3

EXPERIMENTAL PROCEDURE

The development of new therapeutics for treating *Pseudomonas aeruginosa* infections has been considerably hampered by its numerous drug-resistance mechanisms (Thi *et al.,* 2020). The National Institute of Health (NIH) declared that nearly eighty percent of infections caused by microbes in humans are due to biofilms. The infections of biofilms include infections of the kidneys, endocarditis, osteomyelitis, non-healing chronic wounds, cystic fibrosis, rhinosinusitis, prosthetic device-related infections, and periodontitis (Khatoon *et al.,* 2018; Gupta *et al.,* 2016). Biofilm formation and dispersion are regulated by multifactorial processes involving exopolysaccharides (EPS), quorum sensing (QS) systems, and c-di-GMP (Koo and Yamada, 2016).

An intercellular signaling and communication system called QS coordinates group behaviours in *Pseudomonas aeruginosa* biofilm communities and regulates expression of genes with respect to population density (Floyd *et al.,* 2017). QS controls more than 10% of the genes in *Pseudomonas aeruginosa,* which are in charge of motility, biofilm formation, generation of virulence factors, development of antibiotic resistance, and modifications of metabolic pathways. Researchers have suggested that interference with the QS system and manipulating QS pathways could pave the way for generating a powerful therapeutic strategy against *Pseudomonas aeruginosa* infections (Puiu *et al.,* 2017). Various methods, including the promotion of biofilm dispersion, focusing on biofilm components, and inhibition of QS and iron metabolism, have been so far used by researchers to combat infections caused by *Pseudomonas aeruginosa* biofilms. However, the prevalence of its infections is still increasing due to the increase in multidrug-resistant strains, the lack of valid biomarkers, and the bacteria's high drug tolerance (Paharik and Horswill, 2016).

Considering the above aspects, the present study entitled **"Structure Based Virtual Screening and Validation of Potential Quorum Sensing Inhibitors Against LasR in *Pseudomonas aeruginosa*"** focused on the following.

- Virtual screening of inhibitors against LasR of *Pseudomonas aeruginosa* and molecular dynamics simulation study
- Assessment of antibiofilm and antiquorum sensing activity of LasR inhibitors against *Pseudomonas aeruginosa*
- Evaluation of compounds on quorum sensing mediated virulence factors production in *Pseudomonas aeruginosa*
- Investigation of the influence of selected compounds on the expression profile of quorum sensing regulatory genes

The experimental design and procedures adopted in this study are given in the flowchart (Figure 10), and the study was divided into four phases, as discussed below.

3.1. Phase I: Virtual screening of inhibitors against LasR of *Pseudomonas aeruginosa* and molecular dynamics simulation study

3.1.1. High Throughput Virtual Screening

3.1.1.1. Protein preparation

3.1.1.2. Receptor grid generation

3.1.1.3. Structure based virtual screening

3.1.2. Calculation of binding free energy of the docked complexes

3.1.2.1. Prime MM-GBSA

3.1.3. ADMET and drug-likeness prediction

3.1.4. Molecular dynamics simulation

3.1.5. Cytotoxicity study

3.1.5.1. MTT assay

3.2. Phase II: Assessment of antibiofilm and antiquorum sensing activity of LasR inhibitors against *Pseudomonas aeruginosa*

3.2.1. Media and chemicals

3.2.2. Bacterial strains and maintenance of cultures

3.2.3. Determination of Minimum Inhibitory Concentration

3.2.3.1. Micro broth dilution

3.2.4. Assessment of antibiofilm activity

3.2.4.1. Biofilm inhibition assay

3.2.4.2. Eradication of preformed matured biofilm

3.2.4.3. Exopolysaccharide inhibition assay

3.2.4.4. Alginate inhibition assay

3.2.4.5. Biofilm total protein assay

3.2.5. Evaluation of metabolic activity of viable cells in biofilm

3.2.5.1. MTT reduction assay

3.2.6. Assessment of antiquorum sensing activity

3.2.6.1. Violacein inhibition assay in *Chromobacterium violaceum*

3.2.6.2. Swimming motility assay

3.2.6.3. Swarming motility assay

3.3. Phase III: Evaluation of compounds on quorum sensing mediated virulence factors production in *Pseudomonas aeruginosa*

3.3.1. Phenotypic detection of quorum sensing regulated virulence factors

3.3.1.1. Pyocyanin assay

3.3.1.2. Rhamnolipid assay

3.3.1.3. Total protease assay

3.3.1.4. *lasB* elastase analysis

3.3.1.5. Alkaline protease assay

3.3.1.6. Lipase assay

3.3.2. Morphology analysis

3.3.2.1. Field emission scanning electron microscopy

3.4. Phase IV: Investigation of the influence of selected compounds on the expression profile of quorum sensing regulatory genes

3.4.1. Quantification of relative gene expression of quorum sensing regulatory genes

3.4.1.1. Quantitative real-time reverse transcription PCR

3.4.2. Evaluation of antagonistic efficacy of LasR inhibitors

3.4.2.1. β-galactosidase reporter gene assay

3.5. Statistical analysis

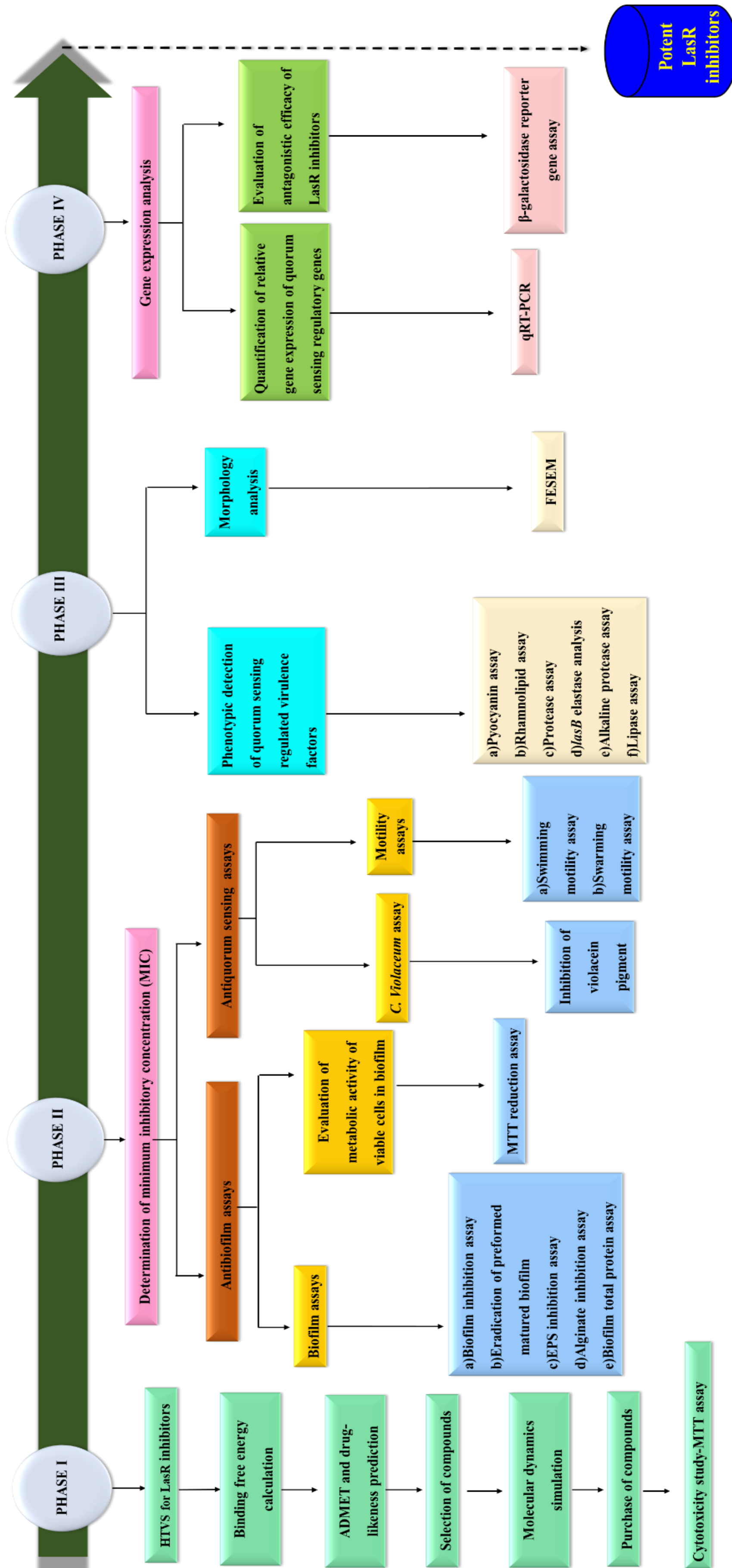

Figure 10

Overall workflow depicting the process in screening of potential LasR inhibitors against *Pseudomonas aeruginosa*

3.1. Phase I: Virtual screening of inhibitors against LasR of *Pseudomonas aeruginosa* and molecular dynamics simulation study

Phase I adopted a molecular docking-based virtual screening to find a novel class of inhibitors for LasR that might act as potent antagonists against *Pseudomonas aeruginosa.* All computational analysis was done using Maestro v11.8 (Schrödinger, LLC, New York, NY) on a CentOS release 6.10 Linux x86_64 platform in IBM. In addition, the cytotoxicity of the compounds on human peripheral blood lymphocyte (PBL) cells was also analysed for their effective utilization in clinical fields and drug development processes.

3.1.1. High Throughput Virtual Screening

3.1.1.1. Protein preparation

The three-dimensional (3D) crystallized structure of the LasR was obtained from Protein Data Bank (PDB) (https://www.rcsb.org/). The number of 3D X-ray crystal structures of LasR deposited in PDB as of February 2021 is 23. We selected LasR protein with **PDB ID: 3IX4** and resolution 1.8 Å for this study (Zou and Nair, 2009). The Protein Preparation Wizard panel of the Schrödinger software was used to process the protein structure. Hydrogen atoms that were lacking were added, accurate orders for bonds were given, and appropriate ionization states were produced during the preparation process via the OPLS-2005 force field. The heavy atoms were employed to restricted minimization until they reached a root mean square deviation (RMSD) of 0.30 Å to perform an ideal docking investigation.

3.1.1.2. Receptor grid generation

A grid box was generated over the bound ligand molecule in LasR using the Glide v8.1 module's Receptor Grid Generation panel in Schrödinger that enables docking into the ligand binding site. The centroid of the bound ligand served as the center of a cubic box with x, y, and z coordinates of 3.336, 15.597, and 4.868, respectively. The cutoff for partial charge was held constant at 0.25 with no restrictions, and the scaling factor of *van der Waals* radius was set to 1.0 Å.

3.1.1.3. Structure based virtual screening

When designing a drug or an inhibitor, structure-based virtual screening (SBVS) helps to identify efficient lead compounds. It searches through millions of compounds in a vast ligand database to find compounds that interact and adequately pack into the active site

of the target (Dhasmana *et al.,* 2019). Three steps of high throughput virtual screening (HTVS) were employed on the Schrödinger small molecule library, including SBVS, standard precision (SP) docking, and extra precision (XP) docking.

Virtual screening workflows and all docking computations are included in the Schrödinger Glide v8.1 module. It uses a ligand docking method employing a grid to produce several ligand postures. A range of hierarchical filters is then used to examine the ligand-receptor interactions. The initial filters assessed the ligand's spatial fit into the active site. It also looked at the complementarity in the interaction between ligand and receptor using the empirical ChemScore algorithm. Only those postures that passed the screening process are taken to the last stage of the algorithm.

Initially, a broad range of compounds from the Schrödinger small molecule library that interacted with LasR was subjected to docking in HTVS mode. The SP docking mode analysed the top 10% of the HTVS mode compounds to identify potential binders. Followed by Glide XP docking was used to examine the top scoring 10% of compounds from SP docking mode more intensively. False positives introduced by SP were removed by XP docking, and ligands that did not fit the active site of the target structure were penalized. The top hits with the finest docked postures and the greatest docking scores were chosen for further investigation.

3.1.2. Calculation of binding free energy of the docked complexes

3.1.2.1. Prime MM-GBSA

The post-docking analysis was carried out to confirm the binding affinities of the docked complexes using the Prime Molecular Mechanics/Generalized Born and Surface Area (MM-GBSA) technique in Maestro v11.8 of the Schrödinger module. To ascertain the binding affinities, the OPLS-2005 force field, the polar implicit solvation model VSGB 2.0, solvent accessible surface area (SASA) and *van der Waals* interactions categorized as nonpolar solvation energies were used. The following equation assessed the binding affinity of the docked complex.

$$\Delta G_{bind} = \Delta G_{complex} - (\Delta G_{receptor} + \Delta G_{ligand})$$

3.1.3. ADMET and drug-likeness prediction

The ADMET and drug-likeness characteristics of the top hits were evaluated using Schrödinger's QikProp v5.8 module. The most crucial pharmacological characteristics were investigated to determine the druggable effects and acceptance of the top hits.

3.1.4. Molecular dynamics simulation

Molecular dynamics (MD) simulation was done using the Desmond v5.6 package to examine the stability of the top-scoring hits in the active pocket of LasR. The single point charge (SPC) water model from the OPLS-AA force field was used in an explicit solvent. It provided information on the atom movements within the complexes in a specified environment. The default relaxation procedure in Desmond was used to pre-equilibrate and minimize complex systems after adding the SPC water molecules. The box wall and complex were separated by 10 Å. To simulate the systems and to achieve a stabilized system, a time step set to 100 ns was utilized at a pressure of 1 atm with intervals of 4.8 ps and a temperature of 300 K. Before simulation, the model system was relaxed. The root mean square deviation (RMSD) and root mean square fluctuation (RMSF) for the protein side chain and backbone were used to describe the stability of the complexes. The protein's compactness was ascertained by computing the radius of gyration (Rg) during the process of simulation. In addition, the exact binding of the ligand molecules into the target's binding site is greatly facilitated by hydrogen bonding. Hence, the count of hydrogen bond contacts in the complexes was also examined.

3.1.5. Cytotoxicity study

3.1.5.1. MTT assay

Cytotoxicity of the compounds was studied on human PBL cells. The cells were cultured in RPMI 1640 medium with fetal bovine serum (FBS) (10%), penicillin-streptomycin (0.5%) and phytohemagglutinin. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reported by Igarashi and Miyazawa, 2001 whose procedure is provided in Appendix I.

3.2. Phase II: Assessment of antibiofilm and antiquorum sensing activity of LasR inhibitors against *Pseudomonas aeruginosa*

Phase II involved the *in vitro* validation of the top hits for their antibiofilm and antiquorum sensing efficacy against *Pseudomonas aeruginosa.* The hit compounds were purchased from Mcule (https://mcule.com/), Hungary and Life Chemicals (https://lifechemicals.com/), Canada. The methodology adopted for this phase of the study is discussed below.

3.2.1. Media and chemicals

The media used in this study was HiMedia grade Nutrient agar (Peptone - 10 g/L; Beef extract - 10 g/L; Sodium chloride - 5 g/L; Agar - 12 g/L) and Nutrient broth (Peptone - 10 g/L; Beef extract - 10 g/L; Sodium chloride - 5 g/L; Agar - 12 g/L) for isolation and maintenance of bacterial strains. HiMedia Luria-Bertani (LB) broth (Tryptone - 10 g/L; Yeast extract - 5 g/L; Sodium chloride - 10 g/L) was used to produce inoculum. All the chemicals used were of an analytical grade standard.

3.2.2. Bacterial strains and maintenance of cultures

The wild-type model bacterial strain *Pseudomonas aeruginosa PAO1* (MTCC 2453) was provided by the Microbial Type Culture Collection and Gene Bank (MTCC) located in Chandigarh, India. The culture was maintained on nutrient agar at 4°C with periodic subculturing. Stock cultures were preserved in 50% glycerol at -20°C. Before each experiment, a single colony of bacteria was cultured in LB broth for 16-18 hrs at 37°C. The overnight grown culture was diluted using LB broth at a ratio of 1:100 to an appropriate optical density (OD) of 0.5 at 600 nm that corresponds to 1×10^5 colony forming unit (CFU/ml). For the antiquorum sensing study, a biomarker strain *Chromobacterium violaceum* (MTCC 2656) was procured and maintained, as mentioned above.

3.2.3. Determination of Minimum Inhibitory Concentration

3.2.3.1. Micro broth dilution

The Minimum Inhibitory Concentration (MIC) of the top hits against *Pseudomonas aeruginosa PAO1* was identified by the method of micro broth dilution as recommended in guidelines of the Clinical and Laboratory Standards Institute (CLSI). Initially, two-fold serial dilution of the compounds whose concentrations ranged between 1.95-4000 μM was

performed in LB broth. In each well of a 96-well microtiter plate containing serially diluted compounds, 100 µl of the overnight culture of *Pseudomonas aeruginosa PAO1* (diluted 1:100) was added. The plate was kept at 37°C for 18 hrs. The MIC was recorded as the minimum dosage of the compounds that completely lacked growth of bacterial cells in LB broth. The ½ & ¼ subinhibitory concentration (sub-MIC) of the selected compounds was used for subsequent analysis. These sub-MICs are considered for inhibition studies as they do not affect bacterial growth but can alter the functions of bacteria that lead to the reduction of bacterial virulence.

3.2.4. Assessment of antibiofilm activity

3.2.4.1. Biofilm inhibition assay

The assay for biofilm inhibition was done employing the method of crystal violet staining stated by Lee *et al.* (2011). The overnight culture (diluted 1:100) of *Pseudomonas aeruginosa PAO1* was incubated in the 96-well microtiter plate with and without compounds at 37°C for 24 hrs. Before the biofilm assay, the growth of the bacteria in presence of compounds was assessed by measuring their absorbance at 600 nm. The detailed procedure for biofilm inhibition assay is provided in Appendix II.

3.2.4.2. Eradication of preformed matured biofilm

The eradication potential of the compounds on the preformed developed biofilm was performed with the overnight culture (diluted 1:100) of *Pseudomonas aeruginosa PAO1* in LB broth contained in the 96-well microtiter plate. The plate was kept at 37°C for 24 hrs. Free-floating cells were removed, and all the wells were rinsed with LB broth. To each well of the plate, fresh LB broth with and without compounds was added. After 24 hrs of incubation, biofilm was measured according to the protocol detailed in Appendix II.

3.2.4.3. Exopolysaccharide inhibition assay

The EPS production by *Pseudomonas aeruginosa PAO1* in untreated and treated conditions was evaluated using the method of phenol-sulphuric acid reported by Rasamiravaka *et al.* (2015b). The diluted (1:100) overnight culture of *Pseudomonas aeruginosa PAO1* was incubated with and without compounds at 37°C for 24 hrs. Inhibition of EPS production by the compounds was evaluated after 24 hrs following the procedure in Appendix III.

3.2.4.4. Alginate inhibition assay

The alginate production by *Pseudomonas aeruginosa PAO1* in untreated and treated conditions was assayed using the procedure detailed in Appendix IV.

3.2.4.5. Biofilm total protein assay

The amount of extractable protein in untreated and treated biofilms of *Pseudomonas aeruginosa PAO1* was estimated following the procedure mentioned by Das *et al.* (2016). To estimate the concentration of total extractable protein, an overnight (diluted 1:100) culture of *Pseudomonas aeruginosa PAO1* was incubated for 48 hrs with and without compounds. The free-floating cells were discarded, and the attached biofilm cells were gently rinsed with PBS. Followed by the cells were boiled (30 minutes) with 5 ml of 0.5 N sodium hydroxide (NaOH). The contents were centrifuged (10,000 × g) for 5 minutes, and the clear supernatants were obtained. From the supernatant, the concentration of protein was determined by Lowry's method, whose procedure is detailed in Appendix V.

3.2.5. Evaluation of metabolic activity of viable cells in biofilm

3.2.5.1. MTT reduction assay

MTT reduction assay was employed to assess the metabolic activity of viable biofilm cells. Initially, *Pseudomonas aeruginosa PAO1* biofilms were formed and treated with compounds as described in Section 3.2.4.3. The amount of metabolically active biofilm cells in untreated and treated *Pseudomonas aeruginosa PAO1* cultures was determined according to the procedure given in Appendix VI.

3.2.6. Assessment of antiquorum sensing activity

3.2.6.1. Violacein inhibition assay in *Chromobacterium violaceum*

The antiquorum sensing ability of the compounds was observed by agar disc diffusion assay using a protocol detailed in Appendix VII.

3.2.6.2. Swimming motility assay

The swimming motility of *Pseudomonas aeruginosa PAO1* was observed in accordance with the protocol described by Packiavathy *et al.* (2014). Swimming agar medium (tryptone (1.0%), NaCl (0.5%), and agar (0.3%)) was point inoculated with

overnight grown culture (diluted 1:100) of *Pseudomonas aeruginosa PAO1* treated with and without LasR inhibitors. The inoculated plates were kept for 24 hrs at 37°C under static condition. The swimming zone diameter was measured using a transparent ruler in millimeters (mm).

3.2.6.3. Swarming motility assay

The swarming motility behavior of *Pseudomonas aeruginosa PAO1* was observed using the procedure reported by Packiavathy *et al.* (2014). For investigation of swarming motility, the overnight culture (diluted 1:100) of *Pseudomonas aeruginosa PAO1* treated with and without compounds was inoculated at the center of the swarming medium (peptone (1.0%), NaCl (0.5%), agar (0.5%), and filter sterilized glucose (0.5%)). The inoculated plates were kept for 24 hrs at 37°C, and the swarming zone diameter (mm) was measured using a transparent ruler.

3.3. Phase III: Evaluation of compounds on quorum sensing mediated virulence factors production in *Pseudomonas aeruginosa*

In Phase III, the compounds were evaluated for their effect on quorum sensing controlled production of virulence factors by *Pseudomonas aeruginosa PAO1*. The morphology and architectural changes in *Pseudomonas aeruginosa PAO1* biofilms on treatment with LasR inhibitors were examined by field emission scanning electron microscopy (FESEM).

3.3.1. Phenotypic detection of quorum sensing regulated virulence factors

Phenotypic detection of essential virulence factors, namely pyocyanin, rhamnolipid, total protease, *lasB* elastase, alkaline protease and lipase in *Pseudomonas aeruginosa PAO1* was carried out in untreated and treated conditions. Before each assay, overnight culture (diluted 1:100) of *Pseudomonas aeruginosa PAO1* (OD_{600}=0.5) was grown in LB broth with and without compounds and kept at 37°C for 24 hrs. The bacterial cells were subjected to centrifugation (10,000 × g) at 4°C for 8 minutes. The obtained supernatant fraction was filtered using a syringe filter (0.22 μm). Then the various biochemical assays were subsequently carried out using the filter-sterilized filtrate to study the effectiveness of the compounds on virulence factors production.

3.3.1.1. Pyocyanin assay

The untreated and treated cell-free supernatants of *Pseudomonas aeruginosa PAO1* were added to same volume of chloroform and mixed for extraction of pyocyanin. The procedure followed for pyocyanin extraction is detailed in Appendix VIII.

3.3.1.2. Rhamnolipid assay

Production of rhamnolipid by *Pseudomonas aeruginosa PAO1* was assayed from the filter-sterilized cell-free supernatants obtained after incubation for 24 hrs at 37°C. The assay was done according to Kim *et al.* (2015), whose procedure is given in Appendix IX.

3.3.1.3. Total protease assay

The impact of compounds on the total protease production in *Pseudomonas aeruginosa PAO1* was assayed using skim milk agar plates, whose procedure is detailed in Appendix X.

3.3.1.4. *lasB* elastase analysis

The activity of *lasB* elastase in *Pseudomonas aeruginosa PAO1* treated with compounds was analysed by qRT-PCR. The detailed procedure adopted and the respective primer used are given in Appendix XI & XII, respectively.

3.3.1.5. Alkaline protease assay

The activity of alkaline protease was measured using the culture filtrates of *Pseudomonas aeruginosa PAO1* with and without compounds. The protocol used for the measurement of alkaline protease is provided in Appendix XIII.

3.3.1.6. Lipase assay

Production of lipase by *Pseudomonas aeruginosa PAO1* treated with and without compounds was assayed based on the method of Alhajlan *et al.* (2013), whose procedure is detailed in Appendix XIV.

3.3.2. Morphology analysis

3.3.2.1. Field emission scanning electron microscopy

The architecture and structure of *Pseudomonas aeruginosa PAO1* biofilm in the untreated and treated states were observed by field emission scanning electron microscopy

(FESEM) using a modified protocol of Singh *et al.* (2017). *Pseudomonas aeruginosa PAO1* was cultured on glass slides immersed in LB broths with and without compounds. They were allowed to form biofilms by keeping the slides for 48 hrs at 37°C. Thereafter, glass slides with biofilms were initially prefixed by overnight incubation (4°C) with glutaraldehyde (2.5%). After fixation with glutaraldehyde, they were subjected to dehydration using an ethanol series gradient (10%-95%) for 10 minutes at each concentration. The dried slides were then sputter-coated with gold and observed under FESEM (Sigma Carl Zeiss, Jena, Germany).

3.4. Phase IV: Investigation of the influence of selected compounds on the expression profile of quorum sensing regulatory genes

In this phase, the gene expression pattern of quorum sensing regulatory genes were examined using quantitative real-time reverse transcription PCR (qRT-PCR). Antagonistic efficacy of LasR inhibitors at the transcriptional level was studied using *Escherichia coli* DH5α cells harboring plasmid pKDT17.

3.4.1. Quantification of relative gene expression of quorum sensing regulatory genes

3.4.1.1. Quantitative real-time reverse transcription PCR

qRT-PCR was done to examine the changes in gene expression patterns in *Pseudomonas aeruginosa PAO1* quorum sensing regulatory genes, namely *lasI, lasR, rhlI, rhlR, pqsA* and *pqsR*. For this purpose, *Pseudomonas aeruginosa PAO1* biofilms were grown with and without compounds for 24 hrs at 37°C. Subsequently, the cultures were subjected to RNA extraction, and qRT-PCR was performed according to the procedure detailed in Appendix XI. The primers used in this study are given in Appendix XII.

3.4.2. Evaluation of antagonistic efficacy of LasR inhibitors

3.4.2.1. β-galactosidase reporter gene assay

For the reporter gene assay, *Escherichia coli* DH5α cells harboring plasmid pKDT17 (gift from Peter Greenberg; Addgene plasmid #27503; https://www.addgene.org/; RRID: Addgene 27503)) was utilized to investigate the capacity of the compound to either activate or inactivate LasR mediated transcription. By fusing the *lasB::lacZ* translational fusion with the LasR gene, the plasmid pKDT17 functions as a reporter strain (Pearson *et*

al., 1994). The protocol adopted by Kim *et al.* (2005) was followed with minor changes to measure LasR activity as the β-galactosidase enzyme activity as in Miller assays (Miller, 1972). The procedure for β-galactosidase activity measurement is provided in detail in Appendix XV.

Initially, overnight culture of *Escherichia coli* DH5α cells containing the plasmid pKDT17 was diluted 1:100 in LB broth containing ampicillin (50 µg/ml) and was kept at 37°C till it reached an OD_{600} of 0.2. The antagonistic effect of the selected inhibitors was assessed using 50 nM AHL (Acyl homoserine lactone). The β-galactosidase enzyme activity in the treated cultures was determined after 2.5 hrs of constant agitation (150 rpm) at 37°C.

3.5. Statistical analysis

Statistical differences were determined by performing one-way ANOVA in Microsoft Excel, where p values < 0.05 and < 0.01 defined its statistical significance.