Al-Rabia *et al. AMB Express* (2024) 14:87
https://doi.org/10.1186/s13568-024-01740-1

AMB Express

ORIGINAL ARTICLE Open Access

Cilostazol is a promising anti-pseudomonal virulence drug by disruption of quorum sensing

Mohammed W. Al-Rabia[1], Hani Z. Asfour[1], Nabil A. Alhakamy[2,3,4], Mohammed A. Bazuhair[5], Tarek S. Ibrahim[6], Hisham A. Abbas[7], Basem Mansour[8,9], Wael A. H. Hegazy[7,10*] and Noura M. Seleem[7]

Abstract
Resistance to antibiotics is a critical growing public health problem that desires urgent action to combat. To avoid the stress on bacterial growth that evokes the resistance development, anti-virulence agents can be an attractive strategy as they do not target bacterial growth. Quorum sensing (QS) systems play main roles in controlling the production of diverse virulence factors and biofilm formation in bacteria. Thus, interfering with QS systems could result in mitigation of the bacterial virulence. Cilostazol is an antiplatelet and a vasodilator FDA approved drug. This study aimed to evaluate the anti-virulence activities of cilostazol in the light of its possible interference with QS systems in *Pseudomonas aeruginosa*. Additionally, the study examines cilostazol's impact on the bacterium's ability to induce infection in vivo, using sub-inhibitory concentrations to minimize the risk of resistance development. In this context, the biofilm formation, the production of virulence factors and influence on the in vivo ability to induce infection were assessed in the presence of cilostazol at sub-inhibitory concentration. Furthermore, the outcome of combination with antibiotics was evaluated. Cilostazol interfered with biofilm formation in *P. aeruginosa*. Moreover, swarming motility, biofilm formation and production of virulence factors were significantly diminished. Histopathological investigation revealed that liver, spleen and kidney tissues damage was abolished in mice injected with cilostazol-treated bacteria. Cilostazol exhibited a synergistic outcome when used in combination with antibiotics. At the molecular level, cilostazol downregulated the QS genes and showed considerable affinity to QS receptors. In conclusion, Cilostazol could be used as adjunct therapy with antibiotics for treating Pseudomonal infections. This research highlights cilostazol's potential to combat bacterial infections by targeting virulence mechanisms, reducing the risk of antibiotic resistance, and enhancing treatment efficacy against *P. aeruginosa*. These findings open avenues for repurposing existing drugs, offering new, safer, and more effective infection control strategies.

Keywords *Pseudomonas aeruginosa*, Cilostazol, Quorum sensing, Anti-virulence, Healthcare, Antimicrobial resistance

*Correspondence:
Wael A. H. Hegazy
waelmhegazy@daad-alumni.de

Full list of author information is available at the end of the article

Introduction

Pseudomonas aeruginosa is an aerobic non-fermenting Gram-negative bacterium that is characterized by its blue-green pyocyanin pigment (Lee et al. 2006; Stover et al. 2000). In addition to natural resources such as soil and surfaces in aqueous environments (Diaz et al. 2018; Sadikot et al. 2005) P. *aeruginosa* is present on the skin of healthy people (Horcajada et al. 2019; Lila et al. 2023; Maurice et al. 2018; Sadikot et al. 2005). *P. aeruginosa* is a common healthcare-associated bacterium and is listed among the most concerning nosocomial pathogens. Its notorious reputation stems from its remarkable propensity to resist a wide range of antibiotics and its formidable capacity to cause infections in various host tissues (Daikos et al. 2021; Horcajada et al. 2019; Skindersoe et al. 2008; Strateva and Mitov 2011). This biological flexibility enables *P. aeruginosa* can cause a plenty of infections particularly in patients with immunity disorders (Sathe et al. 2023). These infections include nosocomial pneumonia, wounds and burns infections, blood stream and urinary tract infections as well as infections affecting bones and joints (Lila et al. 2023; Qin et al. 2022). Among patients with cystic fibrosis, *P. aeruginosa* is commonly implicated in lower respiratory tract infections (West et al. 2002).

The antimicrobial resistance in *P. aeruginosa* is a great challenge due to its ability to develop resistance in addition to its intrinsic one (Carattoli 2013; Khayat et al., 2022; Qin et al. 2022). The very slow introduction of novel antibiotics into the market worsens the situation. This complicated challenge of antibiotic resistance makes it necessary to search for other therapeutic options such as repurposing of drugs for targeting bacterial virulence (Abbas and Hegazy 2017; Khayat et al. 2022a, c; Rajab and Hegazy 2023; Rangel-Vega et al. 2015). Anti-virulence agents interfere with virulence factors expression that is controlled by the well-reported communication systems termed quorum sensing (QS) (Alotaibi et al. 2023; Chadha et al. 2023; Khayat et al. 2022). QS is simply a system that enables bacteria to sense their numbers that are proportional to the concentration of chemical autoinducers or signaling molecules, and then shift the expression of virulence genes (Chen et al. 2011; Hegazy et al. 2020; Venturi 2006). The autoinducers in *P. aeruginosa* are mainly N-acylated homoserine lactones (AHLs) (Smith and Iglewski 2003; Venturi 2006). The QS systems are LasI-LasR that is responsible for secretion and binding with N-(3-oxododecanoyl)-L-homoserine or C12-HSL autoinducer. RhlI-RhlR is responsible for synthesis and sensing of N-butyryl-L-homoserine lactone or C4-HSL autoinducer, while PQS-MvfR system that includes 2-heptyl-3-hydroxy-4(1 H) quinolone autoinducer that binds with MvfR receptor (Hentzer et al. 2003; Li and Tian 2012; Smith and Iglewski 2003). The merit of anti-virulence agents over the classical antibiotics is the disarming of bacterial virulence with nothing to do with bacterial growth; a character that minimizes the selection pressure and the emergence of resistant mutants (Garcia-Contreras 2016; Khayat et al. 2023b; Rutherford and Bassler 2012).

Cilostazol is a quinolone derivative that is approved by FDA for treating intermittent claudication caused by peripheral vascular disease (Niu et al. 2016). Other indications for cilostazol are secondary prophylaxis in patients with previous transient ischemic attacks or non-cardioembolic ischemic stroke (Ahmad et al. 2012). Cilostazol promotes vasodilation and exerts antiplatelet activity and it can decrease plasma triglycerides and elevate HDL cholesterol (Elam et al. 1998; Shattil et al. 1998). Quinolone moiety has been used as a scaffold for development of antibacterial, antifungal, anti-parasitic, antiviral and anti-virulent drugs as reviewed (Senerovic et al. 2020). Quinolone at sub-inhibitory concentrations significantly diminished the bacterial virulence and interfere with the type three secretion system in *Salmonella* (Askoura and Hegazy 2020; Sonstein and Burnham 1993). Bearing in mind that *P. aeruginosa* employs its own very vital non-Lux-type signaling network Pseudomonas quinolone signal (PQS) QS (Nalca et al. 2006; Venturi 2006), which could indicate the possible anti-QS activity of quinolone like structures. In this context, it is aimed to to investigate the anti-virulence anti-QS activities of cilostazol against *P. aeruginosa* PAO1 strain.

Materials and methods

Bacterial strain and chemicals

P. aeruginosa PAO1 standard strain was used. All used media were purchased from Oxoid (Hampshire, UK). Cilostazol was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Detection of minimum inhibitory concentration (MIC) of cilostazol and antibiotics

The broth microdilution method was used to determine the MIC according to the guidelines of clinical and laboratory standards institute (CLSI) with modifications as previously detailed (Khayat et al. 2022a, c; Nazeih et al. 2023).

Evaluation of the cilostazol influence on bacterial growth

Sub-inhibitory concentrations of cilostazol [1/4 MIC (250 µg/mL) and 1/8 MIC (125 µg/mL)] were used to inhibit virulence of PAO1. To exclude any influence on bacterial growth, the optical density of the PAO1 grown in the presence of cilostazol at sub-MIC was detected and compared to untreated PAO1 growth (Gomaa et al. 2024; Khayat et al. 2023).

Inhibition of biofilm and virulence factors

The ability of sub-MICs of cilostazol to affect biofilm (Cavalu et al., 2022; Thabit et al. 2022a), swarming motility (Elfaky et al. 2023; Koshak et al. 2023), protease activity (Alotaibi et al. 2023; Maan T. Khayat, Tarek S. Ibrahim, et al., 2022), sensitivity to oxidative stress (Hegazy et al. 2020) and pyocyanin production (Almalki et al. 2022; Badr-Eldin et al. 2024) was assessed against *P. aeruginosa* PAO1. The detailed procedures are detailed in the supplementary.

Determination of MIC of antipseudomonal antibiotics when combined with cilostazol at sub-MIC

The MIC of different antipseudomonal antibiotics were detected in the presence of ¼ MIC of cilostazol by broth microdilution procedure (Khayat et al. 2022a, c; Nazeih et al. 2023). The antibiotics used were cefepime, cefotaxime and tobramycin to investigate possible synergy. The fractional inhibitory concentration (FIC) was calculated (MIC antibiotic with cilostazol/ MIC antibiotic alone) (Elfaky et al. 2024; Koshak et al. 2023).

In vivo survival test and histopathological study

A murine model simulating peritoneal sepsis caused by PAO1 was established through the intra-peritoneal injection of bacteria into mice (Almalki et al. 2022; Khayat et al. 2023b). PAO1 overnight cultures were grown with and without cilostazol (at ¼ MIC), and incubated at 37 °C for 18 h. The resulting suspensions were adjusted to reach a concentration of 2.5×10^7 CFU/ml. Subsequently, 100 µL portions of these suspensions were administered via intraperitoneal injection into female Swiss albino mice aged four to five weeks and weighing between 15 and 20 g. Two negative control groups were employed, with one group receiving 100 µl aliquots of PBS via injection, while the other group remained uninoculated. At the end of the study period, the mice were euthanized via cervical dislocation to extract the liver, spleen, and renal tissues. The tissues were prepared for histopathological examinations as described (Khayat et al. 2023b; Nazeih et al. 2023).

Quorum sensing genes expression analysis

The RNA was extracted from PAO1 cultured in the presence and absence of cilostazol at ¼ MIC and the rt-PCR was conducted as previously described (Askoura et al. 2022; Hegazy and Henaway 2015). The primers used in this study were listed in Table **S1** (Khayat et al. 2022a, b, c, 2023b; Nazeih et al. 2023). The expressions of QS encoding genes were normalized to the housekeeping gene *ropD*. The details of the used kits and procedure were provided in the supplementary data.

Virtual study of the potential affinity of cilostazol to LasR, RhlR and PqsR receptors

The crystal structures of *P. aeruginosa* quorum-sensing receptors; namely LasR (PDB: 6MVN) (McCready et al. 2019), RhlR (PDB: 7R3H) (Borgert et al. 2022) and PqsR (MvfR) (PDB: 6Q7U) (Zender et al. 2020), were recovered from protein data bank. The natural ligands N-3-oxo-dodecanoyl-homoserine lactone, N-butyrylhomoserine lactone and 2-heptylquinolin-4(1 H)-one in addition to cilostazol were sketched into Marvin Sketch of Marvin suite (http://www.chemaxon.com) and the lowest energy three-dimensional conformer for each, was generated then saved as Mol2 format. The natural ligands and cilostazol were docked into the active site of the quorum sensing receptors and the docking study was performed as described previously (Saqr et al. 2021).

Statistical analysis

To test the extent of significance of the activities of cilostazol against growth, protease, biofilm formation, swarming, tolerance to oxidative stress, pyocyanin and downregulation of quorum sensing genes, one way ANOVA test followed by Dunnett posttest was used, p value <0.05 indicated significant difference.

Results

Antibacterial activity of cilostazol and effect of sub-MICs of cilostazol on growth of PAO1

Cilostazol could inhibit the growth of PAO1 at a concentration equivalent to 1 mg/mL. Cilostazol was used at ¼ MIC (0.25 mg/ml) and 1/8 MIC (0.125 mg/ml) to inhibit PAO1 virulence. It was important to make sure that sub-inhibitory concentrations of cilostazol had no effects on the growth of the bacterium, so any inhibitory activities were due to targeting virulence factors away from the inhibition of growth. When PAO1 was grown in the presence of sub-MICs of cilostazol, there was no significant difference between the turbidities of PAO1 control and treated PAO1 with sub-inhibitory concentrations of cilostazol (Fig. 1A).

Cilostazol inhibited PAO1 biofilm formation

The anti-biofilm activity of cilostazol was assayed by the spectrophotometric method using crystal violet as a dye for adherent cells. Cilostazol showed good biofilm inhibiting activities (Fig. 1B). At ¼ MIC, cilostazol inhibited biofilm by 43.28% and at 1/8 MIC, it inhibited biofilm by 37.42%, respectively.

Cilostazol inhibited PAO1 swarming motility

To investigate the ability of cilostazol to decrease swarming motility, swarming agar plates containing cilostazol and control plates were used. Cilostazol successfully decreased the diameter of swarming zones (Fig. 1C). The

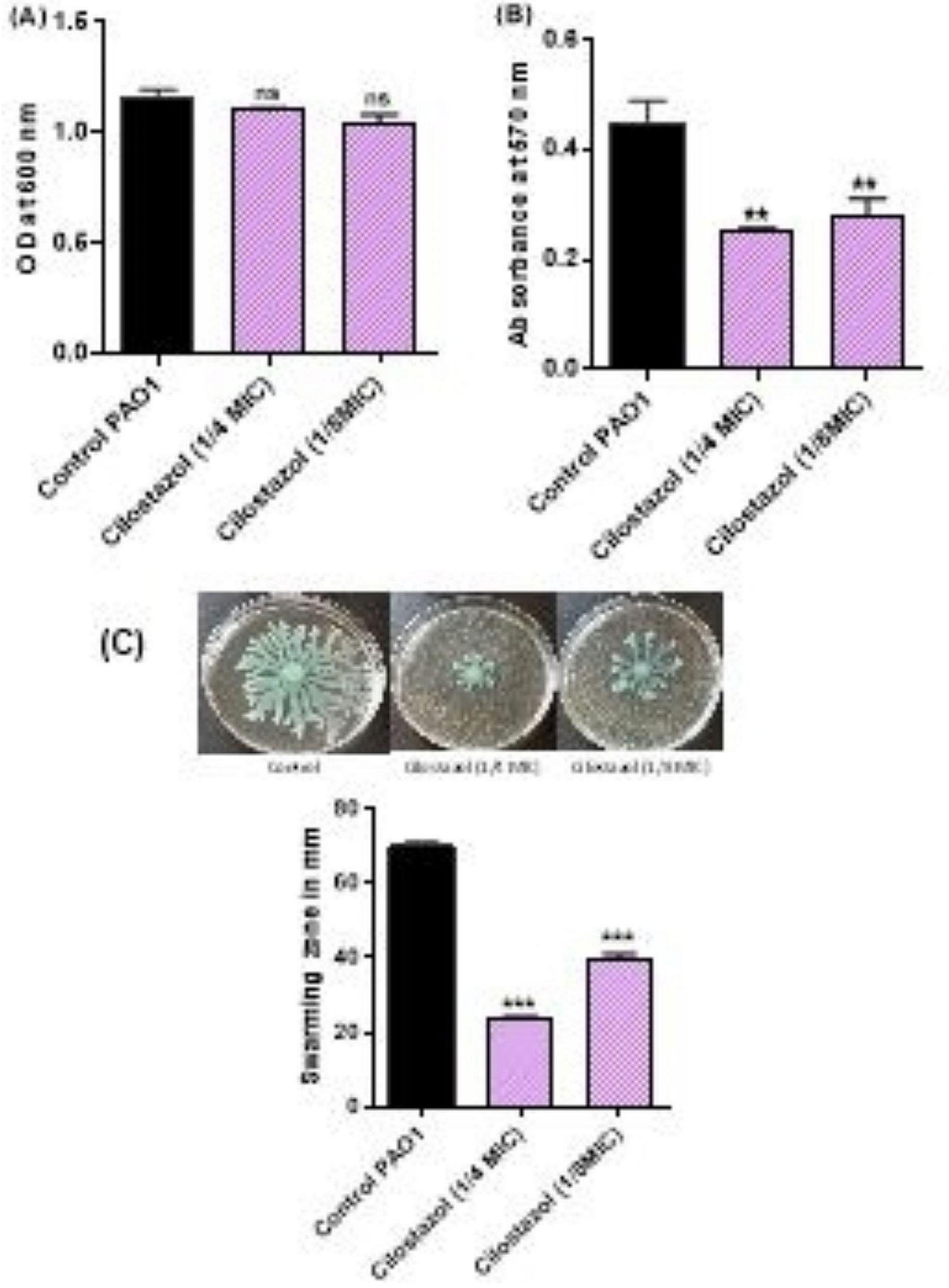

Fig. 1 Cilostazol diminished the PAO1 motility and ability to form biofilms. (**A**) Growth of PAO1 with and without sub-inhibitory concentrations of cilostazol. No statistically significant differences were found in turbidity of overnight cultures of PAO1 in the presence or absence of cilostazol (ns: non-significant $p > 0.05$). (**B**) Cilostazol at sub-MIC significantly inhibited biofilm formed by PAO1 ($p < 0.01$). (**C**) Cilostazol at sub-MIC significantly reduced swarming zones formed in swarming agar plates ($p < 0.001$)

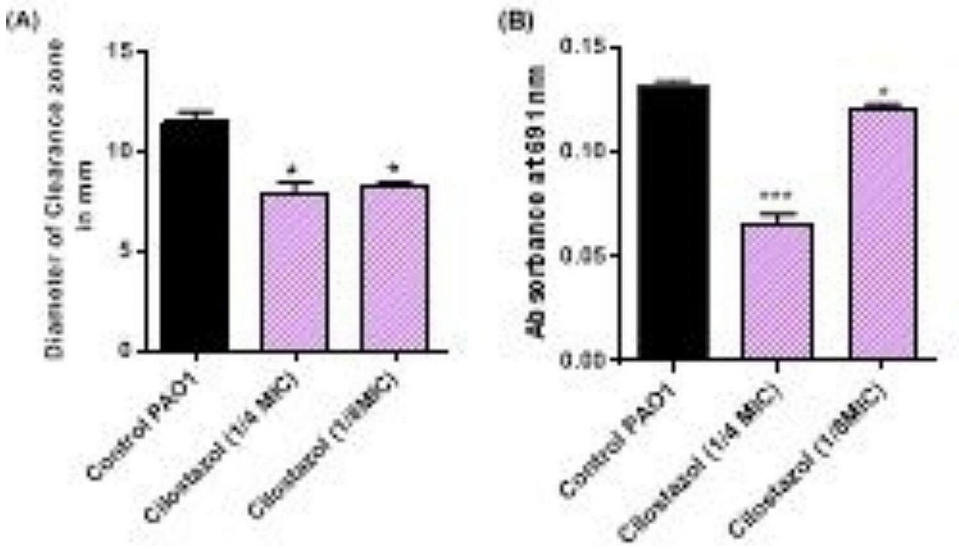

Fig. 2 Cilostazol significantly reduced the production of virulence factors. (**A**) Anti-proteolytic activity of cilostazol. Cilostazol significantly reduced clearance zones around the wells in skim milk agar plates (p <0.05). (**B**) Cilostazol reduced the pyocyanin production to a significant extent (*p < 0.05; *** p< 0.001)

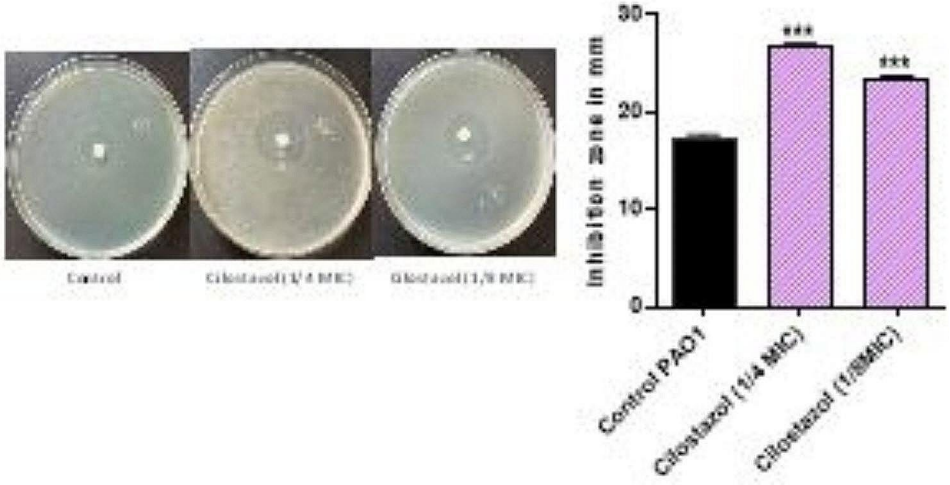

Fig. 3 Augmentation of oxidative stress exerted by hydrogen peroxide. Cilostazol significantly potentiated hydrogen peroxide as seen by the increase in the diameter of inhibition zone (p < 0.001)

inhibition of swarming was 66.19% and 42.86% at ¼ MIC and 1/8 MIC, respectively.

Anti-proteolytic activity of cilostazol

To investigate if cilostazol inhibits protease activity, the skim milk agar procedure was employed. The diameters of clear zones formed around the wells to which the supernatants of PAO1-treated cilostazol were statistically higher than the diameters of control PAO1 (Fig. 2A). Protease activity was reduced by 27.27% at ¼ MIC and 25% at 1/8 MIC.

Cilostazol decreased the production of pyocyanin

The blue green pyocyanin pigment of PAO1 production was quantified in both control PAO1 and cilostazol-treated PAO1. Cilostazol was found to significantly reduce pyocyanin secretion by 49.91% at ¼ MIC, but the reduction was much lesser at 1/8 MIC (8.62%) (Fig. 2B).

Cilostazol decreased tolerance of PAO1 to oxidative stress

Cilostazol enhanced the sensitivity to oxidative stress by potentiation of hydrogen peroxide inhibitory activity against PAO1. Cilostazol remarkably augmented the sensitivity to oxidative stress by 35.02% at ¼ MIC and 25.72% at 1/8 MIC (Fig. 3).

Synergy between cilostazol and some antibiotics

To test the possible synergy between cilostazol and some anti-pseudomonal antibiotics if sued in combination, the MICs of the antibiotics was determined both in the presence or absence of ¼ MIC of cilostazol. Cilostazol decreased the MIC of cefepime by 8 folds, cefotaxime and tobramycin by 4 folds indicating synergy if cilostazol is used with antibiotics (Table S2). The FIC values were lower than 0.5 indicating synergistic outcome.

Cilostazol downregulated the expression of QS genes

For confirmation of the diminishing activities of cilostazol against biofilm and virulence factors, the relative expressions of QS genes encode LasI-LasR, RhlI-RhlR, *and* PqsA-PqsR QS systems were assessed by quantitative real-time PCR in cilostazol treated and untreated PAO1. Cilostazol was able to decrease the expression of all tested genes (Fig. 4). It downregulated the genes *LasI*, and *RhlR* by 48%, each and *PqsR*, *PqsA* and *RhlI* by 58%, 60% and 66%, respectively. On the other hand, *LasR* was almost completely downregulated (99.5%).

Protection of mice against pathological change sin liver, spleen and kidney tissues

Cilostazol could alleviate the pathogenesis produced by PAO1 in mice as shown in Figs. 8, 9 and 10. The liver tissues from the control uninfected group exhibited typical tissue architecture and cellular features. In contrast, liver tissues from mice injected with untreated PAO1 displayed focal areas with inflammatory cell infiltration (Fig. 5A), pronounced congestion of hepatic blood vessels (Fig. 5B), and diffuse vacuolation of certain hepatocytes (Fig. 5C). However, hepatic tissues from mice injected with cilostazol-treated PAO1 showed apparently normal hepatic parenchyma and cellular details (Fig. 5D and E) and mild focal area of cellular infiltration (Fig. 5F).

In Fig. 6, unlike the renal tissues in the uninfected mice that showed normal renal cortex with intact glomeruli and renal tubules, the kidney tissues of the mice injected with PAO1 showed congestion of renal blood vessel (Fig. 6A), degenerative changes of some renal tubules represented in cloudy swelling with hyper cellularity of some glomeruli in the renal cortex (Fig. 6B), cystic dilation of some renal tubules with cloudy swelling of others (Fig. 6C) and atrophy of some glomeruli with degeneration of some renal tubules (Fig. 6D). When injected with cilostazol-treated PAO1, the renal tissues exhibited apparently normal renal cortex with normal renal tubules and glomeruli (Fig. 6E) and normal parenchyma with both white and red pulps (Fig. 6F).

Concerning the spleen tissues removed from mice in the uninfected group, they showed normal red and white pulps, the tissues from the mice injected with control PAO1, congestion of splenic blood vessel was found (Fig. 7A), focal area of extravasated blood (hemorrhage) (Fig. 7B), and depletion of lymphocytes from white pulp (Fig. 7C). On the other hand, spleen tissues from mice injected with treated PAO1 showed apparently normal parenchyma with both white and red pulps (Fig. 7D) and mild depletion of lymphocytes from white pulps (Fig. 7E).

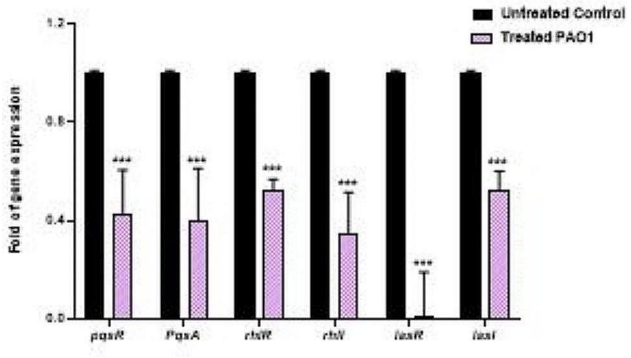

Fig. 4 Downregulation of QS genes by cilostazol. Cilostazol significantly reduced the expression of QS genes ($p < 0.001$)

Binding of cilostazol to quorum sensing receptors in the docking study

In order to investigate the possible binding of cilostazol with QS receptors LasR, RhlR, and PqsR, docking study was used. Regarding LasR receptor (Fig. 8) the tetrazolyl moiety of cilostazol formed an arene- arene bond with Trp88, the sp2-hybridized nitrogen atom at position-3, constructed a bifurcated H-bond with Tyr56 and Ser129. Also, the fused benzene ring in the 3,4-dihydro-2(1 H)-quinolinone moiety formed an arene-arene bond with Tyr64. Moreover, the strong hydrophobic/hydrophilic interactions revealed from the cyan-shaded amino acids from the receptor side, and blue-shaded parts from the ligand side improved the overall recognition and steered the ligand into a well-fitted pose of free binding energy −13.3477879 Kcal/mol. The internal ligand (N-3-oxo-dodecanoyl-homoserine lactone) displayed a bifurcated H-bond with Tyr56 and Ser129 and H-bond with Trp60, sp3-hybridized nitrogen atom displayed a H-bond with Asp73, and the hydrogen atom attached to C-5 of the tetrahydrofuran-3-yl moiety displayed a H-arene bond with Trp88 with free binding energy of −13.3930901 Kcal/mol.

Concerning the binding of cilostazol to RhlR receptor, it was found that Trp68 exhibited a H-bond with sp2-hybridized nitrogen atom at position-3 of the tetrazol-5-yl moiety, whereas Tyr72 displayed an arene-arene bond with tetrazole ring (Fig. 9). Furthermore, the sp2-hybridized oxygen atom attached to C-2 of the 3,4-dihydro-2(1 H)-quinolinone moiety received a H-bond from the backbone of Trp108 to stabilize the ligand/ receptor complex to score −10.4613638 Kcal/mol.

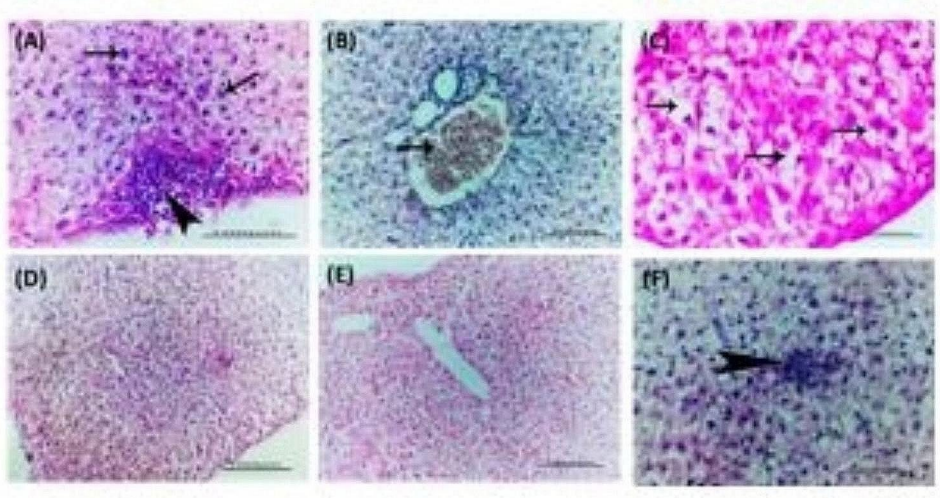

Fig. 5 Cilostazol alleviates pathogenesis of PAO1 in liver tissues. PAO1 caused infiltration of inflammatory cells, severe congestion of hepatic blood vessel and diffuse vacuolation of hepatocytes (**A–C**). With cilostazol, normal parenchyma and cellular details are maintained with only mild cellular infiltration (**D**–**F**).

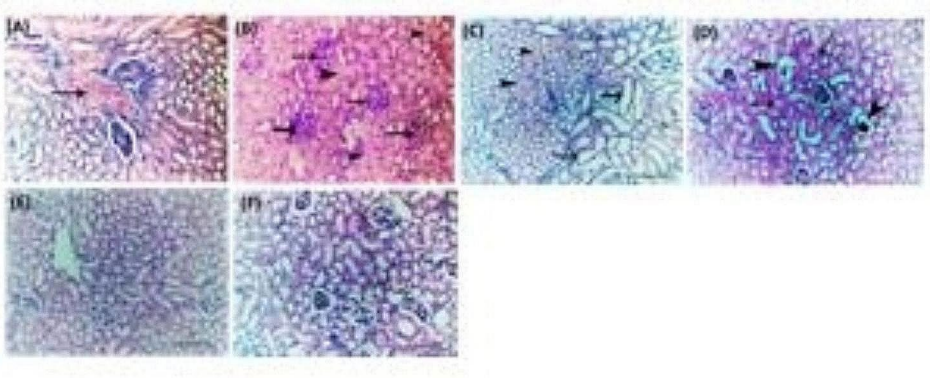

Fig. 6 Cilostazol alleviates pathogenesis of PAO1 in renal tissues. PAO1 caused renal congestion, renal tubules degeneration, cystic dilation of renal tubules and atrophy of some glomeruli (**A**–**D**). The renal tissues exhibited normal renal cortex with normal renal tubules and glomeruli and normal parenchyma with both white and red pulps (**E** and **F**)

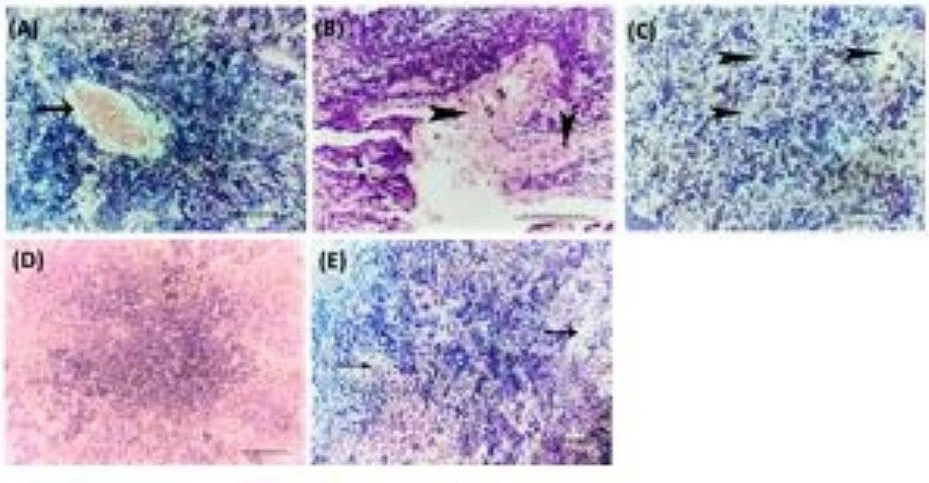

Fig. 7 Effect of cilostazol on histology of spleen tissues. PAO1 caused congestion of splenic blood, hemorrhage and depletion of lymphocytes from white pulp (**A**–**C**). Normal spleen parenchyma with mild depletion of lymphocytes from white pulps when injected with cilostazol treated PAO1 (**D** and **F**)

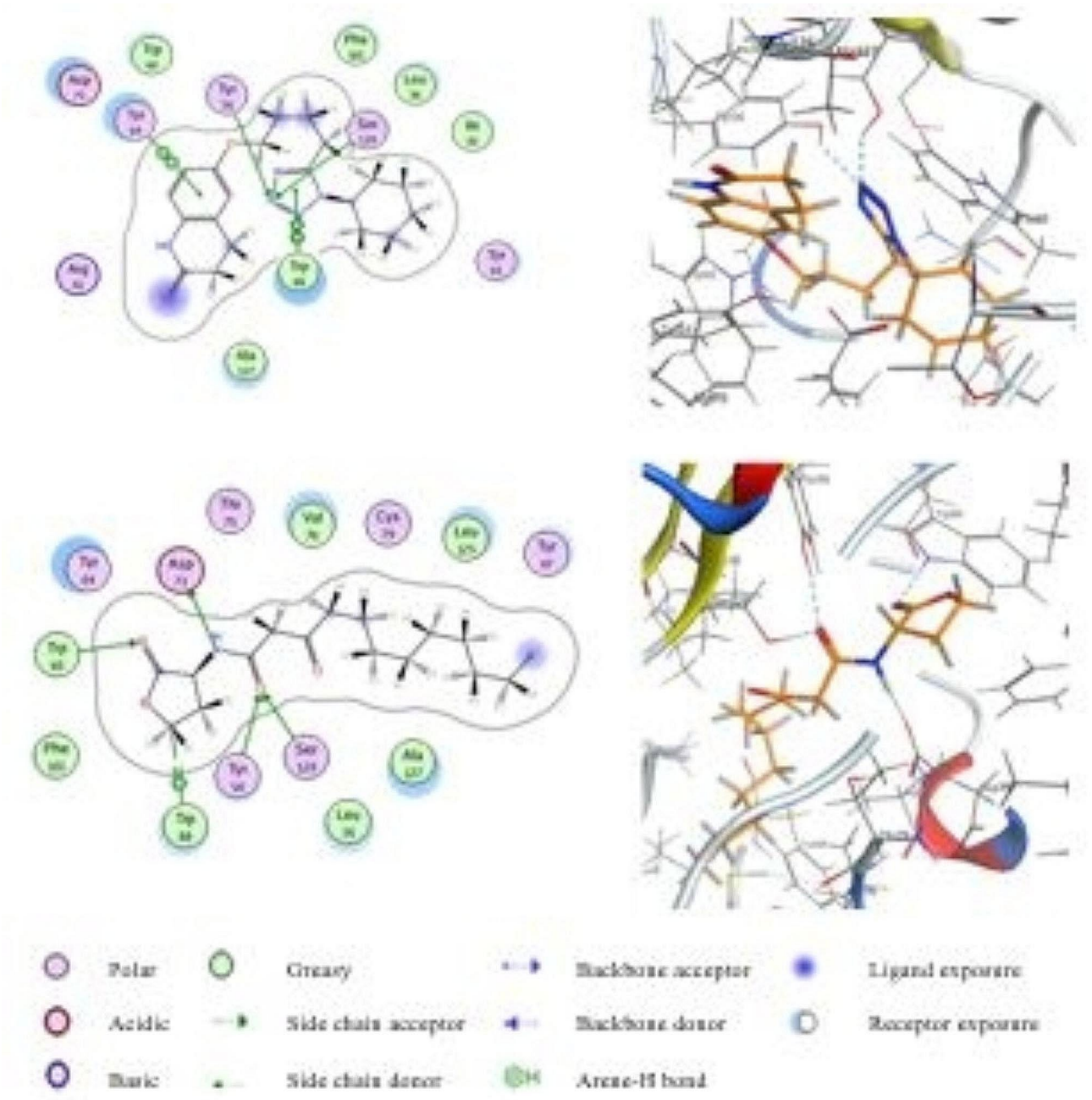

Fig. 8 The putative binding mode (2D&3D) of Cilostazol (upper panel) versus the internal ligand (lower panel) against the crystal structure of *P. aeruginosa* LasRquorum-sensing receptor, (PDB: 6MVN)

However, docking results of the native autoinducer C4-HSL showed that the ligand via its two classical Lewis bases sp2-hybridized oxygen atoms of the butyramide and furanone moieties formed two H-bonds from Tyr64 and Trp68, respectively. Besides, the sp3-hybridized nitrogen atom formed a H-bond with Asp81 that augmented the free binding energy to score up to -10.4964447 Kcal/mol.

Finally, cilostazol could bind to PqsR (MvfR) by an arene-H bond between the fused benzene ring in the 3,4-dihydro-2(1 H)-quinolinone moiety and Val211, in addition to H-bond between the hydrogen atom attached to C-1 of the buotoxy moiety and the backbone of Leu207, giving rise to free binding energy score up to -10.057127 Kcal/mol. On the other hand, the internal ligand of PqsR receptor displayed two H-bonds; the first was between the H atom on N-1 of the 3,4-dihydro-2(1 H)-quinolinone

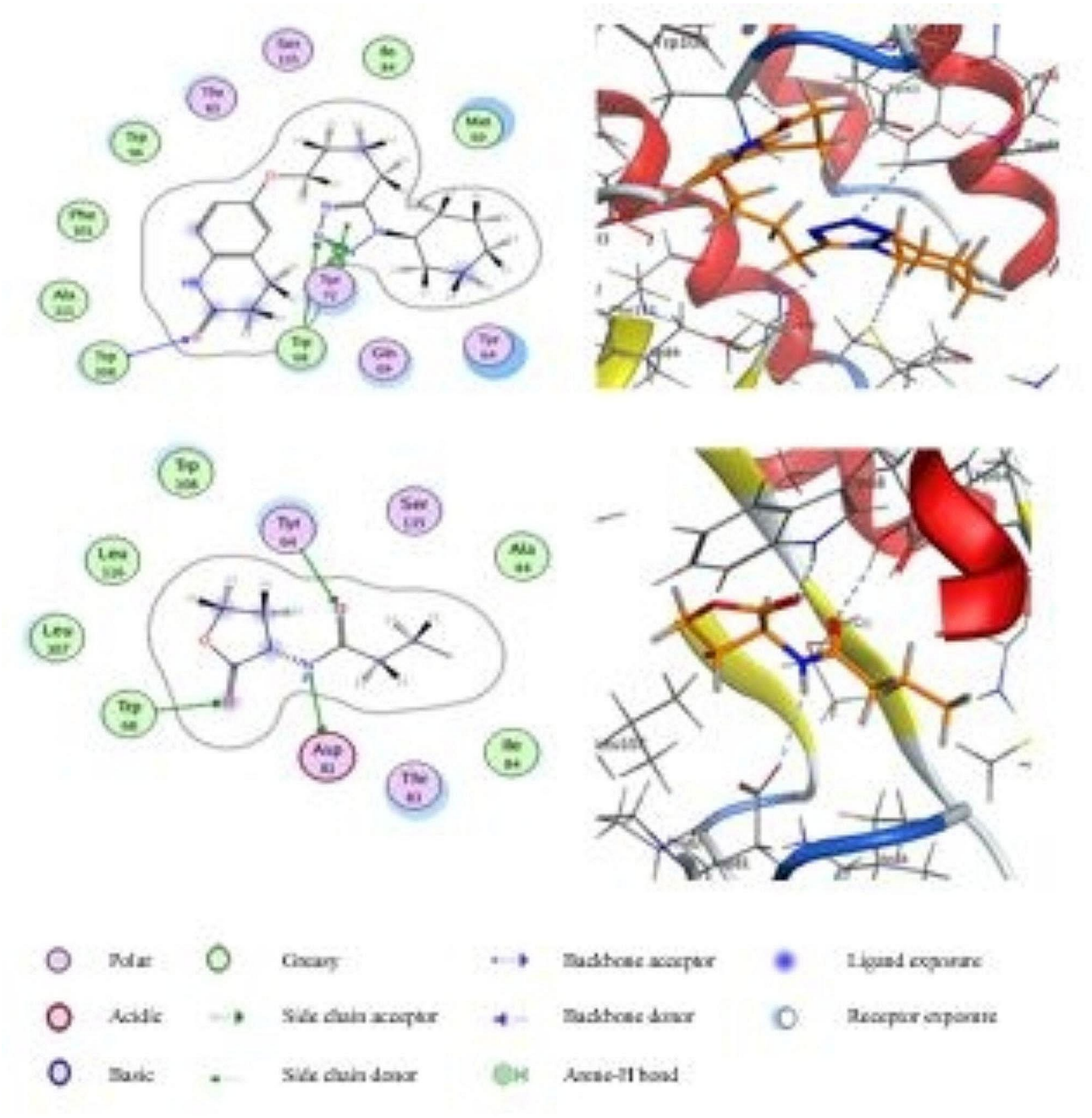

Fig. 9 The putative binding mode (2D&3D) of Cilostazol (upper panel) versus the native autoinducer C4-HSL ligand (lower panel) against the crystal structure of *P. aeruginosa* PROSS optimized variant of RhlR (75 mutations), (PDB: 7R3H)

moiety and Leu207, while the other H-bond was between the sp2-hybridized oxygen atom attached to C-4 of the same moiety and Tyr258, paving the way to achieve free binding energy score up to −10.20279621 Kcal/mol (Fig. 10).

Discussion

Resistance to antibiotics has increasingly spread either in hospitals or in community to constitute a great problem that affects public health. This problem is heightened by continuous emergence of resistant strains and the great decrease in investments in discovery of new antibiotics by pharmaceutical companies due to rapid development of antibiotic resistance that will result in great decrease in profits (Marston et al. 2016; Ventola 2015). This necessitates urgent actions to discover new agents for treating infections (Agha et al. 2016; Rudramurthy et al. 2016). To cause infection, bacteria such as *Pseudomonas* aeruginosa employ virulence factors including hydrolytic enzymes and bacterial toxins that help in spreading of

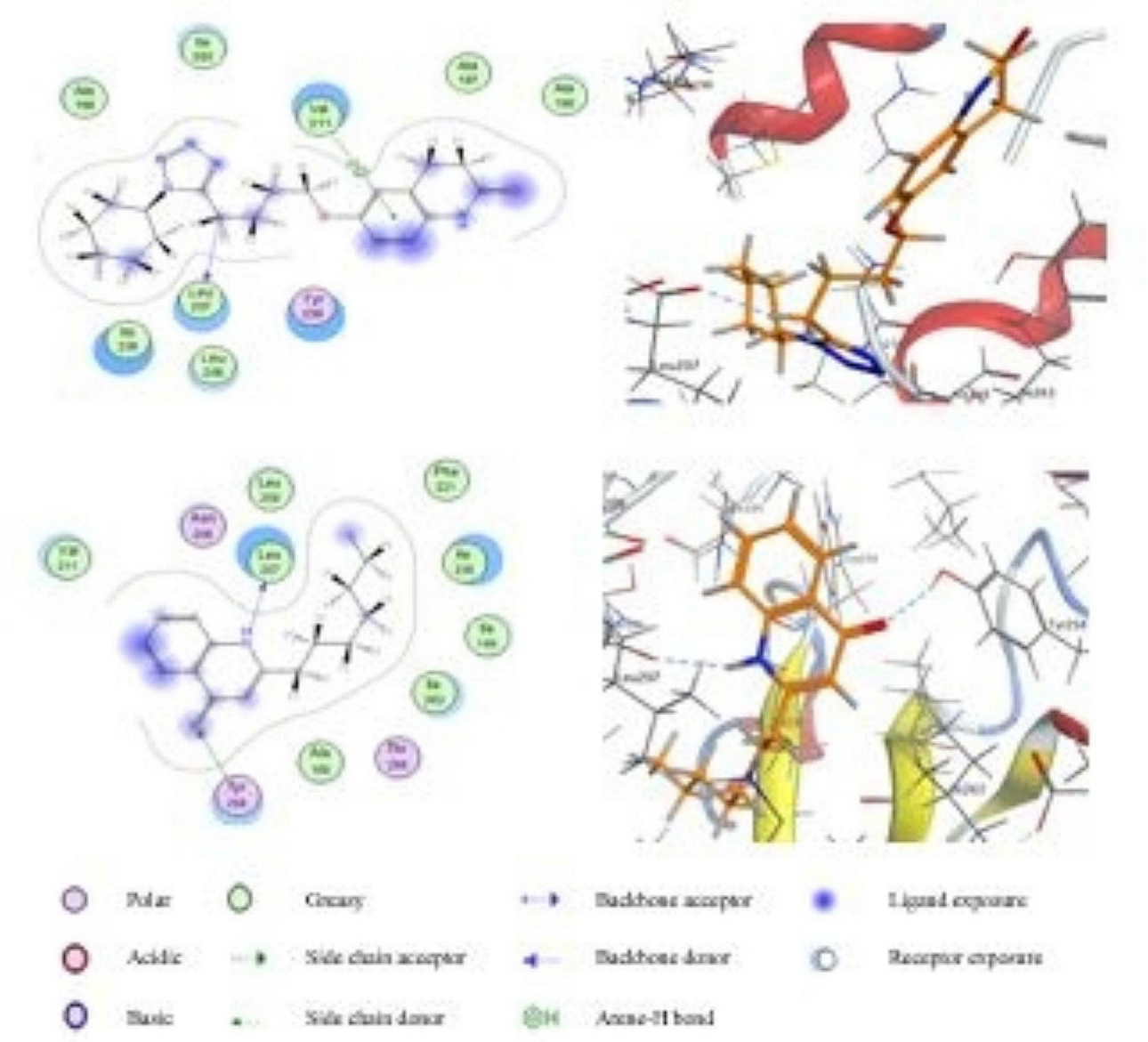

Fig. 10 The putative binding mode (2D&3D) of Cilostazol (upper panel) versus the native HHQ ligand (lower panel) against the crystal structure of *P. aeruginosa* PqsR (MvfR) ligand-binding domain (PDB: 6Q7U)

bacteria into the host tissues, in addition to biofilm formation that hinders the penetration of antibiotics and complicates the therapy (Elfaky et al. 2022; Hall and Mah 2017; Thabit et al. 2022b). *P. aeruginosa* virulence is under the harmonical control of QS (Lee and Zhang 2015). As a result, targeting QS and virulence can be an alternative or supporting approach to antibiotics in treating infections. Furthermore, QS inhibition is selective because its components are absent in humans (El-Mowafy et al. 2019).

In this study, cilostazol was investigated for its potential inhibitory activity against quorum sensing (QS) and virulence in *P. aeruginosa.* Cilostazol was chosen because it is a quinolone compound that is homologous in structure to the autoinducer of the QS system termed Pseudomonas quinolone signal (PQS), so it is expected to compete with the autoinducer for binding with the PqsR receptor (Juhas et al. 2005; Pesci et al. 1999). Cilostazol was used at ¼ MIC and 1/8 MIC corresponding to 0.25 and 0.125 mg/ml, respectively as a potential anti-virulence agent. It was important to ensure the lack of effect of these concentrations on bacterial growth by incubation of *P. aeruginosa* in presence and absence of the tested concentrations and it was found that the growth was not statistically different either with or without cilostazol.

Bacterial cells that grow forming biofilms are much more resistant than planktonic cells. It is expected that

deactivation of QS mechanism could much decrease biofilm formation due to the central role of QS in biofilm formation. Cilostazol exerted antibiofilm activity at both sub-inhibitory concentrations (Kirisits and Parsek 2006; Moser et al. 2017). *P. aeruginosa* swarming motility enables bacterial dissemination in host tissues and biofilm formation. Also, swarmer cells can produce virulence factors more potently and exhibit higher rate of antibiotic resistance (Coleman et al. 2021; Cavalu et al. 2022; Floyd et al. 2016). Cilostazol could block swarming motility; a result that explains the anti-biofilm activity of cilostazol. In our study, cilostazol decreased protease activity. Protease has a role in spreading of *P. aeruginosa* in the host tissues (Ahmed et al. 2019). Also, protease can decompose the host lung elastin immunoglobulins and fibrin in addition to epithelial tight junctions (Kipnis et al. 2006).

Cilostazol reduced the production of the blue green pigment pyocyanin. Pyocyanin can destroy the host cells as it has both oxidative and cytotoxic damaging effects (Ahmed et al. 2019). In our study, cilostazol significantly increased the sensitivity of *P. aeruginosa* to oxidative stress. This may be explained by anti-QS activity of cilostazol. It was reported that inhibiting phagocytosis and intracellular killing by oxygen radicals in neutrophils is related to QS (Hassett et al. 1999). This is confirmed by the finding that mutants deficient in QS had lower capacity of producing catalase and superoxide dismutase and higher susceptiblity to oxidative stress than the wild strain (Bjarnsholt et al. 2005). The promising phenotypic results were further confirmed by investigating the effect of cilostazol on the expression of QS genes in *P. aeruginosa* by quantitative real-time PCR. Cilostazol considerably decreased the expression of all tested QS encoding genes *LasI*, *LasR*, *RhlI*, *RhlR*, *PqsA* and *PqsR*.

The protective effects of cilostazol against the pathologic effects of PAO1 in mice was tested. In contrary to the infiltration of inflammatory cells, severe congestion of hepatic blood vessel and diffuse vacuolation of hepatocytes in mice injected with PAO1, those injected with cilostazol-treated PAO1, normal parenchyma and cellular structures were found with only minimal cell infiltration. Furthermore, Injection of PAO1 in mice caused renal congestion, renal degeneration, cystic dilation of renal tubules and atrophy of some glomeruli. Also, congestion of splenic blood, hemorrhage and depletion of lymphocytes from white pulp were found. However, when injected with cilostazol-treated PAO1, the mice renal tissues, renal cortex, tubules and glomeruli and the spleen parenchyma were normal.

The docking study revealed the ability of cilostazol to bind with LasR quorum sensing receptor by arene-arene binding, hydrogen bonding and hydrophobic interactions with binding energy very close to that of the natural ligand indicating the strong possible interference with binding of the natural ligand to LasR receptor. Similarly, H bonding and arene-arene binding of cilostazol with RhlR receptor were found with a binding energy similar to that of the natural ligand reflecting the possible competition between cilosatzol and the natural ligand on binding with RhlR receptor. Furthermore, arene-H bond and H binding was found with the QS receptor PqsR with more or less similar binding energy to that of the natural ligand. It is noteworthy that there is structural similarity between the internal ligand of PqsR receptor and cilostazol as both are quinoline-based. Interestingly, despite of the different sizes and steric effects of the three internal ligands and consequently the different cavity sizes, cilostazol was able to keep up with the three ligands with very close free binding energy scores along docking process. Undoubtedly, this is ascribed to the presence of the free rotating butoxy spacer and flexible structure of cyclohexane. The tetrazole ring displayed a fundamental role in enhancing ligand/receptor interactions specially in receptors complexed with furanone-based ligands. From these results, strong competitive inhibitory effect for all the aforementioned receptors was found with cilostazol making it a recommendable drug to increase the activity of co-administered antipseudomonal antibiotics.

In the context of repurposing FDA approved drugs to serve as antibiotic adjuvants, cilostazol showed considered anti-QS and anti-virulence activities. There are several studies were conducted to evaluate the anti-QS activity of antidiabetic gliptins drugs (Maan et al. 2022), adrenoreceptor antagonists (Almalki et al. 2022). The current findings suggest another chemical moiety, quinolone, as a pharmacophore to develop new effective anti-QS candidates showing considerable activity as well as gliptins and adrenoreceptor antagonists. In conclusion, cilostazol quinolone chemical moiety acquire a considered affinity to QS receptors, furthermore, it lessened the expression of the genes encode the main three QS systems in *P. aeruginosa*. Bearing in mind the pivotal role of QS in controlling the production of diverse virulence factors and biofilm. The anti-QS activities could explain the significant anti-virulence activities of cilostazol at sub-MIC. Cilostazol significantly diminished the biofilm formation, the production of protease, pyocyanin, and significantly increased the sensitivity to oxidative stress in *P. aeruginosa*. In compliance with the in vitro findings, the histopathological examination of the liver, spleen and kidney tissues showed the diminishing effects of cilostazol to the *P. aeruginosa*-induced pathogenesis. Moreover, cilostazol at sub-MIC lowered the MICs of anti-pseudomonal antibiotics showing synergistic outcome by combination. These findings greatly support the anti-QS and anti-virulence activities of cilostazol to be

used in combination with antibiotics in treatment of *P. aeruginosa* serious infections.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13568-024-01740-1.

Supplementary Material 1

Acknowledgements
The Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, Saudi Arabia has funded this project under grant №. (RG-7-166-43). The authors, therefore, acknowledge with thanks the DSR for technical and financial support.

Author contributions
Conceptualization, H. A. A. and W. A. H. H.; methodology, M. W. A., H. Z. A. and N. M. S.; validation, N. (A) A., M. A. (B) and T. S. I.; formal analysis, N. M. S., B. M and M. W. A.; investigation, N. M. S., B. M., H. Z. A; resources, T. S. I.; data curation, M. B. A.; writing—original draft preparation, N. M. S., B. S., H. A. A. and W. A. H. H.; writing—review and editing, H. A. A. and W. A. H. H.; visualization, N. M. S.; supervision, H.A. A.; project administration, W. A. H. H.; funding acquisition, T. S. I. All authors have read and agreed to the published version of the manuscript.

Funding
The Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia has funded this project, under grant № (RG-7–166 – 43). The authors, therefore, gratefully acknowledge DSR technical and financial support.

Data availability
All data supporting the reported results are included within the article and its supplementary materials.

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
Experimental design and animal handling procedures were performed according to the guidelines for animal use of the Ethical Committee of the Faculty of Pharmacy, Zagazig University (Approval number: ZU-IACUC/3/F/254/2023).

Author details
[1]Department of Clinical Microbiology and Immunology, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[2]Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[3]Center of Excellence for Drug Research and Pharmaceutical Industries, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[4]Mohamed Saeed Tamer Chair for Pharmaceutical Industries, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[5]Department of Clinical Pharmacology, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[6]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[7]Microbiology and Immunology Department, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt
[8]Pharmaceutical Chemistry Department, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt
[9]Department of Pharmaceutical Chemistry, Kut University College, Al Kut, Wasit 52001, Iraq
[10]Department of Pharmaceutical Sciences, Pharmacy Program, College of Health Sciences, 113 Muscat, Oman

Received: 1 April 2024 / Accepted: 3 July 2024
Published online: 01 August 2024

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