

Perturbation of Quorum Sensing in *Pseudomonas aeruginosa* by Febuxostat

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Abstract

Quorum sensing is a signal-based communication system in bacteria. It is an attractive target because it regulates the production of virulence factors in *Pseudomonas aeruginosa*. As a result, interference with quorum sensing could result in inhibition of virulence of *Pseudomonas aeruginosa* with the merit of lack of selective pressure on growth that leads to development of resistance. This study investigated the anti-quorum sensing and anti-virulence activities of febuxostat in *Pseudomonas aeruginosa* PAO1 strain. At 1/8 MIC of febuxostat, the production of the quorum-sensing regulated violacein pigment of *Chromobacterium violaceum* CV026 was significantly reduced. Moreover, it markedly reduced pyocyanin, hemolysin, protease and elastase production. Significant inhibitory activities were also found against biofilm, swimming, twitching and swarming motilities. Molecular docking showed the ability of febuxostat to inhibit quorum sensing by competing with the autoinducers to bind with LasR and RhIR receptors. Febuxostat could bind to both receptors by hydrogen bonding and hydrophobic interaction. From the Molecular docking scores, febuxostat is a very promising quorum sensing inhibitor. Febuxostat could also significantly decrease the level of expression of all QS genes LasI, LasR, RhII, RhIR, PqsA and PqsR that regulate the production of virulence factors as confirmed by qRT-PCR.

Keywords

Febuxostat, *Pseudomonas aeruginosa*, Quorum Sensing, Virulence Inhibition, Molecular Docking

1. Introduction

Pseudomonas aeruginosa is a frequent opportunistic and nosocomial pathogen

that affects infecting patients with lowered immunity [1]. A wide range of infections can be caused by *P. aeruginosa* such as those of urinary tract, respiratory tract in addition to wound and burn infections [2] [3].

P. aeruginosa has the ability to form biofilms; this biofilm forming capacity renders the biofilm cells much more resistant to antimicrobial agents and represents a therapeutic dilemma [4]. As a result, biofilm inhibiting agents are a necessity to help treat *P. aeruginosa* infections. Biofilm formation is under the controlling power of quorum sensing (QS). QS can be regarded as a system through which bacterial cells communicate with each other. This relies on the number of bacterial cells. To know their number, bacterial cells employ autoinducers as signaling molecules whose concentration reflects the number of the secreting cells. At certain threshold concentration, the autoinducers bind to their cognate receptors to trigger changes in gene expression including genes that encode virulence factors [5]. *P. aeruginosa* among other Gram-negative bacteria use N-acylated homoserine lactones (AHLs) as signaling molecules. The QS machinery in *P. aeruginosa* is composed of three systems; namely LasI-LasR, RhlI-RhlR, and PQS-MvfR. In LasI-LasR system, Las I regulates the secretion of N-(3-oxododecanoyl)-L-homoserine signal molecule that can bind the transcriptional regulator LasR. On the other hand, RhlI is responsible for the production of the N-butyryl-L-homoserine lactone autoinducer that is recognized by its transcriptional regulator RhlR [6] [7]. Moreover, 2-heptyl-3-hydroxy-4(1H) quinolone (PQS) is the autoinducer in the PQS-MvfR system. Binding of this signal molecule to its transcriptional regulator; MvfR regulates the transcription of downstream targets [8]. A fourth QS signal was also discovered. This signal is 2-(2-hydroxyphenyl) thiazole-4-carbaldehyde (IQS) that is linked to phosphate-stress response in *P. aeruginosa* [9].

The underlying reason for targeting QS to interfere with bacterial pathogenesis is the control of biofilm formation and virulence factors by QS genes [10].

Many QS inhibitors were investigated such as synthetic furanones. Synthetic furanones are competitive inhibitors of autoinducers, so they interfere with their binding to *P. aeruginosa* LasR and/or RhlR [11].

Febuxostat is a non-purine selective inhibitor of xanthine oxidoreductase that is used in the treatment of gout due to its effect in reducing the urate concentration [12] [13].

The objective of this study is the investigation of the possible quorum sensing inhibition by febuxostat and the effect on virulence factors of *P. aeruginosa* PAO1 strain.

2. Materials and Methods

2.1. Media and Chemicals

The media used in this study were Luria-Bertani (LB) broth, LB agar and tryptone (Lab M Limited, Lancashire, United Kingdom), Mueller Hinton broth, Mueller Hinton agar, and tryptone soya broth (Oxoid, Hampshire, UK). Dimethyl sul-

phoxide (DMSO), febuxostat, elastin congo red, and N-hexanoylhomoserine lactone were the products of Sigma (St. Louis, USA). Other chemicals were of pharmaceutical grade.

2.2. Bacterial Strains

Pseudomonas aeruginosa PAO1 strain and *Chromobacterium violaceum* CV026 mutant strain were obtained from the Department of Microbiology, Faculty of Pharmacy, Mansoura University, and the Department of Microbiology, Faculty of Pharmacy, Ain Shams University, respectively.

2.3. Determination of Minimum Inhibitory Concentration (MIC) of Febuxostat

In order to determine the minimum inhibitory concentration of febuxostat, the broth microdilution method was used [14]. Two fold serial dilutions of febuxostat in Mueller-Hinton broth were prepared and then added to the wells of 96-wells microtiter plate in 100 μ l aliquots. PAO1 suspension in Mueller-Hinton broth was prepared from an overnight culture with approximate cell density of 1×10^6 CFU/ml and 100 μ l of the prepared suspension were added to each dilution. The minimum inhibitory concentration was calculated as the lowest concentration of the drug that showed no visible growth after 20 h of incubation of the plate at 37°C.

2.4. Effect of Sub-Inhibitory Concentration of Febuxostat on Bacterial Growth

To guarantee that febuxostat has no effect on the growth of PAO1, the turbidities of PAO1 in the presence and absence of 1/8 MIC of febuxostat was determined according to Nalca *et al.* [15]. Overnight culture of PAO1 was prepared and used to inoculate LB broth with and without febuxostat. LB broth tubes were incubated at 37°C for overnight and the turbidities of LB cultures treated with febuxostat and control LB cultures were measured at 600 nm by using Biotek Spectrofluorimeter (Biotek, USA).

2.5. Violacein Inhibition Assay

The ability of febuxostat to inhibit the quorum sensing-regulated violacein pigment was assessed using the method of Choo *et al.* [16]. *Chromobacterium violaceum* CV026 cultured in LB broth and incubated overnight. The bacterial suspension optical density was adjusted to 1 at 600 nm and aliquots of 100 μ l of LB broth with the autoinducer N-hexanoyl homoserine lactone in the presence and absence of 1/8 MIC of febuxostat were delivered to the wells of a 96-well microtiter plate followed by the addition of aliquots of 100 μ l of the prepared suspension. The plate was incubated for 16 h at 28°C and was then left at 60°C until complete dryness. Violacein pigment was eluted by addition of aliquots of 100 μ l of DMSO and incubation at 30°C with shaking. Negative control was also

prepared by using DMSO alone and the absorbance at 590 nm was determined by using Biotek Spectrofluorimeter (Biotek, USA).

2.6. Assay of Biofilm Inhibition

The potential biofilm inhibition by febuxostat was estimated according to the modified method of Stepanovic *et al.* [17]. Overnight culture of PAO1 in TSB was prepared and diluted to achieve an approximate cell density of 1×10^6 CFU/ml. Aliquots of 0.1 ml of the bacterial suspension were delivered into the wells of 96 well sterile microtiter plate in the presence and absence of 1/8 MIC of febuxostat. The plate was incubated at 37°C for 24 h and then the free swimming planktonic cells were aspirated. The wells were washed 3 times using sterile phosphate buffered saline (PBS, pH 7.2) and the adherent cells were fixed by methanol (99%) for 20 minutes. The wells were stained with crystal violet (1%) for a period of 20 minutes. Excess stain was washed off with distilled water and glacial acetic acid (33%) was used to elute crystal violet after air-drying of the plate. The absorbance of solubilized stain was measured at 590 nm with Biotek spectrofluorimeter (Biotek, USA).

2.7. Microscopic Visualization of Biofilm Inhibition

In order to further analyze the inhibition of biofilm formation by febuxostat, biofilms were formed on sterilized cover slips placed in 50 ml centrifuge tubes containing TSB with and without 1/8 MIC of febuxostat and inoculated with *P. aeruginosa* PAO1 suspension adjusted to optical density of 600 nm at 1. After incubation of the tubes at 37°C for 16 h, the cover slips were removed and washed with phosphate-buffered saline to remove any planktonic cells. The attached biofilms were fixed with methanol, stained with crystal violet (1%) and examined under the light microscope using the high power (400× magnification).

2.8. Swimming and Twitching Motilities Assay

To detect the possible inhibitory activities of febuxostat on swimming and twitching motilities, the modified method of Rashid and Kornberg was used [18]. Swimming agar plates (tryptone 1%, sodium chloride 0.5% and agar 0.3%) containing febuxostat (1/8 MIC) and control plates were prepared. The plates were stabbed in the center with 5 µl of diluted overnight culture of PAO1 in tryptone broth. After incubation of the plates for 24 h at 37°C, the swimming zones were measured. For assay of twitching motility inhibition, 2 µl of the prepared culture were used to stab-inoculate LB agar plates (1%) with and without febuxostat (1/8 MIC) and incubated at 37°C for 48 h. To measure the twitching zones, the agar was removed, stained with crystal violet after air-drying of the plates. The stain was removed; the plates were washed with water and dried.

2.9. Protease Assay

The ability of febuxostat to inhibit the proteolytic activity of PAO1, the skim

milk agar method was used [19]. Overnight cultures of *P. aeruginosa* PAO1 in LB broth in the presence and absence of 1/8 MIC of febuxostat were centrifuged at 10,000 rpm for 15 min and the supernatants were separated. Aliquots of 100 μ l of the supernatants were added to the wells made in 5% skim milk agar plates. After overnight incubation of the plates at 37°C, the clear zones around the wells were measured in order to detect the protease inhibitory activity of febuxostat.

2.10. Elastase Assay

The possible elastase inhibition by febuxostat was assessed by the elastin congo red assay [20]. Supernatants of untreated and febuxostat treated *P. aeruginosa* PAO1 cultures were added in aliquots of 500 μ l to elastin congo red reagent tubes. The tubes were incubated for 6 h at 37°C with shaking and any insoluble elastin congo red was removed by centrifugation and the absorbance was measured at 495 nm using Biotek Spectrofluorimeter (Biotek, USA).

2.11. Pyocyanin Assay

The reduction in production of the blue-green pyocyanin pigment by febuxostat treated PAO1 was estimated according to Das and Manefield [21]. PAO1 was cultured in LB broth and incubated overnight. The bacterial suspension was diluted to achieve an approximate optical density of 0.4 at 600 nm. LB broth tubes each contains 1 ml with 1/8 MIC of febuxostat and control LB broth tubes were prepared and inoculated with 10 μ l of the prepared suspension. After incubation at 37°C for 48 h, the tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatants were separated. The pyocyanin was estimated by measuring the absorbance at 691 nm by Biotek Spectrofluorimeter (Biotek, USA).

2.12. Hemolysin Assay

Hemolytic activity of PAO1 in the presence and absence of febuxostat was determined according to Rossignol *et al.* [22]. The prepared cell free supernatant (500 μ l) was mixed with 700 μ l of fresh 2% saline suspension of erythrocytes in saline. After incubation at 37°C for 2 h, the hemoglobin released from lysed erythrocytes was separated by centrifugation at 2500 g for 5 minutes at 4°C and measured at 540 nm. The hemoglobin released was compared with positive control prepared by addition of 0.1% SDS to erythrocyte suspension to cause complete hemolysis and negative control prepared by incubation of erythrocytes in LB broth at the same conditions. To calculate percentage hemolysis, the following formula was used:

% hemolysis = $[X - B/T - B] \times 100$, (X: the treated and untreated samples, B: the negative control, T: the positive control). The hemolysis of febuxostat treated cultures was expressed as % compared to hemolysis of untreated culture.

2.13. Total RNA Extraction for qRT-PCR

RNA of febuxostat-treated and untreated PAO1 cultures was extracted using

GeneJET RNA Purification Kit (Thermoscientific, USA) according to manufacturer instructions. Overnight cultures of PAO1 in the presence and absence of 1/8 MIC of febuxostat were prepared and centrifuged at 12,000 g for 2 minutes to collect the pellets. The Pellets were then resuspended in 100 μ l of Tris-EDTA buffer containing lysozyme. After incubation for 5 minutes at 25°C, the lysis buffer containing β -mercaptoethanol was added and mixed thoroughly. After purification, RNA was eluted by addition of 100 μ l of nuclease-free water and stored at -70°C until use.

2.14. Quantitative RT-PCR of QS Genes

To determine the effect of febuxostat on quorum sensing genes, the relative expression levels of QS genes were analyzed in PAO1 strain treated and untreated with febuxostat by qRT-PCR. RopD gene was used to normalize the relative expression level of each gene because the expression levels of this gene undergo no change in febuxostat treated and untreated PAO1 cultures. **Table 1** shows the primers used in this study. The protocol described in SensiFAST™ SYBR® Hi-ROX One-Step Kit (Bioline, UK) was used for this analysis employing StepOne Real-Time PCR system (Applied Biosystem, USA). Agarose gel electrophoresis and a melting curve analysis of products were used to confirm the specific PCR amplification according to the manufacturer recommendation. The relative gene expression was calculated by using the comparative threshold cycle ($\Delta\Delta C_t$) method [23].

2.15. Docking Study

Docking study was used to determine the molecular interaction of febuxostat with the LasR, RhlR receptors. The crystal structure of *P. aeruginosa* LasR ligand

Table 1. Primers used in RT-PCR [24].

Gene name	Primer sequence	Amplicon size (bp)
RopD (F)	5'-CGAACTGCTTGCCGACTT-3'	131
RopD (R)	5'-GCGAGAGCCTCAAGGATAC-3'	
LasI (F)	5'-CGCACATCTGGGAACTCA-3'	176
LasI (R)	5'-CGGCACGGATCATCATCT-3'	
LasR (F)	5'-CTGTGGATGCTCAAGGACTAC-3'	133
LasR (R)	5'-AACTGGTCTTGCCGATGG-3'	
RhlI (F)	5'-GTAGCGGGTTTGC GGATG-3'	101
RhlI (R)	5'-CGGCATCAGGTCTTCATCG-3'	
RhlR (F)	5'-GCCAGCGTCTTGTTCCG-3'	160
RhlR (R)	5'-CGGTCTGCCTGAGCCATC-3'	
PqsA (F)	5'-GACCGGTGTATTTCGATTC-3'	74
PqsA (R)	5'-GCTGAACCAGGAAAGAAC-3'	
PqsR (F)	5'-CTGATCTGCCGGTAATTGG-3'	142
PqsR (R)	5'-ATCGACGAGGAACTGAAGA-3'	

*F = Forward, **R = Reverse.

binding domain and the rhlr receptor model (ID: P54292.1) were obtained from Protein Data Bank (PDB ID: 2UV0) [24] and the protein model portal [25] respectively. Febuxostat and the co-crystalized natural ligand, 3-oxo-dodecanoylhomoserine lactone were docked into the receptor active site using Molegro Virtual Docker (MVD Version 6.0). Febuxostat was drawn into Marvin Sketch V5.11.5 [26] and the most energetically favored conformer was saved as (*mol²) file format for docking. The optimal geometry of the ligand was determined during the docking process. The E monomer was selected for analysis, water was discarded, cavities were determined and the search area was set to be 9 Å from the active site center. Docking process was performed by using MolDock optimizer algorithm with 10 runs/ligand, 150 population size, 4000 max iteration and 5 poses for each ligand. MolDock docking engine [27] using docking template and the optimized ligands was executed. At last, the top returned poses were selected for analysis. For docking febuxostat and the autoinducer, butanoyl homoswerine lactone (C4-HSL), into the active site of the rhlr receptor model, the same procedure was used.

2.16. Statistical Analysis

In unpaired t tests, Graph Pad Prism 5 was used to investigate the significance of the inhibitory activities of febuxostat against swarming, swimming and twitching motilities. In paired t tests, Graph Pad Prism 5 was used to detect the significance of the effects of febuxostat against biofilm formation and eradication, protease, violacein, pyocyanin, elastase and hemolysin.

P values < 0.05 were considered statistically significant.

3. Results

3.1. Determination of Minimum Inhibitory Concentration of Febuxostat against *Pseudomonas aeruginosa* PAO1

Febuxostat showed growth inhibitory effect against *Pseudomonas aeruginosa* PAO1 at a concentration of 8 mg/ml. Then, 1/8 MIC (1 mg/ml) was used to assess the activity of febuxostat against quorum sensing and virulence factors of PAO1.

3.2. Growth Inhibition Effect of Sub-MIC of Febuxostat

Potential inhibition of QS and virulence by febuxostat may be due to its effect on growth of PAO1. To avoid such possibility, PAO1 overnight cultures in LB broth with and without 1/8 MIC of febuxostat were prepared and the turbidities of the bacterial suspensions were measured at 600 nm. The difference in turbidities as a measure of growth rates in febuxostat treated and untreated PAO1 cultures was not statistically significant.

3.3. Anti-Biofilm Activities of Febuxostat

Febuxostat exerted a significant biofilm inhibitory activity. Biofilm formation by

PAO1 was reduced by 64.4% (**Figure 1(a)**). In order to further investigate the Antibiofilm effect of febuxostat, biofilms formed on sterile glass cover slips in the absence and presence of febuxostat were stained with crystal violet and examined under the light microscope. The febuxostat treated sample showed much fewer scattered cells.

3.4. Inhibition of Violacein Production

As the production of the intracellular violacein pigment in the reporter strain *C. violaceum* CV026 is well reported to be regulated by the quorum sensing machinery, the anti-quorum sensing activity of febuxostat was evaluated by measuring the reduction in violacein pigment. At first the effect of sub-MIC of febuxostat on growth of *C. violaceum* CV026 was investigated and no significant difference in growth was found between the treated and untreated samples. Febuxostat at 1/8 MIC diminished violacein production in *C. violaceum* CV026 by 71.61% (**Figure 1(b)**).

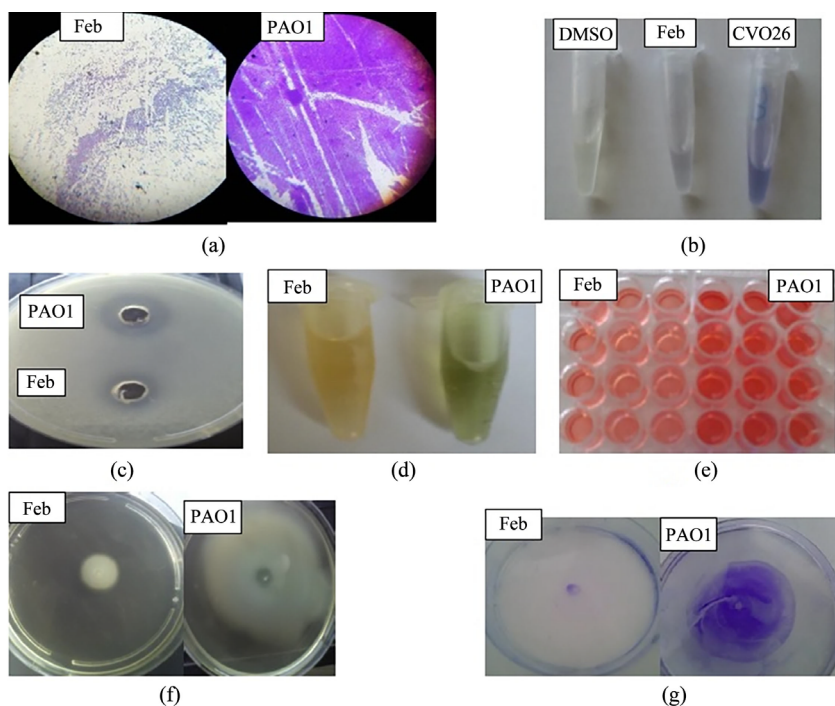


Figure 1. (a) Inhibition of biofilm formation of PAO1 by 1/8 MIC of febuxostat (i), microscopic visualization of biofilm under the light microscope ($\times 400$) (ii); biofilm in the treated culture (left) and untreated (right). *Significant $P < 0.05$. (b) Effect of febuxostat on growth of *Chromobacterium violaceum* CV026. No statistically significant difference was found in the presence or absence of febuxostat, while inhibition of production of violacein pigment of *Chromobacterium violaceum* CV026 by 1/8 MIC of febuxostat. *Significant $P < 0.05$; (c) Inhibition of protease production by 1/8 MIC of febuxostat by the skim milk agar method. *Significant $P < 0.05$; (d) Pyocyanin production in PAO1 untreated and febuxostat treated. *Significant $P < 0.05$; (e) Inhibition of elastase activity by 1/8 MIC of febuxostat. *Significant $P < 0.05$; (f) Inhibition of swimming motility of PAO1 by 1/8 MIC of febuxostat. *Significant $P < 0.05$; (g) Inhibition of twitching motility of PAO1 by 1/8 MIC of febuxostat. *Significant $P < 0.05$.

3.5. Proteolytic Activity Inhibition Assay

The supernatant of the febuxostat treated and untreated cultures were added to the wells in skim milk agar and the clear zones around them were measured. Febuxostat could inhibit protease by 55.18% (**Figure 1(c)**).

3.6. Pyocyanin Inhibition Assay

The blue green pyocyanin pigment is a redox metabolite in *P. aeruginosa* that has pulmonary cytotoxic effect [28]. In the presence of 1/8 MIC of febuxostat, pyocyanin production was lowered by 63.63% (**Figure 1(d)**).

3.7. Elastolytic Activity Inhibition Assay

The ability of elastase enzyme produced by febuxostat treated and untreated PAO1 culture supernatant to decompose elastin congo red was assayed. Elastolytic activity was reduced with febuxostat by 60.96% (**Figure 1(e)**).

3.8. Inhibition of Swimming and Twitching Motilities

Febuxostat at 1/8 MIC reduced the ability to PAO1 to swim, twitch and swarm by 70.12% (**Figure 1(f)**), 83.72% (**Figure 1(g)**), and 85.17% (**Figure 2(a)**), respectively.

3.9. Inhibition of Hemolytic Activity

The hemolytic activity of untreated PAO1 and febuxostat treated culture supernatants was assayed quantitatively and the hemoglobin release was measured spectrophotometrically. Febuxostat could inhibit hemolytic activity of PAO1 by 73.94% as compared to the untreated control.

3.10. Effect of Febuxostat on the Expression of Quorum Sensing Genes in *Pseudomonas aeruginosa*

To investigate the effect of febuxostat on the expression levels of QS genes of PAO1, qRT-PCR was performed (**Figure 2(b)**). The expression of QS genes was assessed in febuxostat treated and untreated PAO1 using $2^{-\Delta\Delta Ct}$ method. Significant decrease in the expression levels of LasI, LasR, RhlI, RhlR, PqsA and PqsR was found as compared to control PAO1 culture. The relative expression of virulence factors regulating genes were decreased from 100% in untreated PAO1 to 39.54% for LasI, 43.27% for LasR, 45.19% for RhlI, 69.87% for RhlR, 61.52% for PqsA and 28.24% for PqsR.

3.11. Docking Study

The molecular docking study included the quorum sensing proteins LasR and rhlR as targets for *P. aeruginosa* virulence inhibition. The natural ligand and febuxostat were docked into LasR active site. The binding interaction with the receptor is shown in (**Figure 3(a)**). The MolDock scores for febuxostat and the natural ligand along with the interacting residues are shown in **Table 2**. The in-

hibitor lacks the hydrophobic side chain that induces the correct formation of the hydrophobic core of LasR. The natural ligand is a LasR inducer that can stabilize the conformational change through its long acyl hydrophobic chain. Febuxostat has no acyl hydrophobic chain thus act as LasR inhibitor. The high docking score indicates a promising LasR antagonist activity for febuxostat.

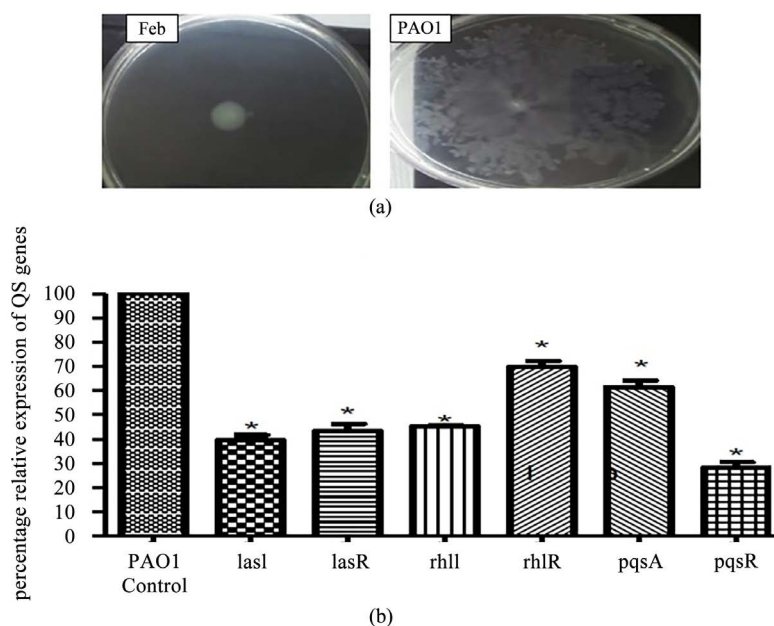


Figure 2. (a) Inhibition of swarming motility of PAO1 by 1/8 MIC of febuxostat. *Significant $P < 0.05$; (b) Reduced expression of QS genes by RT-qPCRAs compared to untreated PAO1. The data shown are the means \pm standard errors. $P < 0.05$ (One Way ANOVA) was considered significant.

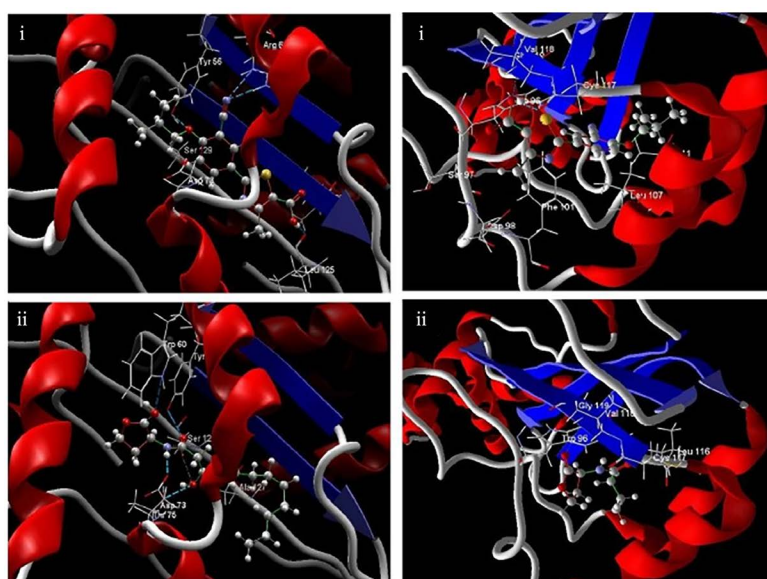


Figure 3. (a) The Molecular docking of (i) Febuxostat, (ii) Natural ligand inducer and (iii) C30 furanone into the active site of LasR enzyme; (b) The Molecular docking of (i) Febuxostat, (ii) C4-BHL into the active site of rhIR receptor model.

Table 2. The binding mode of ligands with the different residues inside the active site of LasR enzyme.

Ligand	H-Bonding	Hydrophobic Interaction	MolDock Score
LasR enzyme natural ligand	Trp60, Tyr56, Thr75, Ser129, Asp73	Ala50, Ala105, Cys79, Gly38, Ile52, Leu36, Leu39, Leu40, Leu110, Phe51, Phe101, Thr80, Thr115, Trp88, Tyr47, Tyr64, Tyr93, Val76, Ala127, Gly126, Leu125	-134.274
Febuxostat	Arg61, Tyr56, Leu125	Gly38, Leu36, Leu39, Leu40, Leu110, Phe101, Thr75, Thr115, Trp60, Trp88, Tyr47, Tyr64, Tyr93, Val76, Ala127, Gly126, Ser129	-133.876

Febuxostat was docked into rhlr receptor model active site. The binding interaction with the rhlr receptor is shown in **Figure 3(b)**. It showed similar interaction to the autoinducer C4-HSL as shown in **Table 3**. The important hydrophobic acyl group of C4-HSL is responsible for conformational change to act as inducer that is absent in febuxostat which make it a possible inhibitor with a promising high antagonist activity as indicated from high docking score.

4. Discussion

Quorum sensing system plays a vital role in regulation of production of virulence factors linked with various pathogenic phenotypes such as the ability of bacteria to adhere to tissues and to form biofilms [29]. Inhibition of QS has attracted much attention during the search for novel solutions to the problem of development of antibiotic resistance [30].

Unfortunately, many tested QS inhibitors were found to be highly toxic and so they cannot find their way to clinical application [31]. As a result, many reports tested the possibility of finding QS inhibitors among FDA approved drugs to make their use possible on clinical background. This hypothesis was found to be true in our previous studies [32] [33].

In this study, the hyporecemic drug febuxostat was investigated for anti-quorum sensing activity. At first, its antibacterial activity was evaluated and its MIC was 8 mg/ml. Sub-MIC (1/8 MIC or 1 mg/ml) was used in further work on quorum sensing and virulence inhibition. This concentration was found to have no significant effect on growth of PAO1 or *Chromobacterium violaceum* CV026 reporter strain. As a result, any potential QS inhibiting activity is not due to effect on growth.

The biosensor *Chromobacterium violaceum* CV026 strain was used to quantify quorum sensing due to its ability to produce the violacein pigment as a response to the presence of acylhomoserine lactones in the media and under the regulation of CVi/R QS system [34]. Sub-inhibitory concentration of febuxostat was capable of significantly decreasing the production of violacein. Moreover, molecular docking study was made to analyze the ability of febuxostat to competitively bind to the LasR and RhlR. Febuxostat could bind with both LasR and

Table 3. The binding mode of each ligand with the different residues inside the active site of rhIR receptor model.

Ligand	H-Bonding	Hydrophobic interaction	MolDock Score
C4-BHL	Gly119	Ala111, Arg112, Arg138, Asp98, Cys117, Leu107, Leu116, Phe101, Ser97, Trp96, Trp108, Val118	-70.064
Febuxostat	Gly119, Cys117	Ala83, Ala111, Arg112, Asp81, Asp98, Glu110, Leu107, Leu116, Phe101, Ser97, Trp68, Trp96, Trp108, Tyr64, Tyr72, Val118	-84.131

RhIR through hydrogen bonding and hydrophobic interaction. The docking score of febuxostat was nearly the same as the natural ligand dodecanoyl homoserine lactone and lower than that of butanoyl homoserine lactone; a finding that indicates a highly promising QS inhibiting activity of febuxostat.

Further assessment of quorum sensing regulated virulence factors of PAO1 in the presence of febuxostat was performed. Pyocyanin is a key virulence factor in *Pseudomonas aeruginosa* involved in formation of reactive oxygen species via oxidation of reduced cellular glutathione with simultaneous oxygen reduction [30]. Febuxostat showed a remarkable ability to diminish pyocyanin. Febuxostat could also significantly inhibit biofilm formation activity. QS is a key regulator of biofilm development [35]. Moreover, adhesion and biofilm formation is affected by motility, which in turn is controlled by QS LasI/R and RhlI/R [36]. Furthermore, biofilms formed by QS-deficient strains that are impaired in motilities are only thin and disperse [37]. In our study, febuxostat showed a marked inhibiting activities against swarming, swimming and twitching motilities of PAO1.

In our study, hemolysin, elastase and protease were significantly lowered by febuxostat. These hydrolytic enzymes enhance the spread of bacteria inside the tissues of the host and resistance to the host defense [38].

In addition to the molecular docking study that proved the ability of febuxostat to bind the LasR and RhlR receptors by competitive inhibition of the autoinducers dodecanoyl homoserine lactone and butanoyl homoserine lactone, respectively, febuxostat can also be a competitive inhibitor of the signaling molecule 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde of the new integrated QS system because febuxostat has the thiazole moiety [12].

In this study, qRT-PCR was used to further confirm the anti-QS activity of febuxostat by estimation of the levels of the relative expression of QS genes; namely LasI, LasR, RhlI, RhlR, PqsA and PqsR. Interestingly, febuxostat showed a significant down regulation of the expression of these QS genes; a finding that provides a basis to explain the remarked decrease in the production of QS regulated virulence factors. Significant down regulation of the key LasI/LasR QS regulator system was achieved in our study. This, in turn, affected the expression of the other QS systems; rhl and pqs systems with a net result of significant anti-virulence capacity of febuxostat.

In summary, febuxostat could be considered as a novel inhibitor of quorum sensing in PAO1 that can be used as virulence inhibitor for treating *P. aeruginosa* infection.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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