For example, Yangui and coworkers identified ten phenolic compounds in leaves extract of three Tunisian genotypes of *M. communis*: five flavonols and five hydrolysable tannins. It was also noticed that the flavonol myricetin derivatives represent more than 40 % of total polyphenols in *M. communis* leaves [66]. Two myricetin glycosides with antioxidant activity were reported from another Tunisian *M. communis* leaves extract [67]. 3,5-di-*O*-galloylquinic acid and four myricetin glycosides were isolated from the leaves of *M. communis* collected in Japan [68]. Several myricetin glycosides were identified from *M. communis* leaves extract collected in Portugal displaying antioxidant activity [22]. Furthermore, myricetin glycosides and other flavonols were the major phenolic compounds identified in a *M. communis* pericarp extract from Algeria using an optimized ultrasound-assisted extraction [69].

On the other hand, the antibacterial activity of flavonoid myricetin has been demonstrated in previous studies. The ability of myricetin to inhibit the bacterial growth of various Gram-negative bacteria such as *Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa,* and *Shigella flexineri* at a concentration of 30 μg/mL was reported [70]. Taheri et *al* outlined its ability to inhibit the bacterial growth of *P. aeruginosa* ATCC 27853 at MIC of 256 μg/mL [71]. The significant inhibitory effect of myricitrin on *P. aeruginosa* biofilm formation has been reported by Motlhatlego and coworkers in 2020 [72]. Additionally, Nain et *al.* (2021) [73] reported that myricetin isolated from *Gynura procumbens* leaves, exhibited antibacterial activity along with a promising anti-QS potency. Myricetin was also reported to effectively inhibit various virulence factors in *P. aeruginosa* MZ4A, including biofilm formation, swarming motility, pyoverdine, and pyocyanin production, as well as proteolytic activity [73]. In relation to quinic acid, it was found to exert inhibitory effect on *P. aeruginosa* biofilm formation by modulating the transcriptional regulation of genes involved in the quorum sensing pathway when it was isolated from *Lonicerae japonicae Flos* [74]. Quinic acid was also reported to display a competitive binding inhibition of signalling molecules in Rhl system related to biofilm formation [74].

Regarding the QS system in *P. aeruginosa*, *Las* and *RhI* systems regulate the expression of various genes. Targeting them could be a potential strategy for controlling *P. aeruginosa* infections [75]. In this context, compounds that interfere with these systems have been identified and tested in preclinical studies, showing promising results in reducing virulence factor production and biofilm formation. In this way, we found that the F2-2 active fraction of *M. communis* leaves ethanolic extract effectively inhibited the expression of all the tested autoinducer synthase genes (*lasI* and *rhlI*), their cognate receptor genes (*lasR* and *rhlR),* and the *pqsA* genes at sub-inhibitory concentration, corresponding to 500 μg/mL. These findings are consistent with prior studies in which plant-derived phenolic compounds were observed to decrease virulence phenotypes and related genes [20]. Previous studies also outlined an overall reduction of the expression of QS regulatory genes by six medicinal plants from South Florida. Among them, *Callistemon viminalis* which belongs to the Myrtaceae family, reduced the expression of *lasI, lasR, rhlI* and *rhlR* genes in PAO1 [76]. Natural plant-derived compounds *trans*-cinnamaldehyde and salicylic acid significantly inhibit the expression of QS regulatory and virulence genes in *P. aeruginosa* at sub-inhibitory concentrations without affecting bacterial growth [63]. Paeonol, one of the main compounds in traditional Chinese medicine have shown a significant inhibitory effect on the quorum-related genes *lasI/R, rhlI/R, pqs/mvfR*, and their mediated virulence factors, LasA, LasB, RhlA, RhlC, PhzA, PhzM, PhzH, and PhzS and therefore down-regulated the virulence phenotypes [20].

The bacterium *C. violaceum* produces natural violet pigment violacein, and its production is regulated by quorum sensing system that consists of *CviI*/CviR, a LuxI/LuxR homolog [77]. Therefore, the QS receptor CviR could serve as a target model to identify promising molecules that can disrupt the quorum sensing system in *C. violaceum* [77]. Several investigations applied in silico computational studies to select potential inhibitors of quorum sensing, using the quorum sensing receptor CviR of *C. violaceum* as a model target [27,77]. In this context, molecular docking studies were conducted to evaluate the interactions of compounds **1**–**3** identified in this work in *Myrtus communis* leaves ethanolic extract with the 3QP8 structure of the CviR protein, and the results demonstrated a high binding affinity with 3,5-di-*O*-galloylquinic acid (**1**). This interaction was even higher than that of the inducer 3-OHC10-HSL to the 3QP8 structure. It was previously reported that the plant compound Z-phytol showed a higher binding affinity level than the inducer compound for the 3QP8 structure of the CviR protein [27]. Moreover, 3,5-di-*O*-galloylquinic acid (**1**) binds to residues in the 3QP8 protein that are similar to those of the inducer compound, specifically: A/ILE.57, A/LEU.85, A/TYR.88, A/SER89, and D/LEU.48. As previously noted, compounds binding to positions close to the amino acid sequence between residues 80 and 94, similarly to the inducer 3-OHC10-HSL, have the potential to compete for the binding site with the inducer molecule. Consequently, this competition could lead to the inhibition of the QS mechanism [27]. A different study highlighted that phenolic compounds, specifically quercetin aglycone and quercetin 3-β-D-glucoside, have a shared binding site with 3-OHC10-HSL within the 3QP8 structure. This shared site elucidates the inhibitory impact these compounds have on the production of violacein [78].

5. Conclusion

This study has uncovered the promising anti-QS capabilities of the ethanolic extract of *Myrtus communis* leaves. This extract exhibits a broad-spectrum effect against QS and effectively reduces the virulence factors in both *P. aeruginosa* PAO1 and *C. violaceum* 6267 strains. Three phenolic compounds were identified as the major components of the active subfaction: 3,5-di-*O*-galloylquinic acid (**1**), myricetin 3-*O*-*α*-L-rhamnopyranoside (myricitrin) (**2**), and myricetin-3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside (**3**). These compounds have shown a significant binding affinity with the QS receptor CviR, with 3,5-di-*O*-galloylquinic acid (**1**) being particularly effective. The potential of the *Myrtus communis* ethanolic leaves extract and its active fractions to inhibit QS, represents a promising strategy for combating bacterial infections, especially those caused by resistant strains. As far as we know, this is the first study that highlights the potential of *Myrtus communis* ethanolic leaves extract and its active fraction containing 3,5-di-*O*-galloylquinic acid, myricetin 3-*O*-α-L-rhamnopyranoside (myricitrin), and myricetin

3-*O*-(2″-*O*-galloyl)-ß-D-galactopyranoside in inhibiting QS in *P. aeruginosa* PAO1 and *C. violaceum* 6267 strains. However, further *in-vivo* studies are needed to evaluate the efficacy of *M. communis* extract and its active compounds as therapeutic agents against drug-resistant pathogens.

CRediT authorship contribution statement

Nadine Khadraoui: Writing – original draft, Methodology, Investigation, Conceptualization. **Rym Essid:** Methodology, Investigation, Data curation. **Bilel Damergi:** Software, Methodology, Investigation. **Nadia Fares:** Methodology, Investigation. **Dorra Gharbi:** Methodology, Investigation. **Abel Mateo Forero:** Methodology, Investigation. **Jaime Rodríguez:** Writing – review & editing, Investigation. **Ghassen Abid:** Methodology, Investigation. **Erika-Beáta Kerekes:** Methodology. **Ferid Limam:** Writing – review & editing, Conceptualization. **Carlos Jiménez:** Writing – review & editing, Investigation, Conceptualization. **Olfa Tabbene:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Carlos Jiméenez reports financial support was provided by Spanish State Agency for Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100205.

References

[1] Preda VG, Săndulescu O. Communication is the key: biofilms, quorum sensing, formation and prevention. Discoveries 2019;7(3):e100.
[2] Shukla V, Bhathena Z. Broad spectrum anti-quorum sensing activity of Tannin-rich crude extracts of indian medicinal plants. Scientifica 2016;2016:5823013.
[3] Asfour HZ. Anti-Quorum sensing natural compounds. J Microsc Ultrastruct 2018;6(1):1–10.
[4] Liao C, Huang X, Wang Q, Yao D, Lu W. Virulence factors of *Pseudomonas aeruginosa* and antivirulence strategies to combat its drug resistance. Front Cell Infect Microbiol 2022;12:926758.
[5] Wilder CN, Diggle SP, Schuster M. Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the las, rhl and pqs quorum-sensing systems. ISME J 2011;5:1332–43.
[6] Wu D, Huang W, Duan Q, Li F, Huijuan Cheng H. Sodium houttuyfonate affects production of N-acyl homoserine lactone and quorum sensing-regulated genes expression in *Pseudomonas aeruginosa*. Front Microbiol 2014;5:2014.
[7] Ding F, Oinuma KI, Smalley NE, Schaefer AL, Hamwy O, Greenberg EP, Dandekar AA. The *Pseudomonas aeruginosa* orphan quorum sensing signal receptor QscR regulates global quorum sensing gene expression by activating a single linked operon. mBio 2018;9(4):e01274. 18.
[8] Wang S, Feng Y, Han X, Cai X, Yang L, Liu C, Shen L. Inhibition of virulence factors and biofilm formation by wogonin attenuates pathogenicity of *Pseudomonas aeruginosa* PAO1 via targeting *pqs* quorum-sensing system. Int J Mol Sci 2021;22(23):12699.
[9] Wang Y, Gao L, Rao X, Wang J, Yu H, Jiang J, Zhou W, Wang J, Xiao Y, Li M, Zhang Y, Zhang K, Shen L, Hua Z. Characterization of lasR-deficient clinical isolates of *Pseudomonas aeruginosa*. Sci Rep 2018;8:13344.
[10] Kanak KR, Dass RS, Pan A. Anti-quorum sensing potential of selenium nanoparticles against LasI/R, RhlI/R, and PQS/MvfR in *Pseudomonas aeruginosa*: a molecular docking approach. Front Mol Biosci 2023;10:2023.
[11] Singh VK, Mishra A, Jha B. Anti-quorum sensing and anti-biofilm activity of *Delftia tsuruhatensis* extract by attenuating the quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa*. Front Cell Infect Microbiol 2017;7:337.
[12] Beury-Cirou A, Tannières M, Minard C, Soulère L, Rasamiravaka T, Dodd RH, Queneau Y, Dessaux Y, Guillou C, Vandeputte OM, Faure D. At a Supra-Physiological concentration, human Sexual Hormones Act as quorum-sensing inhibitors. PLoS One 2013;8:e83564.
[13] Naga NG, Zaki AA, El-Badan DE, Rateb HS, Ghanem KM, Shaaban MI. Inhibition of *Pseudomonas aeruginosa* quorum sensing by methyl gallate from *Mangifera indica*. Sci Rep 2023;13:17942.
[14] Damergi B, Essid R, Fares N, Khadraoui N, Ageitos L, Ben Alaya A, Gharbi D, Abid I, Alothman MR, Limam F, Rodríguez J, Jiménez C, Tabbene O. *Datura stramonium* flowers as a potential natural resource of bioactive molecules: identification of anti-inflammatory agents and molecular docking analysis. Molecules 2023;28(13):5195.
[15] Lafi O, Essid R, Lachaud L, Jimenez C, Rodríguez J, Ageitos L, Mhamdi R, Abaza L. Synergistic antileishmanial activity of erythrodiol, uvaol, and oleanolic acid isolated from olive leaves of cv. Chemlali. 3 Biotech 2023;13:395.
[16] Gorjian H, Khaligh NG. Myrtle: a versatile medicinal plant. Nutrire 2023;48:10.
[17] Harassi Y, Tilaoui M, Idir A, Frédéric J, Baudino S, Ajouaoi S, Mouse HA, Zyad A. Phytochemical analysis, cytotoxic and antioxidant activities of *Myrtus communis* essential oil from Morocco. J Complement Integr Med 2019;16(3):20180100.
[18] Alipour G, Dashti S, Hosseinzadeh H. Review of pharmacological effects of *Myrtus communis* L. and its active constituents. Phytother Res 2014;28(8):1125–36.
[19] Raoof M, Khaleghi M, Siasar N, Mohannadalizadeh S, Haghani J, Amanpour S. Antimicrobial activity of methanolic extracts of *Myrtus communis* L. and *Eucalyptus galbie* and their combination with calcium hydroxide powder against *Enterococcus faecalis*. J dent 2019;20(3):195–202.
[20] Yang D, Hao S, Zhao L, Shi F, Ye G, Zou Y, Song X, Li L, Yin Z, He X, Feng S, Chen H, Zhang Y, Gao Y, Li Y, Tang H. Paeonol attenuates quorum-sensing regulated virulence and biofilm formation in *Pseudomonas aeruginosa*. Front Microbiol 2021;12:692474.
[21] Adeyemo RO, Famuyide IM, Dzoyem JP, Joy ML. Antibiofilm, antibacterial, and anti-quorum sensing activities of selected south african plants traditionally used to treat diarrhoea. Evid Based Complement Alternat Med 2022;2022:1307801.
[22] Pereira P, Cebola M-J, Oliveira MC, Gil MGB. Antioxidant capacity and identification of bioactive compounds of *Myrtus communis* L. extract obtained by ultrasound-assisted extraction. J Food Sci Technol 2017;54(13):4362–9.
[23] Romani A, Pinelli P, Galardi C, Mulinacci N, Tattini M. Identification and quantification of galloyl derivatives, flavonoid glycosides and anthocyanins in leaves of *Pistacia lentiscus* L. Phytochem Anal 2002;13:79–86.
[24] Souza JNS, Silva EM, da Silva MN, Arruda MSP, Larondellea Y, Rogez H. Identification and antioxidant activity of several flavonoids of *Inga edulis* leaves. J Braz Chem Soc 2007;18(6):1276–80.
[25] Yang Z-G, Jia L-N, Shen Y, Ohmura A, Kitanaka S. Inhibitory effects of constituents from *Euphorbia lunulata* on differentiation of 3T3-L1 cells and nitric oxide production in RAW264.7 Cells. Molecules 2011;16(10):8305–18.
[26] Devescovi G, Kojic M, Covaceuszach S, Cámara M, Williams P, Bertani I, Subramoni S, Venturi V. Negative regulation of violacein Biosynthesis in *Chromobacterium violaceum*. Front Microbiol 2017;8:349.
[27] Vargas ELG, de Almeida FA, de Freitas LL, Pinto UM, Vanetti MCD. Plant compounds and nonsteroidal anti-inflammatory drugs interfere with quorum sensing in *Chromobacterium violaceum*. Arch Microbiol 2021;203(9):5491–507.
[28] Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 2002;50(10):3010–4.
[29] Julkunen-Titto R. Phenolics constituents in the leaves of northern willos: methods for the analysis of certain phenolics. J Agric Food Chem 1985;33(2):213–7.
[30] Kefi S, Essid R, Mkadmini K, Kefi A, Mahjoub Haddada F, Tabbene O, Limam F. Phytochemical investigation and biological activities of *Echium arenarium* (Guss) extracts. Microb Pathog 2018;118:202–10.
[31] Khadraoui N, Essid R, Jallouli S, Damergi B, Ben Takfa I, Abid G, Jedidi I, Bachali A, Ayed A, Limam F, Tabbene O. Antibacterial and antibiofilm activity of *Peganum harmala* seed extract against multidrug-resistant *Pseudomonas aeruginosa* pathogenic isolates and molecular mechanism of action. Arch Microbiol 2022;204(2):133.
[32] Chenia HY. Anti-quorum sensing potential of crude *Kigelia africana* fruit extracts. Sensors 2013;13(3):2802–17.
[33] Zhou JW, Ji PC, Wang CY, Yang YJ, Zhao XY, Tang HZ. Anti-virulence activity of dihydrocuminyl aldehyde and nisin against spoilage bacterium *Pseudomonas aeruginosa* XZ01. LWT 2023;177:114573.
[34] 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(4):4016–30.

[35] Gharbi D, Karkouch I, Ben Slimene I, Coquet L, Azaiez S, Tabbene O, Cosette Pascal, Jouenne T, Limam F. Purification and characterization of a novel high molecular weight alkaline protease produced by an endophytic *Bacillus halotolerans* strain CT2. Int J Biol Macromol 2018;111:342–51.

[36] Essid R, Gharbi D, Abid G, Karkouch I, Ben Hamouda T, Fares N, Trabelsi D, Mhadhbi H, Elkahoui S, Limam F, Tabbene O. Combined effect of *Thymus capitatus* and *Cinnamomum verum* essential oils with conventional drugs against *Candida albicans* biofilm formation and elucidation of the molecular mechanism of action. Ind Crops Prod 2019;140:111720.

[37] Mangoni ML, Rinaldi AC, Di Giulio A, Mignogna G, Bozzi A, Barra D, Simmaco M. Structure-function relationships of temporins, small antimicrobial peptides from amphibian skin. Eur J Biochem 2000;267(5):1447–54.

[38] Hossain MA, Lee SJ, Park NH, Mechesso AF, Birhanu BT, Kang J, Reza MA, Suh JW, Park SC. Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. Sci Rep 2017;7(1):10618.

[39] Terefe EM, Ghosh A. Molecular docking, validation, dynamics simulations, and pharmacokinetic prediction of phytochemicals isolated from croton dichogamus against the HIV-1 reverse transcriptase. Bioinform Biol Insights 2022;16:1–20.

[40] Zhou L, Zhang Y, Ge Y, Zhu X, Pan J. Regulatory mechanisms and promising applications of quorum sensing-inhibiting agents in control of bacterial biofilm formation. Front Microbiol 2020;11:589640.

[41] Raju DV, Nagarajan A, Pandit S, Nag M, Lahiri D, Upadhye V. Effect of bacterial quorum sensing and mechanism of antimicrobial resistance. Biocatal Agric Biotechnol 2022;43:102409.

[42] Qin S, Xiao W, Zhou C, Pu Q, Deng X, Lan L, Liang H, Song X, Wu M. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct Targeted Ther 2022;7(1):199.

[43] García-Contreras R. Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections? Front Microbiol 2016;7:1454.

[44] Miao L, Xu J, Yao Z, Jiang Y, Zhou H, Jiang W, Dong K. The anti-quorum sensing activity and bioactive substance of a marine derived *Streptomyces*. Biotechnol Biotechnol Equip 2017;31(5):1007–15.

[45] Bouyahya A, Dakka N, Et-Touys A, Abrini J, Bakri Y. Medicinal plant products targeting quorum sensing for combating bacterial infections. Asian Pac J Trop Med 2017;10(8):729–43.

[46] Amensour M, Sendra E, Abrini J, Pérez-Alvarez JA, Fernández López J. Antioxidant activity and total phenolic compounds of myrtle extracts. J Food 2010;8(2):95–101.

[47] Tuberoso CIG, Rosa A, Bifulco E, Melis MP, Atzeri A, Pirisi FM, Dessi MA. Chemical composition and antioxidant activities of *Myrtus communis* L. berries extracts. Food Chem 2010;123(4):1242–51.

[48] Bouyahya A, Abrini J, Ouardy Khay E, Charfi S, Boujida N, EL Harsal A, Talbaoui A, Et-Touys A, Bakri Y, Dakka N. In vitro antibacterial activity of organic extracts from north-west Moroccan medicinal plant *Myrtus communis* (L.). Biotechnol J Int 2016;16(4). BJI.28500.

[49] Hennia A, Miguel MG, Nemmiche S. Antioxidant activity of *Myrtus communis* L. and *Myrtus nivellei* Batt & Trab extracts: a brief review. Medicines 2018;5(3):89.

[50] Aleksic V, Knezevic P. Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. Microbiol Res 2014;169(4):240–54.

[51] Mir MA, Bashir N, Alfaify A, Oteef MDY. GC-MS analysis of *Myrtus communis* extract and its antibacterial activity against Gram-positive bacteria. BMC complement med ther 2020;20(1):86.

[52] Mansouri S, Safa A, Najar S, Najar AG. Inhibitory activity of Iranian plant extracts on growth and biofilm formation by *Pseudomonas aeruginosa*. Malays J Microbiol 2013;9:176–83.

[53] Poli JP, Guinoiseau E, de Rocca Serra D, Sutour S, Paoli M, Tomi F, Quilichini Y, Berti L, Lorenzi V. Anti-quorum sensing activity of 12 essential oils on *chromobacterium violaceum* and specific action of *cis-cis-p*-menthenolide from Corsican *Mentha suaveolens* ssp. Insularis. Molecules 2018;23(9):2125.

[54] Oulahal N, Degraeve P. Phenolic-rich plant extracts with antimicrobial activity: an alternative to food preservatives and biocides? Front Microbiol 2022;12:753518.

[55] Alyousef AA, Mabood Husain F, Arshad M, Rizwan Ahamad S, Shavez Khan M, Abul Qais F, Khan A, Alqasim A, Almutairi N, Ahmad I, Albalawi T, husain P, Khan S. *Myrtus communis* and its bioactive phytoconstituent, linalool, interferes with Quorum sensing regulated virulence functions and biofilm of uropathogenic bacteria: in vitro and in silico insights. J King Saud Univ Sci 2021;33(7):101588.

[56] Givskov M. Beyond nutrition: health-promoting foods by quorum-sensing inhibition. Future Microbiol 2012;7(9):1025–8.

[57] Moradi F, Hadi N, Bazargani A. Evaluation of quorum-sensing inhibitory effects of extracts of three traditional medicine plants with known antibacterial properties. New microbes new infect 2020;38:100769.

[58] Venkatramanan M, Sankar Ganesh P, Senthil R, Akshay J, Veera Ravi A, Langeswaran K, Vadivelu J, Nagarajan S, Rajendran K, Shankar EM. Inhibition of quorum sensing and biofilm formation in *Chromobacterium violaceum* by fruit extracts of *Passiflora edulis*. ACS Omega 2020;5(40):25605–16.

[59] Samreen Qais FA, Ahmad I. Anti-quorum sensing and biofilm inhibitory effect of some medicinal plants against gram-negative bacterial pathogens: *in vitro* and *in silico* investigations. Heliyon 2022;8(10):e11113.

[60] Hall S, McDermott C, Anoopkumar-Dukie S, McFarland AJ, Forbes A, Perkins AV, Davey AK, Chess-Williams R, Kiefel MJ, Arora D, Grant GD. Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*. Toxins 2016;8(8):236.

[61] Sankar Ganesh P, Ravishankar Rai V. Attenuation of quorum-sensing-dependent virulence factors and biofilm formation by medicinal plants against antibiotic resistant *Pseudomonas aeruginosa*. J Tradit Complement Med 2017;8(1):170–7.

[62] Lakshmanan D, Nanda J, Jeevaratnam K. Inhibition of swarming motility of *Pseudomonas aeruginosa* by methanol extracts of *Alpinia officinarum* Hance. and *Cinnamomum tamala* T. Nees and Eberm. Nat Prod Res 2018;32(11):1307–11.

[63] Ahmed SAKS, Rudden M, Smyth TJ, Dooley JSG, Marchant R, Banat IM. Natural quorum sensing inhibitors effectively downregulate gene expression of *Pseudomonas aeruginosa* virulence factors. Appl Microbiol Biotechnol 2019;103(8):3521–35.

[64] Naga NG, Zaki AA, El-Badan DE, Rateb HS, Ghanem KM, Shaaban MI. Methoxyisoflavan derivative from *Trigonella stellata* inhibited quorum sensing and virulence factors of *Pseudomonas aeruginosa*. World J Microbiol Biotechnol 2022;38(9):156.

[65] Gadouche L, Zidane A, Khayra Z, Karima A, Saadia B. Cytotoxic effect of *Myrtus communis*, Aristolochia longa, and Calycotome spinosa on human erythrocyte cells. Foods Raw Mater 2021;9:379–86.

[66] Yangui I, Younsi F, Ghali W, Boussaid M, Messaoud C. Phytochemicals, antioxidant and anti-proliferative activities of *Myrtus communis* L. genotypes from Tunisia. S Afr J Bot 2021;137:35–45.

[67] Hayder N, Bouhlel I, Skandrani I, Kadri M, Steiman R, Guiraud P, Mariotte AM, Ghedira K, Dijoux-Franca MG, Chekir-Ghedira L. In vitro antioxidant and antigenotoxic potentials of myricetin-3-o-galactoside and myricetin-3-o-rhamnoside from *Myrtus communis*: modulation of expression of genes involved in cell defence system using cDNA microarray. Toxicol Vitro 2008;22(3):567–81.

[68] Yoshimura M, Amakura Y, Tokuhara M, Yoshida T. Polyphenolic compounds isolated from the leaves of *Myrtus communis*. J Nat Med 2008;62(3):366–8.

[69] Bouaoudia-Madi N, Boulekbache-Makhlouf L, Madani K, Silva AMS, Dairi S, Oukhmanou-Bensidhoum S, Cardoso SM. Optimization of ultrasound-assisted extraction of polyphenols from *Myrtus communis* L. Pericarp. Antioxidants 2019;8(7):205.

[70] Semwal DK, Semwal RB, Combrinck S, Viljoen A. Myricetin: a dietary molecule with diverse biological activities. Nutrients 2016;8(2):90.

[71] Taheri Y, Suleria HAR, Martins N, Sytar O, Beyatli A, Yeskaliyeva B, Seitimova G, Salehi B, Semwal P, Painuli S, Kumar A, Azzini E, Martorell M, Setzer WN, Maroyi A, Sharifi-Rad J. Myricetin bioactive effects: moving from preclinical evidence to potential clinical applications. BMC complement med ther 2020;20(1):241.

[72] Motlhatlego KE, Abdalla MA, Leonard CM, Eloff JN, McGaw LJ. Inhibitory effect of Newtonia extracts and myricetin-3-o-rhamnoside (myricitrin) on bacterial biofilm formation. BMC complement med ther 2020;20(1):358.

[73] Nain Z, Mansur FJ, Syed SB, Islam MA, Azakami H, Islam MR, Karim MM. Inhibition of biofilm formation, quorum sensing and other virulence factors in *Pseudomonas aeruginosa* by polyphenols of *Gynura procumbens* leaves. J Biomol Struct Dyn 2022;40(12):5357–71.

[74] Lu L, Zhao Y, Yi G, Li M, Liao L, Yang C, Cho C, Zhang B, Zhu J, Zou K, Cheng Q. Quinic acid: a potential antibiofilm agent against clinical resistant *Pseudomonas aeruginosa*. Chin med 2021;16(1):72.

[75] Venturi V. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiol Rev 2006;30(2):274–91.

[76] Adonizio A, Kong KF, Mathee K. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by South Florida plant extracts. Antimicrob Agents Chemother 2008;52(1):198–203.

[77] Martins FG, Melo A, Sousa SF. Identification of new potential inhibitors of quorum sensing through a specialized multi-level computational approach. Molecules 2021;26(9):2600.

[78] Quecan BXV, Santos JTC, Rivera MLC, Hassimotto NMA, Almeida FA, Pinto UM. Effect of quercetin rich onion extracts on bacterial quorum sensing. Front microbial 2019;10:867.