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INTRODUCTION

One of the biggest challenges faced by healthcare sectors and a significant health concern worldwide is the rising incidences of microbial resistance to conventional antibiotics. Numerous resistant bacterial species have emerged due to the widespread microbial resistance brought about by the overuse and misuse of traditional antibiotics (Dhingra *et al.,* 2020). Due to the untreatable nature of bacterial infections caused by multidrug-resistant (MDR) strains, these infections continue to be the most significant cause of morbidity worldwide. These are also responsible for many acute and chronic infections (Uddin *et al.,* 2021). Antibiotic resistance is generated as a result of the exponential growth in antibiotic production and the extensive usage of antibiotics, making the management of infectious diseases much more difficult (Gupta *et al.,* 2023).

Globally, illnesses brought on by MDR pathogenic microorganisms claim the lives of about 700,000 people each year. By 2050, it is expected to result in 10 million annual deaths (Ponyon *et al.,* 2022). According to an assessment report by the UK government, mortality rates brought by antimicrobial resistance (AMR) may surpass cancer deaths by 2050 (Ahmed *et al.,* 2019). An epidemiological report surveyed annually by the European Center for Disease Prevention and Control (ECDC) stated that infections caused by antibiotic-resistant strains contributed to around 670,000 infections in the nations of the European Union or European Economic Area (EU/EEA) in 2019 (ECDC, 2020).

Low-income and middle-income nations in Asia are disproportionately impacted by the direct and indirect effects of antibiotic resistance. Among them, the highest rates of infectious disease mortality is in South Asia, and the prevalence of antibiotic resistance are concerning (Manesh and Varghese, 2021). India has become the world leader and a key contributor to the rise of antimicrobial resistance, with 10.7 units of antibiotics used per person (Taneja and Sharma, 2019). In response to this, the Antimicrobial Resistance

Surveillance Research Network was set up by the Indian Council of Medical Research to assess the burden of resistance and to launch stewardship initiatives (Walia *et al.,* 2019).

According to the Antimicrobial Resistance Surveillance Research Network assessment, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter sp*., were reported as the six concern ESKAPE pathogens (Chadha *et al.,* 2022). It was reported that 929 million deaths were caused by AMR and 357 million deaths were linked to AMR in 2019 (Antimicrobial Resistance Collaborators, 2022).

According to a data from the UN Ad hoc Interagency Coordinating Group on Antimicrobial resistance, drug-resistant diseases would lead to 10 million annual fatalities by 2050 and would put 24 million people in extreme poverty by the year 2030 (World Health Organization, 2019). These bacteria develop such resistant nature through biofilm formation, persister cells, exopolysaccharides (EPS) matrix protection, limited antibiotic penetration, slower growth rate, efflux pumps and horizontal gene transfer. Among them, biofilm development is one of the most critical resistance mechanisms.

The National Institute of Health (NIH) estimated that bacteria found in biofilms contributed to around eighty percent of all human microbial illnesses (Schulze *et al.,* 2021). Biofilms are formed by microbial communities that are adhered to a living or inert surface and enclose themselves within a self-secreted EPS matrix material (Banerjee *et al.,* 2020). These biofilms serve as repository for harmful pathogens and a point of the breakout for majority of infectious disorders, including cystic fibrosis, otolaryngologic infections, bacterial endocarditis, non-healing wounds, and osteomyelitis (Vestby *et al.,* 2020). The innate antibiotic resistance mechanism of these biofilms contribute to wide range of acute and chronic illnesses, particularly in immune suppressed people (Zhang *et al.,* 2020).

Pseudomonas aeruginosa is a major human pathogen and a virulent Gram-negative bacterium that forms stable biofilm structures. It is responsible for infections in patients with cystic fibrosis (CF), nosocomial infections, and infections of immune suppressed individuals. The inherent resistance to conventional antibiotics and the adaptable nature of *Pseudomonas aeruginosa* has increased the risk of death due to its infections (Tuon *et al.,* 2022). It is considered significant as it is the main cause of death and morbidity in individuals suffering from cystic fibrosis and one of the commonest bacteria

affecting hospitalized patients with their inherent resistance to wide variety of medications (Spagnolo *et al.,* 2021).

Pseudomonas aeruginosa produces multiple virulence factors, which are responsible for the progression of diseases through mechanisms, namely modification of immune response, enforcement of adhesion ability, evasion of phagocytosis, and destruction of host tissues (Liao *et al.,* 2022). It infects people by expressing virulence traits, including lipopolysaccharides, elastase, pyocyanin, cyanide, rhamnolipids, exotoxins, alginate, antimicrobial resistance, and flagellar motility. The secreted virulence factors enable *Pseudomonas aeruginosa* to establish a stable structural architecture called biofilms in its associated infections (Parasuraman *et al.,* 2020).

International Nosocomial Infection Control Consortium reported that nosocomial infections caused by *Pseudomonas aeruginosa* are becoming a primary global healthcare concern (Reynolds and Kollef, 2021). *Pseudomonas aeruginosa* is among one of the "top 10" prevalent hospital "superbugs" for more than decades due to its multiple antibiotic-resistant strains, which result in potentially fatal consequences (Behzadi *et al.,* 2021). It is the third most common pathogen contributing to nosocomial infections, accounting for about 57% of cases (Rashiya *et al.,* 2021). *Pseudomonas aeruginosa* strains with high levels of antibiotic resistance are responsible for about 11% of hospital-acquired infections and 61% of fatality rate (Pachori *et al.,* 2019). Additionally, it has evolved as a constant companion for patients undergoing medical treatment since it can grow on both abiotic and biotic surfaces, such as those found on medical instruments, resist cleaning agents and transmit between patients (Funari and Shen, 2022).

Antibiotic resistance among bacteria in biofilms is higher than that of organisms in planktonic states due to differences in their physiology and phenotypic characteristics. Mainly, the enhanced tolerance to antibiotics and more resistant nature to host responses have made clearance of *Pseudomonas aeruginosa* biofilms challenging and their associated infections persistent (Olivares *et al.,* 2020). MDR strains of *Pseudomonas aeruginosa* have been resistant against almost all class of regularly used antibiotics, including aminoglycosides, fluoroquinolones, cephalosporins, and carbapenems (Pang *et al.,* 2019).

The capacity of *Pseudomonas aeruginosa* to establish biofilms, which protect them from environmental stressors and prevent phagocytosis, gives them the capability for

colonization and long-term survival that further complicates the treatment of its associated infections. Research on other therapeutic strategies, such as focusing on social behaviours involved in pathogenesis as well as bacteriophages and vaccines, are therefore being driven against infections caused by variant strains of *Pseudomonas aeruginosa* (Qin *et al.,* 2022).

The pathogenesis of *Pseudomonas aeruginosa* is chiefly dependent on the virulence factors production and the biofilm development (Wang *et al.,* 2021). An integrated signaling network referred to as quorum sensing (QS) mainly owes to the pathogenesis of *Pseudomonas aeruginosa* (Jurado-Martín *et al.,* 2021). QS is a type of cellular communication that mediates group behaviour in response to stress from the outside environment or changes in cell density. Autoinducers (AIs), a class of secreted signaling molecules required for QS, are quantified and specifically recognised by a subset of transcriptional regulators (Thi *et al.,* 2020).

The quorum sensing network of *Pseudomonas aeruginosa* is interconnected. It has four crucial systems, namely *las*, *rhl*, Pseudomonas quinolone signal (*pqs*), and integrated QS (*iqs*), all of which are controlled by different QS signaling molecules (Zhu *et al.,* 2021). LasR and RhlR are the homologous LuxR-type receptors of these systems that recognize the autoinducer molecules generated by *las* and *rhl* QS systems, respectively. The transcriptional factor PqsR, which is unrelated to LuxR-type receptors, binds the autoinducer molecule of the *pqs* QS system (Kumar *et al.,* 2022). The three transcriptional receptor proteins, which include LasR, RhlR, and PqsR, sense their corresponding AIs, namely N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), N-butyryl-L-homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively (Li *et al.,* 2022).

At their critical concentrations, the AIs of the QS systems activate a group of genes that governs formation of biofilm and other activities like the release of host-microbe interactions, release of virulence factors, metabolic changes, and stress tolerance. The transformation of an acute infection into a chronic infection depends critically on the expression of these genes since QS controls more than 10% of the genes in *Pseudomonas aeruginosa* (Pena *et al.,* 2019). When the autoinducer of the *lasI/R* system, namely OdDHL, binds to LasR, it induces the downstream genes, such as *lasI* synthase and some of the other genes of the *rhl* system. The LasR-OdDHL complex regulates the expression of *rhlI, rhlR, pqsR,* and *pqsABCDE* genes (Simanek and Paczkowski, 2022).

The other system, *rhl*, whose receptor protein RhlR on binding to C4-HSL, drives up the genes necessary for biofilm development and the production of virulence factors. Finally, *pqs* utilize its autoinducer and control *lasI/lasR* system to induce *rhlI/rhlR* expression (Bernabé *et al.*, 2022). These QS systems influence and coordinate primary physiological functions that contribute to pathogenicity by controlling the genes that generate biofilms and produce virulence factors. Thus, as the QS systems in *Pseudomonas aeruginosa* are crucial for the generation of virulence factors and the development of biofilm, these systems are viewed as excellent targets for developing antibiotics to fight against its associated infections (Wang *et al.,* 2021).

An established finding that *Pseudomonas aeruginosa* regulates virulence traits by QS offered a new strategy for the discovery of a robust and novel drug target. Many researchers have also reported that QS suppression would effectively reduce *Pseudomonas aeruginosa* pathogenicity and the development of biofilms (Hemmati *et al.,* 2020). Quorum sensing inhibitors (QSIs) developed may appear to have reduced risk of resistance in contrast to existing antibiotics since they decrease the pathogenicity of bacteria without affecting its growth pattern (Gupta and Kumar, 2022). Therefore, targeting QS-mediated gene expression would be one potential technique to be considered for treating infections associated with biofilms and biofilm development by multi-drug resistant (MDR) *Pseudomonas aeruginosa* (Kalia *et al.,* 2019).

Generally, there are two ways to interfere with QS: enzyme degradation or small molecule binding. The latter has been frequently employed by using acyl homoserine lactone (AHL) analogs to bind the QS receptor region. Additionally, various research has suggested that *Pseudomonas aeruginosa* QS suppression through small molecule leads has remarkably reduced the development of biofilms (Coquant *et al.,* 2020). In this context, though each of the QS systems in *Pseudomonas aeruginosa* are distinct, the systems are arranged hierarchically, with the *las* system controlling the other two systems. The respective receptor protein of the *las* system, LasR, is considered as a critical protein involved in regulating a variety of virulence-related phenotypes and occupying the top position in the hierarchy.

LasR is considered the principal protein as it controls various virulence traits, including biofilm formation, pyocyanin, protease, and rhamnolipid synthesis in *Pseudomonas aeruginosa* (Jurado-Martín *et al.*, 2021). LasR comprises of two separately

folded domains: an amino-terminal ligand binding domain (LBD) and a C-terminal DNA binding domain (DBD). The monomeric form of the OdDHL, dimerizes the two LasR subunits when it binds to LasR. The ensuing homodimer gains the DNA binding capability and initiates transcriptional modifications (Elnegery *et al.,* 2021). Numerous researchers have also shown that the QS circuit regulates a wide range of physiological processes and that explicitly targeting the *las* system may be a valuable strategy to hinder the pathogenesis of *Pseudomonas aeruginosa* (Duplantier *et al.,* 2021).

Computer-aided drug discovery (CADD) has become a crucial tool for drug discovery in the pharmaceutical and biotechnology sectors over the past few decades. Although drug discovery is laborious and time-consuming, using *in silico* methods would enable the identification of more effective hits and scaffolds for a target (Ejalonibu *et al.,* 2021). *In silico* methods can examine novel compounds before their synthesis, opening up the possibility of early hazard identification (Fan *et al.,* 2019). Homology modeling, molecular docking, virtual screening, pharmacophore modeling, and molecular dynamics simulation, are a few techniques that have been used successfully in drug discovery (Liu *et al.,* 2020). Using computer-aided drug design, it is possible to predict the pharmacokinetic features, including absorption, distribution, metabolism, excretion, and toxicity (ADMET) of a compound (Opo *et al.,* 2021).

The use of molecular docking technique opens the venture to "virtual" or "structure-based" searches for compounds that are compatible with the structure of the protein. Virtual screening techniques are nowadays widely employed in drug discovery procedures due to their time and financial efficiency. Virtual screening refers to the application of *in silico* methods to look for small ligand molecules in the vast library of compounds to find structures that may specifically bind to a target structure. The technique is further divided as ligand and structure based approaches (Kiriiri *et al.,* 2020).

The objective of the structure-based virtual screening (SBVS) method which incorporates molecular docking, is to forecast the preferred binding mode and affinities of the compounds to a therapeutic target. Ligand-based virtual screening (LBVS) includes pharmacophore modeling to compare the similarity in structures of unknown and known compounds with known active ligands as a query (Vázquez *et al.,* 2020). If molecules with undesirable features can be ruled out using *in silico* methods, significant resources can be

saved wherein the "prescreened" molecules could be progressed to more expensive *in vitro* screenings (Fan *et al.,* 2019).

The rising need for *in vitro* assays and the growing list of potential targets have paved the way for tools like ligand- and structure-based virtual screens to support and optimize the profiling process. Due to increased structural data accessibility and the availability of high-performance computer platforms, the virtual screening process has lately grown (Nain *et al.,* 2020). High-throughput screening procedures work well with SBVS since it has been adopted as a successful paradigm for lead discovery. Numerous compounds identified by the virtual screening process have demonstrated potential efficacies. Also, several chemical scaffolds obtained from the virtual screening procedures have been suggested as prospective leads for the continued generation of novel therapeutic agents against many disorders. In addition, the investigation of structure-activity relationships (SARs) could be the foundation for developing novel compounds and structural alterations for identifying active compounds (Suay-García *et al.,* 2022).

Various *in silico* approaches have been preferred over traditional methods for developing effective QSIs against LasR over the past 15 years to decrease the effort, time, and cost. Although vast antagonists that target LasR is currently known, molecular docking and X-ray crystallographic studies have demonstrated that structurally distinct antagonists and activators bind to the exact binding site of LasR and mimic the effects of either an antagonist or an agonist (Sadiq *et al.,* 2020; McCready *et al.,* 2019; Luise and Robaa, 2018; Choi *et al.,* 2017).

Numerous biofilm and QSIs that researchers have identified include naringenin (Hernando-Amado *et al.,* 2020), salicylic acid (Ahmed *et al.,* 2019), 5-hydroxymethylfurfural **(**Rajkumari *et al.,* 2019), 6-gingerol (Kim *et al.,* 2015), hordenine (Beury-Cirou *et al.,* 2013), rhubarb (Chu *et al.,* 2013), meta-bromo-thiolactone (O'Loughlin *et al.,* 2013), trans-cinnamaldehyde and ajoene (Jakobsen *et al.,* 2012), and 7-fluoroindole (Lee *et al.,* 2012). In addition, several researchers have also looked into the *in silico* identification of QSIs for the LasR receptor (Magalhães *et al.,* 2022; Nain *et al.,* 2020; Sadiq *et al.,* 2020; Kalia *et al.,* 2017; Tan *et al.,* 2013).

Despite various attempts to discover a lead molecule with antibiofilm and antiquorum sensing activities, bacterial resistance is still growing at an alarming rate. With

this background, the present study focused on identifying potential QSIs with antibiofilm and antiquorum sensing properties and could serve as a molecule that can act as lead for the design of novel antibiofilm agent against LasR in *Pseudomonas aeruginosa*. The present research work was designed with the following objectives:

- To identify and analyze antagonists against LasR in *Pseudomonas aeruginosa* through virtual screening and molecular dynamics simulation
- To assess the antibiofilm and antiquorum sensing activity of the selected LasR inhibitors against *Pseudomonas aeruginosa*
- To examine the effect of LasR inhibitors on quorum sensing mediated virulence factors production in *Pseudomonas aeruginosa*
- To investigate the influence of selected compounds on the expression profile of quorum sensing regulatory genes