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Identifying Multitargeting Inhibitors against Targets Involved in the Quorum-Sensing Pathway of *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant gram-negative bacterium where the quorum-sensing (QS) circuit coordinates biofilm formation, virulence, and drug resistance. Given the complexity of the QS circuit, targeting a single receptor becomes highly inefficient. To address these challenges, 11 QS receptors from the LAS, RHL, PQS, and IQS circuits were computationally studied and targeted via structure-based virtual screening. From the Indian Medicinal Plants, Phytochemistry and Therapeutics (IMPPAT) natural compound library, some multitarget inhibitors were selected for further computational validation using molecular dynamics and free-energy calculations analysis. This demonstrates the untapped potential of natural products derived from Indian medicinal plants to act as multitarget QS inhibitors. The integrated computational framework, including molecular docking, molecular dynamics simulations, and free energy calculations, showed that calamansanin, a phenolic natural product, and clivimine, an alkaloid, exhibit the best multitargeting potential among the others. Thus, computational studies on the development of QS inhibitors against antibiotic-resistant *P. aeruginosa* indicate that the QS network can serve as a therapeutic target. The study reveals the novel chemistry and druglike properties of compounds isolated from natural sources, including Indian medicinal plants, as potent antivirulence agents.

KEYWORDS

Quorum sensing, *Pseudomonas aeruginosa*, IMPPAT database, biofilm formation, homology modeling, multidrug resistance, MD simulations, and multitarget inhibitors.

Highlights

- A modular, reproducible computational pipeline was developed, comprising homology modeling, structure-based virtual screening, molecular dynamics simulations, and free energy calculations.
- A dataset containing 17,967 phytochemicals from the IMPPAT database was screened against eleven QS receptors from the LAS, RHL, PQS, and IQS QS systems.
- Calamansanin and clivimine were found to be potent multitargeting QS inhibitors, a conclusion further substantiated by structural analysis and MD simulations.

- The findings demonstrate that the multitarget approach to inhibit QS networks will be effective at counteracting the redundancy and compensatory mechanisms in these signaling systems.
- The study reveals the novel chemistry and druglike properties of compounds isolated from natural sources, including Indian medicinal plants, as potent antivirulence agents.

1. Introduction

Infectious diseases, particularly those caused by gram-negative pathogens resistant to antibiotics such as *P. aeruginosa*, are becoming a growing global health risk (Terreni et al. 2021). Conventional antibiotic therapies are hindered by bacterial resistance, which is often facilitated by biofilm formation and the controlled production of virulence factors (Quave 2016; Odularu et al. 2022). The core concept underlying these phenomena is QS, a process in which cells communicate based on population density (Kleerebezem et al. 1997; Asfour 2018).

In QS, chemical substances called autoinducers, including acyl-homoserine lactones (AHLs) in gram-negative bacteria and oligopeptides in gram-positive bacteria, trigger a set of genes encoding receptors that regulate gene expression (Asfour 2018; Verbeke et al. 2017). In *P. aeruginosa*, four hierarchically organized and interlinked QS pathways, LAS, RHL, PQS, and IQS, coordinate the bacterium’s virulence repertoire. These signaling systems make use of QS enzymes, a term that includes not only the autoinducer synthases (e.g., LasI, RhII, and PqsA), but also their receptors/transcriptional regulators (LasR, RhIR, and PqsR/MvfR, among others), which transform population-wide chemical signals to changes in gene regulation, as shown in Figure 1.

Traditional methods targeting QS involve inhibiting a single enzyme or signaling pathway and often fail due to high levels of compensatory crosstalk among QS pathways in *P. aeruginosa* (Koul et al. 2016; Kalia et al. 2014). The redundancy of pathways in this regard confers upon the bacterium a high ability to escape single-target therapy. Mutations that confer resistance to single-target approaches, upregulate efflux pumps, and activate alternative pathways can undermine the durability of single-target approaches (Kalia et al. 2014; Kalia 2013). As a result, broad-spectrum, multitarget approaches are essential by simultaneously disrupting multiple QS subsystems. Multitarget inhibitors are much more likely to cause a global loss of virulence, collapse the bacterium’s social signaling network, and drastically reduce the likelihood of resistance development (Odularu et al. 2022; Alibi et al. 2020). Multitargeting also raises the possibility of combinatorial inhibitory effects across multiple QS circuits, thereby improving disruption of biofilm formation and virulence phenotypes. Recently, it has been demonstrated that β -lactam antibiotics, including azlocillin and moxalactam, can form stable complexes with several QS-related proteins, such as LasI, LasA, and PqsR (Anwer 2024). This allows them to efficiently target and disrupt several levels of the QS network in *P. aeruginosa*. By reducing escape mechanisms common in monotherapy, this targeted multitargeting not only lessens bacterial toxicity but also offers a possible way to overcome antimicrobial resistance (Qais et al. 2024).

Over the past two decades, significant screening efforts for QS inhibitors derived from natural sources and those developed synthetically have been conducted, including halogenated furanones, flavonoids, autoinducer analogs, and imidazole derivatives. Their activity has been successfully demonstrated against several pathogenic bacteria (Asfour 2018; Arendse et al. 2022; Ćirić et al. 2019). This effort has been predominantly fueled by

in silico molecular docking and virtual screening techniques (Rather et al. 2022; Zhong and He 2021). Two major disadvantages of such screening techniques exist, however: most in silico screenings for QS inhibitors are still "single-target," not taking into account network-level inhibition and multitarget effect, and virtual computational libraries of molecules tend to be dominated by synthetic chemicals available for laboratory research and to ignore natural substances, especially plant-based ones (Zhong and He 2021; Yang et al. 2020). There has been no approved use of QS inhibitors in medicine by the FDA until now in 2025; new antibiotic drug candidates continue to obtain regulatory approvals ahead of QS inhibitors (e.g., plazomicin, cefiderocol, eravacycline), which interfere with but do not disrupt QS systems (Terreni et al. 2021; Quave 2016). The focus on traditional antibiotics failed to stop the rise of multidrug-resistant bacteria, and often, newly developed drugs provided only slight improvements and quickly became obsolete due to resistance. Despite considerable success in QS inhibitor development, their single use may lead to rapid resistance development, as with traditional antibiotics (Kalia et al. 2014). Recent research indicates that certain antibiotics with appropriate ADMET properties and multitarget interaction with important QS proteins, namely, LasI, LasA, and PqsR, including azlocillin and moxalactam, could be repurposed (Qais et al. 2024).

The IMPPAT (version 2.0) database, a repository of natural product diversity from plant sources, contains 17,967 phytochemicals from 4,010 Indian medicinal plants (Vivek-Ananth et al. 2023). The vast pool of chemically diverse structures is classified into 24 superclasses, with the most dominant being lipids (33%), phenylpropanoids and polyketides (19%), and organoheterocyclics (12%). The said classes of compounds are especially relevant to the topic of QS inhibition because they represent widely prevalent natural pharmacophores, such as phenolic, alkaloid, and terpenoid derivatives, with immunomodulatory activity and antiseptic potential to disrupt bacterial regulatory networks (Asfour 2018; Rather et al. 2022; Yang et al. 2020; Vivek-Ananth et al. 2023). It is interesting that very few of the constituents of the IMPPAT database match any FDA-approved drugs, while nearly 10% meet the required drug-likeness criteria (Glen and Lamont 2021).

Some essential oils and plant extracts recorded in the IMPPAT database exhibit considerable potential to act against QS systems and biofilms, making them relevant candidates as antivirulence drugs (Alibi et al. 2020; Rather et al. 2022; Manner and Fallarero 2018). By utilizing such a rich chemical dataset, especially targeting multitarget effects, computational approaches such as docking and simulation can be used to discover broad-spectrum QS inhibitors to overcome antibiotic resistance and bacterial virulence (Koul et al. 2016; Kalia et al. 2014).

Past research on QS inhibition in *P. aeruginosa* has generally focused on specific agents or a small set of chemicals, often synthetic drugs or a few classes of antibiotics (Terreni et al. 2021; Quave 2016; Kalia 2013). However, unlike past studies that used a single target or restricted screening (Alibi et al. 2020; Arendse et al. 2022), the current research employs a multifunctional, multitarget approach that examines 11 key QS enzymes across the interlinked LAS, RHL, PQS, and IQS pathways. This is done through extensive screening that considers the diversity of chemicals in the IMPPAT database. Following the virtual screening, we have also performed molecular dynamics and free-energy calculations to further validate the inhibitory potential of the phytochemicals.

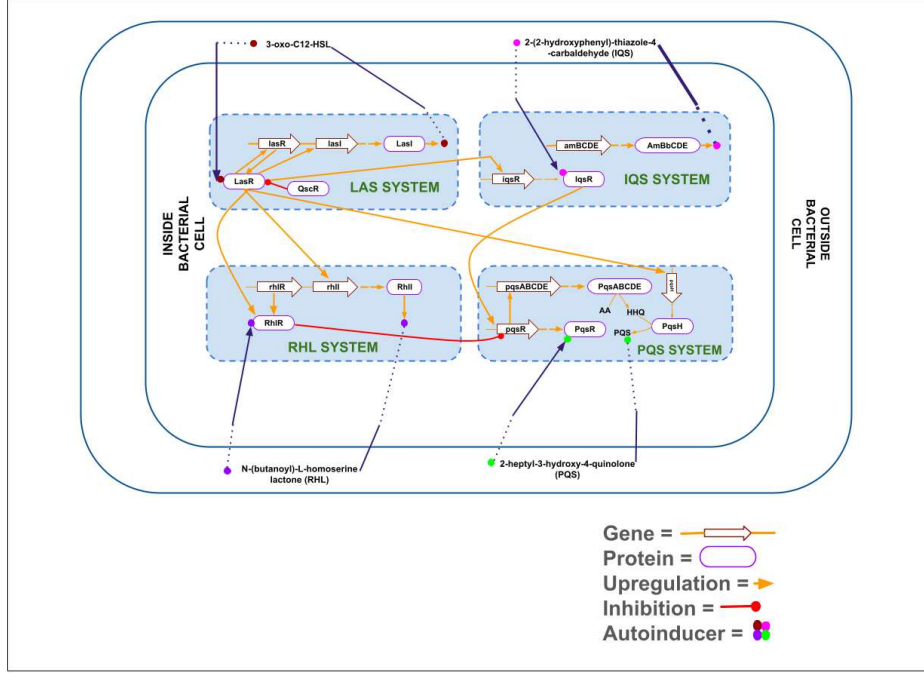


Figure 1. Schematic representation of the QS signaling pathways in *Pseudomonas aeruginosa*. The network comprises four major interconnected systems: Las, Rhl, Pqs, and Iqs. Each system contains specific autoinducers that regulate signaling, virulence factor production, and biofilm formation.

2. Computational Method

In *P. aeruginosa*, a comprehensive structural study of QS receptors was conducted following a thorough literature review, which identified the primary QS pathways as LAS, RHL, PQS, and IQS. While RhlR was identified as the primary receptor of the RHL pathway, LasI and LasR were identified as important proteins in the LAS pathway within these systems. PqsE, PqsD, PqsA, PqsH, and MvfR (also known as PqsR) were among the many receptors that comprised the PQS system, whereas AmbE and AmbB formed the IQS pathway, and the LuxR-type regulator QscR is the orphan QS signal receptor (Ding et al. 2018). The RCSB-PDB database (Berman et al. 2000) was used to obtain the three-dimensional structures of 10 of these 11 receptors, using their published PDB IDs (1RO5, 5HIO, 4NG2, 6CC0, 7QA0, 8DQ0, 7R9X, 7X17, 3H77, 5OE6, and 3C96).

Table 1. List of targets with their UniProt IDs, PDB IDs, classification, and co-crystallized ligands or Ions used in this study.

S.No	Target	PDB ID	UniProt ID	Classification	Ligand/Substrate/Ions
1	LasI	1RO5	P33883	Protein (Signaling)	SO4 ion, Zn ion
2	PqsE	5HIO	P20581	Protein (Metal-binding)	61M
3	LasR	4NG2	P25084	Protein (Transcriptional regulator)	OHN
4	QscR	6CC0	Q9RMS5	Protein (Transcriptional regulator)	EWM
5	MvfR	7QA0	Q9I4X0	Protein (Gene regulation)	9ZL
6	RhlR	8DQ0	P54292	Protein (Transcription)	K5Z
7	AmbE	7R9X	Q9I1H3	Protein (Biosynthetic)	Iodide ion, Sodium ion
8	AmbB	7X17	Q9I1H0	Protein (Biosynthetic)	868
9	PqsD	3H77	P20582	Enzyme (Transferase)	COW
10	PqsA	5OE6	Q9I4X3	Enzyme (Ligase)	9SN
11	PqsH	5CHH	Q9I0P9	Enzyme (Oxidoreductase)	MSE

Since PqsH lacked an experimental structure, a homology model was generated using the UniProt sequence (Ahmad et al. 2025) and the SWISS-MODEL server (Waterhouse et al. 2018), yielding a structural similarity of nearly 99%. With the same approach, missing residues in specific receptors were also modeled. Using the CASTp server (Tian et al. 2018), active sites were found for proteins without annotated binding pockets, as shown in Table 1. To ensure structural integrity, PyMOL (DeLano 2002) was utilized for structural visualization and validation, as shown in Figure 2.

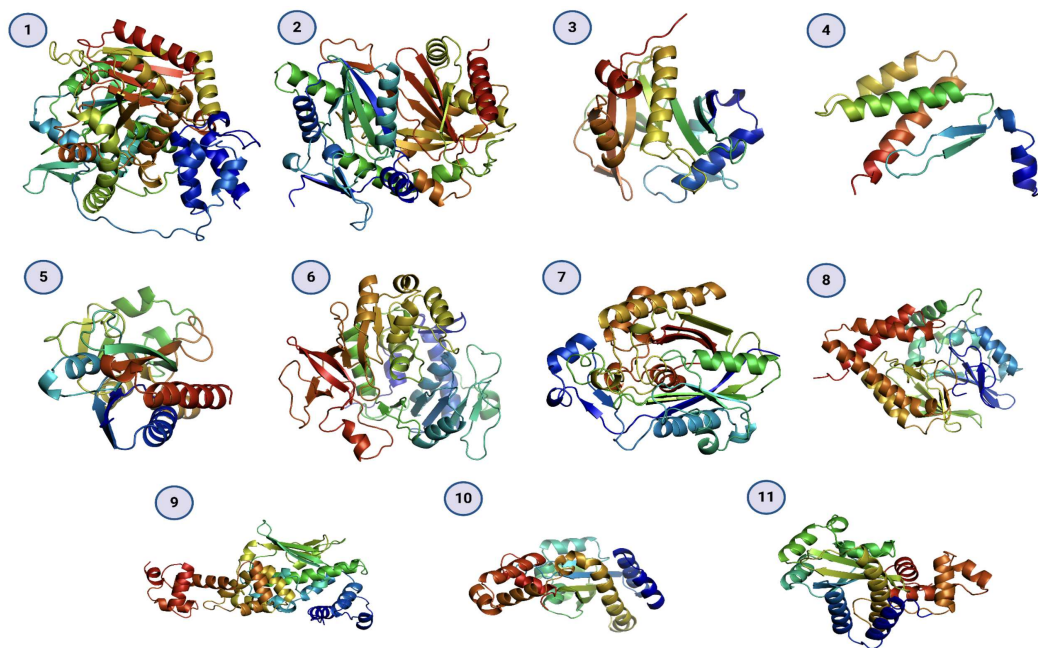


Figure 2. Ribbon structures of target proteins: (1) AmbB, (2) AmbE, (3) LasI, (4) LasR, (5) MvfR, (6) PqsA, (7) PqsD, (8) PqsE, (9) PqsH, (10) QscR, and (11) RhlR. Proteins are displayed in a rainbow color scheme from the N-terminus (blue) to the C-terminus (red).

The IMPPAT database to identify potential natural compounds for inhibitor screening. To ensure compatibility with molecular docking tools, all compounds were exported in SDF format, converted to PDBQT files using Open Babel (O’Boyle et al. 2011), and were then ready for docking. Similar optimizations were made to receptor architectures for precise docking performance.

The initial screening for potential interactions between natural compounds and QS targets was performed using molecular docking. Eleven target structures—LasI, LasR,

RhlR, PqsE, PqsD, PqsA, PqsR (MvfR), PqsH, AmbE, AmbB, and QscR were subjected to docking using AutoDock Vina (Trott and Olson 2010). Grid boxes were generated for each protein target, ensuring that the corresponding binding sites were fully covered when preparing receptor and ligand structures in PDBQT format using the Molecular Graphics Lab (MGL) tool. The top 100 compounds with the highest binding affinities were retained for in-depth analysis, and the best-scoring compound for each target protein is reported in Table 2.

Table 2. Best-scoring ligand complexes identified for each QS target based on molecular docking.

S.No	Complex with best BA	Binding affinity (kcal/mol)
1	AmbB + IMPHY007725	-14.75
2	AmbE + IMPHY007982	-13.56
3	LasR + IMPHY004849	-13.19
4	PqsA + IMPHY009219	-12.79
5	RhlR + IMPHY000058	-12.77
6	PqsD + IMPHY007982	-12.47
7	MvfR + IMPHY014605	-12.22
8	PqsE + IMPHY005634	-12.21
9	QscR + IMPHY004244	-11.93
10	PqsH + IMPHY009207	-11.48
11	LasI + IMPHY007725	-10.91

The SwissADME (Daina et al. 2017) and pkCSM (Pires et al. 2015) programs were used to estimate the pharmacokinetics and toxicity of selected natural compounds from the IMPPAT database. Key parameters such as hepatotoxicity, Gastrointestinal (GI) absorption, aqueous solubility, blood-brain barrier (BBB) penetration, Ames mutagenicity, and hERG channel inhibition were some of those considered. The above tests helped determine which compounds had desirable safety and pharmacological properties. Compounds with high GI absorption, proper solubility, and low toxicity were selected for further testing to determine effective QS inhibitors.

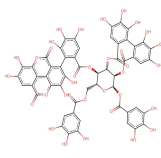
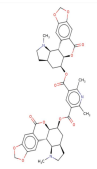
To further evaluate the stability of the selected ligand–receptor complexes, molecular dynamics (MD) simulations were performed for the two most promising multitarget compounds, calamansanin and clivimine, against all eight of their respective shared target proteins. For each ligand, two representative complexes were selected for extended production runs of 500 ns, while the remaining six complexes were simulated for 300 ns. For calamansanin, the AmbB and MvfR complexes were carried to 500 ns; for clivimine, the PqsA and PqsD complexes were carried to 500 ns. All simulations were performed using the GROMACS package with an appropriate force field (Justin 2018). Ligand topologies were generated prior to simulation, and each protein–ligand complex was solvated in a cubic water box followed by ion neutralization. Energy minimization was carried out to remove steric clashes, followed by equilibration in the NVT and NPT ensembles before the production run. Structural stability and dynamic behavior of the complexes were analyzed using root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and radius of gyration (Rg).

Binding free energy calculations were further performed using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) and Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) approaches to estimate the binding strength of ligand–receptor complexes. Energy components, including van der Waals interactions, electrostatic interactions, polar solvation energy, and solvent-accessible surface area energy (associated with nonpolar solvation energy), were calculated to determine the overall binding free energy (ΔG) of the complexes.

3. Results and Discussion

A search was conducted to identify common ligands (IMPPAT compounds) with multi-targeting activity. The results were filtered to include only those ligands associated with more than two targets. The analysis revealed that two ligands were shared across eight targets. Among these, seven targets were common, with one differing target for each ligand; specifically, RhlR was uniquely associated with calamansanin, whereas PqsE was uniquely associated with clivimine, as summarized in Table 3 and illustrated in Figure 3.

Table 3. Common multitarget ligands identified across QS targets with their corresponding binding affinities.

Ligand ID	Ligand Name	Ligand structure	Binding affinity with different targets (kcal/mol)
IMPHY007725	Calamansanin		PqsA = -12.9 AmbE = -12.5 PqsD = -11.5 MvfR = -10.8 PqsH = -10.5 AmbB = -9.8 LasI = -9.7 RhlR = -7.8
IMPHY013460	Clivimine		PqsA = -12.8 PqsD = -11.5 AmbE = -11.3 PqsE = -10.8 PqsH = -10.7 MvfR = -9.9 LasI = -9.3 AmbB = -8.5

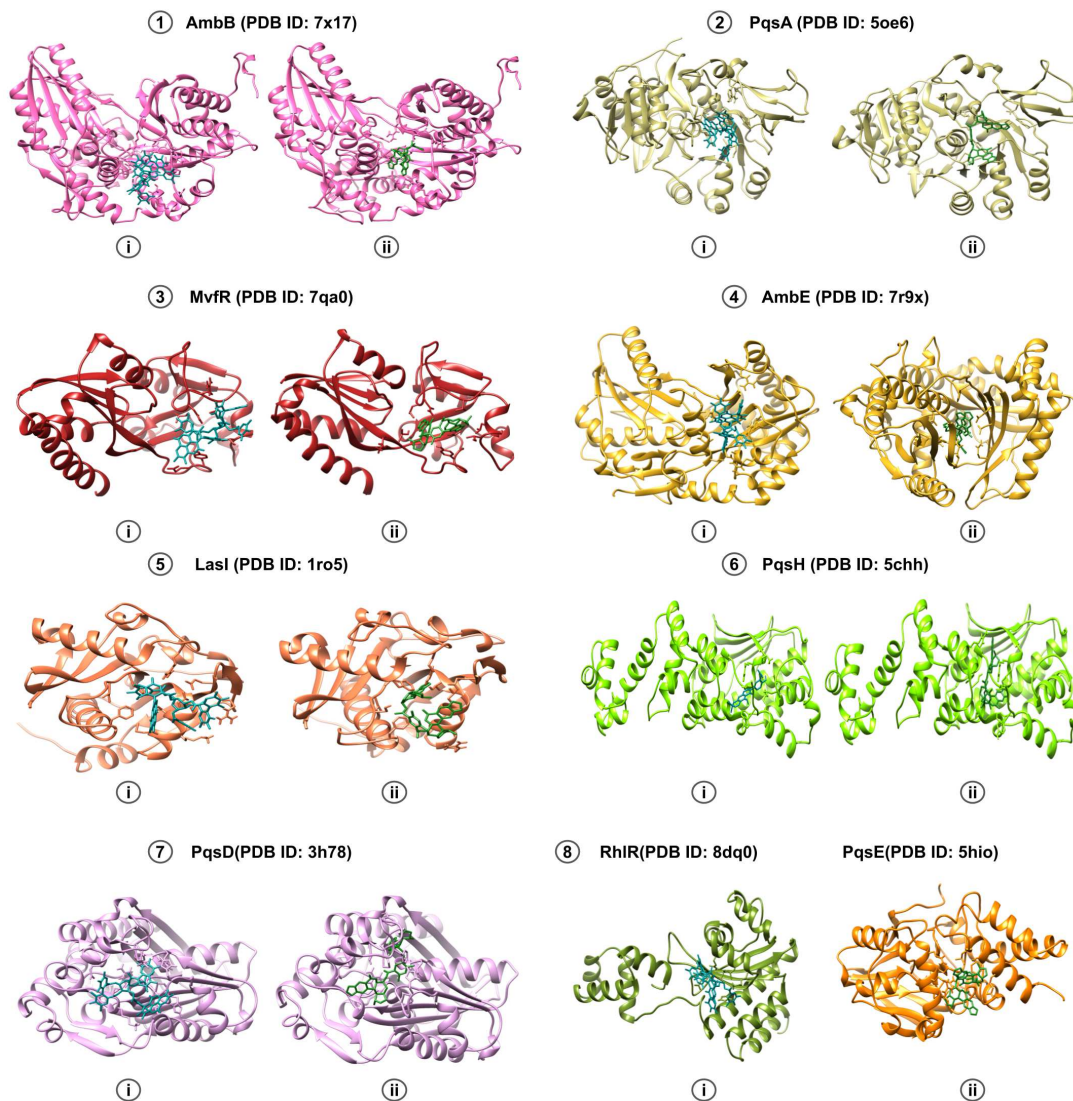


Figure 3. The figure shows three-dimensional ribbon models of eight QS-associated target proteins- AmbB (PDB ID: 7x17), PqsA (PDB ID: 5oe6), MvfR (PDB ID: 7qa0), AmbE (PDB ID: 7r9x), LasI (PDB ID: 1ro5), PqsH (PDB ID: 5chh), PqsD (PDB ID: 3h78), RhlR (PDB ID: 8dq0), and PqsE (PDB ID: 5hio)—with two multitarget ligands from the IMPPAT database docked into their binding pockets. For each protein, (i) depicts calamansanin (shown in dark cyan), whereas (ii) depicts clivimine (shown in forest green). Both ligands bind to similar regions within the active sites of seven out of eight common targets, suggesting a strong multitargeting potential. A notable difference in target preference was observed for one protein in each case. Calamansanin showed a stronger, more specific interaction with RhlR, whereas Clivimine displayed selectivity for PqsE.

The computational results were then validated by comparing the predicted binding affinities with the native ligands, as shown in Table 4. Only 5 of the 8 target proteins under investigation have experimentally characterized native ligands, allowing comparison between the predicted and reference binding affinities for these targets.

Table 4. Comparison of predicted binding affinities of calamansanin and clivimine with experimentally characterized native ligands.

Natural ligand complex (kcal/mol)	Calamansanin (kcal/mol)	Clivimine (kcal/mol)
PqsA (9SN) = -10.5	PqsA = -12.9	PqsA = -12.8
MvfR (9ZL) = -9.2	MvfR = -10.8	MvfR = -9.9
AmbB (868) = -5.1	AmbB = -9.8	AmbB = -8.5
RhlR (K5Z) = -7.3	RhlR = -7.8	–
PqsE (61M) = -6.8	–	PqsE = -10.4

LigPlot+ (Wallace et al. 1995) was used to investigate receptor-ligand complexes in more detail and to clarify the key chemical interactions that control binding. To interfere with the interrelated signaling pathways that regulate *P. aeruginosa* virulence and biofilm formation, putative multitarget inhibitors must interact with numerous QS receptors, as demonstrated by our investigation. The docking data revealed a variety of interaction patterns and enabled the identification of key receptor residues that stabilize binding. Only five of the eight QS-related sites under investigation have experimentally reported native ligands; therefore, comparative LigPlot+ analyses were generated only for these targets. However, for the other three proteins, no data were available on the native ligands, so comparisons are not possible. Referring to the LigPlot+ structures presented in Figure 4, it can be seen that amino acids marked with red circles are frequently associated with interactions with both the native ligand and the IMPPAT molecules, meaning the plant compounds are appropriate substitutes for the ligands.

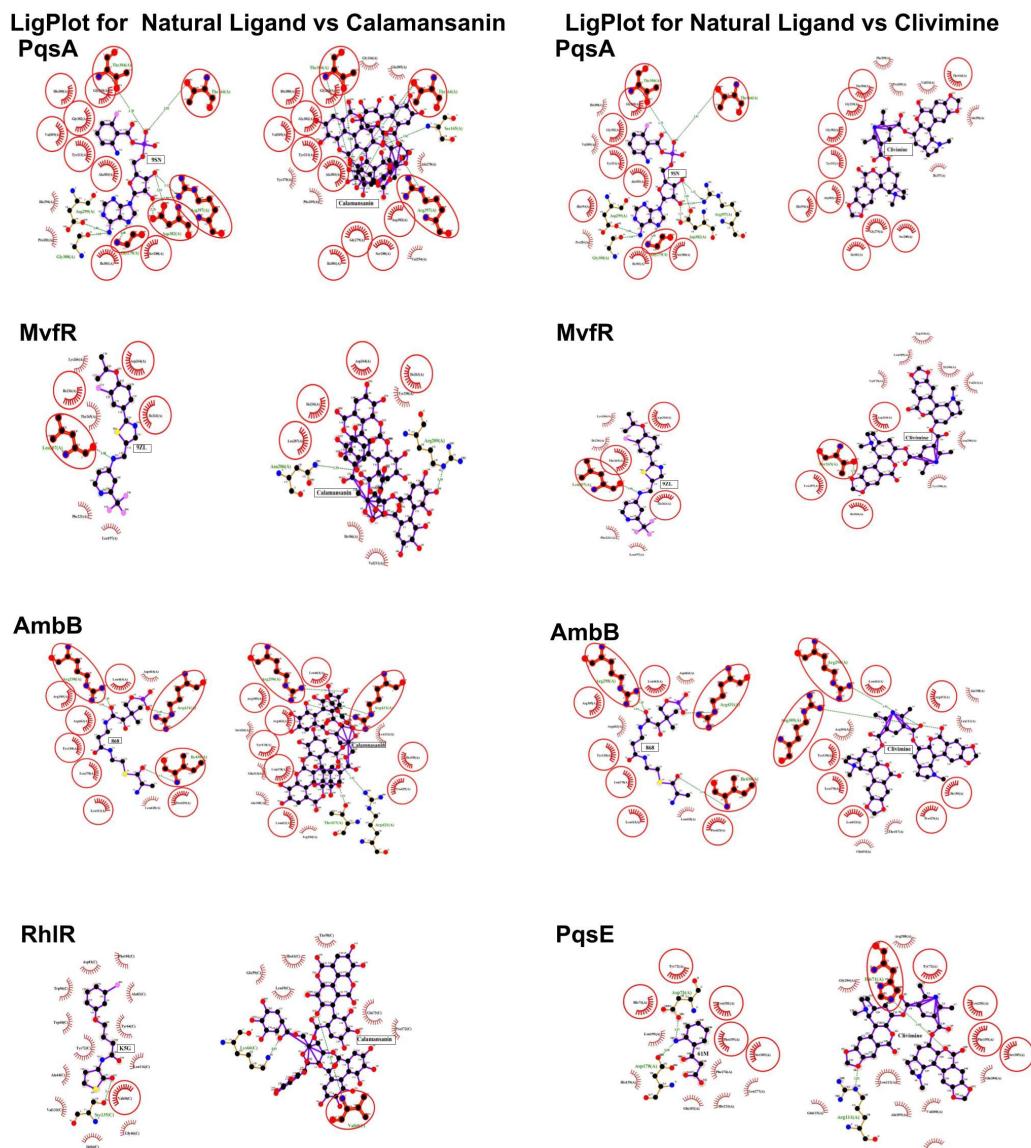


Figure 4. The comparative LigPlot+ analyses were carried out to visualize and compare the binding interactions of native ligands with the IMPPAT molecule-derived multitarget inhibitors, calamansanin and clivimine, against key QS and virulence-associated proteins of *P. aeruginosa*. For each target protein (PqsA, MvfR, AmbB, RhlR, and PqsE), the native ligand interaction map is presented alongside the corresponding inhibitor map to clearly illustrate similarities and differences in binding behavior. Green dashed lines represent hydrogen bonding, while hydrophobic interactions are represented by red spoked arcs around the interacting residues. Important interacting residues are labeled to emphasize both shared and distinct contacts that contribute to ligand stabilization within the binding site.

The drug-likeness profiles of calamansanin and clivimine differ considerably based on their physicochemical and pharmacokinetic properties. Clivimine showed comparatively better drug-like characteristics, violating only two drug-likeness criteria, namely molecular weight and topological polar surface area (TPSA), according to Lipinski's and Ghose's rules. In contrast, calamansanin violated multiple parameters, including molecular

weight, number of hydrogen bond donors/acceptors, and TPSA, which may limit its oral bioavailability. The predicted Gastro-intestinal (GI) absorption for calamansanin and clivimine is low. There is a lower risk of adverse effects on the central nervous system, as both substances are substrates of P-gp, which can alter their cellular retention. However, it is doubtful that they will cross the blood-brain barrier, as shown in Table 5.

Table 5. Drug-likeness and pharmacokinetic properties of selected multitarget inhibitors.

IMPPAT ID	Compound	Lipinski violation	Ghose violation	GI absorption	BBB permeant	P-gp substrate
IMPHY007725	Calamansanin	3	3	Low	No	Yes
IMPHY013460	Clivimine	2	3	Low	No	Yes

Based on ADMET safety analysis, the two compounds are relatively safe, as evidenced by their non-mutagenic properties in the Ames test and their lack of cardiotoxic potential due to hERG inhibition. In addition, clivimine demonstrates CYP3A4 inhibition; hence, there is a risk of potential drug interactions, whereas calamansanin, owing to its lower toxicity scores, including an LD₅₀ estimate, has a relatively higher safety margin. Based on the toxicity scores, calamansanin poses relatively low environmental toxicity, compared to the higher Minnow toxicity score associated with clivimine. Based on the findings of Tables 6 and 7, calamansanin is the best choice in future studies on QS inhibition approaches.

Table 6. ADME properties comparison of selected multitarget inhibitors.

IMPPAT ID	Compound	Intestinal absorption (%)	Water solubility (log mol/L)	VD _{ss} (log L/kg)	FUH (Fu)	CYP3A4 substrate	CYP3A4 inhibitor	R-OCT2 substrate
IMPHY007725	Calamansanin	54.713	-2.892	0.014	0.381	No	No	No
IMPHY013460	Clivimine	100	-3.049	-0.030	0.299	No	Yes	No

Table 7. Toxicity comparison of selected multitarget inhibitors.

IMPPAT ID	Compound	AMES tox	hERG I inhibitor	ORAT (LD50) (mol/kg)	Skin sens.	TPT (log ug/L)	Minnow toxicity (log mM)
IMPHY007725	Calamansanin	No	No	2.5	No	0.285	19.859
IMPHY013460	Clivimine	No	No	2.8	No	0.285	-0.918

MM-GBSA and MM-PBSA analyses confirmed energetically stable, multitarget interactions for both clivimine and calamansanin across the QS-associated proteins of *P. aeruginosa*. For clivimine, the strongest binding was seen with AmbB ($\Delta G = -67.32$ kcal mol⁻¹, 300 ns), PqsD ($\Delta G = -64.90$ kcal mol⁻¹, 500 ns), PqsA ($\Delta G = -59.32$ kcal mol⁻¹, 500 ns), and PqsH ($\Delta G = -51.99$ kcal mol⁻¹, 300 ns), driven mainly by favorable van der Waals and electrostatic contributions. For calamansanin, the most stable complexes were MvfR ($\Delta G = -60.49$ kcal mol⁻¹, 500 ns), AmbB ($\Delta G = -59.45$ kcal mol⁻¹, 500 ns), and RhlR ($\Delta G = -49.77$ kcal mol⁻¹, 300 ns). In both ligand series, polar solvation energy opposed complex formation while non-polar solvation energy favored it. As expected, MM-PBSA yielded systematically less negative ΔG values than MM-GBSA, owing to larger polar solvation contributions in the Poisson-Boltzmann treatment, but the two methods agreed on the relative ranking of complexes across both the 500 ns and 300 ns runs. Full energy decompositions are given in Tables 8–11.

Table 8. Binding free energy terms for clivimine–receptor complexes calculated using MM-GBSA and MM-PBSA methods (500ns).

Complex	MM-GBSA					MM-PBSA		
	ΔE_{vdw} (kcal/mol)	ΔE_{elec} (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{SUR} (kcal/mol)	ΔG (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{NP} (kcal/mol)	ΔG (kcal/mol)
PqsA	-67.88	-76.75	94.11	-8.80	-59.32	102.14	-6.62	-49.12
PqsD	-86.40	-21.66	52.96	-9.80	-64.90	69.37	-7.97	-46.66

Table 9. Binding free energy terms for clivimine–receptor complexes calculated using MM-GBSA and MM-PBSA methods (300ns).

Complex	MM-GBSA					MM-PBSA		
	ΔE_{vdw} (kcal/mol)	ΔE_{elec} (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{SUR} (kcal/mol)	ΔG (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{NP} (kcal/mol)	ΔG (kcal/mol)
AmbB	-84.61	-15.03	41.75	-9.42	-67.32	65.93	-6.91	-40.63
AmbE	-62.16	-15.88	40.96	-7.45	-44.53	50.64	-6.40	-33.81
LasI	-63.04	-3.65	22.58	-6.95	-51.07	29.50	-5.17	-42.37
MvfR	-52.88	-32.95	48.05	-6.57	-44.35	54.43	-5.09	-36.50
PqsE	-41.14	-4.79	19.19	-4.82	-31.55	22.96	-4.07	-27.03
PqsH	-66.80	-6.31	28.48	-7.36	-51.99	36.33	-5.39	-42.17

Table 10. Binding free energy terms for calamansanin–receptor complexes calculated using MM-GBSA and MM-PBSA methods (500ns).

Complex	MM-GBSA					MM-PBSA		
	ΔE_{vdw} (kcal/mol)	ΔE_{elec} (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{SUR} (kcal/mol)	ΔG (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{NP} (kcal/mol)	ΔG (kcal/mol)
AmbB	-94.55	-18.62	65.54	-11.81	-59.45	68.00	-8.99	-54.16
MvfR	-58.28	-122.59	129.18	-8.87	-60.49	135.69	-7.03	-52.15

Table 11. Binding free energy terms for calamansanin–receptor complexes calculated using MM-GBSA and MM-PBSA methods (300ns).

Complex	MM-GBSA					MM-PBSA		
	ΔE_{vdw} (kcal/mol)	ΔE_{elec} (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{SUR} (kcal/mol)	ΔG (kcal/mol)	ΔE_{PS} (kcal/mol)	ΔE_{NP} (kcal/mol)	ΔG (kcal/mol)
AmbE	-74.72	-17.75	62.53	-10.32	-40.27	58.01	-7.34	-41.80
LasI	-48.10	-8.91	34.19	-6.67	-29.49	33.15	-4.99	-28.84
PqsA	-49.35	-63.61	78.86	-7.29	-41.39	81.29	-6.08	-37.76
PqsD	-76.59	-24.10	62.87	-10.13	-47.95	58.76	-6.97	-48.90
PqsH	-51.09	-9.93	29.07	-6.35	-38.29	35.13	-4.93	-30.82
RhlR	-75.67	-17.34	52.87	-9.63	-49.77	52.45	-7.31	-47.88

Both compounds formed stable complexes with all eight QS targets, with RMSD values remaining below approximately 1.0 nm throughout the simulations, indicating that ligand binding did not induce major conformational rearrangement. For calamansanin, the two complexes carried to 500 ns, AmbB and MvfR, showed RMSD values in the 0.25–1.0 nm range, with MvfR fluctuating somewhat more than AmbB; the remaining six calamansanin complexes (AmbE, LasI, PqsA, PqsD, PqsH, and RhlR), simulated for 300 ns, settled into a tighter 0.2–0.7 nm range, with RhlR showing the largest deviations within this group. For clivimine, the two 500 ns complexes, PqsA and PqsD, likewise stayed within 0.25–1.0 nm, with PqsA the more stable of the two; the remaining six clivimine complexes (AmbB, AmbE, LasI, MvfR, PqsE, and PqsH), run for 300 ns, ranged between 0.2–0.7 nm, with PqsE showing the lowest RMSD of any complex in the study, as shown in Figures 5–8. Taken together, the clivimine-bound complexes trended toward marginally lower RMSD values than the calamansanin-bound complexes, pointing to slightly greater rigidity upon clivimine binding.

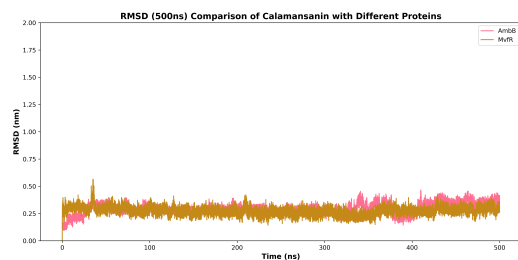


Figure 5. RMSD profiles of calamansanin bound to two QS receptors during 500ns. AmbB (pink) and MvfR (orange) remained stable, with RMSD values ranging from 0.25–1.0 nm; MvfR showed marginally greater fluctuation than AmbB throughout the run.

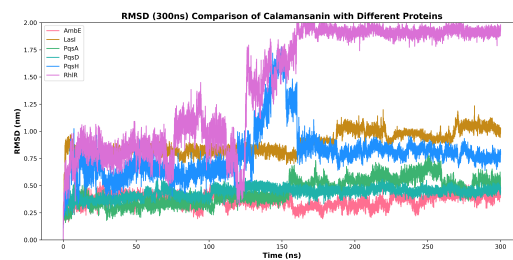


Figure 6. RMSD profiles of calamansanin bound to six QS receptors during 300ns. All complexes remained within 0.2–0.7 nm; RhlR (pink) showed the largest fluctuations in the group, while LasI (orange) and PqsA (green) were the most stable.

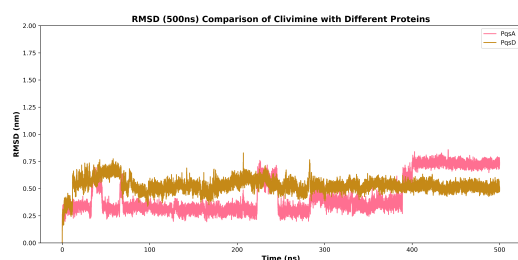


Figure 7. RMSD profiles of clivimine bound to two QS receptors during 500ns. PqsA (pink) and PqsD (orange) stayed within 0.25–1.0 nm, with PqsA showing the more stable trajectory of the two.

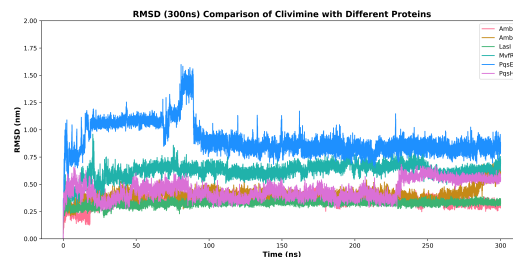


Figure 8. RMSD profiles of clivimine bound to six QS receptors during 300ns. All complexes ranged between 0.2–0.7 nm, with PqsE (blue) showing the lowest RMSD of any complex in the study, indicating the most rigid binding conformation overall.

Residue-level dynamics were assessed from the RMSF profiles of each complex (Figures 9–12). Calamansanin-bound receptors showed generally low RMSF values across both the 500 ns (AmbB, MvfR) and 300 ns (AmbE, LasI, PqsA, PqsD, PqsH, RhlR) simulations, consistent with stable, well-anchored binding. Fluctuations were mostly confined to loop and terminal segments; AmbE and MvfR showed a modest increase in flexibility, likely reflecting local conformational adjustment around the ligand, while PqsH and RhlR fluctuated more at their termini even though their core folds remained intact. Clivimine-bound receptors, by contrast, showed higher RMSF values overall across both the 500 ns (PqsA, PqsD) and 300 ns (AmbB, AmbE, LasI, MvfR, PqsE, PqsH) simulations, pointing to greater backbone mobility, particularly in loop and terminal regions associated with ligand recognition and downstream signaling. PqsD and PqsH stood out with the most pronounced fluctuations upon clivimine binding, although in both cases the protein core remained structurally stable.

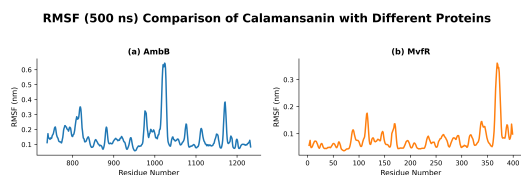


Figure 9. Comparison of RMSF profiles of calamansanin–protein complexes (500ns): Fluctuations were low overall and confined mainly to loop and terminal residues; MvfR (orange) showed slightly greater local flexibility than AmbB (blue), consistent with conformational adjustment around the bound ligand.

RMSF (300 ns) Comparison of Calamansanin with Different Proteins

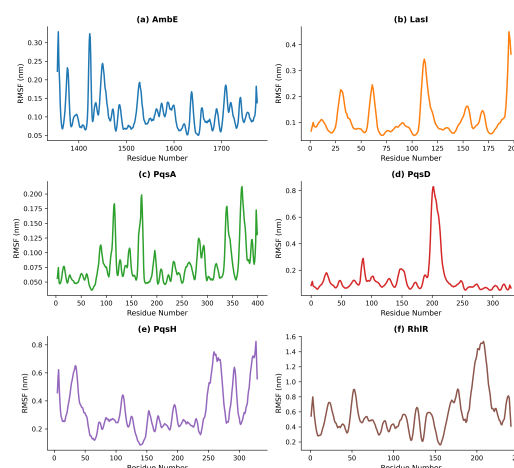


Figure 10. Comparison of RMSF profiles of calamansanin–protein complexes (300ns): Overall fluctuations were low; AmbE (blue) showed a modest increase in flexibility, while PqsH (purple) and RhlR (brown) showed higher fluctuations restricted to their terminal regions, with core folds otherwise preserved.

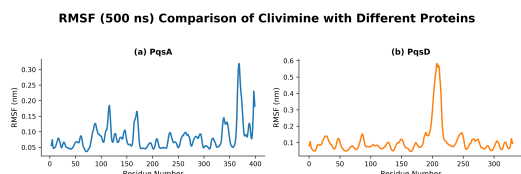


Figure 11. Comparison of RMSF profiles of clivimine–protein complexes (500ns): Both complexes showed higher RMSF than their calamansanin counterparts, particularly in loop regions linked to ligand recognition, with PqsD (orange) showing the more pronounced fluctuation of the two.

RMSF (300 ns) Comparison of Clivimine with Different Proteins

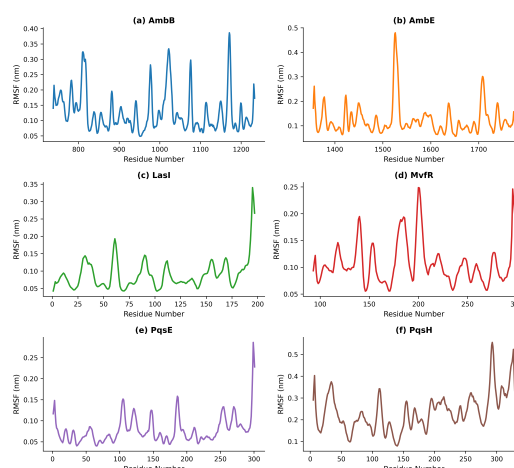


Figure 12. Comparison of RMSF profiles of clivimine–protein complexes (300ns): Fluctuations were elevated relative to the calamansanin-bound complexes; PqsH (brown) showed the most pronounced fluctuation, concentrated in loop and terminal regions, while its protein core remained structurally intact.

Rg was used to assess overall complex compactness (Figures 13–16). For calamansanin, Rg remained essentially flat across both the 500 ns (AmbB, MvfR) and 300 ns (AmbE, LasI, PqsA, PqsD) simulations, indicating no meaningful unfolding, whereas PqsH and RhlR showed larger Rg excursions, mirroring the higher flexibility already seen in their RMSF profiles. Clivimine-bound complexes followed the same pattern, with stable Rg values for most targets across both simulation lengths except PqsH, which again showed the most pronounced variation and is thus consistently identified — across RMSD,

RMSF, and Rg — as the most conformationally flexible target in the panel. Overall, the Rg data corroborate the RMSF results and suggest that calamansanin produces a marginally more compact complex architecture than clivimine.

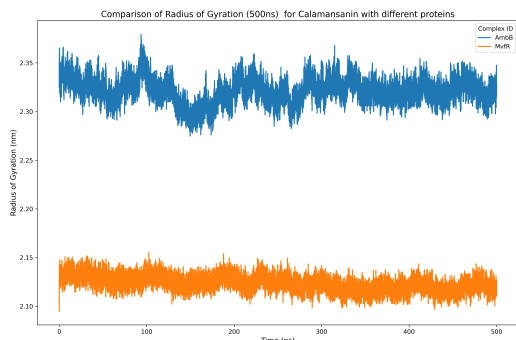


Figure 13. Calamansanin bound to QS receptors (500ns): Rg remained stable for both targets throughout the simulation, indicating no unfolding upon calamansanin binding.

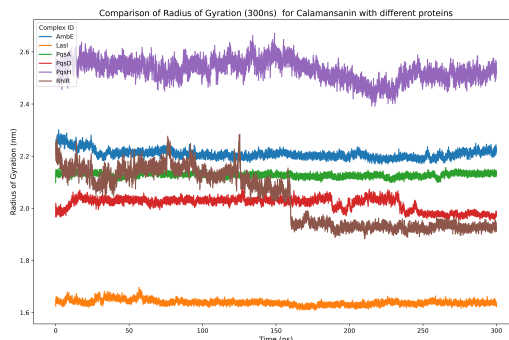


Figure 14. Calamansanin bound to QS receptors (300ns): Rg was stable for AmbE (blue), LasI (orange), PqsA (green), and PqsD (red), whereas PqsH (purple) and RhlR (brown) showed larger fluctuations, consistent with the higher flexibility seen in their RMSF profiles.

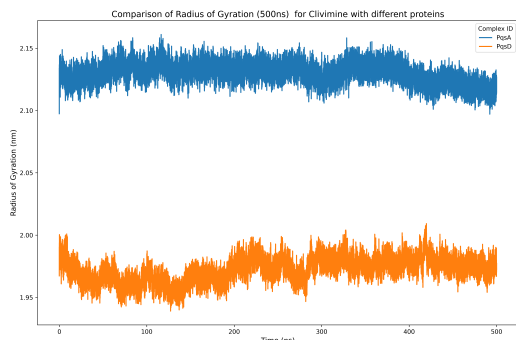


Figure 15. Clivimine bound to QS receptors (500ns): Rg remained stable for both complexes across the trajectory, indicating that clivimine binding did not compromise the compactness of either target.

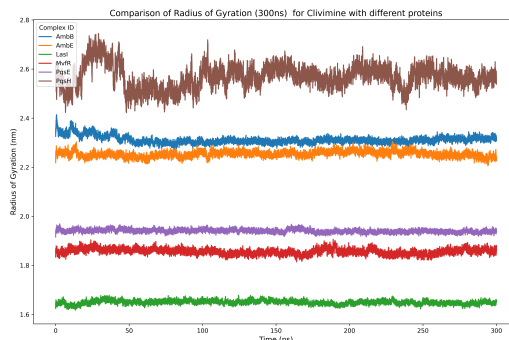


Figure 16. Clivimine bound to QS receptors (300ns): Rg was stable for AmbB (brown), AmbE (orange), LasI (green), MvfR (red), and PqsE (purple), whereas PqsH (brown) again showed pronounced variation.

4. Conclusion

An integrated computational workflow has been employed in the present work to identify natural QS inhibitors against the multidrug-resistant pathogen *P. aeruginosa*. In docking-based virtual screening, a total of 11 QS receptor targets belonging to four different QS systems, namely LAS, RHL, PQS, and IQS, were selected. Screening of natural products from the IMPPAT database identified ligands with strong binding affinity toward multiple receptors. Among these natural compounds, calamansanin (a phenolic compound) and clivimine (an alkaloidal compound) showed the highest binding affinities toward the receptors investigated in the present study. The predicted binding energy values ranged from -7.8 to -12.9 kcal/mol.

The selected compounds interacted with QS-associated proteins and receptors in a manner comparable to their corresponding native ligands. To probe this further, MD

simulations were extended to 500 ns for two priority targets per ligand (AmbB and MvfR for calamansanin; PqsA and PqsD for clivimine) and run for 300 ns for the remaining six targets in each series. MM-GBSA and MM-PBSA analyses across both timescales demonstrated energetically favorable and thermodynamically stable ligand-receptor interactions, with binding free energies ranging from approximately -30 to -68 kcal/mol across the studied complexes. Clivimine showed its strongest binding with AmbB, PqsD, PqsA, and PqsH, primarily driven by favorable van der Waals and electrostatic interactions, while calamansanin showed its most stable interactions with MvfR, AmbB, and RhlR through a balance of electrostatic and non-polar solvation contributions. MM-PBSA consistently yielded less negative binding free energies than MM-GBSA, reflecting larger polar solvation contributions, although both methods agreed on the relative ranking of complexes at both the 500 ns and 300 ns timescales. These trends were corroborated by RMSD values that remained below the ~ 1.0 nm stability threshold across all complexes and both simulation lengths, indicating that ligand binding did not induce major conformational changes in any of the receptors. RMSF analysis indicated that calamansanin conferred greater rigidity on the core regions of its bound receptors, whereas clivimine increased flexibility in loop and terminal regions linked to ligand recognition. PqsH emerged as the most conformationally flexible target for both ligands, with RhlR also exhibiting comparatively higher flexibility in the calamansanin-bound systems. Radius of gyration (Rg) profiles confirmed the absence of global unfolding throughout all simulations, supporting the RMSD and RMSF observations. Collectively, these findings, consistent across both the extended (500 ns) and standard (300 ns) simulations, suggest that both ligands may effectively interfere with virulence- and biofilm-associated QS pathways that are essential for the survival and pathogenicity of *P. aeruginosa*.

A major strength of this work is the integration of homology modeling, molecular docking, dynamic simulations, and free energy calculations into a unified framework. Together, these approaches provide a reliable strategy for designing multitarget inhibitors. The use of the IMPPAT database further adds value by linking traditional medicinal knowledge with modern drug discovery and enabling exploration of a diverse set of compounds from Indian medicinal plants.

However, certain limitations persist. Some receptor structures were derived through homology modeling, which may not fully represent their true conformations. Docking methods can also lack precision, and relatively short simulation times may limit the capture of complete molecular behavior. Although these methods improve prediction confidence, experimental validation remains necessary to confirm the effectiveness of the identified natural inhibitors. Future work will focus on experimental validation which will strengthen the rational design of plant-derived antivirulence agents and support efforts to combat antibiotic resistance in *P. aeruginosa* and other opportunistic pathogens.

5. Author Contribution

Virendra Kumar: data curation, formal analysis, investigation, methodology, visualization, validation, writing – original draft, writing – review and editing. Sriram K: investigation, project administration, supervision, writing – review and editing. D. K. Sharma: supervision, writing - review. N Arul Murugan: conceptualization, investigation, project administration, supervision, writing – review and editing.

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