J Comput Aided Mol Des
DOI 10.1007/s10822-012-9599-1

Computational discovery of putative quorum sensing inhibitors against LasR and RhlR receptor proteins of *Pseudomonas aeruginosa*

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Received: 1 February 2012 / Accepted: 30 August 2012

Abstract Drugs have been discovered in the past mainly either by identification of active components from traditional remedies or by unpredicted discovery. A key motivation for the study of structure based virtual screening is the exploitation of such information to design targeted drugs. In this study, structure based virtual screening was used in search for putative quorum sensing inhibitors (QSI) of *Pseudomonas aeruginosa*. The virtual screening programme Glide version 5.5 was applied to screen 1,920 natural compounds/drugs against LasR and RhlR receptor proteins of *P. aeruginosa*. Based on the results of *in silico* docking analysis, five top ranking compounds namely rosmarinic acid, naringin, chlorogenic acid, morin and mangiferin were subjected to in vitro bioassays against laboratory strain PAO1 and two more antibiotic resistant clinical isolates, *P. aeruginosa* AS1 (GU447237) and *P. aeruginosa* AS2 (GU447238). Among the five compounds studied, except mangiferin other four compounds showed significant inhibition in the production of protease, elastase and hemolysin. Further, all the five compounds potentially inhibited the biofilm related behaviours. This interaction study provided promising ligands to inhibit the quorum sensing (QS) mediated virulence factors production in *P. aeruginosa*.

Keywords Virtual screening · Molecular docking · Quorum sensing inhibitors · *Pseudomonas aeruginosa* · Clinical isolates · Pathogenesis

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Introduction

The most common intercellular communication system that exists in bacteria is "quorum sensing" (QS). The bacteria initiate coordinated activities only when a "quorum" of population density is reached [11, 35]. At present, the QS system in *Pseudomonas aeruginosa* has been most intensively studied by researchers, for the reason that this bacterium is an opportunistic human pathogen, which is responsible for the death of thousands of cystic fibrosis sufferers and many other immunocompromised patients. The success of this bacterium is largely due to the production of QS mediated virulence factors such as LasA protease, LasB elastase, pyoverdin, pyocyanin and its ability to form intractable biofilm [20, 32]. There are two well known QS systems present in *P. aeruginosa* such as Las and Rhl, which are controlled by the genes namely *las* and *rhl,* respectively. These systems utilize self secreted autoinducer (AI) molecules such as 3-oxo-dodecanoyl acyl homoserine lactone (3-oxo-C12 HSL) and N-butanoyl acyl homoserine lactone (C4-HSL), respectively. These AI molecules at higher concentrations dock with their cognate receptor proteins such as LasR and RhlR and form a signal-receptor complex, which inturn regulates the expression of various genes responsible for biofilm formation and virulence factor production [32]. In addition to these two QS systems, a third system of QS known as Pseudomonas Quinolone Signal (PQS) has been reported in this organism [22, 31]. It is also known that the PQS, Las and Rhl systems are interdependent to each other [26]. Over all, QS in *P. aeruginosa* controls probably 350 genes, of which ~30 % encode virulence factors production [25]. In the treatment of *P. aeruginosa* infections, the current conventional antibiotic therapies lack efficacy, partly because the bacterial pathogens create and inhabit surface—associated biofilm conferring increased resistance to

Published online: 18 September 2012

antibiotics and host immune responses [4]. Therefore, there is an urgent requirement for the discovery of novel treatment measures to counter *P. aeruginosa* pathogenesis.

Computer based molecular docking analysis are now widely used to discover new ligands for targets of known protein structures. So far, only a limited number of ligands for the known protein structures have been reported by this molecular docking approach [16]. The molecular docking programme finds application in 'virtual' or 'structure based' screening of ligands to fit into the protein structure and evaluating them for structural complementarities. Using virtual screening, several molecules could be docked into the structure of the target protein and according to the docking score function those molecules that fit the best will be subjected to the further experimental analysis [16]. However, identification of suitable candidates with quorum sensing inhibitory (QSI) potential through Computer Aided Drug Discovery (CADD) is currently limited. Moreover, most of the identified QSI compounds are found to be unsuitable for medicinal applications [24]. Therefore, we aimed to develop potential QSIs from natural edible resources by generating molecular information of *P. aeruginosa* QS regulators such as LasR and RhlR.

In the present study, 1,500 natural compounds were chosen from the Interbioscreen database (www.ibscreen.com). In addition, using drawn function (Chemsketch), antagonistic structures of AI were drawn in work space and hits were searched in the whole database. Based on the antagonistic specificity of AI, 420 new compounds were obtained from the database. Totally, 1,920 natural compounds were subjected to the docking analysis against LasR and RhlR receptor proteins of *P. aeruginosa.* Based on the docking scores, five top ranking compounds such as rosmarinic acid, mangiferin, morin, chlorogenic acid and naringin were selected to assess their QSI potential using in vitro experimental assays.

Materials and methods

Molecular docking studies

Molecular docking study was performed using Glide version 5.5. All ligands were docked flexibly to LasR and RhlR receptor proteins. The LasR protein (ID: 2UV0) structure was downloaded from http://www.pdb.org. The modelled RhlR protein (ID: P54292.1) was obtained from http://www.proteinmodelportal.org/query/uniprot/P54292 [23]. The typical protein structure file from the PDB is not suitable for immediate use in molecular docking studies. Hence, the optimization and minimization of the proteins were performed using protein preparation wizard tool. Initially, the bond orders were assigned, hydrogen atoms were added and all the crystallographic waters without hydrogen bond interactions with protein residues were removed. Minimization was performed until the average root-mean-square-deviation (RMSD) of the non-hydrogen atoms reached 0.3Å. Grid generation was performed for both proteins with site-map predicted residues namely PHE 40, TYR 42, GLU 32, THR 63, TYR 77, GLU 59, THR 58, ASP 168, SER 16, GLY 119 and TRP 124 for Rhl R protein as well as TYR 47, ASP 46, SER 106, ALA 50, GLU 89, VAL 111 and PHE 51 for Las R protein. A library of 1,920 natural compounds selected from Interbioscreen database (www.ibscreen.com) were compiled together to form a stand-alone library. These compounds were prepared via Lig-Prep 2.4 with optimized potential for liquid simulation (OPLS)-2005 force field to retain original state of ligand and chirality. The prepared ligands were docked against the generated grid by the step wise process using the Glide standard precision (SP), extra precision (XP) and high throughput virtual screening (HTVS) mode at default settings [29].

QSI compounds preparation

The compounds such as rosmarinic acid, morin, naringin, chlorogenic acid and mangiferin were selected on the basis of the docking results and were tested for their ability to inhibit LasR and RhlR dependent virulence factors production in *P. aeruginosa* PAO1 and the clinical isolates AS1 and AS2. All the test QSI compounds used in this study were purchased from Sigma, St. Louis, MO, USA. The stock solutions of rosmarinic acid, naringin and chlorogenic acid were prepared in 50 % (*w/v*) ethanol to the concentration of 50 mg/mL, whereas morin and mangiferin (50 mg/mL) were dissolved in 50 % (*w/v*) methanol and dimethyl sulfoxide (DMSO), respectively.

Bacterial strain and culture conditions

The laboratory strain *P. aeruginosa* PAO1 was used as a marker strain. Two *P. aeruginosa* clinical isolates were collected from a clinical laboratory, Madurai, India. Through 16S rRNA gene sequence, both the clinical isolates AS1 and AS2 were identified as *P. aeruginosa*. The 16S rRNA gene sequences of AS1 and AS2 were deposited in NCBI genbank under the accession numbers GU447237 and GU447238, respectively. For the study, all three bacterial strains were cultured aerobically and maintained in Luria–Bertani (LB) broth (Himedia, India) at their optimum temperature of 37 °C under 150 rev/min.

Measurement of virulence factors production

Overnight (O/N) culture of PAO1, clinical isolates AS1 and AS2 strains were inoculated in LB medium and

allowed to grow until reach an OD of 1.5 at 600 nm (HITACHI, U-2800, Japan). In each experiment, 1 % of the above mentioned test bacterial strains (OD adjusted to 0.8 at 600 nm) were added to the fresh LB medium and cultivated in the presence and absence of test QSI compounds. The compounds namely rosmarinic acid, chlorogenic acid, naringin and mangiferin were tested at various concentrations such as 250, 500, 750 and 1,000 µg/mL. Morin was tested at 10, 20, 30, 40 and 50 µg/mL concentrations in order to find out the effective inhibitory concentration (EIC). After 18 h of growth at 37 °C, the treated and untreated cultures were centrifuged at 10,500*g* for 10 min and the cell-free culture supernatant was subjected to following enzymatic assays. Estimation of exogenous proteolytic activity was carried out as described by Musthafa et al. [18]. Briefly, 100 µL of culture supernatant was added to 500 µL of 0.3 % azocasein (Sigma, St. Louis, MO, USA) along with 0.05 M Tris–HCl, 0.5 mM $CaCl_2$ (pH 7.5) and incubated at 37 °C for 30 min. Enzymatic reaction was stopped by the addition of ice cold trichloroacetic acid (10 %, 0.5 mL) followed by centrifugation 10,500*g* for 10 min and the supernatant containing released free azo dyes were measured at OD_{400nm}. The elastolytic activity of the cell-free culture supernatants of *P. aeruginosa* was determined according to the method of Kessler et al. [15] by using elastin congo red (ECR; Sigma, St. Louis, MO, USA) as the substrate. 100 µL of treated and untreated *P. aeruginosa* supernatant was mixed with 900 µL of ECR buffer containing 100 mM Tris, 1 mM $CaCl_2$ (pH 7.5) and 20 mg of ECR substrate. The reaction was incubated with shaking at 37 °C for 3 h. Further, 1 mL of 0.7 M sodium phosphate buffer (pH 6.0) was added to stop the reaction and the tubes were placed in an ice water bath for 30 min. The insoluble ECR was removed by centrifugation at 10,500 g for 10 min and the absorbance was measured at OD_{495nm}. Hemolysin assay was performed to determine the potential of compounds in reducing the hemolysin production in PAO1, AS1 and AS2. Cell-free supernatants (100 µL each) of both compounds treated and untreated were subjected to hemolysin assay with 900 µL of 2 % washed sheep erythrocytes containing 20 mM $CaCl_2$, 10 mM Tris and 160 mM NaCl (pH 7.4). The mixture was incubated in an ice for 20 min. After incubation, the reaction mixture was centrifuged and the released hemoglobin in the supernatant was measured at OD_{530nm}. The results were expressed as percent lysis compared with the lysis of erythrocytes in distilled water [3].

Biofilm inhibition assay

The efficiency of test QSI compounds to prevent the biofilm formation in test organisms was assessed by following the method of Annapoorani et al. [2]. Briefly, the biofilms were allowed to grow on glass slides (1 × 1 cm) in 24 well MTP (Greiner Bio-One) containing 1 mL of LB inoculated with 1 % of O/N culture of PAO1, AS1 and AS2 ($OD_{600nm} = 0.8$) along with and without test QSI compounds at their respective EICs. Culture set up was incubated without agitation at optimum temperature 37 °C for 18 h. After incubation, adherent cells in glass slides were gently rinsed twice with double distilled water (MilliQ, Millipore) and allowed to air dry before being stained. The biofilm was stained with 0.1 % acridine orange solution for 2 min and rinsed twice with double distilled water. The stained glass slides were observed at magnification of 20 × under the confocal laser scanning microscope (CLSM) (LSM 7010, Carl Zesis, Germany). The reduction in biofilm thickness was assessed by CLSM Z-stack analysis using Zen 2009 software through the process of optical sectioning.

Swarming assay

Swarming assay was performed as previously described by the method of Bosgelmez and Ulusoy, [6] with slight modification. Swarm plates were prepared with the media containing 1 % of peptone, 1 % NaCl, 0.5 % beef extract, 0.5 % glucose and 0.5 % agar. 10 µL of compounds treated and untreated cultures ($OD_{600nm} = 1$) of PAO1, AS1 and AS2 were placed at the centre of the swarm agar plates and were incubated in an upright position at 37 °C for 24 h.

Protein degradation assay

Protein degradation assay was performed in two different experimental set up in order to determine the effect of test QSI compounds on virulence enzymes production. In set 1: the test QSI compounds were added to the culture at 0 h of incubation, in set 2: the test QSI compounds were added to the culture at 18 h of incubation and further it was extended up to 24 h. At the end of incubation, cell-free supernatant of both the cultures were obtained and subjected to protein degradation assay, in order to analyse the binding and degrading efficiency of QSI compounds to the enzymes.

Growth inhibition analysis

The effect of test QSI compounds on bacterial survival was assessed by Growth inhibition analysis on PAO1, AS1 and AS2. Briefly, 1 % O/N culture of the test bacterial strains ($OD_{600nm} = 0.8$) were inoculated to growth medium consisting test QSI compounds at the tested EIC and were incubated for 24 h at 37 °C. After incubation, the growth OD was measured at 600 nm.

Table 1 Cumulative results for the compounds docked against LasR and RhlR receptor proteins

Name	Docking score	Energy	Efficiency	H bonds
3-Oxo-C12 HSL	−8.14	−49.78	−0.38	2
Rosmarinic acid	−7.01	−45.27	−0.26	2
Naringin	−8.83	−33. 82	−0.44	1
Morin	−10.83	−36.839	−0.49	3
Mangiferin	−10.82	−40.33	−0.360	2
C4-BHL	−4.727	−32.07	−0.363	2
Rosmarinic acid	−11.32	−48.70	−0.43	3
Chlorogenic acid	−12.49	−53.69	−0.49	4
Mangiferin	−11.75	−45.05	−0.391	3

Statistical analysis

All the experiments were performed in triplicates to validate reproducibility and the *P* values were calculated statistically by Student's *t* test. Values were expressed as mean ± SD. Comparison analysis was performed between tests and control.

Results

Virtual screening for LasR and RhlR QSI candidates

Totally, 1,920 natural compounds from database along with the AIs such as 3-oxo-C12-HSL and C4-HSL were docked against the ligand binding domain of LasR and RhlR using the docking programme Glide 5.5. The docking scores of these natural compounds were compared with the docking scores of AI molecules. The docked compounds, which showed better docking scores than that of AI, were chosen for the in vitro analysis. In this manner, the five top-ranking compounds such as rosmarinic acid, naringin, morin, mangiferin and chlorogenic acid were shortlisted to perform the in vitro bioassays. The interactions of aforementioned compounds with LasR and RhlR were shown in Table 1 as well as in Figs. 1 and 2.

Determination of EIC of test compounds against *P. aeruginosa*

The dose dependent activity of the selected compounds on the growth and QSI activity at different concentrations were observed. Except morin all other test QSI compounds were assessed at four different concentrations such as 250, 500, 750 and 1,000 µg/mL. Rosmarinic acid and chlorogenic acid exhibited significant QSI activity only at 750 µg/mL and both the compounds showed antibacterial activity at 1,000 µg/mL. But, no effective QSI activity was found below 500 µg/mL concentration. In naringin and mangiferin, the QSI activity was observed at 1,000 µg/mL concentration without any growth inhibition. Morin was tested at 10, 20, 30, 40 and 50 µg/mL concentrations. At higher concentrations, morin exhibited induction of virulence enzyme production. Based on the obtained results, it was found that EIC for rosmarinic acid 750 µg/mL, chlorogenic acid—750 µg/mL, naringin—1,000 µg/mL, mangiferin—1,000 µg/mL and morin—30 µg/mL. Hence, the respective EICs alone were selected for further virulence assays analyses (Tables 3, 4).

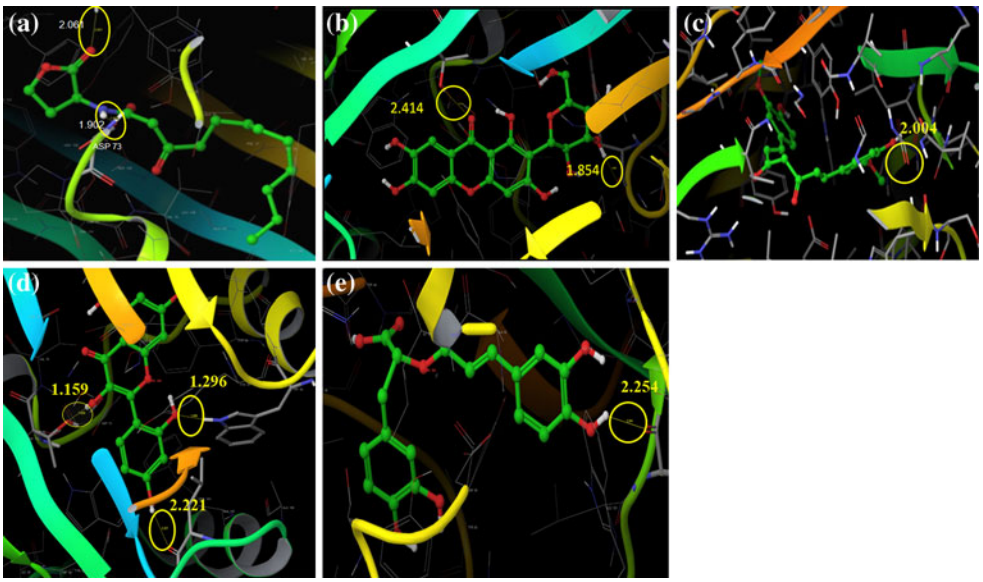

Fig. 1 Interaction of LasR receptor with **a** 3-Oxo-C12-HSL, **b** Mangiferin, **c** Naringin, **d** Morin, **e** Rosmarinic acid

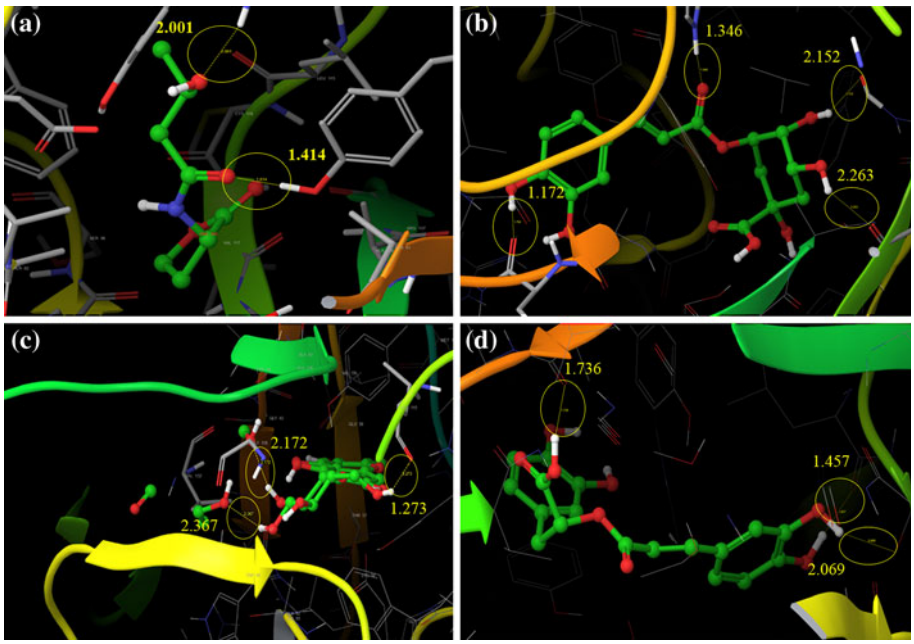

Fig. 2 Interaction of RhlR receptor with **a** C4-BHL, **b** Cholorogenic acid, **c** Mangiferin, **d** Rosmarinic acid

Total proteolytic assay

In total proteolytic assay, the QSI potential of rosmarinic acid, morin, naringin, chlorogenic acid and mangiferin were assessed. Rosmarinic acid showed consistent reduction in PAO1, AS1 and AS2 to the maximum of 87, 68 and 54 %, respectively. Naringin showed 51, 38 and 30 % reduction, respectively. In case of morin, a minimal level of inhibition such as 20, 22 and 26 %, respectively were observed. Chlorogenic acid inhibited the protease production to the level of 68, 30 and 64 %, respectively. The compound mangiferin did not show any inhibitory pattern in protease production (Table 3).

LasB elastase assay

In LasB elastase assay, rosmarinic acid showed a significant reduction 88 % in AS1, whereas, in PAO1 and AS2, the compound showed only to the level of 67 and 58 %, respectively. In case of naringin, a substantial reduction was observed in PAO1, AS1 and AS2 to the level of 64, 71 and 53 %, respectively. Further, morin exhibited better inhibition pattern in PAO1, AS1 and AS2 to the level of 56, 50 and 34 %, respectively. Chlorogenic acid showed inhibition to the level of 65, 37 and 54 %, respectively. The compound mangiferin did not show any inhibitory pattern in elastase production (Table 3).

Hemolysin assay

Hemolysin assay was performed to determine the potential of selected compounds in reducing the hemolysin production of PAO1, AS1 and AS2. A gradual increase in the viability of sheep erythrocytes were observed at their respective EICs of each QSI compound. Reduction in the sheep erythrocyte lysis in the presence and absence of compounds was compared with that of 100 % distilled water lysis. In this assay, rosmarinic acid showed a significant reduction in AS1 79 %, whereas, in PAO1 and AS2 showed a reduction to the level of 60 and 19 %, respectively. In case of naringin, a substantial reduction was observed in PAO1, AS1 and AS2 to the level of 63, 83 and 20 %, respectively. Morin exhibited better inhibition pattern in PAO1, AS1 and AS2 to the level of 64, 80 and 19 %, respectively. In case of chlorogenic acid treatment, PAO1 showed inhibition to the level of 15 %, no inhibition was observed in AS1 and AS2. Mangiferin also exhibited minimal inhibition in PAO1 and AS2 to the level of 28 and 7 % respectively, no inhibition was observed in AS1 (Table 4).

Inhibition of biofilm related behaviours

The CLSM biofilm images evidenced that these compounds effectively disrupted the biofilm architecture. A visible reduction in the biofilm formation was observed in the treated slides than the untreated control. The disruption of biofilm architecture and reduction in biofilm formation were shown as 3D images of CLSM, analysed with Zen 2009 software (Table 2; Fig. 3). The reduction of swarming motility evidenced that the factor required for the biofilm formation has been inhibited by the test QSI compounds (Fig. 4).

Table 2 CLSM image analysis of reduction in biofilm thickness of compounds treated and untreated bacterial pathogens

Compounds used	PAO1 (μm)	AS-1 (μm)	AS-2 (μm)
Control	100	60	80
Rosmarinic acid	30	25	25
Morin	25	45	40
Naringin	40	35	30
Chlorogenic acid	30	40	30
Mangiferin	60	30	30

The biofilm thickness was measured by CLSM Z-stack analysis through the process of optical sectioning using Zen 2009 software

Effect of QSI compounds on virulence enzymes production

In experiment setup 1: when compared to the control, a reduction in virulence enzyme activity was noticed in the bioassays such as protease, elastase and hemolysin degradation assay. The obtained results revealed that the test QSI compounds possibly inhibit the production of virulence enzymes (Tables 3, 4).

In set 2: since there was no exposure to test QSI compounds till 18th h, it is believed that the cell-free supernatant consists of virulence enzymes. If the test QSI compound has the ability to bind with the enzymes and to inhibit their activity, then the addition of QSI compounds at 18th h should inhibit the enzyme activity. But no such reduction in the enzyme activity was observed even after 24 h exposure (Fig. 5). Hence, the inhibitory activity of virulence enzymes was due to the QSI potential of test compounds and it was not due to the binding or degradation activity with enzymes.

Growth inhibition analysis

The effect of test QSI compounds on cell proliferation was determined by monitoring the growth of PAO1, AS1 and AS2. In growth inhibition analysis, the bacterial cultures treated with these compounds did not show any variation in the cell densities of all the three strains of *P. aeruginosa* when compared to the untreated controls. These results confirmed that the potential of these compounds were not due to antibacterial activity, but possibly through QSI activity. The growth pattern at their respective EICs with four different time intervals was given in Fig. 6.

Discussion

Pseudomonas aeruginosa is the most common Gram-negative bacterium found in hospital-acquired infections and is responsible for nosocomial pneumonia, urinary tract infections and bloodstream infections [33]. The increase in antibiotic resistance of *P. aeruginosa* [8, 9] and their ability

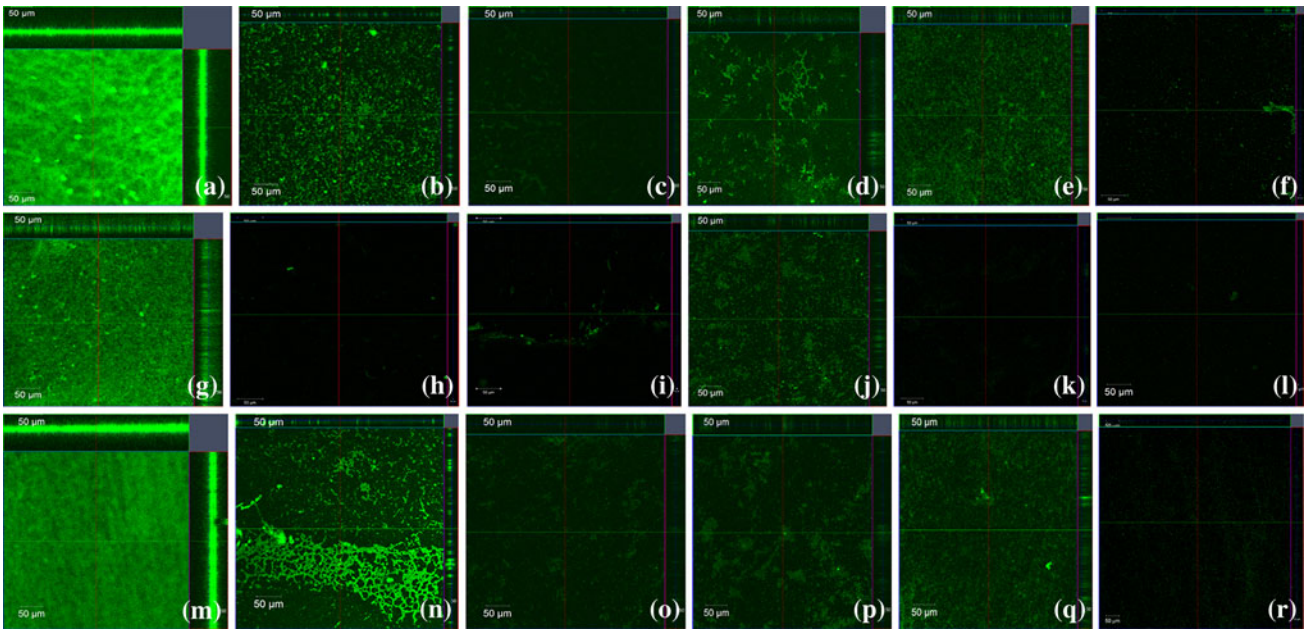

Fig. 3 Representation of CLSM ortho images by the process of optical sectioning (*Scale bar* 50 μM) of compounds treated and untreated PAO1, AS1 and AS2 biofilm analysed with Zen 2009 software. (*a*, *g*, *m*) Control PAO1, AS1 and AS2, respectively, (*b*, *h*, *n*) Rosmarinic acid 750 μg/mL treated, (*c*, *i*, *o*) Naringin 1,000 μg/mL treated, (*d*, *j*, *p*) Morin 30 μg/mL treated, (*e*, *k*, *q*) Chlorogenic acid 750 μg/mL treated, (*f*, *l*, *r*) Mangiferin 1,000 μg/mL treated

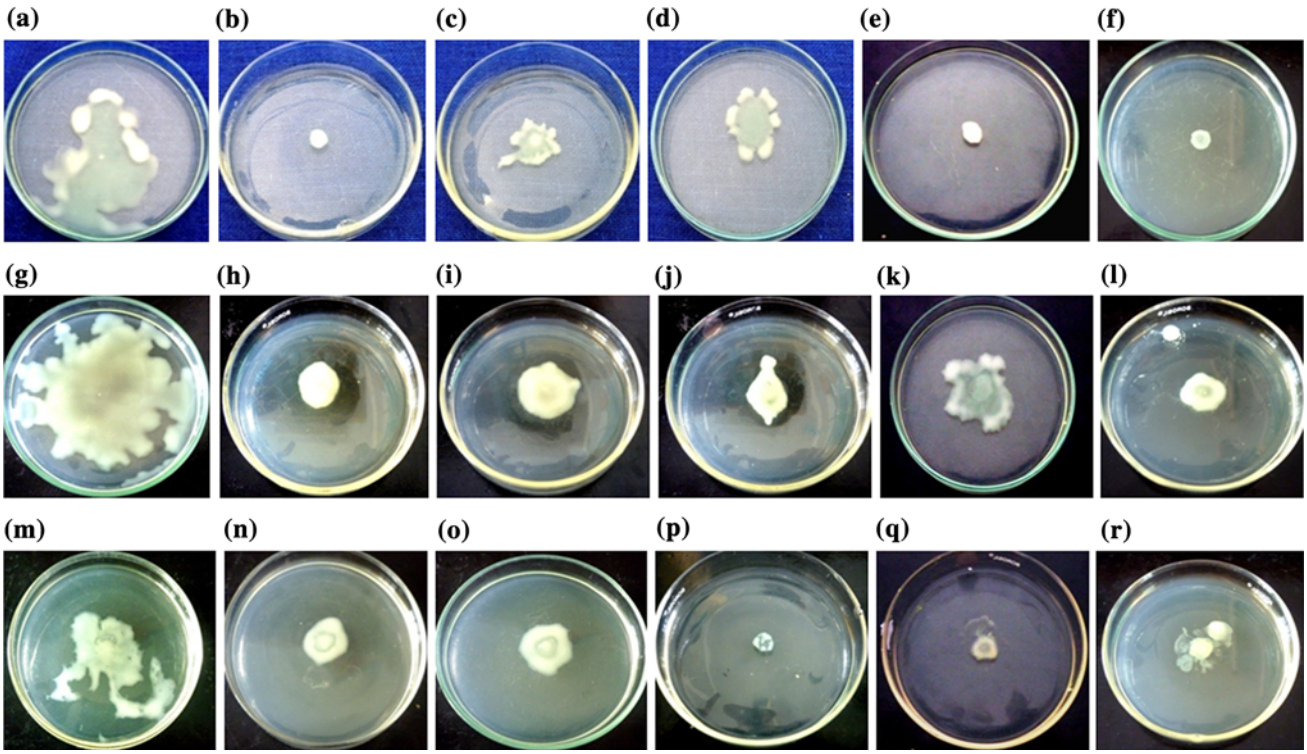

Fig. 4 Effects of test QSI compounds on swarming motility in *P. aeruginosa* PAO1, AS1 and AS2. All the compounds effectively inhibited the flagellar and pili mediated motility with respect to all strains. (*a*, *g*, *m*) Control PAO1, AS1 and AS2, respectively, (*b*, *h*, *n*) Rosmarinic acid 750 µg/ml treated, (*c*, *i*, *o*) Morin 30 µg/ml treated, (*d*, *j*, *p*) Naringin 1,000 µg/ml treated, (*e*, *k*, *q*) Chlorogenic acid 750 µg/ml treated, (*f*, *i*, *r*) Mangiferin 1,000 µg/ml treated

Table 3 Effect of test QSI compounds against protease and elastase virulence enzymes production in *P. aeruginosa* PAO1, AS1 and AS2

Tested compounds	Protease assay			Elastase assay		
	PAO1	AS1	AS2	PAO1	AS1	AS2
Control	1.2 ± 0.021	1.129 ± 0.268	1.421 ± 0.167	0.211 ± 0.005	0.216 ± 0.007	0.364 ± 0.014
Rosmarinic acid 750 µg/mL	0.155 ± 0.039[c]	0.363 ± 0.034[e]	0.65 ± 0.071[d]	0.069 ± 0.023[d]	0.027 ± 0.006[c]	0.152 ± 0.035[d]
Morin 30 µg/mL	0.965 ± 0.015[d]	0.878 ± 0.077[e]	1.051 ± 0.091[c]	0.093 ± 0.010[c]	0.108 ± 0.057[d]	0.242 ± 0.042[d]
Naringin 1,000 µg/mL	0.593 ± 0.010[c]	0.698 ± 0.033[e]	1.001 ± 0.082[d]	0.075 ± 0.010[c]	0.062 ± 0.033[d]	0.17 ± 0.044[c]
Chlorogenic acid 1,000 µg/mL	0.375 ± 0.051[c]	0.787 ± 0.039[d]	0.512 ± 0.176[e]	0.074 ± 0.030[c]	0.136 ± 0.025[d]	0.167 ± 0.063[d]
Mangiferin 1,000 µg/mL	1.335 ± 0.071	1.334 ± 0.100	1.413 ± 0.007	0.241 ± 0.035	0.240 ± 0.068	0.452 ± 0.223

[a] Protease activity is expressed as the decrease in OD_{400} per µg of protein

[b] Elastolytic activity is expressed as the absorbance at OD_{495} per µg of protein

[c] Significance *** $p \leq 0.0005$

[d] Significance ** $p \leq 0.005$

[e] Significance * $p \leq 0.25$

to form persistent infections through the formation of biofilm [10, 28] have drawn attention to find alternative measures for the current treatment strategies. A great effort has been made to develop antipathogenic strategies, especially by means of reducing bacterial virulence through QS systems [14, 30]. Evidences showed that the inhibition of LasR and RhlR QS systems could attenuate the pathogenicity of *P. aeruginosa* [13, 14, 19, 21]. Hence, switching off QS in *P. aeruginosa* by the use of QSIs has been shown to be a promising strategy for the treatment of infections [14]. A series of QSIs have been identified by different groups through traditional methods from various natural

Table 4 Effect of test QSI compounds against hemolysin virulence enzyme production in *P. aeruginosa* PAO1, AS1 and AS2

Hemolysis assay[a]	Distilled water lysis 100 % lysis	Control	Rosmarinic acid 750 μg/mL	Morin 30 μg/mL	Naringin 1,000 μg/mL	Chlorogenic acid 750 μg/mL	Mangiferin 1,000 μg/mL
PAO1	2.734 ± 0.037	1.88 ± 0.467	0.247 ± 0.030[b]	0.129 ± 0.006[b]	0.157 ± 0.008[b]	1.475 ± 0.016[c]	1.117 ± 0.006[c]
AS1	2.734 ± 0.037	2.395 ± 0.697	0.238 ± 0.002[b]	0.195 ± 0.018[b]	0.138 ± 0.0005[b]	2.698 ± 0.001	2.639 ± 0.127
AS2	2.734 ± 0.037	0.665 ± 0.273	0.142 ± 0.028[c]	0.133 ± 0.026[c]	0.120 ± 0.012[c]	0.691 ± 0.069	0.455 ± 0.065[d]

[a] Hemolysis activity is expressed as percent lysis compared with the lysis of erythrocytes in distilled water

[b] Significance *** $p \leq 0.0005$

[c] Significance ** $p \leq 0.005$

[d] Significance * $p \leq 0.25$

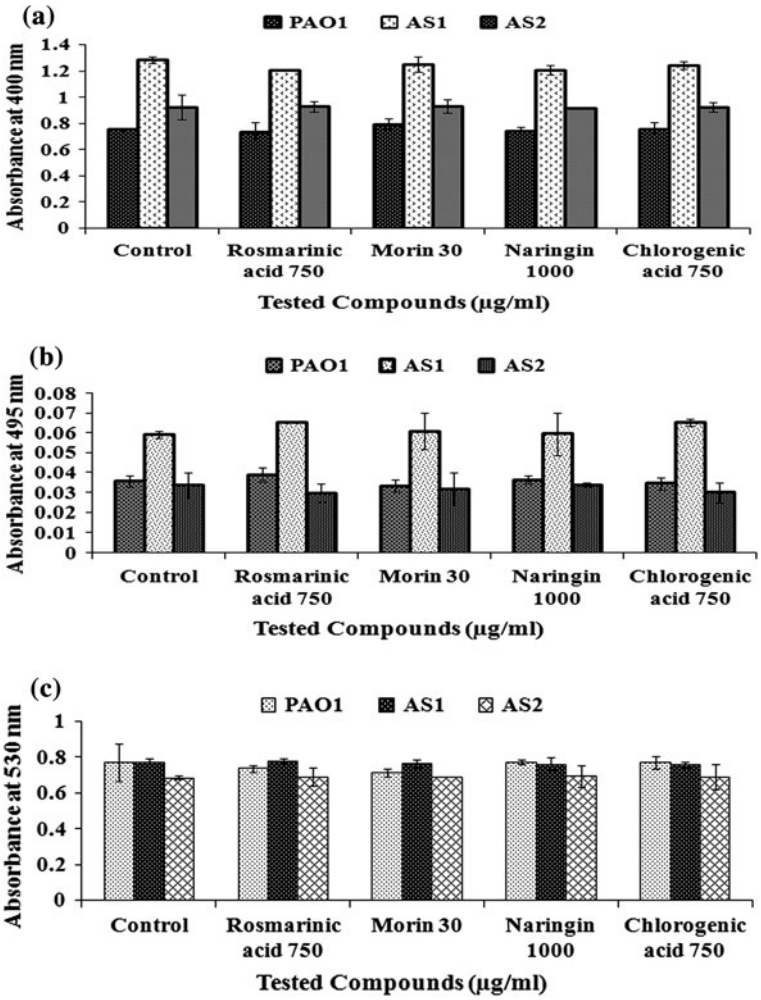

Fig. 5 The effect of test QSI compounds on virulence enzyme production after 18 h in PAO1, AS1 and AS2. **a** Total proteolytic activity. **b** Elastolytic activity. **c** Hemolysis activity

resources [1, 14, 18, 34]. However, the traditional methods have some limitations that can be complemented by novel CADD application [17, 27]. This opens a new avenue for the designing of QSIs, which reduce virulence, pathogenicity and resistance rather than directly inhibiting the bacterial growth.

The core objective of this study was to find out novel and potential QSI compounds through structure based virtual screening against LasR and RhlR receptor proteins of *P. aeruginosa*. Totally, 1,920 natural compounds were docked against aforementioned receptor proteins. Among the 1,920 compounds, five compounds such as rosmarinic acid, mangiferin, chlorogenic acid, morin and naringin were selected for experimental analysis based on the Glide score, energy, interacting efficiency, hydrogen bond of the ligands and Adsorption Distribution Metabolism Excretion

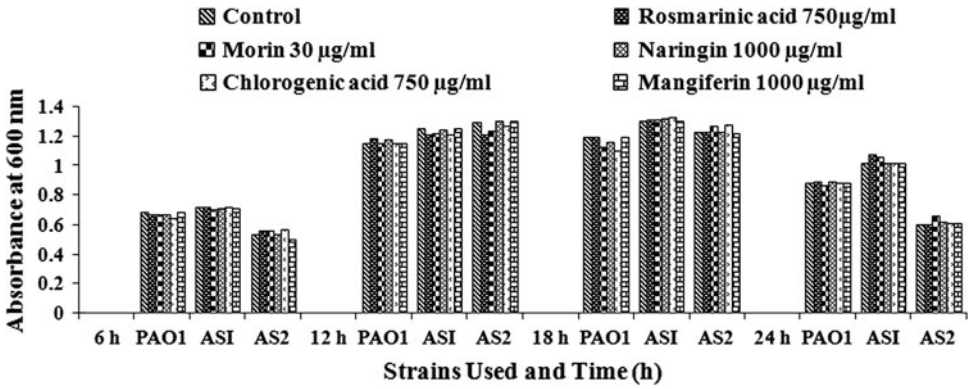

Fig. 6 Effect of test QSI compounds on growth of *P. aeruginosa* PAO1, AS1 and AS2. Compounds were added at 0 h

(ADME) properties (Table 1). The active site residues were predicted using sitemap and these residues were found to be accurately bound in docking. There are certain amino acid residues which showed similarities in the binding interactions between the selected compounds and AI. It has been found that 36 LEU, 56 TYR, 60 TRP, 64 TYR, 73 ASP, 75 THR, 76 VAL and 127 ALA are the key amino acid residues taking part in similar interaction with LasR receptor proteins among the selected compounds and also with the docking result of known ligand namely 3-oxo-C12-HSL. Similarly, in case of RhlR receptor proteins the two main amino acids such as 57 THR and 71 TYR interactions were found to be similar among the selected compounds and with the known ligand namely C4-BHL. The Glide docking scores of five selected compounds and AI are summarized in Table 1. The docking scores and the binding efficiency values of the ligands with receptor proteins of all the selected compounds were better than that of AI. Hence, it is envisaged that all the five selected natural compounds have better docking power to the receptor proteins than the AI. Further, the efficiency of the selected compounds was tested under in vitro condition.

It has been known that among the identified QSIs, few of them were considered as pharmaceutically not relevant due to their toxicity and instability [24]. But the fact behind our study is that the natural compounds used in this study are already approved drugs even for human consumption and has a significant benefit in further application as anti-pathogenic drugs. According to the report of Pubchem database, all five tested compounds possess various biological properties. Rosmarinic acid has anti-inflammatory, anti-viral and anti-oxidants properties. Mangiferin is known to possess a wide range of bioactive potential such as anti-mutagen, anti-tumor, anti-oxidant, anti-allergic, anti-inflammatory, anti-diabetic, anti-viral, anti-fungal and anti-parasitic properties. Naringin is an anti-ulcer, anti-apoptotic and anti-hypercholesterolemia agent. Morin is a potential anti-oxidant and chlorogenic acid acts as a carcinogenic inhibitor. These natural compounds such as rosmarinic acid, naringin, morin, chlorogenic acid and mangiferin present in the natural resources like edible fruits such as *Rubus idaeus*, *Citrus x paradisi*, *Artocarpus heterophyllus*, *Malus domestica* and *Mangifera indica*, respectively. A recent report of Annapoorani et al. [3] evidenced that the compounds used in this study have been proven for their QSI activity against the violacein production in *Chromobacterium violaceum* (ATCC 12472) and also against the virulence of urinary pathogen *Serratia marcescens*. Our previous report also revealed the presence of potential QSIs in the edible fruits and plants [18].

In order to evaluate the QSI efficiency of these compounds, in vitro experimental bioassays were performed to inhibit QS dependent virulence factors production and biofilm formation in both laboratory as well as clinical strains of *P. aeruginosa*. Treatment of *P. aeruginosa* with these QSI compounds resulted in a decreased production of QS-controlled virulence factors. Interestingly, the clinical isolates AS1 and AS2 treated with rosmarinic acid, chlorogenic acid and naringin were showed significant inhibition in both Las and Rhl dependent virulence factors production. Treatment with morin indicated that QS regulation was not completely switched off at the tested concentration, but resulted in a minimal inhibition in Las dependent virulence factors production and when the concentration exceeds the EIC, it inturn enhances the QS dependent phenomenon. In case of mangiferin, no inhibition was observed in protease and elastase virulence enzyme production (Tables 3, 4). In addition, all the five compounds were also found to inhibit biofilm related behaviours in wild type PAO1 as well as in the clinical isolates AS1 and AS2 (Figs. 3, 4). The treated biofilms were thinner and less structured than untreated ones (Table 2). The obtained results of reduction in the biofilm development are comparable with the earlier reports [12, 13], who have noticed reduction in bacterial biofilm by the treatment with garlic extract and furanone of *Delisea pulchra*. Furthermore, alteration of *P. aeruginosa* biofilm through the action of QSI makes the pathogen more sensitive to antibiotics and host immune system [5]. Hence, it is envisaged that the combination of these compounds

along with the antibiotics can have the synergistic effects to inhibit biofilm formation and further, it will pave the way to prevent the biofilm mode of infections.

The protein degradation assay clearly evidenced that test QSI compounds did not degrade the virulence enzymes (Fig. 5). Hence, it is believed that the inhibition mediated through QS and not due to binding and degrading activity of test QSI compounds. It was also observed that the QSI compounds such as rosmarinic acid, morin, naringin, chlorogenic acid and mangiferin that displayed QSI activity at their respective EICs without affecting the bacterial growth (Fig. 6). In support to the present findings, the identified three QSIs through molecular docking programme such as salicylic acid, nifuroxazide and chlorzoxazone displayed QSI activity against LasIR and RhlIR controlled virulence factors of *P. aeruginosa* [36].

The obtained results of bioassays showed excellent consistency and correlation with the molecular docking analysis. The interactions between the selected compounds and AI with receptor proteins are displayed in Figs. 1 and 2. Using this CADD approach and biological assays few more QSI compounds were identified for *Agrobacterium tumefaciens* and *P. aeruginosa* [37]. Therefore, it is envisaged that molecular docking is an effective way to design new QS antagonists. Results of the present study revealed that those compounds exhibited potential to function as an effective antipathogenic drug in computational discovery can be used in the treatment of *P. aeruginosa* infection. Based on the results obtained from in vitro analysis, rosmarinic acid, naringin and chlorogenic acid appears to be fairly potent inhibitors of LasR and RhlR QS dependent mechanisms and possibly act as a LasR and RhlR signals antagonist.

The compounds selected for this study are different non-AHL resembling classes of compounds. The QSI activity of these structured compounds and the obtained results agree with a study of Bottomley et al. [7]. Such efforts are exemplified by the recent identification of specific LasR antagonist TP-5, a triphenyl structure. The combination of virtual screening and a preliminary similarity search for molecules similar to already known QS binding compounds has proven to be successful in the discovery of new QSIs.

Conclusion

In conclusion, despite a rich source of reports available on QS and QSI mechanisms of *P. aeruginosa*, most of the studies were concentrated more on laboratory strains under controlled laboratory condition. But, this may not reflect with what happens in clinical isolates of *P. aeruginosa* [6]. Hence, in the present study the efficiency of QSI compounds were tested with clinical isolates of *P. aeruginosa* AS1 and AS2. Further, it is revealed that molecular docking studies can provide focused finding of compounds to combat bacterial infections and can help in the discovery of new inhibitors.

Acknowledgments We extend our sincere thanks to computational and bioinformatics facility provided by the Alagappa University Bioinformatics Infrastructure Facility (funded by the Department of Biotechnology, Government of India; Grant No. BT/BI/25/001/2006). We thank Schrodinger, LLC, New York, USA for providing free access of their bioinformatics tools. We also extend our sincere thanks to Dr. K. Syed Ibrahim, Research Associate, Department of Biotechnology, Mizoram University, Aizawl and Mr. C. Selvaraj, Research Scholar, Department of Bioinformatics, Alagappa University, Karaikudi for their assistance in the bioinformatics part of this manuscript. A. Annapoorani gratefully acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi for the financial assistance rendered [CSIR SRF No. 9/688 (0014)/2011].

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