Infection and Drug Resistance

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ORIGINAL RESEARCH

Effect of Sub-Minimum Inhibitory Concentrations of Tyrosol and EDTA on Quorum Sensing and Virulence of *Pseudomonas aeruginosa*

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Introduction: *Pseudomonas aeruginosa* is considered a dangerous pathogen, as it causes many human diseases, besides that it is resistant to almost all types of antibacterial agents. So, new strategies to overcome *P. aeruginosa* infection have evolved to attenuate its virulence factors and inhibit its quorum-sensing (QS) activity.

Purpose: This study investigated the effect of tyrosol and EDTA as anti-quorum-sensing and antivirulence agents against *P. aeruginosa* PAO1.

Methods: Anti-quorum activity of sub-minimum inhibitory concentrations (sub-MICs) of tyrosol and EDTA was tested using *Chromobacterium violaceum* (CV 12,472) biosensor bioassay. Miller assay was used to assess the inhibition of QS signal molecules by β-galactosidase activity determination. Also, their effects on the production of protease, lipase, lecithinase, and motility were tested. The inhibitory effects of these molecules on QS regulatory genes and exotoxins genes expression were evaluated by real-time PCR.

Results: Tyrosol and EDTA at sub-MICs inhibited the production of violacein pigment. Both compounds inhibited QS molecules production and their associated virulence factors (protease, lipase, lecithinase, and motility) ($P \leq 0.05$). Besides, the expression levels of QS regulatory genes (*lasI, lasR, rhlI, rhlR, pqsA,* and *pqsR*) and exotoxins genes (*exoS and exoY*) were significantly reduced ($P \leq 0.05$).

Conclusion: Both tyrosol and EDTA can be used to fight *P. aeruginosa* infection as anti-quorum-sensing and antivirulence agents at their sub-MICs.

Keywords: PAO1, *C. violaceum*, signal molecules, real-time PCR, exotoxins, QS regulatory genes

Introduction

P. aeruginosa is an opportunistic Gram-negative bacterium that can cause several lethal infections including urinary tract, burn, respiratory tract and eye infections. Multidrug-resistant pathogens have emerged due to the unlimited use of antimicrobials. A new strategy to control the infections aims to affect and stop the adaptability of microbes to the host environment and prevent their communication with each other rather than affecting their growth.[1,2] Quorum sensing (QS) is defined as communication between cells that relies on the cells density and the concentration of specific signaling molecules known as the autoinducers (AIs).[2] AIs bind to their receptors forming a complex that will bind to a promoter and regulate the QS genes. *P. aeruginosa* contains four distinguished QS systems (Las, Rhl, Pqs and Iqs) that use AIs: N-oxododecanoyl-l-homoserine lactone (OdDHL or 3OC12-HSL), N-butanoyl-

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/IDR.S264805

l-homoserine lactone (BHL or C4-HSL), the Pseudomonas quinolone signal (PQS), and the integrated quorum-sensing signal (IQS), respectively.[1,3] Once significant levels of these signals were reached, they control the transcriptional regulatory protein LasR, RhIR, and Pqs then virulence factors transcription was enhanced.[4]

P. aeruginosa produces various pathogenicity factors and enzymes including pyocyanin, elastases, rhamnolipids, protease and extracellular exotoxin A that assist in microbial dissemination and interfere with the host immune system.[5] Virulence factors expression, production of enzymes, cells adhesion and formation of biofilm are regulated by QS systems.[6,7] Inhibitors of QS are an advanced strategy discovered to decrease *P. aeruginosa* pathogenesis and its virulence, so they can be used in treatment of its infections.[8] QS system inhibitors act by different mechanisms including inhibitors of the AIs biosynthetic pathway, interference with the AIs extracellular accumulation (through using enzymes that degrade or modify the signals, and/or antibodies and synthetic polymers that segregate the signals), interference with signal detection (by using compounds that affect binding of signal to receptors), intervention with the binding of transcription factors to DNA, inhibition the quorum-sensing signal synthesis, and the use of structural analogs to the AIs.[9]

Natural compounds such as ellagic acid,[10] ginseng,[11] eugenol,[12] xanthones,[13] penicillanic acid,[14] and different plant extracts[15,16] inhibit QS associated virulence expression. Halogenated furanones which were isolated from certain macroalgae and different acyl-homoserine lactones (AHLs) analogs were found to inhibit QS signals.[17] Also, Signal-degrading or modifying enzymes like lactonase, oxidase, and paraoxonase have been reported.[18–20] In addition, vaccines that antagonize QS signaling molecules such as C12-HSL-BSA and C12-HSL-r-PcrV have been settled.[21,22]

Tyrosol or 2-(4-hydroxyphenyl)ethanol is a natural compound found in the human diet (olives and olive oil)[23] and is produced by *Candida albicans* as QS molecules.[24,25] It exhibits antioxidant, anti-inflammatory, anticancer, antitrypanosomal and antileishmanial activities.[25] Besides, it has antivirulence activity against *P. aeruginosa* and *Staphylococcus aureus*.[24,26] It has a potent antibacterial activity that was explained by its ability to bind and inhibit bacterial ATP synthase.[27] Ethylenediaminetetraacetic acid (EDTA) which is a polyamine carboxylic acid was utilized as a food preservative,-[28] metal chelator, anticoagulant[29] and in combination with antibiotics and vitamins for several diseases therapy.[30]

This research was conducted to evaluate the anti-quorum-sensing and antivirulence activity of sub-MICs of tyrosol and EDTA and to investigate their effect on the expression levels of QS-regulatory genes and some virulence factors production.

Materials and Methods

Bacterial Strains, Culture Conditions, and Reagents

C. violaceum (CV 12,472) is a wild-type strain that produces quorum-sensing controlled purple pigment, violacein. It responds to AIs molecules C4 and C1 AHLs. *P. aeruginosa* PAO1, wild-type strain, was used to test QS and virulence factors. *P. aeruginosa* PAO-JP2 (QS deficient) (ΔLasI: Tn 10, Tc^r, ΔrhII: Tn 501, Hg^r) was used as QS negative control. *Escherichia coli* MG4/pkDT17 (Las reporter, LasB: LacZ plac-LasR; Ap^r) and *Escherichia coli* DH5α/pECP61.5 (rhI reporter, rhID rhIA: LacZ Ap^r) were used to test QS signal molecules. All strains were grown in LB broth/agar at 37°C for 16–18 h except for *C. violaceum* that was incubated at 28°C.

Tyrosol (Sigma number 79,058) and EDTA (Sigma number 27,285) were used in the preparation of stock solution (500 mM) in water and stored at −20° C.

This research was exempt from approval by the ethics committee of faculty of Pharmacy, Mansoura University, Egypt as it was conducted using reference bacterial strains.

Determination of Minimum Inhibitory Concentrations of Tyrosol and EDTA

The MIC of tyrosol and EDTA were determined by the broth microdilution method.[31] Briefly, *P. aeruginosa* PAO1 overnight culture in LB medium was adjusted to $OD_{600\ nm}$=0.08 to 0.1 and mixed with two-fold serial dilutions of tyrosol (250–16,000 μg mL^{-1}) and EDTA (39–5000 μg mL^{-1}) in 96-well microtiter plate. The plates were incubated at 37°C and the MIC was calculated as the lowest concentration that shows no visible growth of the organism. Sub-MICs (1/2, 1/4, 1/8, 1/16, and 1/32x MIC) were used for further experiments.

Determination of Anti-Quorum-Sensing Activity of Tyrosol and EDTA

The anti-quorum-sensing activity of tyrosol and EDTA was determined against *C. violaceum* ATCC 12,472 by agar well diffusion assay.[32] Briefly, 50 μL of sub-MICs (1/2x −1/32x MIC) of tyrosol and EDTA were added in

wells made into LB agar plates that were seeded with *C. violaceum* (0.5 McFarland) then incubation at 30°C for 48 h was done. The pigment production inhibition without exhibiting antibacterial activity around the well was checked.

Effect of Sub-MICs on the Viability of *P. aeruginosa*

To determine the non-inhibitory action of tyrosol and EDTA sub-MICs on *P. aeruginosa* growth; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was conducted on tyrosol and EDTA treated and untreated cells with the predetermined concentrations. Briefly, suspensions of tested strains (100µL) in phosphate-buffered saline (PBS) containing 0.2% glucose were dispensed in 96-well plates with sub-MICs of tyrosol and EDTA in PBS, incubated at 37°C for 24 h. Then, 10 µL of MTT solution (5 mg/mL) was added to the wells and plates were incubated at 37°C for 30 min with shaking. For solubilization of the formed formazan, 100 µL of dimethylsulfoxide (DMSO) solution was added then plates were left for 3 h at room temperature. Absorbance (A_{540}) was recorded using a microtiter plate reader (BioTek ELx800).[26,33,34]

Inhibition of QS Signal Molecule

Supernatants of *P. aeruginosa* PAO1 overnight cultures treated with sub-MICs (1/4x −1/32x MIC) of tyrosol and EDTA, in addition to the supernatant of untreated cells and PAO1-JP2 (a negative control) were prepared as mentioned previously by Abdel-Rhman and Rizk.[26] Miller assay was used to measure AHLs by β-galactosidase activity determination,[35,36] utilizing *E. coli* DH5α/pECP61.5 and *E. coli* MG4/pkDT17 for measuring C4-HSL and C12-HSL, respectively.

Effect of Tyrosol and EDTA on Pseudomonas Virulence Factors

The effectiveness of tyrosol and EDTA at sub-MICs on the production of total protease, lipase, and lecithinase enzymes by *P. aeruginosa* PAO1 was detected. This was conducted as described previously by utilizing the modified skim milk assay for measuring total protease,[26] p-nitrophenyl palmitate as the substrate for measuring lipase,[24] and egg-yolk tellurite emulsion for lecithinase.[26]

Motility Assays

The swarming, swimming and twitching motilities of *P. aeruginosa* were measured in the presence or absence of tyrosol and EDTA sub-MICs as previously described.[2,6] Reduction in the motility of *P. aeruginosa* indicated QS inhibitory activity of the used compounds.

Quantitative Real-Time PCR

P. aeruginosa was treated with tyrosol and EDTA sub-MICs, then TRI Reagent (T9424 Sigma-Aldrich) was used to extract the total RNA which was converted into cDNA using QuantiTect Reverse Transcription kit (QIAGEN, USA) as previously described.[33] The level of (*lasR, lasI, rhlI, rhIR, pqsR, pqsA, exoS*, and *exoY)* genes expression was measured by RT-PCR utilizing the primer sequences which are listed in Table 1. *rpoD* gene was used as an internal *P. aeruginosa* housekeeping gene. The reaction mixture was prepared using 5x FIREPol EvaGreen, qPCR Mix, ROX Dye; Solis BioDyne; Tartu, Estonia; and the program was run as described previously,[37] using Rotor-Gene Q thermocycler (QIAGEN, Hilden, Germany). The level of expression of the tested genes was measured in treated relative to untreated samples using the $2^{-\Delta\Delta Ct}$ method.[33] Expression in the PAO1-JP2 strain was also assessed.

Statistical Analysis

Results were analyzed using GraphPad Prism 5. One-way analysis of variance (ANOVA) for multiple comparisons, followed by Dunnett's posttest to compare the treated isolates versus the control group. A P value of ≤0.05 was considered statistically significant.

Results

Screening of Anti-Quorum-Sensing Activity of Sub-MICs of Tyrosol and EDTA

The MICs of tyrosol and EDTA were determined to estimate the effect of their sub-MICs on bacterial growth, virulence factors' production, and expression of QS genes. The MICs of tyrosol and EDTA against *P. aeruginosa* PAO1 were 4000 µg mL^{-1} and 1250 µg mL^{-1}, respectively. The sub-MICs of tyrosol and EDTA were used throughout the study.

The quorum-sensing inhibitory (QSI) activity of sub-MICs of tyrosol and EDTA were evaluated against *C. violaceum* ATCC 12,472 by agar well diffusion assay.

Table 1 The Sequence of Primers Used in the RT-PCR Amplification

Target Gene	Primer Sequence 5`-3`	Annealing Temp (°C)	Product Size (bp)	Reference
rpoD	5`–CGAACTGCTTGCCGACTT–3` 5`–GCGAGAGCCTCAAGGATAC–3`	56°C	131	37
lasI	5`–CGCACATCTGGGAACTCA–3` 5`–CGGCACGGATCATCATCT–3`	55–56°C	176	37
lasR	5`–CTGTGGATGCTCAAGGACTAC–3` 5`–AACTGGTCTTGCCGATGG–3`	55°C	133	37
rhlI	5`–GTAGCGGGTTTGCGGATG–3` 5`–CGGCATCAGGTCTTCATCG–3`	58°C	101	37
rhlR	5`–GCCAGCGTCTTGTTCGG–3` 5`–CGGTCTGCCTGAGCCATC–3`	58°C	160	37
pqsA	5`–GACCGGCTGTATTCGATTC–3` 5`–GCTGAACCAGGGAAAGAAC–3`	53.2°C	74	37
pqsR	5`–CTGATCTGCCGGTAATTGG–3` 5`–ATCGACGAGGAACTGAAGA–3`	52.8°C	142	37
exoS	5`–CCATCACTTCGGCGTCACT–3` 5`–GAGAGCGAGGTCAGCAGAG–3`	56–58°C	129	37
exoY	5`–TGCCATAGAATCCGTCCTC–3` 5`–GATGACCGCCGATTATGAC–3`	55°C	145	37

Abbreviation: bp, base pair.

The 1/2x MIC of either tyrosol or EDTA inhibited the production of the purple pigment violacein (QS system indicator) and inhibited the bacterial growth. The other used concentrations (1/4x −1/32x MIC) were found to inhibit the violacein pigment production without inhibiting bacterial growth indicating their anti-quorum-sensing activity.

Effect of Sub-MICs of Tyrosol and EDTA on the Viability of *P. aeruginosa*.

The sub-MICs (1/4x −1/32x MIC) of tyrosol and EDTA were tested for their effect on *P. aeruginosa* PAO1 viability by MTT reduction assay. The results showed that the viability of treated cells with these compounds was not significantly affected as compared to the untreated cells over the whole course of growth indicating that these compounds did not affect the metabolic activity of the strain.

Effect of Tyrosol and EDTA on Quorum-Sensing Signals

The ability of tyrosol and EDTA at sub-MICs to inhibit QS signal molecules (C4-HSL and C12-HSL) was tested using reporter strain assay. Untreated PAO1 cells produce high levels of C4-HSL (2707 Miller units) and C12-HSL (11,120 Miller units). The *P. aeruginosa* double mutant PAO-JP2 did not produce any detectable quantity of QS molecules. On the other hand, tyrosol (Figure 1A) and EDTA (Figure 1B) treated *P. aeruginosa* cells exhibited a significant reduction in C4-HSL ($P< 0.001$) and C12-HSL ($P< 0.05$).

The production of C4-HSL was decreased by 72%, 60%, and 39% with 1/4x, 1/8x, and 1/16x MIC of tyrosol, respectively. While with EDTA, the reduction was 70%, 41%, and 22% with 1/4x, 1/8x, and 1/16x MIC, respectively.

The C12-HSL level was also decreased with all used concentrations of tyrosol and EDTA (P-value<0.001). The reduction was 75% by 1/4x, 1/8x, and 1/16x MIC and 60% by 1/32x MIC of tyrosol. EDTA reduced it by 72%, 70%, 63%, and 40% for 1/4x, 1/8x, 1/16x and 1/32x MIC, respectively.

Effect of Tyrosol and EDTA on the Production of Some Virulence Factors

Tyrosol at 1/4x and 1/8x MIC can significantly ($P<0.001$) reduce the total protease production by 80% and 52%, respectively (Figure 2A). EDTA can only decrease the

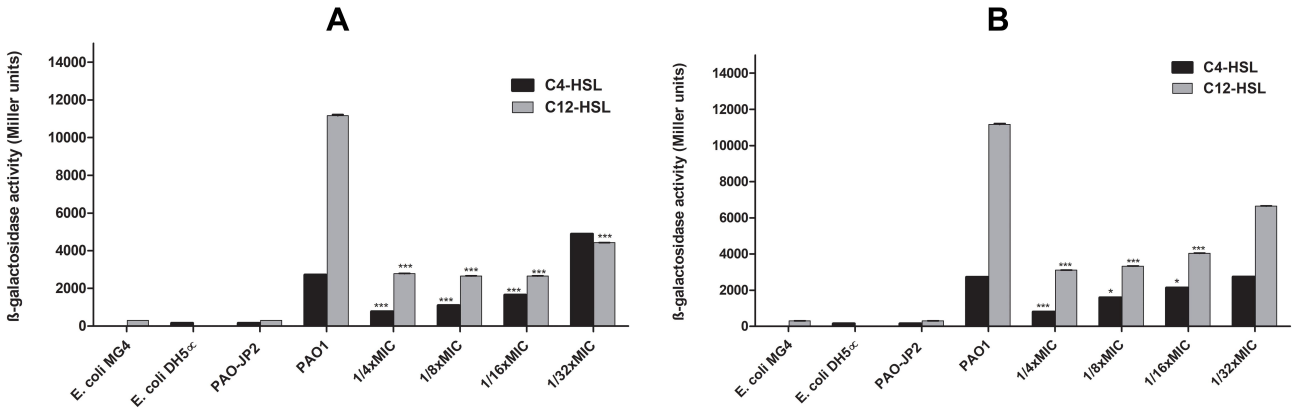

Figure 1 Effect of sub-MICs of tyrosol (**A**) and EDTA (**B**) on quorum-sensing signals molecules using reporter strain assay.
Notes: All used sub-MICs of tyrosol and EDTA caused a significant reduction in the C12-HSL signal. Tyrosol and EDTA sub-MICs (1/4x, 1/8x, and 1/16x MIC) caused significant reduction in the C4-HSL signal. *P< 0.05, ***P< 0.001.
Abbreviations: MIC, minimum inhibitory concentration; HSL, homoserine lactone.

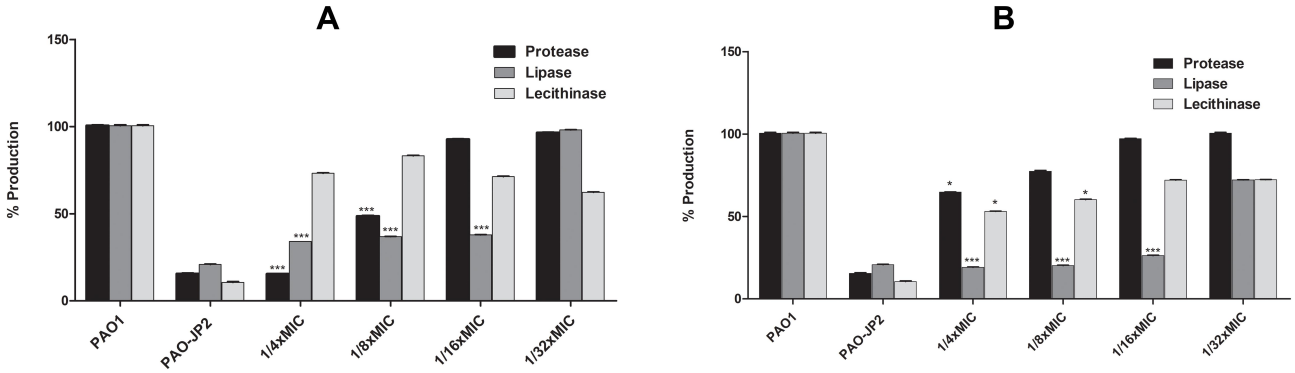

Figure 2 Effect of sub-MICs of tyrosol (**A**) and EDTA (**B**) on the production of lipase, protease, and lecithinase enzymes of PAO1.
Notes: Tyrosol and EDTA (1/4xMIC) caused a significant reduction in Protease and lipase enzymes' production. Only EDTA sub-MICs (1/4x and1/8x MIC) caused a significant reduction in lecithinase production. *P< 0.05, ***P< 0.001.
Abbreviations: MIC, minimum inhibitory concentration; HSL, homoserine lactone.

protease production by 35% at 1/4x MIC (P<0.05) (Figure 2B). Interestingly, tyrosol and EDTA can significantly decrease lipase production by ≥63% at sub-MICs (1/4x, 1/8x, and 1/16x MIC) (P<0.001). EDTA gave a higher effect than tyrosol as the reduction ranged between 81% and 73%. EDTA (1/4x and 1/8x MIC) significantly reduced lecithinase production (P<0.05).

Effect of Tyrosol and EDTA on Motility

The motility (swimming, swarming, and twitching) of PAO1 was measured in the presence and absence of tyrosol and EDTA. Tyrosol can significantly reduce the swarming motility at 1/4x, 1/8x (P<0.0001), and 1/16x MIC (P-value<0.05). EDTA (1/4x and 1/8x MIC) significantly decreased both swimming (P=0.001) and swarming motility (P<0.0001 and P<0.05, respectively). Both tyrosol and EDTA decreased twitching motility but non-significantly as illustrated in Table 2.

Real-Time PCR Analysis of *P. aeruginosa* Exotoxins and QS Regulatory Genes

The expression level of QS regulatory genes was significantly reduced by 80–50% by tyrosol (1/4x and 1/8x MIC). Tyrosol (1/4x MIC) reduced the expression of LasI and LasR by 75% and 80%, respectively; while (1/8x MIC) decreased their expression by 60% and 64%, respectively (Figure 3A). For RhlI/RhIR, tyrosol at 1/4x MIC reduced their expression by 78% and 75%, respectively; while at 1/8x MIC, they were reduced by 60% and 63%, respectively (Figure 3B). PqsA and PqsR were also decreased by tyrosol; 1/4x MIC caused a reduction in expression of PqsA and PqsR by 64% and 70%,

Table 2 Comparison of the Swimming, Swarming and Twitching Motilities Which Were Shown by PAO1 and PAO-JP2 and Treated PAO1 with Sub-MICs of Tyrosol and EDTA

	Strains	Swimming (mm)	Swarming (mm)	Twitching (mm)
Control	PAO1	85 ± 0.07	85 ± 0.070	37 ± 0.35
	PAO-JP2	45 ± 0.14	0.00	15 ± 0.14
Tyrosol sub-MICs	PAO1+1/4xMIC	65 ± 0.70	46 ± 0.70***	25 ± 0.28
	PAO1+1/8xMIC	70 ± 0.77	46 ± 0.70***	27 ± 0.56
	PAO1+1/16xMIC	75 ± 0.70	64 ± 0.70**	27 ± 0.42
	PAO1+1/32xMIC	85 ± 0.84	85 ± 0.070	27 ± 0.56
EDTA sub-MICs	PAO1+1/4xMIC	40 ± 0.70***	18 ± 0.070***	30 ± 0.77
	PAO1+1/8xMIC	55 ± 0.70***	65 ± 0.70***	32 ± 0.56
	PAO1+1/16xMIC	60 ± 2.12	80 ± 0.70	35 ± 0.49
	PAO1+1/32xMIC	75 ± 0.70	85 ± 0.35	38 ± 0.14

Notes: **P< 0.05, ***P< 0.001
Abbreviation: MIC, minimum inhibitory concentration.

respectively, while 1/8x MIC caused reduction by 50% and 57%, respectively (Figure 3C).

EDTA (1/4x and 1/8x MIC) decreased the expression of LasI by 73% and 64%, respectively, and LasR by 75% and 47%, respectively (Figure 3A). While for the other genes, only 1/4x MIC EDTA can significantly reduce the expression of *rhlI*, *rhlR* (Figure 3B), *pqsA* and *pqsR* (Figure 3C) by 71%, 73%, 51%, and 58%, respectively.

The reduction in the QS genes expression level indicated the inhibitory activity of tyrosol and EDTA on the transcriptional level of these genes. For exotoxins, our results revealed that only EDTA (1/4x MIC) resulted in a significant reduction (P< 0.05) in the expression level of ExoS and ExoY (80% and 40%, respectively).

Discussion

The increased prevalence of antibiotic resistance in Gram-negative bacteria necessitates finding new strategies for bacterial infections treatment.[38] New approaches focused on targeting Gram-negative virulence factors to decrease their pathogenicity without causing drug resistance such as inhibition of quorum sensing.[39] QS regulates many bacterial physiological processes including virulence characteristics, production of proteolytic enzymes and motility.[40] Attenuation of microbial virulence can be achieved by disruption of this communication system.[41,42] *P. aeruginosa* is considered a highly pathogenic bacterium because of its high resistance to many classes of antibiotics and development of intrinsic, acquired and adaptive resistance mechanisms,[43,44] as well as secretions of several virulence factors.[45]

A new anti-Pseudomonas strategy is targeting QS to reduce its pathogenicity without affecting microbial resistance inducement.[46] Various QS inhibitors have been reported including natural[47] or synthetic compounds.[17] Several previous studies reported that sub-MICs of ciprofloxacin, ceftazidime, and azithromycin reduces the QS of *P. aeruginosa*.[47,48] Other studies indicated that tyrosol, one of major olive oil phenolic compounds, has potent activity against many bacteria causing respiratory and intestinal infections.[49] EDTA was also reported to have an antibacterial activity by destroying the outer membrane of bacterial cells,[50] and prevention of biofilm formation by chelation of several divalent cations which are essential for stabilization of the biofilm.[51]

This is the first study to investigate the effect of tyrosol and EDTA on QS. Our results showed that tyrosol and EDTA have anti-QS activity at sub-MICs when they were screened using *C. violaceum* (CV 12,472) biosensor bioassay. The selected tested concentrations (1/4x −1/32x MIC) inhibited AHL-mediated violacein production without any bactericidal effect on the cells. To evaluate the impact of tyrosol and EDTA on QS signals and the associated virulence factors of *P. aeruginosa* PAO1 and to be sure that the results are not attributed to a decrease in the cell viability, we studied the effect of sub-MICs on the metabolic activity of *P. aeruginosa* PAO1. Interestingly, no decrease in the viability of tested strains was observed compared to the control untreated cells. This finding was consistent with previous studies reported by Abdel-Rhman et al, 2015 and Abdel-Rhman and Rizk, 2016 who found the same effectiveness of tyrosol sub-MICs on the viability of *P. aeruginosa* and *Staphylococcus aureus*,

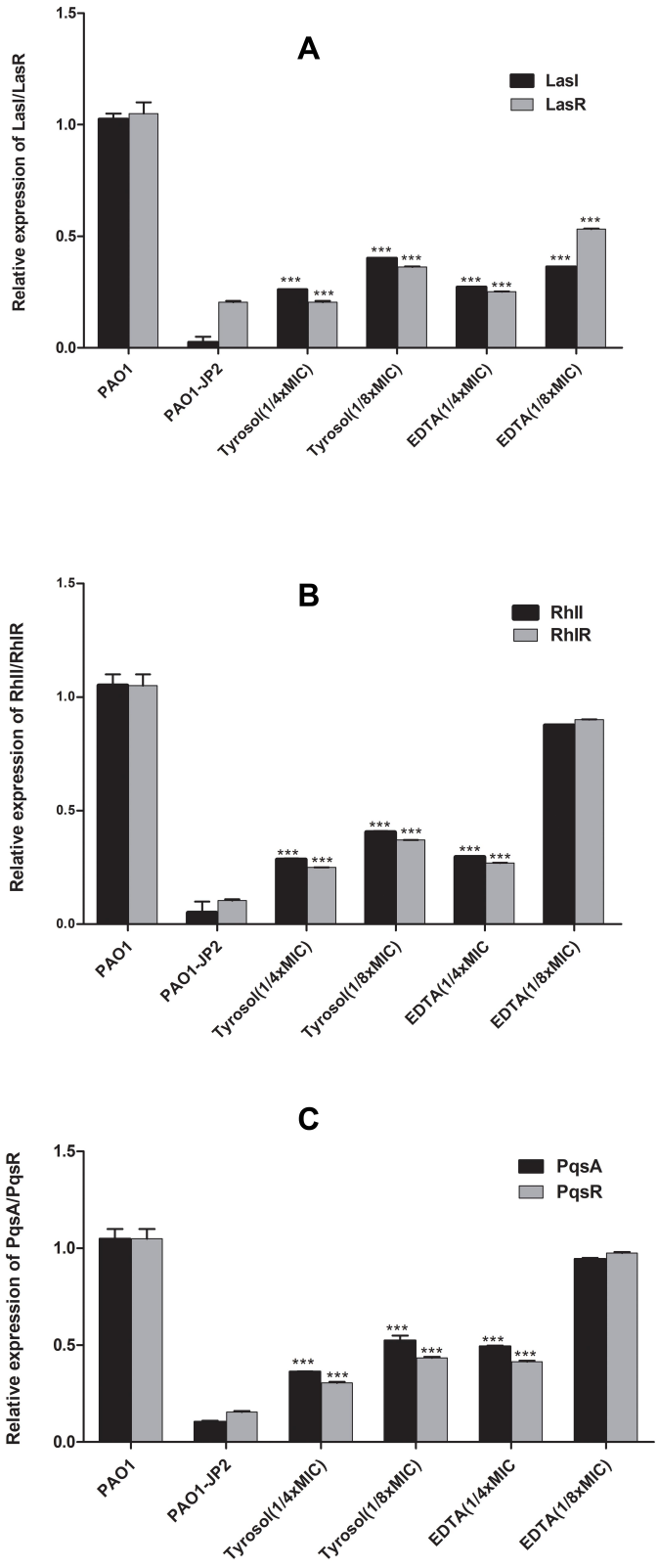

Figure 3 Effect of sub-MICs (1/4x and 1/8x MIC) of tyrosol and EDTA on the level of expression of QS regulatory genes: (**A**) relative expression of *lasI*/*lasR*, (**B**) relative expression of *rhlI*/*rhlR* and (**C**) relative expression of *pqsA*/*pqsR* of treated *P. aeruginosa* PAO1.
Note: ***P<0.0001.
Abbreviations: MIC, minimum inhibitory concentration; QS, quorum sensing.

respectively.[24,26] Also, Aboelenin et al, 2017 reported a similar effect of sub-MICs of EDTA on the viability of *E. coli*.[51]

Many studies reported that several antibacterial agents (tobramycin,[52] ciprofloxacin,[53] azithromycin,[54] ceftazidime,[48] aminoglycosides, tetracyclines[55] and piperacillin-tazobactam[56]), natural plant-derived compounds as gall extract[2,57] and onion extract[58] and drugs such as aspirin[37] can decrease QS signals (HSL) in *P. aeruginosa* at sub-MICs without any bactericidal effect. Our study showed the same result whereas both tyrosol and EDTA at sub-MICs verified a significant reduction of C4-HSL and C12-HSL signal molecules production in *P. aeruginosa* PAO1.

P. aeruginosa produces various exotoxins and enzymes that have important role in tissue injury and causing dissemination of the infection. QS coordinates its virulence factors release.[59] So, inhibition of C4-HSL and C12-HSL signals in *P. aeruginosa* leads to a marked reduction in its QS mediated virulence factors. For this reason, we evaluated the effect of tyrosol and EDTA sub-MICs on the production of some QS regulated virulence factors in *P. aeruginosa* PAO1 strain.

Proteases, lipases, and lecithinase are considered to be remarkable virulence factors of *P. aeruginosa* because they have an important role in Pseudomonas pathogenesis.[60] In the present research, we reported that EDTA and tyrosol at sub-MICs significantly quenched the production of protease. Also, sub-MICs of both compounds significantly reduced the production of lipase enzyme which consequently results in a decreased pathogenicity of the bacterial cells. In accordance with our results, previous studies reported that EDTA had an inhibitory effect on lipase activity.[61]

A significant reduction in lecithinase production was observed only with 1/4x and 1/8x MIC of EDTA. These findings were similar to what was reported by previous studies on other compounds such as that conducted by Mattmann and Blackwell about furanone C-30,[62] Musthafa et al about phenylacetic acid,[63] Quecan et al about onion extract[58] and Ahmed et al about cinnamaldehyde and salicylic acid.[64]

Motility is an important factor in both adhesion and biofilm formation.[65] *P. aeruginosa* has three types of motility that are swimming, swarming and twitching which are positively regulated by both LasIR and RhIR QS systems, where las control swimming, swarming and twitching and rhI regulates swarming and twitching.[66] In this study both swimming and swarming motility were significantly attenuated by tyrosol (1/4x - 1/16x MIC) and EDTA (1/4x and 1/8x MIC). However, both compounds demonstrated no significant reduction of twitching motility. The effect of different compounds on motility varied. Similar to our results, berberine was reported to significantly reduce both swimming and swarming without affecting twitching motility.[67] While azithromycin was reported to affect only swimming motility,[54] other compounds such as tobramycin,[52] FL fraction of *Psidium guajava*[68] and clove oil[69] impair swarming motility. Abbas et al reported a similar effect of Sitagliptin as QS inhibitor of *P. aeruginosa* and it blocked its twitching motility.[70] Gupta et al reported that ciprofloxacin can significantly reduce all three types of motility.[53]

In addition to the role of sub-MICs of tyrosol and EDTA in inhibiting virulence production, it was found that both compounds inhibited the expression of QS regulatory and exotoxin genes. The expression level of *lasR, lasI, rhlI, rhIR, pqsR* and *pqsA* genes was significantly inhibited by tyrosol (1/4x and 1/8x MIC) and EDTA (1/4x MIC), while EDTA (1/8x MIC) significantly reduced the expression of *lasR* and *lasI* genes only. This result was following that found by Ouyang et al and Okusa et al who reported the significant inhibition of QS-related genes expression by quercetin (a naturally occurring flavonol) and *C. gilletii* extracts, respectively.[71,72]

The type III secretion system (T3SS) in *P. aeruginosa* includes four exoenzymes S, U, T, and Y which lead to cell death by the destruction of cellular machinery.[73] In the current study, the expression level of *exoS* and *exoY* genes was significantly reduced by EDTA (1/4x MIC) only. Similarly, naringenin down-regulated type III secretion system genes expression.[74] Although the expression of T3SS in *P. aeruginosa* is negatively regulated by RhlI/RhlR system,[75,76] our results showed that EDTA repressed both RhlI/RhlR system and T3SS exotoxins which suggests the QS independent decrease of their secretion. In agreement, Zhang et al and Kim et al reported that coumarin and 6-gingerol, respectively, are QS inhibitors that repressed T3SS genes.[77,78]

P. aeruginosa combines several QS systems to integrate different signals hierarchically in which four QS systems act in a network.[79] The Las system is at the top of the signaling cascade since LasR/3O-C12-HSL controls the expression of the Rhl and Pqs systems.[80] Tyrosol was found to provide two types of interactions with the LasR receptor, two hydrogen bonds, and the π-π interaction, while EDTA exhibited five intermolecular hydrogen bonds by molecular docking using

MOE v102015.10 software (Data not shown). These interactions between tyrosol and EDTA with the LasR receptor may aid in an explanation of their QS inhibitory effect. Accordingly, tyrosol and EDTA may act by targeting the Las system firstly then the following cascade includes RhI and Pqs systems and as a consequence, the virulence factors production under their control will be reduced. The other possible QS inhibition mechanisms of these compounds necessitate further investigation in future studies.

Conclusion

Our data illustrated that tyrosol and EDTA reduced the production of QS controlled virulence factors (protease, lipase, lecithinase, and motility) and T3SS exotoxins (exoS and exoY). Also, they downregulated the key genes involved in QS (*lasI, lasR, rhlI, rhIR, pqsR and pqsA*). Also, this is the first study that indicates the potential effect of tyrosol and EDTA at sub-MICs as anti-quorum-sensing and antivirulence compounds in *P. aeruginosa*. So the present data suggest the possible application of EDTA and tyrosol in *P. aeruginosa* infections therapy. Further investigations are required to confirm such inhibitory effects in clinical isolates, study their effect on other virulence factors, and the exact mechanism of such inhibitory action at the molecular level.

Acknowledgments

The authors are grateful to Dr. Mona I. Shaaban (Faculty of Pharmacy, Mansoura University, Egypt) who kindly provided standard strains used in this study. We also thank Dr. Youssif M. Ali (School of Biological Science, University of Cambridge, United Kingdom) who kindly copyedited the manuscript for language usage, spelling, and grammar.

Disclosure

The authors report no conflicts of interest in this work.

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