An *In-silico* study of rational drug design for *Pseudomonas aeruginosa* Rhamnosyltransferase A enzyme and transcriptional regulator rhlR enzyme via blocking formation of biofilm

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“And when I am ill, it is (God) who cures me.”

- Quran (26:80)

Dedicated to

all the patients suffering from infections

caused by Pseudomonas aeruginosa.

May Allah bless with health and ease, anyone anywhere fighting

against any illness.

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Abstract

Ubiquitous, mobile, opportunistic pathogen named *Pseudomonas aeruginosa* which is gram negative is well-known for causing a plethora of nosocomial infections. It poses serious threats in immunocompromised hosts and is very difficult to treat in patients. It has become extremely difficult to eradicate it from hospitals due to its resistance towards antibiotics and disinfectants. Therefore, there is a dire need of innovative therapies to treat the multi-drug resistant bacterium. This pathogen easily develops biofilm which shows prominent resistance to antibiotics and this is why WHO has classified it as one of the ESKAPE infections. PAO1 has been of good use in understanding the biology of pseudomonas and is helpful in assessing the novel treatment. We used a rational drug design approach, in this study, to inhibit quorum sensing pathway, rhl, in pseudomonas aeruginosa PAO1 which is responsible for biofilm formation and rhamnolipid synthesis. We did so by suggesting inhibitory molecules against the two enzymes, Rhamnosyltransferase subunit A which is encoded by the gene rhlA and Regulatory protein RHLR encoded by the gene rhlR. Homology modelling was performed to obtain the 3D structure of the target proteins which were then refined. Various tools such as ERRAT and Ramachandran plot were used to assess the quality of the protein. Seventeen compounds for rhlA and eight for rhlR were extracted using Chembl and PubChem. Castp was used to analyze the ligand binding domain. Site specific docking was carried out to find the ligand showing the most negative binding affinity value with the enzyme that has been targeted. Two compounds against each protein were selected with the most negative binding affinity. These compounds were then checked for ADMET properties and bioavailability. Molecular dynamic simulation results further calculated the stability of the complex. The compounds explored are expected to be an effective drug against the target proteins in blocking the rhamnolipid synthesis and biofilm formation. Detailed laboratory experimentation is always necessary to bridge the gap between wet and dry laboratory studies and to ensure the validity of the obtained results.

CHAPTER 1

INTRODUCTION

1. Introduction

The overuse and misuse of antibiotics, including taking them for minor illnesses, self-prescription, and not completing the full course of treatment, has resulted in antibiotic resistance, making drug therapy more complex. To address this issue, the Centers for Disease Control and Prevention (CDC) published Antimicrobial Resistance Threat Report in 2019, which serves as a reference for information on antimicrobial resistance and identifies emerging areas of concern and the need for additional action. The report highlights Pseudomonas aeruginosa as a serious threat. According to the CDC, MDR bacteria are responsible for the loss of 23,000 lives annually. It is suggested that if appropriate measures are not taken, antimicrobial resistance could cause 10 million deaths by 2050. The consumption of antibiotics globally raised by 65% in the years 2000 to 2015, and Pakistan was one of the low-middle income countries with the highest antibiotic consumption in 2015 (Klien et al., 2018). A study published in Pakistan in April 2021 highlighted Pseudomonas *aeruginosa* as the bacteria that are frequently reported drug-resistant in the country's antibiotic stewardship program (Atif, Ihsan & Malik, 2021). The biofilm producing pathogenic bacterium, Pseudomonas *aeruginosa* is opportunistic in nature and lives in water and soil. It can cause diseases in animals and humans. Due to its drug resistance against the conventional antibiotic therapy, it is of high concern in patients with cystic fibrosis and the ones whose immune system is compromised. It also causes chronic lung infections. Traditional antibiotic methods are becoming less effective, making it critical to find alternative therapies. One of the factor that contributes to drug resistance property is the formation of biofilms. Bacterial communities that can adhere to surfaces and produce a matrix of extracellular polymeric substances that can protect the bacteria from antibiotics and detergents are called biofilms. They are bacterial extracellular matrices consisting of cells that attach to each other and to the solid surfaces too. The biofilm then forms a capsule with nuclear material and proteins around the cells of bacteria. This results

in an increased virulence and makes it difficult for antibiotics or immune cells to enter biofilm and get rid of infection (Thornton et al., 2021). Quorum sensing(QS) is one of the factors that contribute and regulate biofilm formation (Yan & Wu, 2019). Cooperation is a general behaviour that benefits other individual and has favourable effects on recipient too. Such is the case with microbes that produce extracellular products, for instance, surfactants, toxins and proteases etc. Such behaviors also include virulence and formation of biofilm. These behaviors in the microbes are mediated by QS (Wilder, Diggle & Schuster, 2011). Quorum sensing is a regulation system controlling the activity of Pseudomonas *aeruginosa* when it produces that are virulent in nature, such factors are synthesis of biofilm, pyocyanin, elastase and rhamnolipids. It is a system that is dependent on cell density.These virulence factors empower host colonisation and adaptation. To reduce virulence, QS inhibitors can be targeted. This approach has enabled to disrupt the QS system either via enzymatic degradation of QS signal molecules or using compounds that inhibit their binding to regulatory proteins. Thus, it has become significant to identify additional compounds with anti-QS abilities. More such compounds can be obtained by computer aided programs that enable structure based virtual screening (Ahmed et al., 2019). Las, rhl and pqs are three QS systems that each consists of an autoinducer (AI) signalling molecule and a regulatory protein. QS system relies on the secretion, processing and sensing of autoinducers which in turn manipulates virulence factors, metabolite production, swimming motility, antibiotic resistance and biofilm maturation. When bacterial density reaches a certain high concentration, the signalling molecule- the autoinducer- binds to its respective receptor to form a complex. This complex controls the gene transcription. Interference with QS regulated signalling disturbs the bacterial communication and constrict virulence. Thus, in antibiotic resistance control, the QS inhibitors are significant. Since resistance of antibiotic and biofilm penetration is a key concern we have targeted rhamnolipids and biofilm formation in this study. Hence, are focus is on rhl system. In rhl system, N-butyryl-

L-homoserine lactone (C4-HSL) signals are generated that activate the process. When the gene rhlR is activated, it further triggers the system to produce C4-HSL. This then combines with Transcriptional regulator (RhlR) protein and increases the rhlR genes expression (Kalaiarasan et al.,2017). C4-HSL determines the rhl system. Rhl system is said to be vital for the bacterial survival during anaerobic biofilm growth mode. C4-HSL also monitors production of rhamnolipids needed for maintenance of biofilm structure (Bonte, Kohler & Delden, 2003).

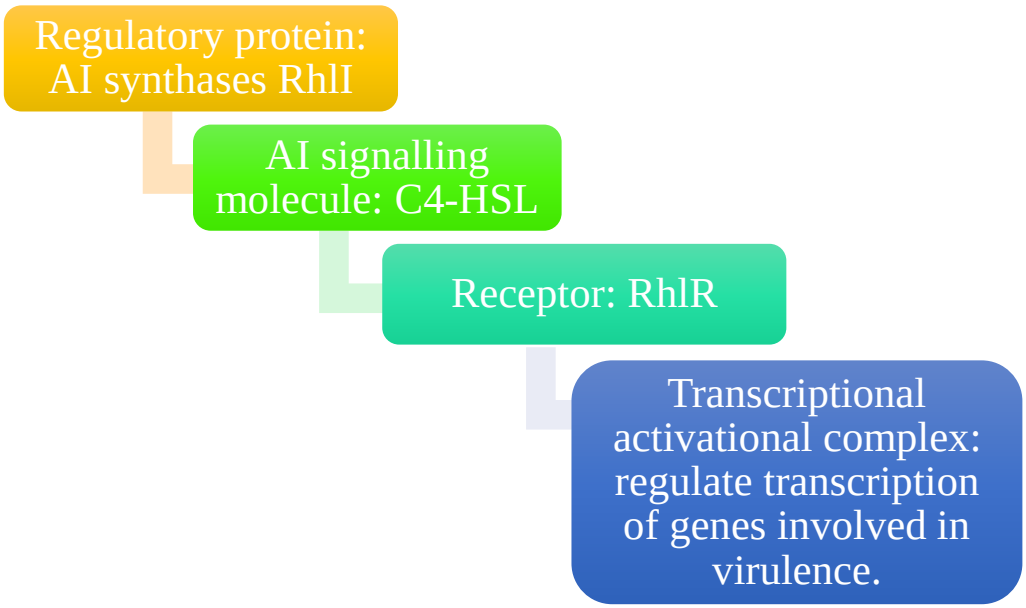


Figure 1.1: rhl system activation

The rhl system, consisting of RhlI and RhlR, is crucial in biofilm formation by producing C4-HSL and activating genes involved in the process. Similarly, rhamnosyltransferase A enzyme (RhlA) plays a significant role in rhamnolipid synthesis. Therefore, inhibiting the activity of RhlA and RhlR enzymes may provide a potential therapeutic option for controlling Pseudomonas *aeruginosa* biofilm formation. Various scientific studies have shown that rhl system has a huge input in pathogenicity. For instance, in fighting the immunity of cells, an investigation about fruit flies mentioned RhlR as a prominent aspect (Limmer et al.,2011). Another study found that the key inhibitor's target to regulate pathogenesis in C. elegans was RhlR (O'Loughlin et al.,2013). In this current study we have targeted RhlA and RhlR in an attempt to disrupt biofilm formation.

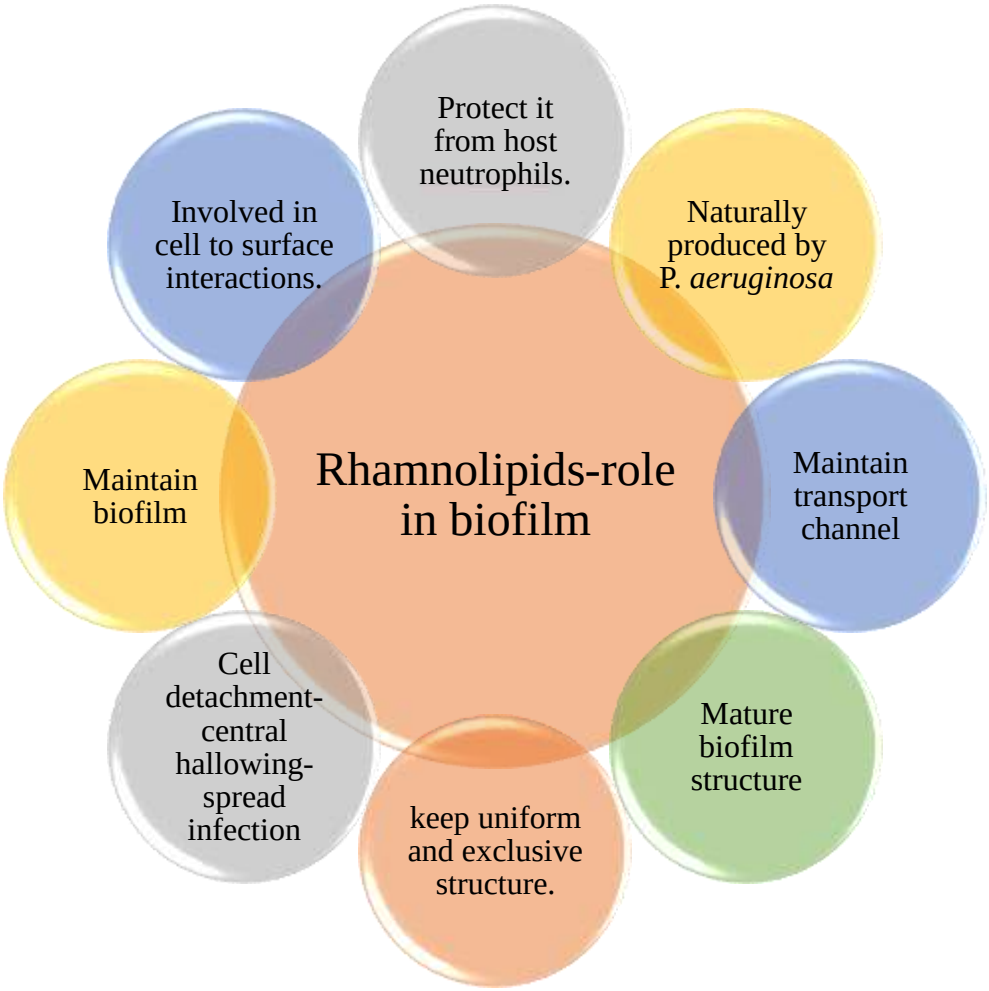


Figure 1.2: Role of rhamnolipids in biofilm

Computer-aided drug design (CADD) techniques are gaining importance in discovery of drugs as they enable the detection of worthwhile drug options economical. In particular, in-silico rational drug design is a type of CADD that allows scientists to predict the properties and behavior of molecules before they are synthesized and tested in the lab. This approach can limit the use of animal models in pharmaceutical research and aid in the proposing of novel and safe drug candidates, while also supporting chemists and pharmacologists during the drug discovery process. By using computational tools and algorithms, researchers can design and optimize drug candidates with desired properties, such as high potency, selectivity, and safety, thus saving time and resources. In addition, such methods can be used for drug repositioning, which is a more cost-effective approach to developing new drugs. Therefore, the use of CADD

methodologies is increasingly important in identifying drug candidates cost-effectively (Brogi et al.,2020). Rational drug design is a computational approach used to discover new drugs by predicting the interaction between small molecules and target proteins. This method has been widely used to design drugs that selectively target specific enzymes or proteins, including those involved in biofilm formation in Pseudomonas *aeruginosa*. Therefore, this study aims to use rational drug design to identify compounds that can inhibit the activity of RhlA and RhlR enzymes, thereby blocking biofilm formation. The study focuses on the RhlA enzyme, which is essential in rhamnolipid synthesis, and RhlR, which activates genes involved in biofilm formation. The study aims to identify potential drug candidates that can selectively inhibit these enzymes, ultimately blocking the formation of biofilms. The approach of the current drug designing study is to inhibit Quorum Sensing in Pseudomonas *aeruginosa* PAO1 as this specie is prevalent in Pakistan, by designing an inhibitory molecule against the enzymes: Rhamnosyltransferase A & Transcriptional regulator RhlR encoded by genes rhlA & rhlR respectively. Pseudomonas *aeruginosa* biofilms are challenging to treat due to their multi drug resistance property. Therefore, there is an immediate need to find novel therapeutic options to overcome Pseudomonas *aeruginosa* infections. Rational drug design offers a promising approach for identifying selective inhibitors of specific enzymes involved in biofilm formation. The findings of this research could add up in the expansion of innovative and helpful methods for treating Pseudomonas *aeruginosa* infections.

1.1 Objectives of the study

The key objectives of the current study are as follows:

- ✓ To acquire the structure of Rhamnosyltransferase subunit A & Regulatory protein RHLR.
- ✓ Targeting genes rhlA and rhlR in rhl system to block the synthesis of biofilm and rhamnolipids.
- ✓ Using databases for virtual screening of ligands to filter out the best candidate.
- ✓ Estimation of binding energies between selected ligands and protein & ADMET properties.
- ✓ To investigate the stability of the complexes via MD simulations.

CHAPTER 2

LITERATURE REVIEW

2. Literature review

2.1 A brief historical perspective

Carle Gessard in 1882, is when the initial isolation of Pseudomonas *aeruginosa* (PA) started. It was from the infections of the wound and collected via bandages of soldiers. They had green and blue color pus like substance on bandages. In 1894, Walter Migula stated that Pseudomonas *aeruginosa* is a genus of gram negative, rod shaped, aerobic bacteria with polar flagella. This broad definition led to addition of as many as hundred unrelated organisms in Bergey's Manual of Systematic Bacteriology in 1984. As there was advancement in molecular based techniques, the species that were earlier classified as Pseudomonas were reclassified by scientists using 16S ribosomal RNA sequencing and comparison. Palleroni split the genus into five divisions known as rRNA divisions I-V in his rRNA-DNA hybridization investigations. Later, groups II to V were reclassified into many genera. Only species from group I remained in the genus Pseudomonas. This group was further categorized into eight various categories (Moore & Flaws, 2011). In 1954 a strain was isolated from a wound in Melbourne, Australia. It was initially called "Pseudomonas *aeruginosa* strain 1". It was the original PAO strain. PAO1 from Bruce Holloway's laboratory is a strain that has been mutated and is now used as a common benchmark strain for the evaluation of function and genetics of the metabolism and morphology of this bacteria. By thorough study of the mechanism of gene exchange that includes conjugation and transduction, a genetic map for PAO1 was made. Later a physical map was made using pulsed-field gel electrophoresis. The knowledge of genetic map and physical map information was then combined. PAO1 strain was sequenced completely by 2000. In order to speed up the identification of therapeutic targets and vaccine candidates of Pseudomonas *aeruginosa*, the genome annotation was continuously updated and the functionality and content of the database were increased. A significant, inversion of 2.2 Mb was found between the PAO1 original strain and the sequenced PAO1-UW strain when the

genome sequence and the physical map were compared, revealing that the genomic sequence of PAO1 sublines maintained throughout the world had transformed the sequence of their genome. By 1970, the reported mutations were stated. It was discovered that two PAO1 sublines from strain collections kept by research teams in Germany and Japan, PAO1-D and PAO1-J respectively, were mutants (Klockgether et al., 2010).

2.2 Prevalence at home and abroad

According to a study, 1.27 million of the 4.95 million deaths in 2019 are related to antimicrobial resistance, with Pseudomonas *aeruginosa* as the number sixth on pathogen's list (Collaborators, A. R., 2022).

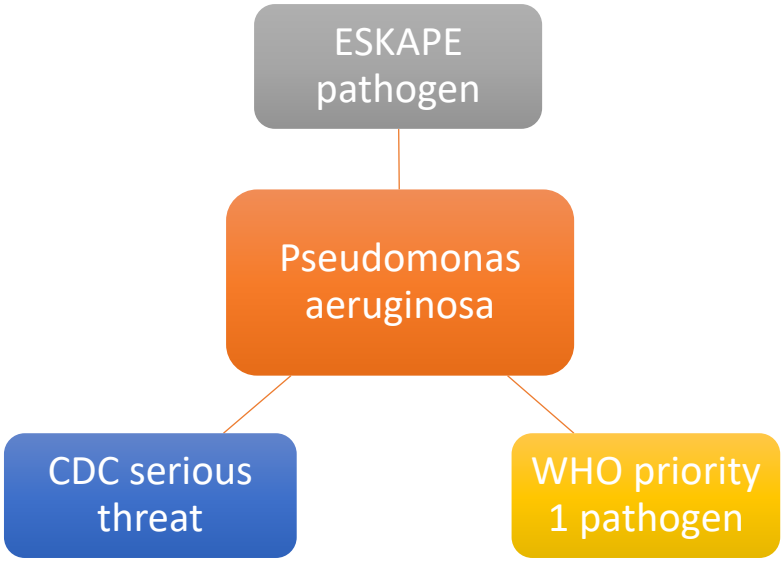


Figure 2.1: Pseudomonas aeruginosa at high alert

Pseudomonas *aeruginosa* PA01 strain is prevalent in Pakistan and thus is of high concern. The World Health Organization (WHO) prepared a global priority pathogen list of multidrug-resistant bacteria. It consisted of bacteria that exists in intensive care unit and are of immense danger to the patients. Based on certain criteria, the bacteria were categorised as critical, high and medium. Pseudomonas *aeruginosa* was categorised as critical (Rello et al., 2019).

In vivo and in vitro data demonstrates that bacterial cell walls in the biofilm are quite resistant to drugs and the defensive immune response because of the matrix protection (Hoiby et al., 2011). The prevalence of P. *aeruginosa* was found to be 44% in a study carried out on samples collected from Faisalabad, Pakistan. This could be brought on by continuous antibiotic misuse and poor hospital hygiene practices (Anam et al., 2018). Pseudomonas *aeruginosa* is of key significance because it's the key reason of infections and death in patients with cystic fibrosis. According to a cohort research, Pseudomonas *aeruginosa* accounted for the majority of healthcare-associated infections in ICU in Europe (Lambert et al., 2011). Pseudomonas *aeruginosa* was enlisted as a life threatening bacteria and was added to the list of pathogens for research and development of new antibiotic's list by World Health Organization in 2017. 90% of the wound infections have biofilms which results in the delayed healing of the wound. Moreover, 6.5 million patients have these wound infections which becomes a huge burden on the economy as it costs more than US$25 billion per year (Thi, Wibowo and Rehm, 2020). According to estimates, 51,000 cases of this infection are found in US annually. This is so because of the ubiquitous nature of Pseudomonas *aeruginosa* in hospitals. The patients in hospitals are carriers during treatments as they can survive on living and non living surfaces that includes the medical equipment, also, they can resist the cleaning techniques and thus can transmit from one patient to another (Russotto et al., 2015).

2.3 Features of Pseudomonas *aeruginosa*

From the Pseudomonadaceae family, omnipresent *Pseudomonas aeruginosa* can withstand various conditions. The genome of Pseudomonas *aeruginosa* is quite big comparatively. It has a size of around 5.5-7 Mbp which is larger than Mycobacterium tuberculosis which has a size of 4.4 Mbp, Escherichia coli which has a size of 4.6 Mbp and Bacillus subtilis with a genome size of 4.2 Mbp. This large genome contributes in encoding a high number of enzymes related

to transportation, metabolism and other necessary functions. There is a genome size variation of 5.5 and 7 Mbp within the species.

An elevated extent of metabolic adaptation and environmental change resistance is made possible by the enhanced coding ability. It is undoubtedly one of the prevalent bacterium causing nosocomial infections and ventilator-associated pneumonia. It has been identified as an opportunistic pathogen that rarely affects healthy people, but has a high morbidity and mortality rate in people with cystic fibrosis, burn victims, those with malignancy or mechanical ventilation and immunocompromised individuals (Pang et al., 2019).

Table 2.1 Infection types caused by Pseudomonas *aeruginosa* (Proctor et al., 2021).

Hospital acquired urinary tract infections.
Infections of respiratory tract.
Osteochondritis and Osteomyelitis.
Infections of central nervous system such as bacterial meningitis and brain abscess.
Infections of ear and eye.
Infections related to skin such as trauma, burns, dermatitis.

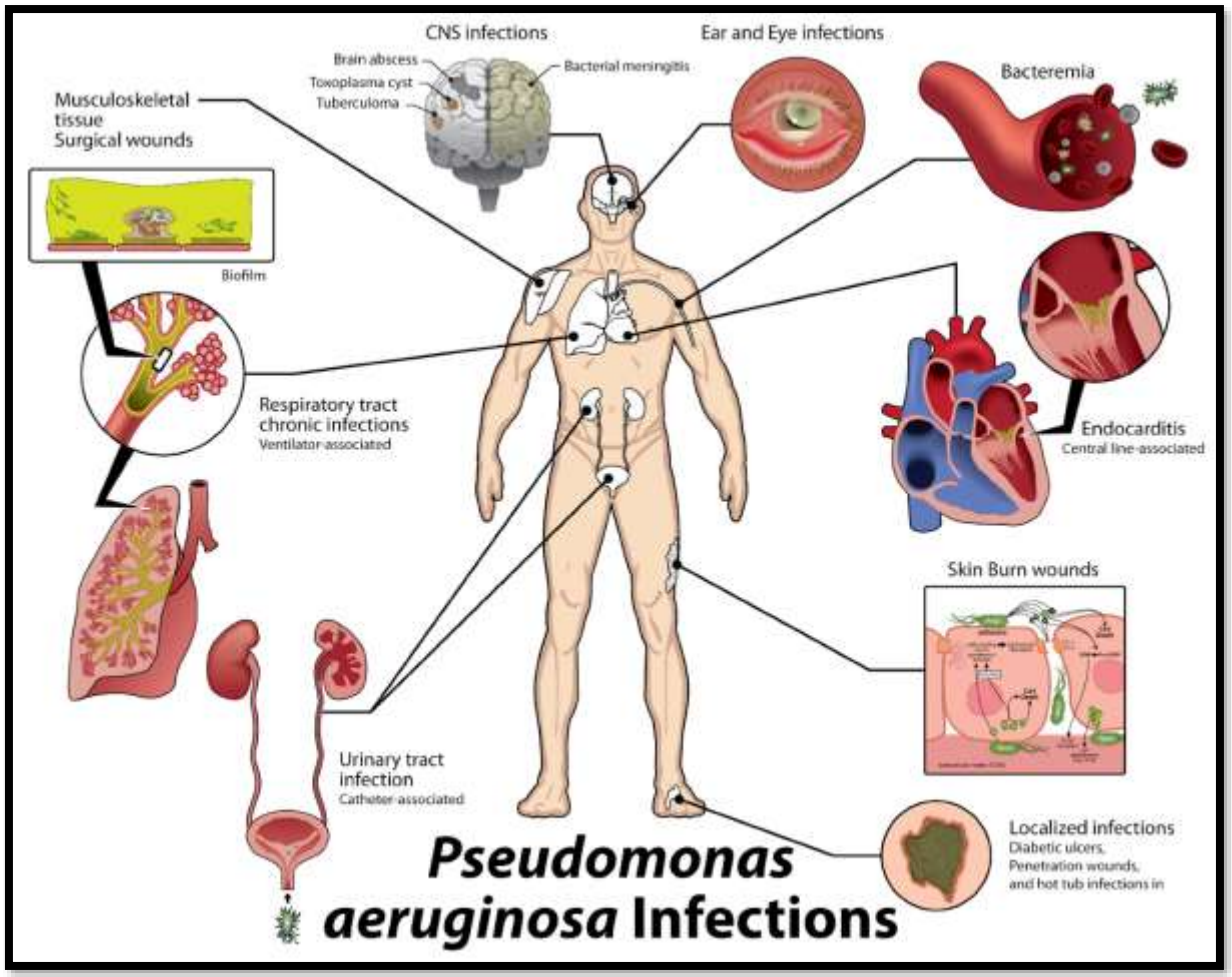


Figure 2.2*:* Infections caused by *Pseudomonas aeruginosa (*Proctor et al., 2021)

Varying number of plasmids and a single circular chromosome make up the genome which accounts for Guanine and Cytosine content to be 65-67%. The genome size is large comparatively reflecting its metabolic diversity to utilise a wide range of nutrients, according to the sequencing of whole genomes or chunks of the accessory genome (Klockgether et al., 2011). P. *aeruginosa* has the ability to survive in anaerobic, aerobic and hypoxic environments, therefore, exhibiting an excellent metabolic adaptability. This feature also makes it a pathogen for all: plants, animals and humans. A key consideration in metabolic studies of Pseudomonas *aeruginosa* is that of the environment whether it is aerobic or anaerobic or both. Under anoxic conditions, Pseudomonas *aeruginosa* depend on nitrate sources thus showing metabolic

flexibility. Metabolic changes and changes in virulence factors are introduced due to this shift from aerobic to anaerobic, as that in the case of Cystic fibrosis lung. Biofilm fitness and antibiotic susceptibility are changed due to such changes (Grace et al., 2022).

Table 2.2 Sources in the environment to extract Pseudomonas *aeruginosa* (Pelegrin et al., 2021).

Hospital settings.
Soil.
Water such as storage tanks, hot tubs, pools, lakes, rivers, ponds.
Plants.
Plumbing systems such as pipes, showerheads, faucets, taps, sinks.
Household environment such as drains, sinks, cleaning equipment like mop and buckets
Potable water, non sterile or contaminated injectable.
Medical devices such as catheters, implants, bronchoscopes, endoscopes, vials.
Humidifiers, air conditioning systems.
Medical facilities (e.g. hydrotherapy pools).
Recreational settings like spas and community reservoirs.
Industrial settings like cooling towers etc.
Contaminated cosmetics such as mascara and eyeliners.
Dental units.
Contaminated food especially raw or undercooked.
Agricultural settings such as livestock farms, poultry houses.

2.4 Characteristics of PAO1

Since PAO1 is widely used as a reference strain in laboratory search, it has some distinct features and characteristics. PAO1 was the first strain for which the complete genome sequencing was performed and is generally widely used in the bacterium's genetics, metabolism and physiology analyses. However, this has created a chance for mutations because of the presence of various genetic and phenotypic differences. It has a 6.264-Mbp circular chromosome that encodes 5,570 predicted protein coding sequences. Inserts of 14kbp or smaller are found in PAO1 genome (Klockgether et al., 2011). The bacterium may directly inject virulence components into host cells due to the T3SS, which increases the pathogenicity of the organism. Additionally, it produces a variety of virulence factors that aid in its capacity to spread infections. Exotoxins which contribute to host cell damage and immune system evasion, are among these factors. Biofilms are intricate microbial communities embedded within a self-produced matrix, and PAO1 is skilled at producing them. The bacterium's capacity to survive in the environment, withstand antimicrobial agents, and produce chronic infections is influenced by biofilms. The difficulties in treating Pseudomonas *aeruginosa* infections are a result of this resistance. A vast variety of carbon and nitrogen sources can be utilised by PAO1 due to its extensive metabolic repertoire. It can adjust to diverse environmental factors, which adds to its adaptability and capacity to thrive in a variety of niches. A single polar flagellum that encourages swimming motility in liquid settings and polar type IV pili that are in charge of twitching motility on solid surfaces are the two surface organelles that P. *aeruginosa* has for motility. PAO1 is able to move in liquid environments because it is motile and has a single polar flagellum. It must possess this quality in order to colonise and spread across host tissues (Murray & Kazmierczak, 2008).

Table 2.3 General features of PAO1.

Source	Wound

Virulence	Moderate
Genome size	5,700 genes
Unique sub elements	0.5%
Pathogenicity Islands	Portion of PAPI-2
Biofilm attachment	Irreversible
Common disease association	Cystic fibrosis

2.5 The Antimicrobial Resistance (AMR) crisis

Acquired, intrinsic and adaptive resistance are the three defense mechanism of Pseudomonas *aeruginosa.*

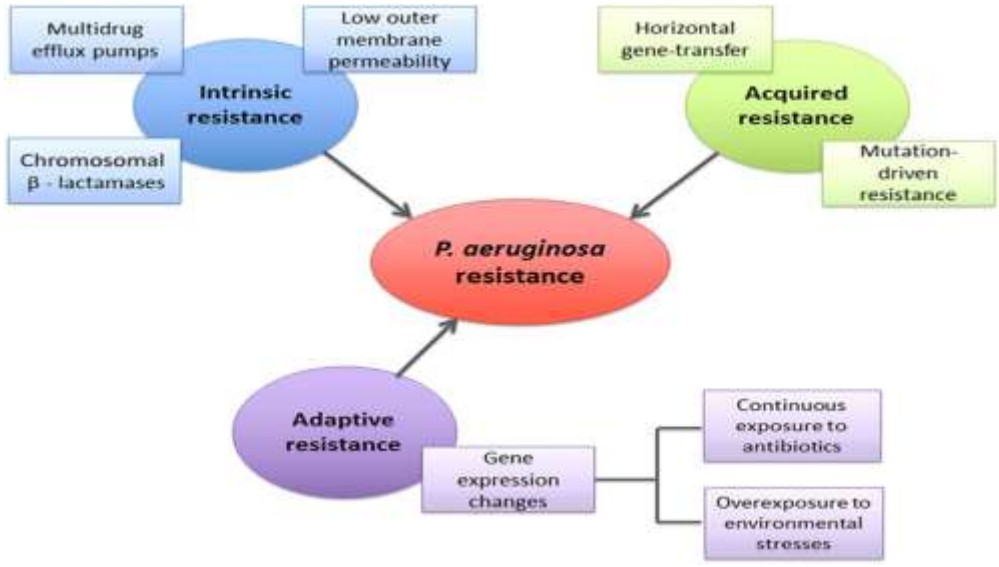


Figure *2.3:* Resistance mechanisms of *Pseudomonas aeruginosa* (Pires et al., 2015)

Intrinsic resistance approach by Pseudomonas *aeruginosa* includes outer membrane permeability to be low. Horizontal gene transfer aids in acquired resistance.It develops adaptive resistance through gene expression changes which is a result of overexposure to environmental stresses and continuous exposure to antibiotics (Breidenstein et al., 2011). In order to prevent antibiotics from reaching the bacterial cells, P. *aeruginosa* forms biofilms in

the lungs of infected patients. These biofilms operate as diffusion barriers limiting antibiotic reach to the cells of bacteria.

Additionally, the biofilm can give rise to multidrug-tolerant persister cells, which can withstand an antibiotic attack and cause infections in CF patients (Mulcahy et al., 2010). Recent years have seen the exploration of newer antibiotics with specific modes of action, as well as novel administration methods and resistance to bacterial enzyme modification. Some of these more recent drugs exhibit great in vitro antibacterial activity against P. *aeruginosa* and have less minimal inhibitory concentrations than the traditional antibiotics (Pang et al., 2019). The rise and spread of antibiotic resistance has become a global healthcare problem. It develops through mutations and horizontal gene transfer and is driven by selective pressure of antibiotic use and misuse. The current AMR crisis has a direct impact on population health, healthcare costs, medical practice and food safety. In Europe alone, AMR causes approximately 33000 deaths annually and costs between €1.5 to €9 billion. Globally, AMR infections already leads to 700,000 deaths annually with an increase expected by 2025. Studies are needed to assess the global burden of AMR. This crisis could lead to global economic recession and affect daily healthcare practices. Also, ESKAPE pathogens play a role in AMR transmission. The Global Antimicrobial Resistance Surveillance System (GLASS) by WHO monitors high-priority pathogens (Pelegrin et al., 2021).

Table 2.4 Therapies for Pseudomonas *aeruginosa* infections (Pelegrin et al., 2021; Pang et al., 2019).

Therapy	Definition	Advantages	Drawbacks

QS inhibitors	Molecules interference with signalling capacity and eliminate its virulence.	Prevent infection from spreading amongst patients.; can be used with other drugs synergistically. Prevention or reduction of biofilm formation, decrease virulence and low risk of development of resistance.	Mostly tested in-vitro, clinical trials required. Further research needed regarding secondary effects and resistance development. Unintentional effect on beneficial bacteria.
Phage therapy (PT)	Lytic bacteriophages to target MDR bacteria.	Target specific. PT can be used as prophylactic therapy (like probiotics). Less side effects, easy administration, delivery of antimicrobial agents to bacteria. Replication at infection site.	Risk of phage resistance development. Immune system of the host clears bacteriophages.

Antimicrobial peptides (AMPs)	Natural or synthetic polycationic peptides with an antimicrobial activity.	Several AMPs in the market, others in phase III clinical trials. Can be used synergistically with other drugs. Enhanced activity against biofilms. low toxicity. Rapid killing kinetics.	Most AMPs in the market are for topical use. Antimicrobial action and cell toxicity need to be further studied. High cost of production.
Antimicrobial nanoparticles (AMNs)	Metallic particles at the nano-metric scale used for their antimicrobial properties.	Silver AMNs have been used to coat indwelling medical devices. Can be used to deliver drugs at infection site. High penetrability into bacterial membrane. Disruption of biofilm formation.	Toxicity for host needs to be further evaluated in clinical trials.
Anti-pseudomonal vaccines and monoclonal antibodies	Vaccines or monoclonal antibodies based on diverse virulence factors used to	Could potentially provide long lasting immunization for risk population. Prophylactic or therapeutic lgA	No anti-pseudomonal vaccine available in the market yet due to low immunogenic response.

	protect patient populations at risk.	administration may help boost patients' immune system.	
Electrochemical scaffold	Use of electrochemical scaffolds to produce a constant concentration of H_2O_2 in order to diminish biofilms.	Disrupts biofilms. Increase of antibiotic penetration.	Difficulty in implantation to clinical trials.
Lectin inhibition	They are outer membrane proteins that allow bacteria to attach to host tissues.	High stability. Low risk of development of bacterial resistance.	Narrow spectrum due to expression of more than one type of adhesion by bacteria.

Recent studies have discovered numerous novel therapeutic approaches to eliminate Pseudomonas *aeruginosa* strains. These innovative non antibiotic therapy strategies offer fulfilling alternatives to conventional antibiotic treatments. By employing these therapeutic modalities, healthcare professionals can complement or even substitute conventional antibiotic treatments, thereby offering new avenues for combating antibiotic resistant Pseudomonas *aeruginosa* strains. (Chatterjee et al., 2016).

2.6 Biofilm's analysis

A biofilm is a collection of bacteria that adhere to one another on a surface, whether it be living or non-living. They form a self-produced matrix consisting extracellular polymeric substances,

including exopolysaccharides, proteins, metabolites and extracellular DNA. This unique matrix provides a protective environment for the microorganisms. In comparison to planktonic cells grown in a free aqueous suspension, microorganisms within biofilm exhibit reduced susceptibility to antimicrobial agents and host immune responses (Stewart and Costerton, 2001). Even bacteria lacking protective mutations or intrinsic resistance can become less vulnerable to drugs when they develop a biofilm. Additionally, when bacteria are exposed to an antibiotic without biofilm protection, the antibiotic sensitivity can be quickly recovered, indicating that the biofilm-mediated resistance is an adaptive mechanism independent of genetic changes. The general mechanisms of biofilm-mediated resistance prevent antibiotic penetration, altered microenvironment encouraging slow growth of biofilm cells, production of an adaptive stress response, and persister cell differentiation protect bacteria against antibiotic attack (Stewart, 2002).

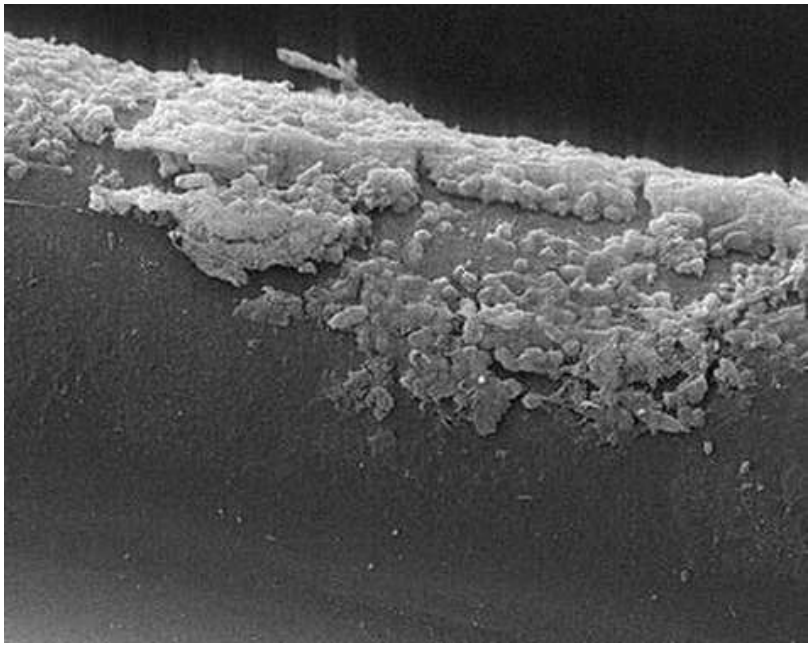


Figure *2.4: Pseudomonas aeruginosa* biofilm formed on a suture (Kievit, 2009)

Production of DNA, proteins and exopolysaccharides help form biofilm on lung epithelial cell surfaces in the CF patients with chronic Pseudomonas *aeruginosa* infection. As biofilm development progresses, they undergo diverse physiological and phenotypic changes. The

regulation of biofilm formation in Pseudomonas *aeruginosa* is a complex process influenced by multiple factors with quorum sensing systems playing a significant role. For instance, P. *aeruginosa* strains change to a mucoid phenotype after chronic CF infection, displaying increased alginate synthesis that is regulated by the CF microenvironment and facilitates the development of biofilm colonies. The P. *aeruginosa* flagellum's capacity to display swarming and twitching motility makes it essential for the beginning of biofilm development. However, P. *aeruginosa* drastically decreases flagellum expression after surface attachment and may even permanently lose the flagellum as a result of genetic changes. This decreases the response produced by the host immune system, allowing P. *aeruginosa* to avoid immune identification and phagocytosis (Jyot et al., 2007).

2.6.1 The composition of biofilm

For survival and adaptation in changing environmental conditions, biofilms rely on self-produced matrix of extracellular polymeric substances. 90% of the biofilm’s mass is of the matrix which consists of extracellular DNA, lipids, proteins and polysaccharides. This matrix has several functions such as facilitates communication between cells, protect bacteria from harsh conditions (antibiotics and immune responses) and provides a scaffold for adhesion to surfaces.

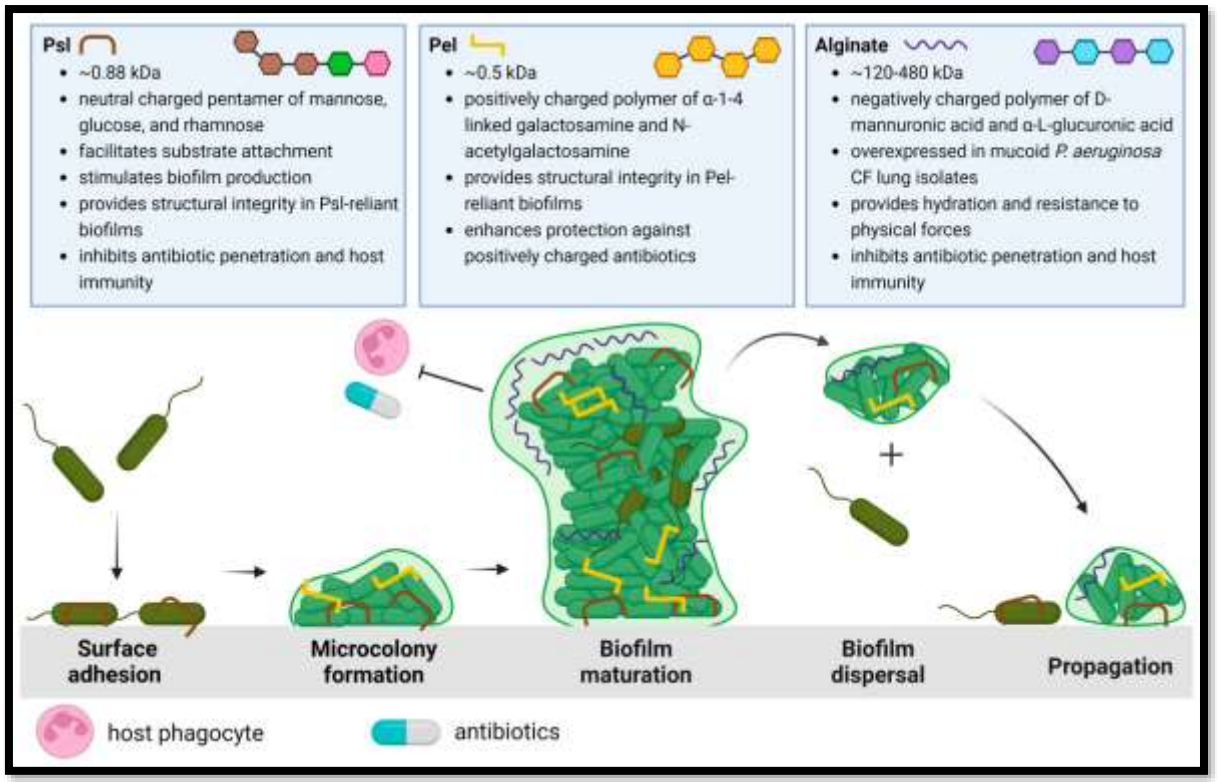


Figure *2.5:* Exopolysaccharides in Biofilm's matrix (Chung et al., 2023)

Three key exopolysaccharides found in the matrix are Psl, Pel and alginate. Psl is important for cell to cell interactions and surface adhesion. It adds up to the structural stability and produces a signalling molecule, c-di-GMP, that enhances the biofilm thickness and resilience. It is also a shield against phagocytosis and antimicrobials. Pel has a similar function and forms biofilm at air-liquid interface and provides resistance to aminoglycoside antibiotics. Alginate is mostly produced in mucoid strains and is found in infections like cystic fibrosis. it has a key role in biofilm maturation, protection and reduced antibiotic diffusion. Each component of the matrix plays distinct roles in antibiotic resistance, biofilm formation and maturity, and interaction with host immune system (Thi, Wibowo and Rehm, 2020).

2.6.2 The development of biofilm

Pseudomonas *aeruginosa* grow slowly in form of unattached cell aggregates in oxygen deprived conditions. They typically develop on non-living surfaces and involves five distint stages.

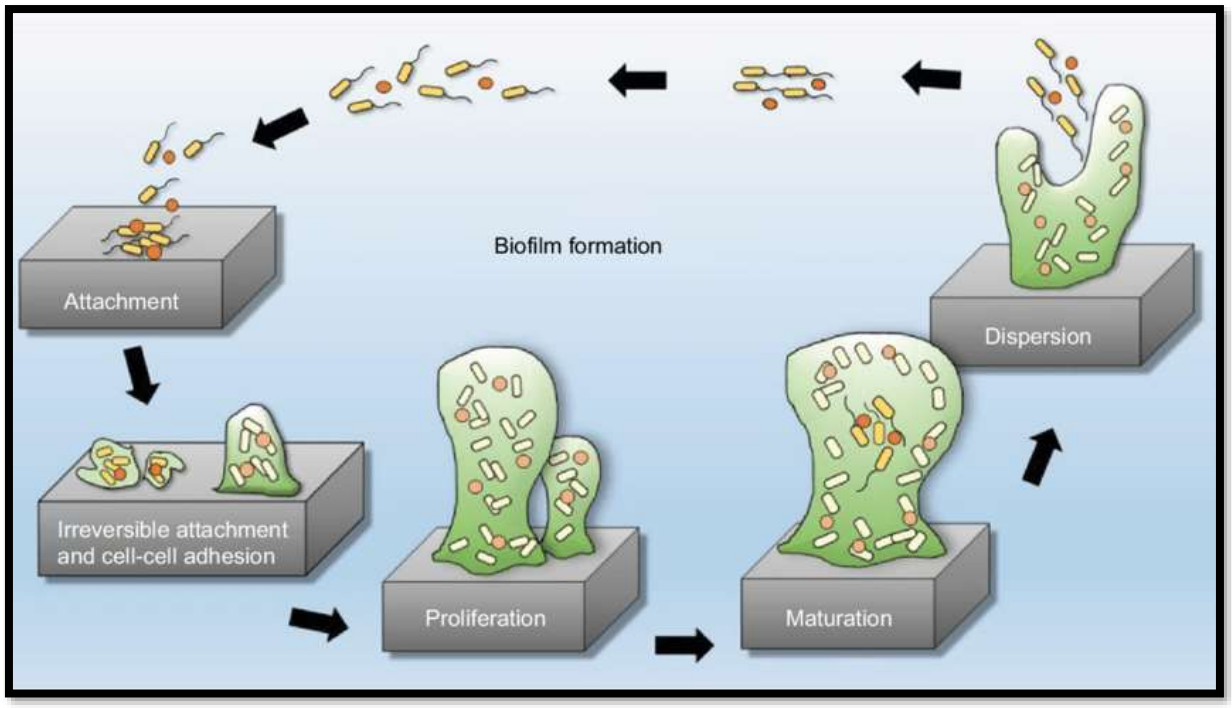


Figure *2.6*: Biofilm development stages (Abu Bakar et al., 2018)

In stage I, bacterial cells attach to surfaces via flagella and type IV pili. This attachment is reversible. In stage II, the attachment becomes irreversible. In stage III, the attached bacteria propagate and form micro-colonies. In stage IV, these micro-colonies mature and form three-dimensional mushroom-like structures. In stage V, autolysis occurs and cells are dispersed initiating a new biofilm cycle. The motility rate is influenced by nutrient availability. The biofilm's structure is influenced by swarming motility. High motile bacteria result in flat biofilms while low motility leads to mushroom shaped biofilms (Conover et al., 2009; Rasamiravaka et al., 2015). In the biofilm formation process, when the population of Pseudomonas *aeruginosa* reaches a critical density, the concentration of C4-HSL produced by RhlA increases. The C4-HSL molecule diffuse and bind to RhlR receptor activating it. Once activated, RhlR binds to specific DNA sequences and triggers the expression of genes involved in biofilm development, such as those responsible for producing EPS and other structural components of biofilm matrix. The EPS produced as a result of RhlA activity provides the

biofilm with stability and structure allowing the bacteria to attach to surfaces and form a community (Ochsner and Reiser, 1995).

2.7 Quorum Sensing

Quorum sensing (QS) regulates bacterial social behaviour through a number of interrelated signalling pathways. It enables communities of bacteria to control a number of biological procedures crucial for bacteria's survival and adaptability. This process basically depends on controlling the expression of particular gene sets in an answer to a crucial limit of signalling molecules called autoinducers. Population density-dependent collective reactions will be mediated by QS, which is advantageous for that population to stay alive. According to a study, cells' reactions to QS signals and the associated gene expression profiles vary depending on the community, which increases fitness and chances of survival (Grote et al., 2015). Bacteria use quorum sensing, a method, to regulate gene expression in a way that depends on cell density. Quorum sensing is used by Pseudomonas *aeruginosa* to control pathogenicity and biofilm development. The production of the N-acyl homoserine lactone (AHL) signal molecules, N-(3-oxododecanoyl)-L-homoserine lactone and N-butanoyl-L-homoserine lactone (C4-HSL) is carried out by P. *aeruginosa* two main quorum-sensing systems, Las and Rhl. The C4-HSL binding activates their respective corresponding transcription factors which causes the development of a variety of virulence factors as well as the creation of biofilms (Rutherford and Bassler, 2012). Pseudomonas *aeruginosa* has four distinct QS systems namely Rhl, Las, PQS and IQS. All the four systems have their own regulatory protein and signals also known as autoinducers. In the Rhl system, the regulatory protein is RhlR and the signal is C4-HSL which is produced by RhlI. To combat biofilm production, autoinducers are targeted in order to inhibit QS system (Wood et al., 2018). It is proposed that a promising approach to treating P. *aeruginosa* infections is to inhibit quorum sensing. This strategy has a low chance of bacterial resistance development, and it can prevent or diminish the production of biofilms

as well as bacterial pathogenicity. Furthermore, this method's narrow spectrum makes it unlikely that it may unintentionally suppress helpful microbes. The quorum sensing inhibitors for the Las and Rhl systems can be either natural or synthetic, and they have the ability to lower AHL synthase activity, prevent the generation of AHLs, degrade AHLs, or compete with AHL receptors for binding. Recent years have seen a lot of research on the management of Pseudomonas *aeruginosa* via the application of quorum sensing inhibitors (Pang et al., 2019).

2.8 Rhamnolipids

Rhamnolipid is one of the virulence factor produced by Pseudomonas *aeruginosa*. They are glycolipids providing surfactant properties. QS is controlled by expression of rhl genes which regulates the production of rhamnolipids. The various functions of rhamnolipids include uptake of hydrophobic substances, enhancing bacterial motility, mediating growth of biofilm, and regulating swarming motility of bacterial colonies which contributes to their virulence and survival. By reducing cell, rhamnolipids may impact the bacteria's ability to interact with its environment. Targeting rhamnolipids in drug development has therapeutic and biological benefits. One of their key function is disrupting the tight junctions between epithelial cells. This disruption has been observed in infections associated with presence of rhamnolipids (Proctor et al., 2021).

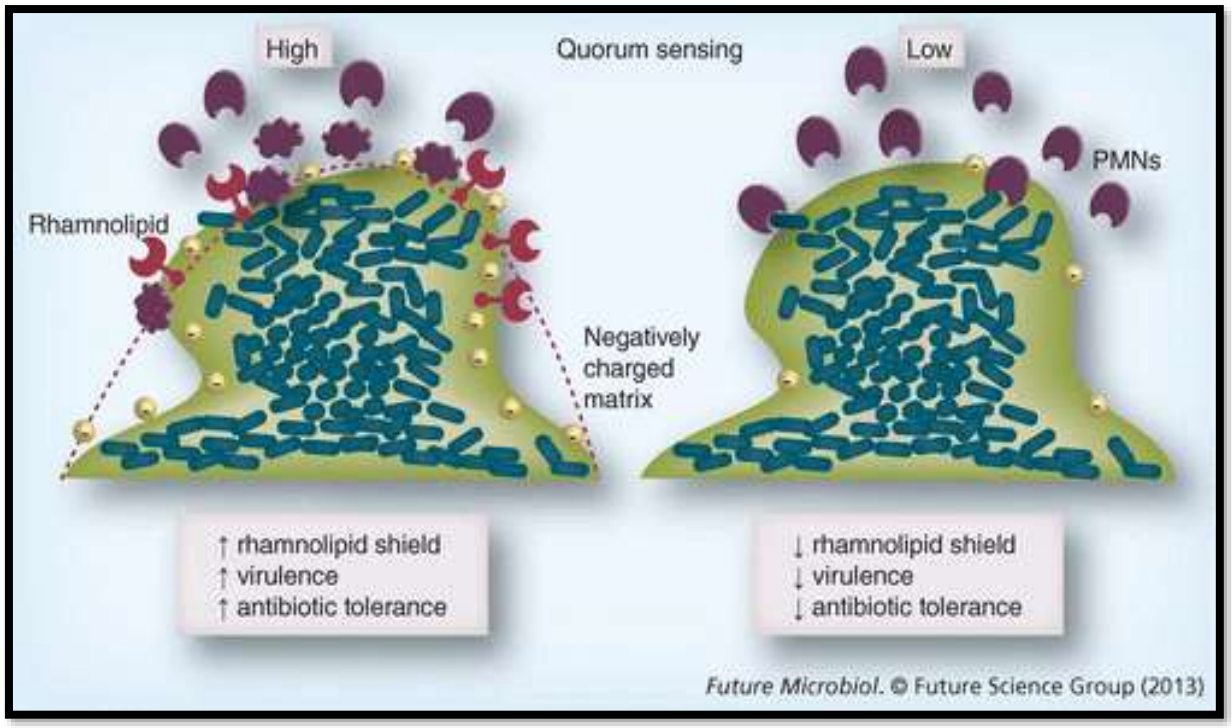


***Figure 2.7*: Rhamnolipids role as a virulence factor (Jakobsen et al., 2013)**

The rhamnolipids production requires various stages. While most bacteria have the necessary enzymes for precursor synthesis the enzymes involved in HAA, mono-rhamnolipid, and di-rhamnolipid synthesis are predominantly found in Pseudomonas and Burkholderia species. Las, rhl and pqs QS systems control the synthesis of rhamnolipids. It involves signal synthases, signal receptor protein and signal molecules. The precursor HAA which contributes to the hydrophobic part of rhamnolipids is produced by rhlA using B-hydroxydecanoyl-ACP from the fatty acid synthesis pathway. The enzymes RhlA, RhlB and RhlC play an important role in rhamnolipid biosynthesis. RhlA catalyzes HAA formation, RhlB is a Rhamnosyltransferase involved in mono-rhamnolipid synthesis, and RhlC is a Rhamnosyltransferase II responsible for di-rhamnolipid production (Chong and Li, 2017).

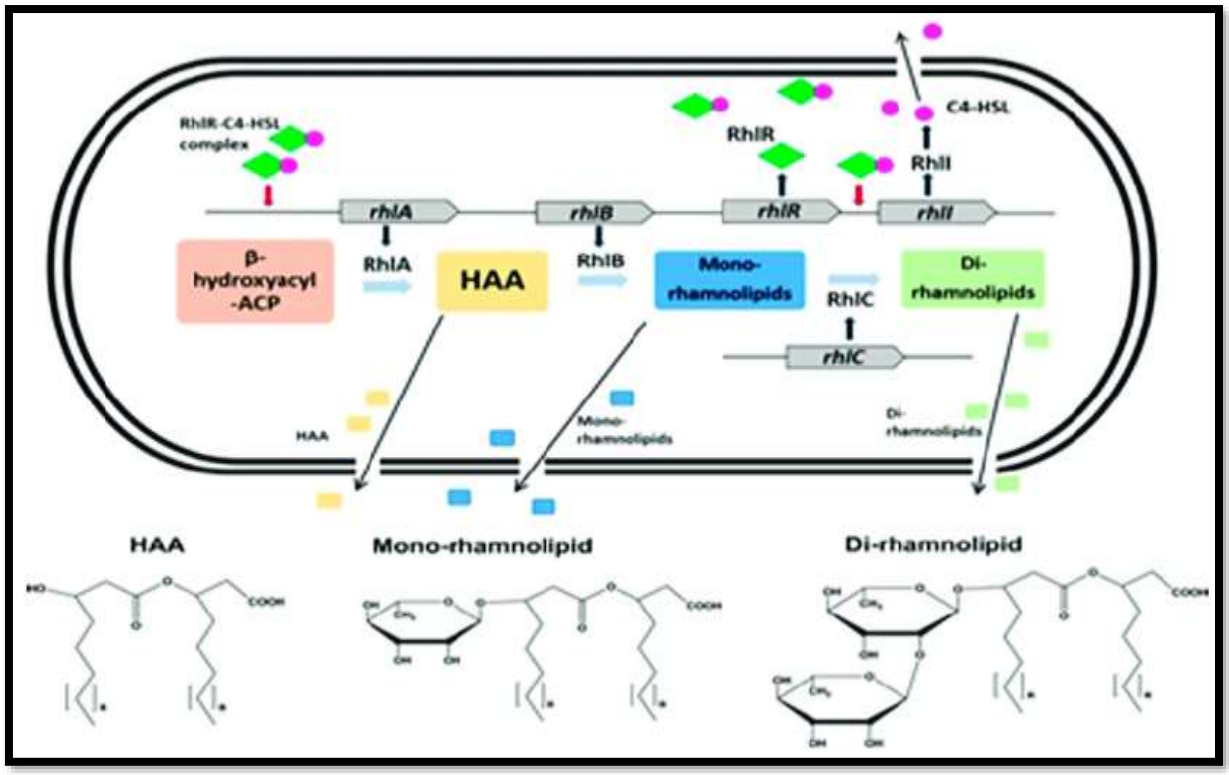


***Figure 2.8*: Synthesis of rhamnolipids (Wood et al., 2018)**

Microscopic analysis of biofilms formed on solid surfaces by Pseudomonas *aeruginosa* has shown cells embedded in EPS matrix containing open channels that facilitate nutrient access, oxygen supply and waste removal. Rhamnolipids have a significant impact on various aspects of Pseudomonas *aeruginosa* biofilm formation, including micro-colony formation, maintenance of open channels, mushroom cap development, and cell detachment. Rhamnolipids are amphipathic glycolipids produced by Pseudomonas *aeruginosa* through the involvement of rhlAB operon and rhlC genes, both regulated by QS (Ochsner and Reiser, 1995).

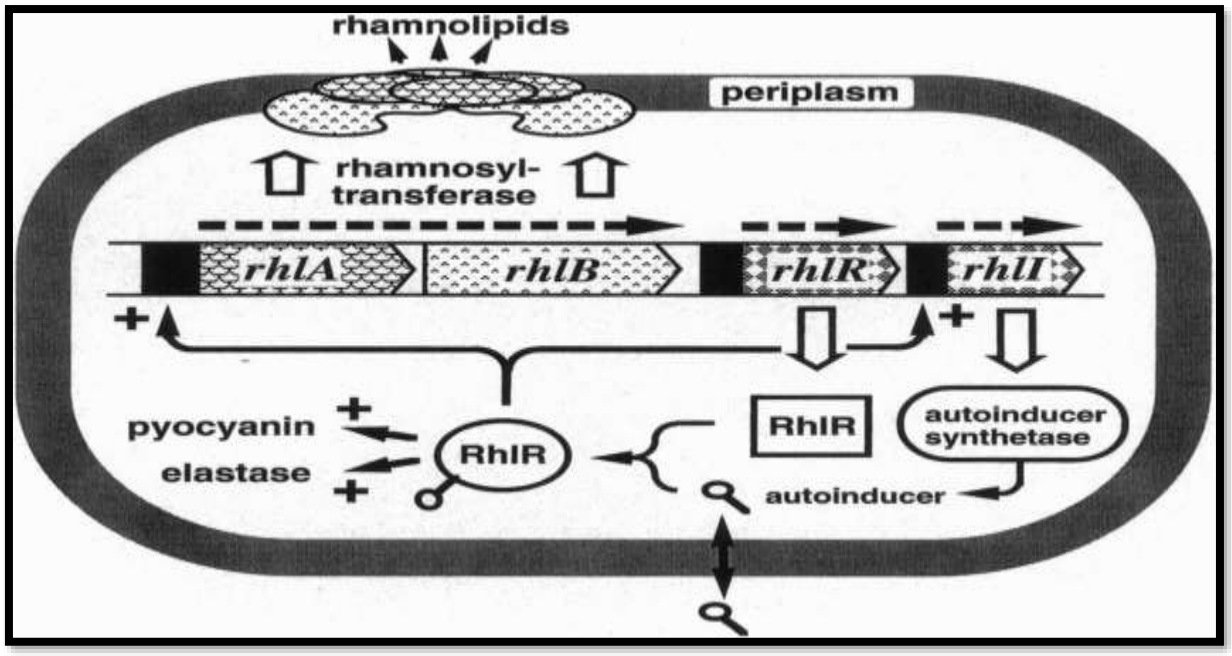


Figure 2.9: Regulation of Rhamnolipids (Ochsner and Reiser, 1995).

2.9 *In silico* approaches for drug designing

The parameters that were calculated in our molecular docking and dynamics investigation were the docking score, binding energy, RMSD (root mean square displacement), and RMSF (root mean square fluctuation). The evaluation of the drugs in relation to one another can be seen in the docking score and binding energy. RMSD demonstrates the docked complex's stability. The complex's conformational flexibility is indicated by the RMSF (Fatriansyah et al., 2022). Molecular dynamic simulation studies are used to investigate the structural stability of the protein and the protein-ligand complexes under physiological settings. For this, MD simulations were performed using the top compounds with the lowest binding energies. In investigations using molecular dynamic simulation, the movements of the atoms are estimated over time using Newton's classical equation of motion. In contrast, molecular docking only offers a static image of the ligand when proteins are actively bound to it and indicates the status of the ligand- protein binding. RMSF is used to investigate fluctuations relative to the overall structure of the protein or to evaluate the flexible region of the protein. At a given pressure and

temperature, it determines the average movement of atoms. A low RMSF value implies that the system will be stable, whereas a high number denotes that the system will be flexible during the MD simulation. The in silico methods, which include drug repositioning, cost reduction, and a shorter time to discovery, have substantially aided in the development of medications for a number of disorders. Numerous potential therapeutic targets for proteins targeted at treating a disease have been discovered through computational tools (Afzaal et al., 2022). Molecular docking and dynamics are strong in silico approaches to explore innovative drugs and have sound application in discovery of drugs (Salmaso and Moro, 2018).

CHAPTER 3

MATERIALS & METHODS

3. Materials and Methods

The methodology employed for designing of the suitable drug is shown in the figure.

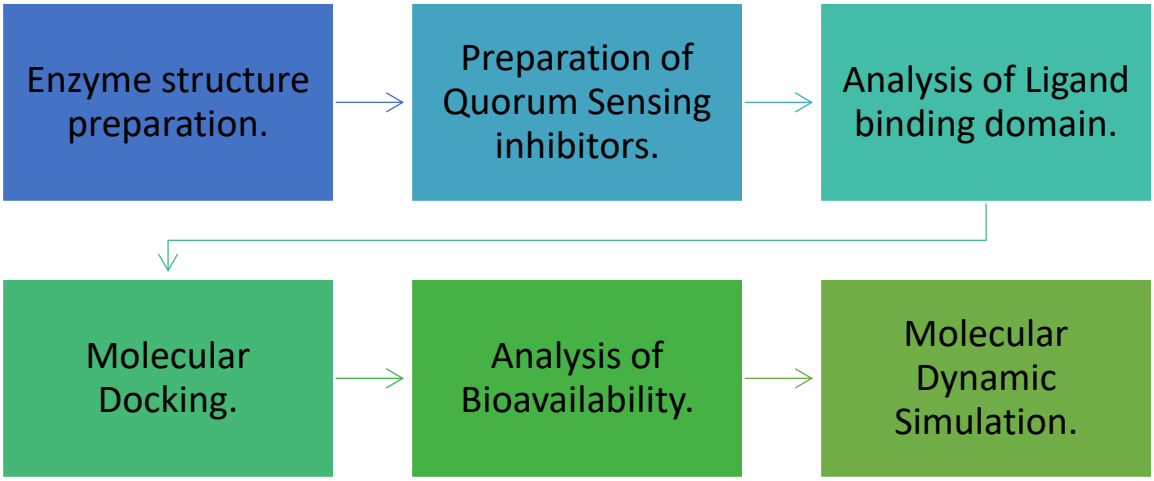


Figure 3.1: Summary of the approach used in the study to identify potential drug targets

Table 3.1: Tools and servers used in *in-silico* study.

Sr. No.	Steps performed	Tools or servers employed
1.	Extracting protein sequences as FASTA files.	UNIPROT
2.	Predicting the structure of proteins.	Phyre2
3.	Protein structure refinement.	GalaxyRefine
4.	Visualization of the refined proteins structure.	UCSF Chimera(v 1.15)
5.	Checking the protein quality	ERRAT
6.	Assessment of the protein structure.	PROCHECK
7.	Inhibitor extraction for both proteins.	ChEMBL

8.	Further Inhibitor extraction.	PubChem
9.	Analysis of ligand binding domain of the protein pocket.	CASTp 3.0
10.	Converting sdf files to pdb files	Open Babel
11.	Converting PDB files to PDBQT files.	Autodock Vina 1.5.7
12.	Site specific docking	PyRx Vina
13.	2D Line model of selected ligands with protein & Visualization of binding poses.	Discovery studio 2.0
14.	Toxicity prediction of the drug.	Pro Tox-II
15.	Determining drug accessibility based on physicochemical properties.	SwissADME
16.	Druggability Analysis.	ADMETlab 2.0
17.	Molecular Dynamic Simulation.	CABS-flex V 2.0

3.1 Enzyme structure preparation

For our scientific study, we have targeted two enzymes of the species Pseudomonas *aeruginosa.* The two enzymes are Rhamnosyltransferase subunit A which is encoded by the gene rhlA and Regulatory protein RHLR that is encoded by gene rhlR.

3.1.1 Extraction of protein sequences

The structure for both the enzyme was unavailable so we predicted the structure of the enzymes in order to understand the interaction of the enzymes with the ligands. Homology modelling is done due to the unavailability of the structure of both the proteins. Homology modelling is one of the most assuring way to narrow down the difference between the known sequences of protein and the structures obtained experimentally. The protein sequence of

Rhamnosyltransferase subunit A (UniProt ID-Q51559) and of Regulatory protein RHLR (UniProt ID: P54292) of Pseudomonas aeruginosa strain PAO1 was saved from the databases online available in FASTA format. UniProt available at https://www.uniprot.org and additional steps were performed for further analysis. Alternative names of both proteins could be found on databases such as Chembl and PubChem with their respective IDs.

3.1.2 Obtaining the structure of the proteins

The protein sequences obtained in FASTA file format were then submitted to the online available protein fold recognition server named Protein Homology/analogy Recognition Engine V 2.0 (Phyre 2). It is a free web-based service for protein structure prediction available at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index. An account was registered on the educational email address. The sequences of the proteins were then submitted separately as different job IDs. It took a few hours for each job to be done and the predicted protein models were obtained via email in PDB format.

3.1.3 Refinement of the protein structures

To bring the predicted modelled proteins closer to their native state, the PDB files of the proteins were submitted to Galaxy Refine (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) in hope to achieve protein structure refinement. The results are obtained in PDB format via email.

3.1.4 Visualization of the proteins

To visualize the modelled protein, the refined modelled structure obtained in the earlier step was subjected to University of California San Francisco (UCSF) Chimera (version 1.15). UCSF Chimera is a program available free of cost and is a source of interactive visualization.

3.1.5 Protein quality verification and structure validation

Several quality assessment tools were used to ensure the confirmation and evaluation of the protein quality models. These included Ramachandran plot evaluation for observing the arrangement in structure of angles with torsion, deviation in the structure, evaluation of

rotamers and clash scores for the entire atoms contact analysis. Such tools were valuable to determine the correctness of the three dimensional protein structure (Praznikar, Tomic, & Turk, 2019). Online server was used to check the protein quality and for structure assessment. The server used was PROCHECK(Laskowski, MacArthur, Moss, & Thornton, 1993) available at https://servicesn.mbi.ucla.edu/PROCHECK/ This was used to analyse the Ramachandran plot. Ramachandran plot gives us a 2D plot of the torsional angles of amino acids in a protein sequence and are considered as a top standard for evaling the value of experimental protein structures (Sobolev et al., 2020). The other server used was ERRAT available at https://servicesn.mbi.ucla.edu/ERRAT/. ERRAT verifies protein structures determined by crystallography and investigates the non-bonded links among atoms of different types based on their comparison with high-resolution refined 3-D structures (Dym, Eisenberg, & Yeates, 2012).

3.2 Preparation of Quorum Sensing inhibitors

The protein models were prepared and it was required to extract the inhibitors for the respective proteins. For a drug to be designed, it is mandatory to investigate protein inhibition pathways or the inhibition of signalling pathways of the protein. It is important to gather knowledge regarding investigations on similarity in ligands or proteins and incorporation of data regarding therapeutics. This connection is built by ChEMBL as it provides vast coverage of various targets, organisms and bioactivity measurements stated in literature (Gaulton et al., 2012). Moreover, ChEMBL is a user-friendly chemical database of molecules that are bioactive and consist of properties similar to drugs. It is available at https://www.ebi.ac.uk/chembl/. Other publicly available servers like PubChem available at https://pubchem.ncbi.nlm.nih.gov supports drug discovery in various ways such as providing opportunities for the examination of pharmacological mechanisms and the genetic basis of diseases which is key for drug repurposing and innovation (Cheng et al.,2014). To extract the inhibitors for both proteins,

ChEMBL and PubChem was used. The inhibitors were shortlisted on the basis of their IC50 value (half-maximal inhibitory concentration). The inhibitors were downloaded in a CSV file in zip form and an excel sheet of all the inhibitors were prepared separately for both the proteins. Moreover, the replicates were deleted after going through the compound IDs thoroughly. A final list of seventeen inhibitors for the protein rhlA and eight inhibitors for the protein RHLR was prepared. These inhibitors were the ligands that would bind to their respective protein in the later stages.

3.3 Analysis of ligand binding domain

To improve the docking algorithms, it is of value to analyse the ligand binding domain. It gives an insight regarding the flexibility of amino acid chain and is useful in studies related to protein engineering. In this step the analysis of the pocket or the domain of the receptor protein with which the ligand (inhibitor) would bind to was done. The structure of the protein is a complex one and has various surface pockets, cross channels and internal cavities. It is because of this key topographical feature of the protein that allows it to carry out ligand binding. Therefore, it is of extreme value to quantify and identify these topographic features and to understand the structure-function relationship of protein in order to develop therapeutic drugs against the protein targets (Tian et al., 2018). The pdb file of the refined protein structure was submitted to Computed Atlas of Surface Topography of proteins (CASTp) 3.0. It is a web server that is freely accessible at http://sts.bioe.uic.edu/castp/index.html?2r7g . CASTp 3.0 provides reliable and all-inclusive quantification and identification of the topography of the respective protein. After the job is completed, it gives out a list of the amino acids with their sequence ID and names. These amino acids represent the ligand binding domain. For both the proteins a list of the ligand binding amino acid was obtained.

3.4 Molecular Docking

Docking represents the interaction at an atomic level between a protein and a molecule. Docking allows us to portray the manner in which a small molecule is bonded in the pocket of a target protein. Moreover, it clarifies the basic chemical processes. Docking encompasses the calculation of the binding affinity and ligand conformation along with ligand's position and orientation within the pocket of the target protein (Meng XY et al.,2011). Docking is a virtual screening of a number of compounds(inhibitors) and their proposed structure of how they fit in with the receptor enzyme target. The results are given as docking score, binding affinity or binding energy. They show the strength of the interaction between the ligand and receptor. The lesser the binding energy, the greater the affinity between the inhibitor and the receptor protein. Binding energy is the energy released due to the bond formation or interaction of protein-ligand. The lesser the energy the tighter the binding and also the binding affinity. The energy of the favorable reaction is negative.

The QS inhibitors selected were on the basis of binding affinity's strength. A stable protein ligand complex is shown by stronger binding affinity.

3.4.1 Processing of files before docking

MGLTools is a software available at https://ccsb.scripps.edu/mgltools/downloads/ For molecular structure's analysis and visualization, this tool has been developed in The Scripps Research Institute's Molecular Grapphics Laboratory (MGL).

One of its application is AutoDock Vina 1.5.7. MGL tools was downloaded. Sdf files of the ligands were extracted from PubChem. Another software used was Open Babel. It is available at https://download.cnet.com/OpenBabel/3000-2054_4-75764428.html . Open Babel was used to convert sdf files into pdb format. AutoDock Vina 1.5.7 was then used to convert the pdb files of the ligands as well as proteins into pdbqt format. Both the ligands(inhibitors) and the receptor protein needs to be processed before docking takes place. In this processing the water

molecules are removed as they could cause hindrance and would not let the ligand sit in the protein pocket properly. Also, hydrogens are added as to make the net charge of the protein zero. Once the proteins and ligands are processed, they are ready for docking.

3.4.2 Site-specific docking

There are fundamentally two types of docking: blind docking and site-specific docking. When carrying out site-specific docking, binding residues and structural details are known whereas they remain unknown in the blind docking. The basic difference lies in the prediction of binding pocket. It is known in the site-specific docking. The ligand is docked in the binding pocket in the site-specific protein, on the other hand, in blind docking the whole protein is docked with ligand. Site-specific docking is considered to have a fast computation time and is precise. It encloses the binding pocket only inside the grid box whereas the entire protein is enclosed inside the grid box. The short listed inhibitors against each receptor protein were docked against their respective proteins by using PyRx as it is able to dock multiple ligands against a specific target simultaneously (Dallakyan & Olson, 2015).

PyRx is available at https://sourceforge.net/projects/pyrx/ . The shortlisted inhibitors, seventeen for rhlA and eight for rhlR were docked against their respective proteins.

3.5 Analysis of bioavailability

Bioavailability of the drug is the part of the drug that arrives in the systemic circulation. Any loss in the drug could be because of metabolism and incomplete absorption (Agoram et al.,2001). The bioavailability analysis is of key importance as it determines the validity of the compound as a drug.

To verify a drug accessibility is it of value to look at the pharmacophoric and toxicological properties. Various physicochemical properties of the inhibitors that were shortlisted to be drugs were predicted. These physicochemical properties included Lipinski's rule of five (H-bond donor <5, H-bond acceptor <10, molecular weight <500 Da and cLogP <5), prediction of

toxicity (mutagenic, irritant and tumorigenic) and drug-likeness (physicochemical properties such as topological polar surface area (TPSA), molecular weight, logP). Three websites that are user friendly and free were used to evaluate various parameters of our predicted drug and their drug-likeness.

3.5.1 SwissADME

SwissADME is a freely available web tool accessible at http://www.swissadme.ch . It is user friendly and the result analysis is easy. SMILES for each inhibitor is submitted and the result comes out in a few minutes. It has non exhaustive various input methods, submission of multiple molecules that can later be displayed, saved and shared as individual molecules, also, in form of interactive graphs (Daina et al.,2017).

i. Drug-likeness

- Lipinski's rule of five.

Lipophilicity is of key importance in drug-likeness and prediction of absorption. LogP is the predictor of lipophilicity (Amezqueta et al.,2020).

- Bioavailability score.

ii. Pharmacokinetics

It is a measure of how good a drug is when it enters our body.

- Blood Brain Barrier (BBB) permeation.
- Skin permeability

iii. Water solubility.

iv. Physicochemical properties

- Molecular weight (g/mol)
- TPSA

TPSA adds to the molecular (usually Van der Waals) surface area of polar atoms, for instance, nitrogen, oxygen and their bonded hydrogens. It looks for the capability of the drug to enter

cells and tissues. Also, measures the solvent accessible surface taken over by hydrogens, oxygen and nitrogen (Prasanna et al.,2009)

v. Medicinal Chemistry

- Synthetic accessibility
- PAINS

Pan Assay Interference (PAINS) are compounds that show strong response in assays regardless of the target protein. They give out false positive biological output. If such fragments are found in the molecule under observation, SwissADME gives a warning (Daina et al.,2017).

3.5.2 Pro Tox-II

Pro Tox-II is a freely available computational toxicity prediction webserver available at https://tox-new.charite.de/protox_II/index.php?site=compound_input which facilitates in prediction of toxicological endpoints. The input is given in form of SMILES or you can also draw the 2D display of the compound. It categorises the results in different toxicity aspects (Banerjee et al.,2018). This study mainly focused on toxicity end points such as cytotoxicity, carcinogenicity and mutagenicity. The results came out as active or inactive along with the confidence score.

3.5.3 ADMETlab 2.0

It is an online server freely available at https://admetmesh.scbdd.com/service/screening/index which evaluates properties such as absorption, distribution, metabolism, excretion and toxicity. The entry is made in SMILES form, once its submitted, the results are generated immediately. This study focused on a few aspects.

i. Toxicity

- Human hepatotoxicity (H-HT).

The results are given off as the probability of being toxic. It ranges from 0 to 1.0 where 0.7 to

1.0 is an indicator of poor drug which can induce liver injury. 0.3 to 0.7 as medium and the range from 0.0 to 0.3 as being excellent.

- Ames Toxicity.

Ames test is a test for recognition of carcinogens by using mutagenicity in bacteria as an end point. Mutagenicity in Ames test and carcinogenicity in animals has shown a correlation. The results are given off as the probability of being toxic. It ranges from 0 to 1.0 where 0.7 to 1.0 is an indicator of poor drug. 0.3 to 0.7 as medium and the range from 0.0 to 0.3 as being excellent.

- Carcinogenicity.

It is a drug's ability to damage the genome or disturb the cellular metabolic pathways. The results are given off as the probability of being toxic. It ranges from 0 to 1.0 where 0.7 to 1.0 is an indicator of poor drug. 0.3 to 0.7 as medium and the range from 0.0 to 0.3 as being excellent.

ii. Absorption.

- Human Intestinal Absorption (HIA).

HIA is a substitute pointer for oral bioavailability. There is a close relationship between intestinal absorption and oral bioavailability.

- Human colon adenocarcinoma (Caco-2) cell lines permeability.

Processes like active transport, passive diffusion and carrier mediated uptake help drug pass through cell membranes of intestines before they reach their destination in systemic circulation. Since Caco-2 has functional and structural similarities to the human intestinal epithelium, it is used to evaluate in vivo drug permeability. Caco-2 permeability is given as the log cm/s.

- Madin-Darby Canine Kidney (MDCK) cells permeability.

It is an in vitro permeability screening model. It evaluates the absorption efficiency of drugs in

the body. It also estimates the effect of the blood brain barrier. The unit for its calculation is cm/s.

3.6 Molecular Dynamic (MD) Simulation

It is an in silico method for the evaluation of the atom location in space. MD simulations was done using CABS-flex V 2.0 available at http://biocomp.chem.uw.edu.pl/CABSflex2 (accessed on 15th November 2022). The server generated an output file and RMSF fluctuation plot. Ten protein-ligand complexes were yielded. The first model is chosen because of its enhanced stability.

CHAPTER 4

RESULTS

4. Results

4.1 Preparation of enzyme structure

The two targeted enzymes of the species Pseudomonas aeruginosa in this study are Rhamnosyltransferase subunit A and Regulatory protein RHLR.

4.1.1 Protein sequences retrieval from UniProt

Since the structure of both the proteins were not present, it was obtained through homology modelling. Protein sequences retrieved from UniProt were saved in FASTA files.

Table 4.1 Primary bioinformatics analysis of the selected protein.

Enzymes	UniProt ID	Length	Biological Process
Rhamnosyltransferase subunit A	Q51559	295	Rhamnolipid biosynthesis
Regulatory protein RHLR	P54292	241	Positive regulation of lipid biosynthetic process.

4.1.2 Protein refinement, verification and structure validation

Various online servers were used to get a protein structure. Phyre2 was used to obtain the structure of the proteins and Galaxy Refine was used to obtain refined protein structure. ERRAT was used to verify protein quality factor. The result of overall quality factor in ERRAT server for both the proteins were:

- Rhamnosyltransferase subunit A =91.837
- Regulatory protein RHLR=89.565

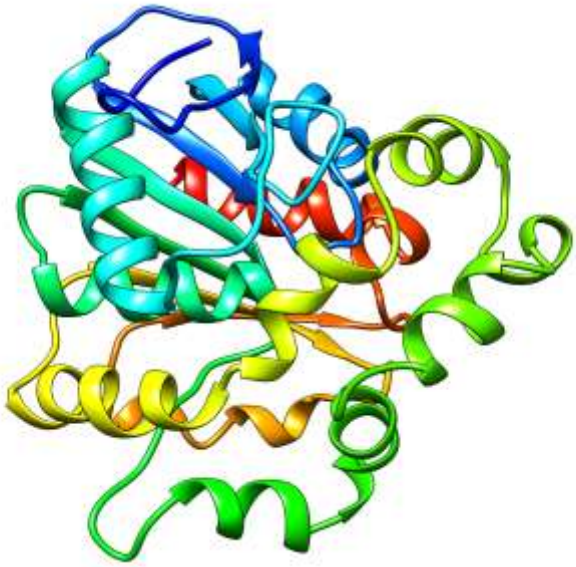


Figure 4.1: Protein before homology modelling and refinement

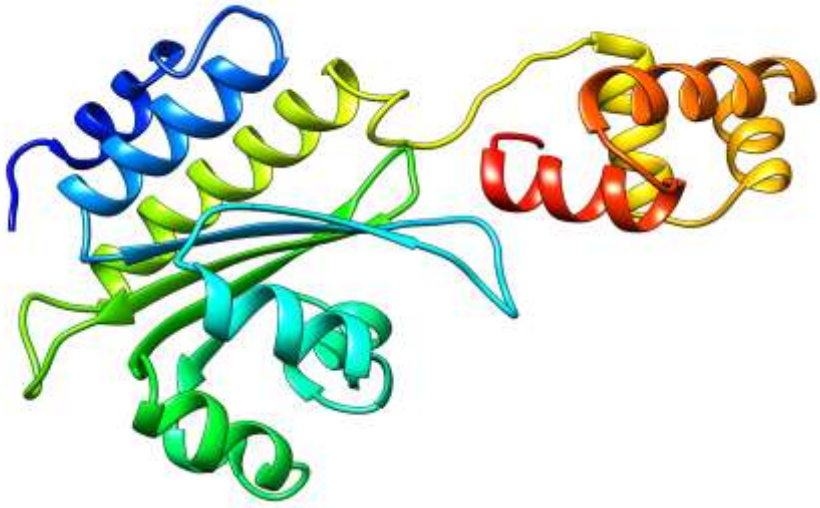


Figure 4.2: Protein after homology modelling and refinement

4.2 Quorum Sensing inhibitors

Online servers such as CHEMBL and PubChem were used to generate a list of inhibitors with certain parameters. The list was refined by deleting the replicates. A final list of seventeen inhibitors for the protein rhlA and eight inhibitors for the protein RHLR was prepared. These inhibitors were the ligands that would bind to their respective protein in the later stages.

Table 4.2 List of inhibitors for Rhamnosyltransferase subunit A.

Compound ID	Compound listed as
24739090	1a
155529330	2a
155529967	3a
102423480	4a
56662633	5a
56666096	6a
56679650	7a
56673001	8a
56666098	9a
56669500	10a
56662632	11a
56659135	12a
56666097	13a
56673000	14a
56666095	15a
56672999	16a
10130163	17a

Table 4.3 List of inhibitors for Regulatory protein RHLR.

Compound ID	Compound listed as
87074839	1r
117976096	2r
154573765	3r
5386591	4r
442793	5r
5317596	6r
3477374	7r
5386591	8r

4.3 Evaluation of the ligand binding domain

It is of key importance to figure out the part of the enzyme where our inhibitors (ligands) would bind to. The analysis of the topographical feature of the enzyme pocket helps in better results obtained when docking is applied. Moreover, it gives an enhance understanding of the relationship of function and structure of the protein and the drug intended to be developed against it.

CASTp 3.0 is an online web server used to evaluate the enzyme pocket. It gave out a list of amino acids with their sequence ID and names. These amino acids represented the pocket of the enzyme where the inhibitor would most likely bind to during docking.

Table 4.4: Amino acids and their sequence ID in the pocket for Rhamnosyltransferase subunit A.

Sequence ID	Amino acid
34	ASN
35	GLY
36	ALA
37	MET
38	ALA
42	SER
43	PHE
77	ILE
101	ALA
102	SER
103	TRP
106	ILE
109	LEU
110	LEU
125	MET
126	ALA
127	PHE
128	ALA
129	PRO
130	GLY

131	LEU
132	ASN
138	TYR
145	LEU
147	GLU
148	LEU
151	LYS
154	ILE
171	LYS
172	ALA
173	SER
174	ASN
177	HIS
181	LEU
186	TYR
190	ARG
192	HIS
193	ILE
195	GLN
196	VAL
199	LEU
200	ASN
203	GLY

224	GLU
225	TYR
226	THR
230	ASP
251	HIS
252	PHE
253	LEU
254	ASP
255	LEU
256	GLU

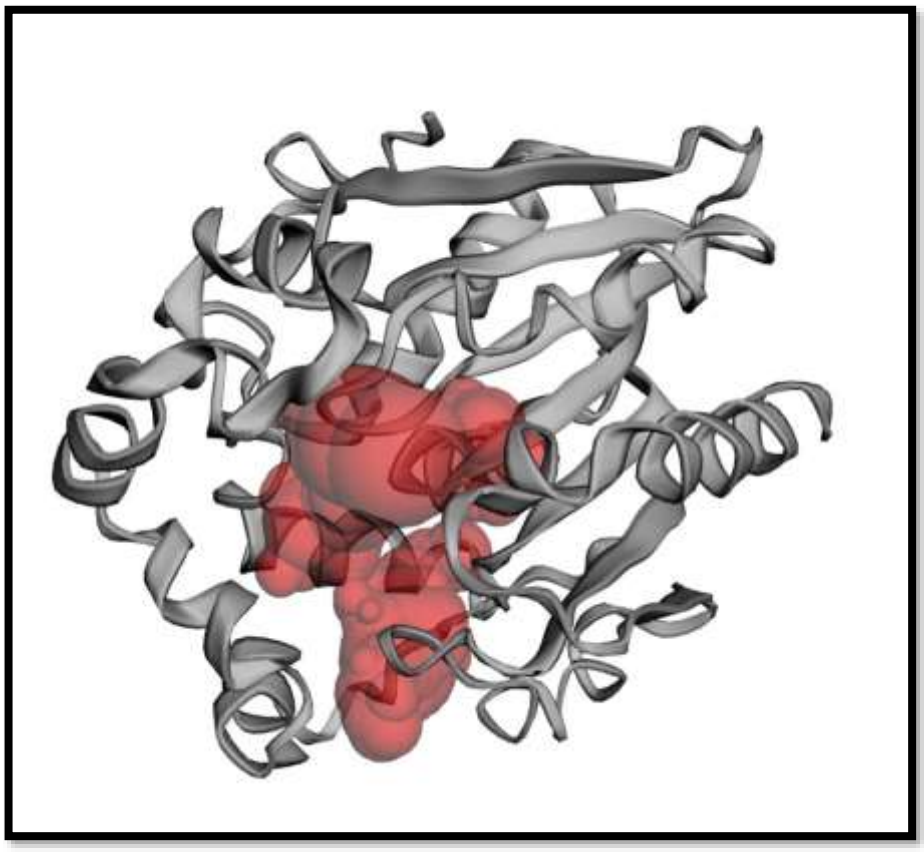


Figure 4.3: The binding pocket in the protein Rhamnosyltransferase subunit A.

Table 4.5 Amino acids and their sequence ID in the pocket for Regulatory protein RHLR.

Sequence ID	**Amina acid**
44	ALA
46	GLY
48	ARG
54	THR
55	ARG
56	PRO
58	THR
60	VAL
62	GLY
64	TYR
68	TRP
69	LEU
72	TYR
73	GLN
76	ASN
78	GLY
79	ALA
81	ASP
83	ALA
84	ILE

96	TRP
101	PHE
107	LEU
108	TRP
111	ALA
133	VAL
135	SER

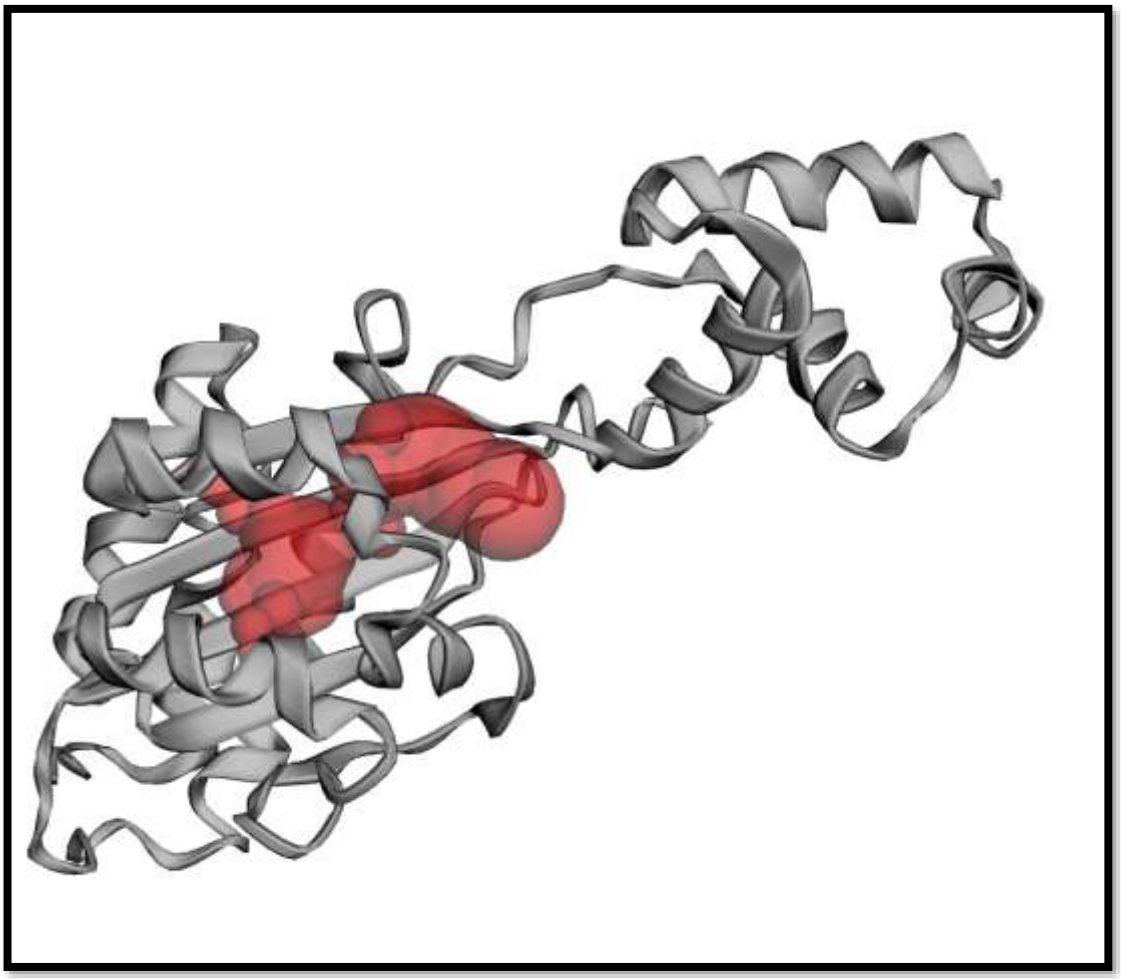


Figure 4.4: The binding pocket in the protein Regulatory protein RHLR.

4.4 Molecular Docking results

Molecular docking is an *in silico* technique that predicts the binding affinity with which a ligand would bind to the groove of the receptor. The orientation and conformation of the ligand-receptor complex is analysed. Before the docking was applied, the inhibitors and the proteins were processed. MGL tools and OpenBabel were used to process these files. AutoDock Vina was used to delete water molecules as they interfere with ligand and will not let it sit in the groove properly. Water molecules can cause hindrance in the binding of the receptor and ligand. Site-specific docking was carried out since it gives out precise results. PyRx was used to carry out docking using multiple ligands at a time.

The results of the docking are given off as docking score, binding affinity or binding energy. PyRx gives result in binding affinity. The affinity between the inhibitor and the receptor protein is more if the binding energy is less. Binding and affinity is stronger and tight when the binding energy is low. The energy of the favorable reaction is negative.

The result gives out ten models for each ligand. The best model with the most negative binding affinity is chosen as the preferred model. Discovery studio 2.0 was used to see the results of the molecular docking. 2D line model of selected ligands with protein and visualization of binding poses was observed via Discovery studio 2.0.

Table 4.6 Docking results for the ligands against Rhamnosyltransferase subunit A.

Ligands	Binding Affinity (kcal/mol)
RHLA_1a	-8.2
RHLA_2a	-7.3
RHLA_3a	-6.2
RHLA_4a	-5.2
RHLA_5a	-4.8
RHLA_6a	-5.8
RHLA_7a	-4.6
RHLA_8a	-5.6
RHLA_9a	-5
RHLA_10a	-5.2
RHLA_11a	-5.8
RHLA_12a	-5.7
RHLA_13a	-5.5
RHLA_14a	-5.1
RHLA_15a	-4.6
RHLA_16a	-5.7
RHLA_17a	-5.7

Table 4.7 Docking results for the ligands against Regulatory protein RHLR.

Ligands	Binding Affinity (kcal/mol)
RHLR_1r	-6.9
RHLR_2r	-6.4
RHLR_3r	-4.5
RHLR_4r	-4.4
RHLR_5r	-6
RHLR_6r	-5.9
RHLR_7r	-5.4
RHLR_8r	-5.4

−4.6 to −7.3 kcal/mol is the binding affinity range for Rhamnosyltransferase subunit A and from -4.4 to -6.9 kcal/mol for Regulatory protein RHLR. The more the negative binding affinity is, the tighter the binding and affinity. Two ligands against each protein were used with the most negative binding affinity.

Table 4.8 Selected ligands of Rhamnosyltransferase subunit A.

Ligand name	Name	Molecular formula
1a	Amorfrutin B	$C_{26}H_{32}O_4$
2a	[2-[4-(Trifluoromethyl)phenyl]quinolin-8-yl] heptanoate	$C_{23}H_{22}F_3NO_2$

Table 4.9 Docking interactions of selected two ligands of Rhamnosyltransferase subunit A.

Common residues are highlighted.

Ligand name	Binding affinity(kcal/mol)	No. of binding residues	Interacting amino acid residues
1a	-8.2	9	HIS251,TYR225,MET37,ALA36,GLU 224,SER102,TRP103,PHE127,LYS15 1
2a	-7.3	6	HIS251,TYR225,MET37,ALA36,ASP 150,PHE252

RMSDs of the docked ligand 1a and 2a was 0.000Å.

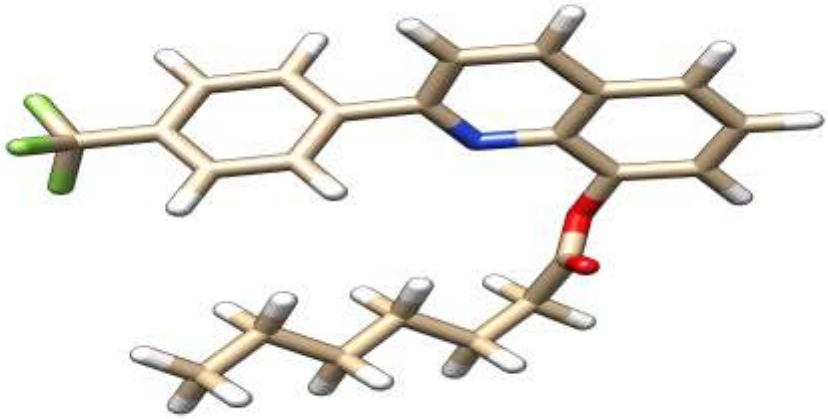


Figure 4.5: 3-D structure of Ligand 1a with Rhamnosyltransferase subunit A.

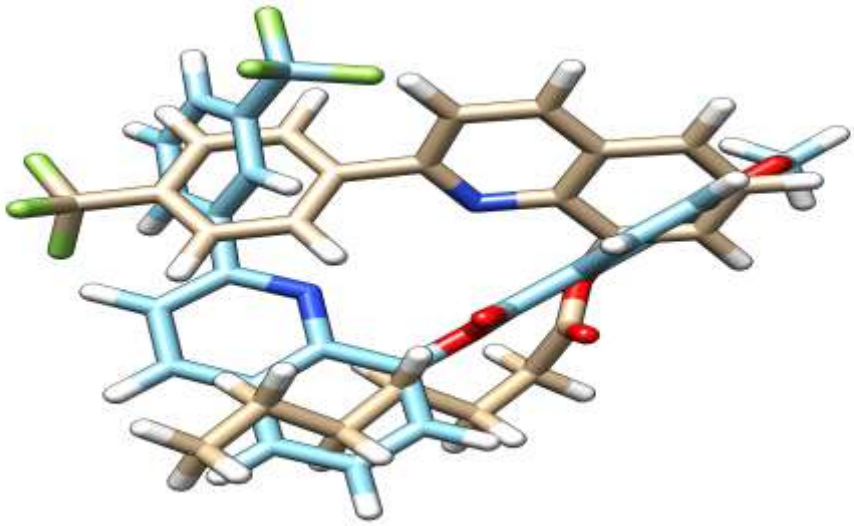


Figure 4.6: 3-D structure of Ligand 2a with Rhamnosyltransferase subunit A.

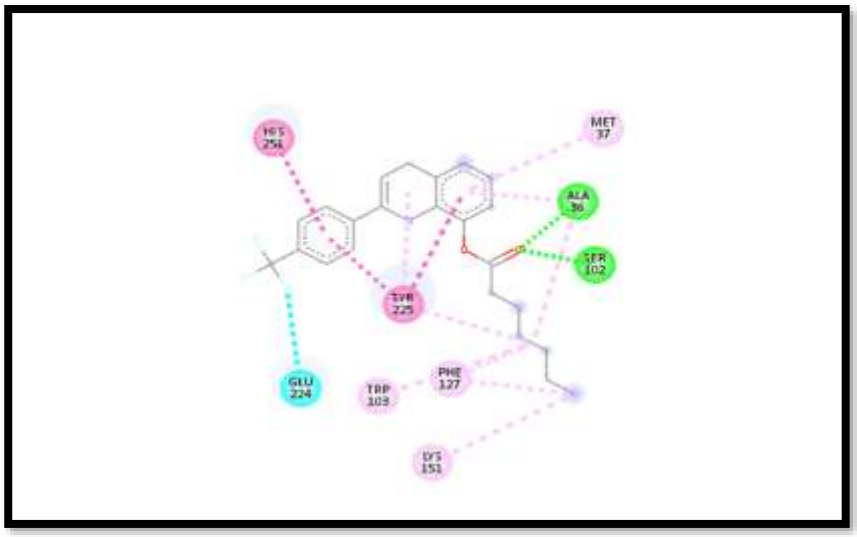


Figure 4.7 (a): Docking model of ligand 1a with Rhamnosyltransferase subunit A.

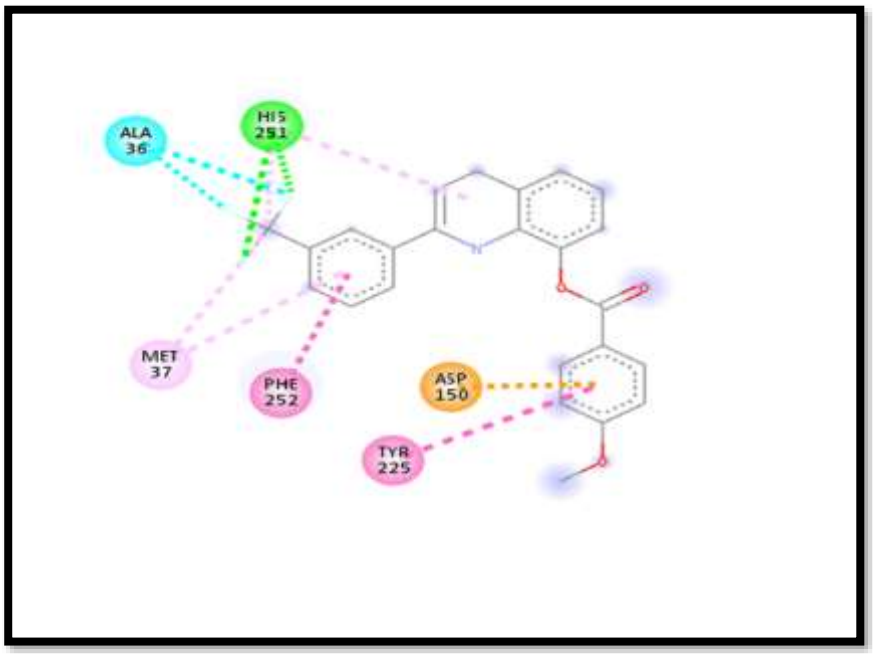


Figure 4.7 (b): Docking model of ligand 2a with Rhamnosyltransferase subunit A.

Ligand 1a possesses two conventional hydrogen bond with Ala36 and Ser102; two pi-pi Stacked interaction with His251 and Tyr225; one halogen interaction with Glu224; Lys 151 show alkyl interaction and three pi-alkyl with Trp103, Phe127 and Met 37.

Ligand 2a possesses one alkyl interaction with Met37, one anion interaction with Asp 150, one halogen interaction with Ala36, one conventional hydrogen bond with His251and two Pi-pi T shaped relation with Phe252 and Tyr225.

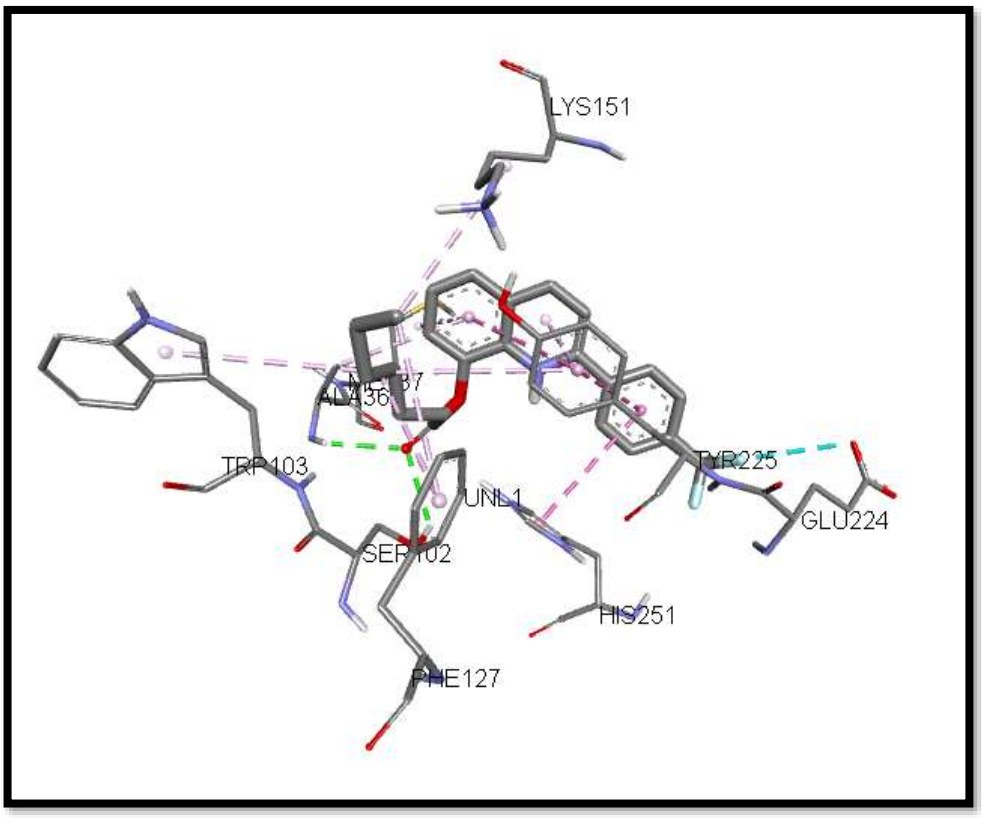


Figure 4.8 (a): Binding poses and interacting residues for ligand 1a for Rhamnosyltransferase subunit A.

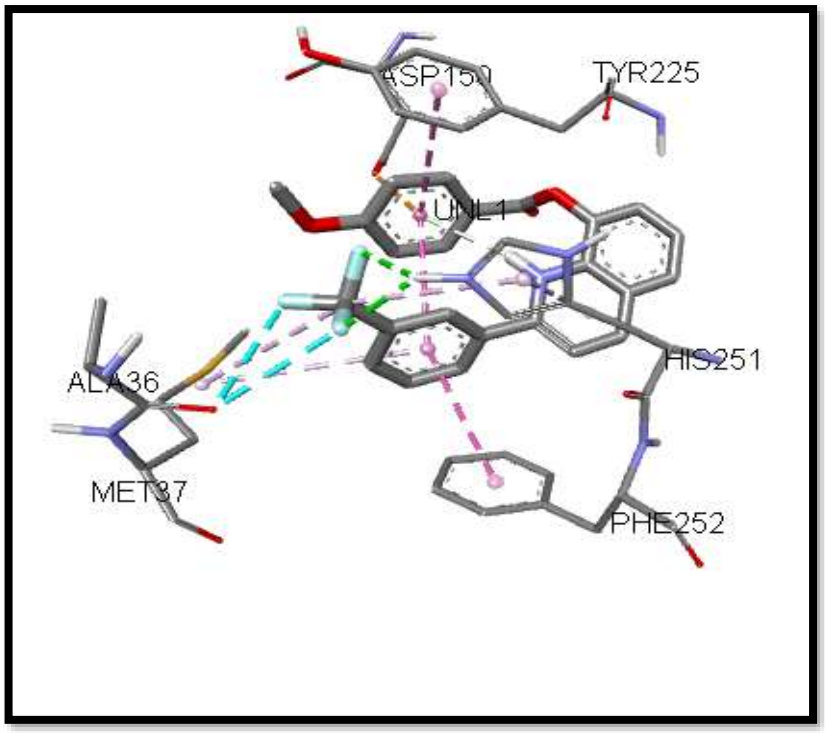


Figure 4.8 (b): Binding poses and interacting residues for ligand 2a for Rhamnosyltransferase subunit A.

Table 4.10 Selected ligands of Regulatory protein RHLR.

Ligand name	**Name**	**Molecular formula**
1r	Allyl benzothiazol-2-yl disulfide	$C_{10}H_9NS_3$
2r	5-(3-bromophenoxy)-N-(2-oxothiolan-3-yl)pentanamide	$C_{15}H_{18}BrNO_3S$

RMSDs of the docked ligand 1r and 2r was 0.000Å. it is an indication that pose of ligand binding and enzyme structure has no noteworthy change. This indicates that there is no significant variability in enzyme structure and ligand binding pose. Six of the interacting residues are same as that of ligand 2r showing that they both connect to the pocket in a manner that is alike.

Table 4.11 Docking interactions of selected two ligands of Regulatory protein RHLR. Common residues are highlighted.

Ligand name	**Binding affinity(kcal/mol)**	**No. of binding residues**	**Interacting amino acid residues**
1r	-6.9	11	ARG55,GLN73,TYR72,ARG48, THR58,ALA44,ILE84,TRP68, VAL60, VAL133, TYR64
2r	-6.4	8	GLY46, GLY78, TYR72, ARG48, ALA44, ILE84, VAL60, VAL133.

Figure 4.9 (a): 3D structure of ligand 1r of Regulatory protein RHLR.

Figure 4.9 (b): 3D structure of ligand 2r of Regulatory protein RHLR.

Ligand 1r possesses four hydrogen bonds with Gln73, Arg48, Arg55, Thr58, and two bonds with Glu305; with Gly279- a carbon hydrogen bond. Three pi-alkyl interactions with Trp68, Val133 and Tyr64; and three alkyl interactions with Ala44, Ile84 and Val60. Tyr72 is involved in unfavorable acceptor-acceptor interaction.

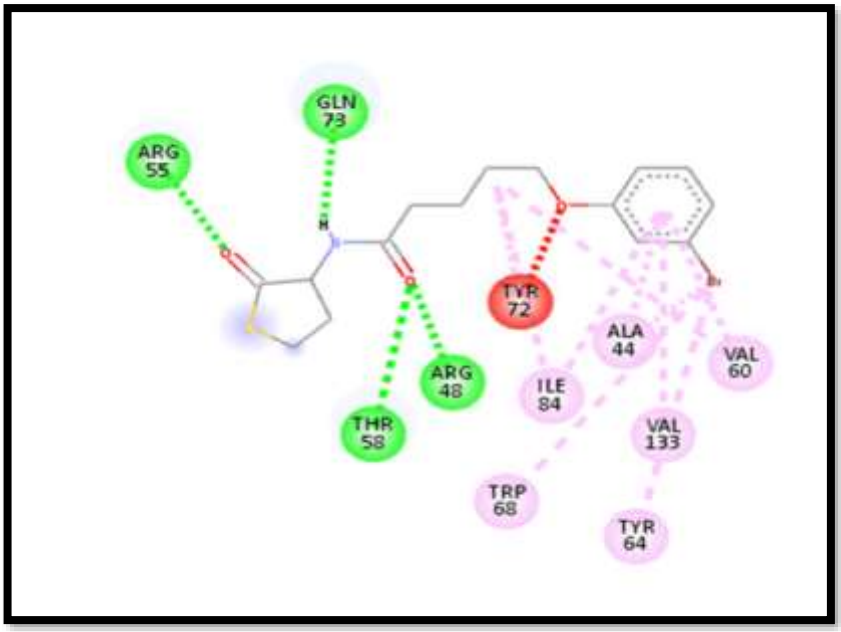


Figure 4.10 (a): Docking model of ligand 1r with Regulatory protein RHLR.

Ligand 2r possesses one conventional hydrogen bond with Arg48, two halogen interactions with Val133, and Ala44, one hydrogen carbon bond with Gly78. It has one pi-alkyl connection with Tyr72 and three pi-sigma interactions for Gly46, Val60, Ile84.

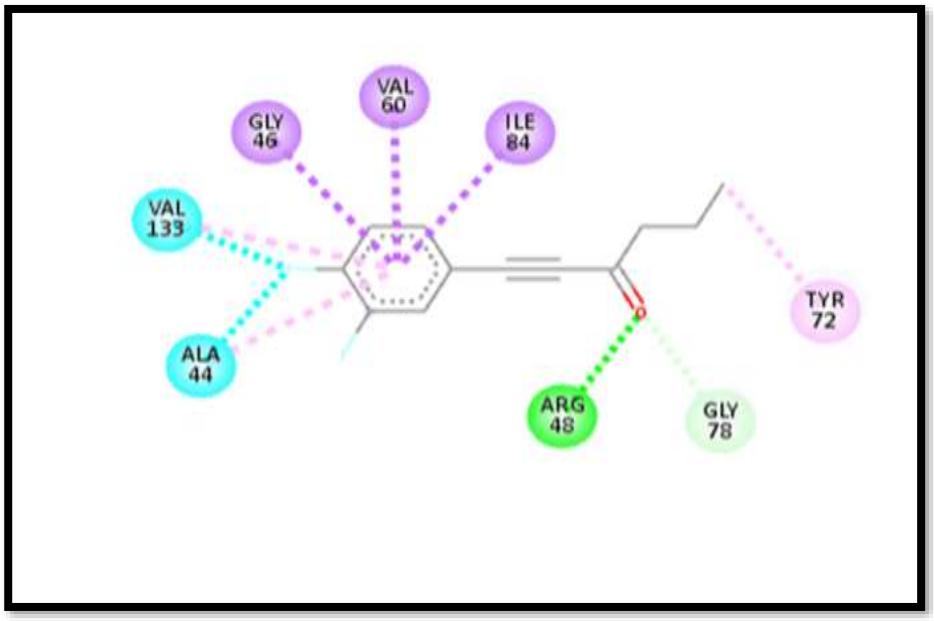


Figure 4.10 (b): Docking model of ligand 2r with Regulatory protein RHLR.

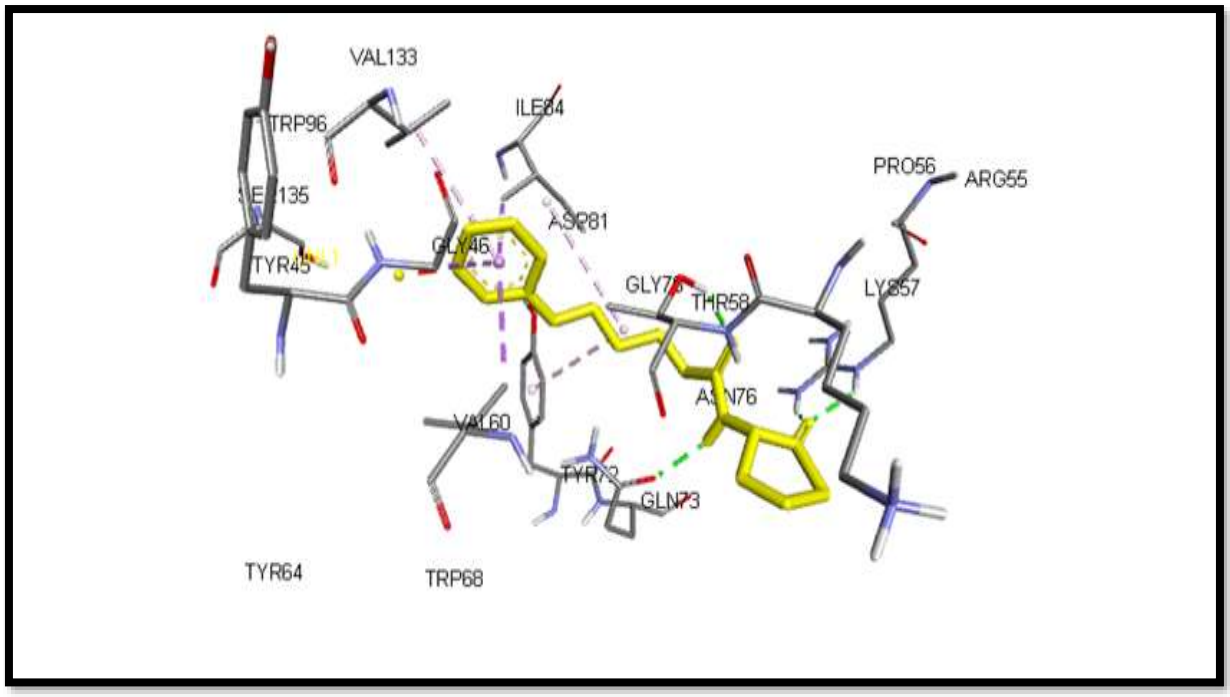


Figure 4.11 (a): Binding poses and interacting residues for ligand 1r for Regulatory protein RHLR.

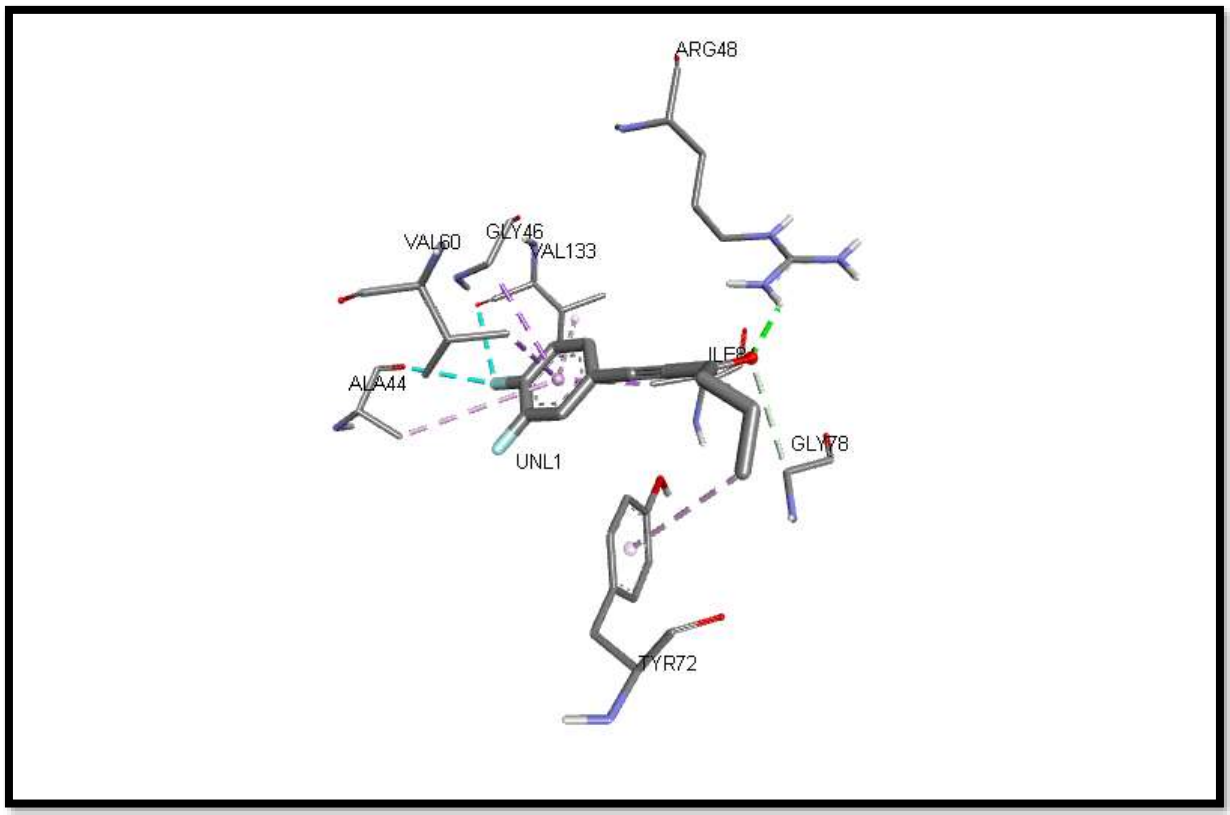


Figure 4.11 (b): Binding poses and interacting residues for ligand 2r for Regulatory protein RHLR.

4.5 Assessment of bioavailability

Various online tools and servers were used to predict the physicochemical properties which included Lipinski's rule of five, prediction of toxicity and drug-likeness.

4.5.1 Lipinski's rule of five.

These are four parameters that state:

i. Hydrogen bond acceptor < 10,

ii. Molecular weight <500 g/mol,

iii. Hydrogen bond donor <5

iv. cLogP < 5.

logP is a predictor of lipophilicity and absorption of a drug. Breaking one or more rule does not mean that a drug is ineffective. These rules aim to predict the absorption and permeability of a drug. SwissADME was used to calculate the result of Lipinski's rule of five.

Table 4.12 Rule of five for the ligands of both the proteins, RHLR & RHLA.

ligands	**1r**	**2r**	**1a**	**2a**
MW < 500 Da	239.38	372.28	408.53	401.42
H-bond donor < 5	0	1	2	0
H-bond acceptor <10	1	3	4	6
clogP <5	3.85	2.93	5.63	6.22
violations	0	0	1	1

4.5.1 Drug-likeness of selected ligands

The online server SwissADME is for the assessment of the selected ligands. Topological polar surface area describes the drug transport regarding absorption in intestines and blood brain barrier. It is ideally between 20 A^2 to 130 A^2. Synthetic accessibility ranges from 1to10. 1 being very easy and 10 being very difficult.

Table 4.13 Properties of selected ligands extracted via SwissADME.

Ligand	**1r**	**2r**	**1a**	**2a**
Physicochemical properties • MW (g/mol) • TPSA (A^2)	 239.38 91.73	 372.28 80.70	 408.53 g/mol 66.76	 401.42 39.19
Medicinal Chemistry • PAINS • Synthetic Accessibility	 0 alert 2.90	 0 alert 3.16	 0 alert 3.62	 0 alert 2.99
Pharmacokinetics • BBB Permeation • Skin Permeability (cm/s)	 No -4.21	 No -6.32	 No -3.38	 No -3.82
Lipophilicity	3.85	2.93	5.63	6.22
Water Solubility	Moderate	Moderate	Poor	Poor
Drug-likeness • Bioavailability Score • Rule of five	 0.55 0 violation	 0.55 0 violation	 0.85 1 violation	 0.55 1 violation

4.5.2 Outcomes of Pro Tox-II

This study mainly focused on toxicity end points such as carcinogenicity, mutagenicity and cytotoxicity. The results came out as active or inactive along with the confidence score.

Table 4.14 Toxicity end point outcomes of the compounds.

Compound	1a	2a	1r	2r
Carcinogenicity	Inactive	Inactive	Inactive	Inactive
Confidence score	0.62	0.62	0.62	0.62
Mutagenicity	Inactive	Inactive	Inactive	Inactive
Confidence score	0.78	0.97	0.97	0.68
Cytotoxicity	Inactive	Inactive	Inactive	Inactive
Confidence score	0.85	0.93	0.93	0.61

4.5.3 Outcomes of ADMETlab 2.0

The result came out as scores. The less the score, the more the ability of a compound for being a drug. The output score is a prediction of toxicity and absorption. It ranges from 0 to 1.0 where 0.7 to 1.0 is an indicator of poor drug. 0.3 to 0.7 as medium and the range from 0.0 to 0.3 as being excellent.

Table 4.15 Compound scores for toxicity.

Compound	1r	2r	1a	2a
Human hepatotoxicity	0.042	0.104	0.924	0.062
Ames	0.965	0.166	0.002	0.057
Carcinogenicity	0.687	0.189	0.092	0.213

HIA, Caco-2 permeability and MDCK permeability test were performed via the ADMETlab server. The results came out as scores. HIA represents the human intestinal absorption. Caco-2 test estimates the in vivo drug permeability and MDCK test assesses the uptake ability of compounds into body. The higher the score, the lower the absorption or poor results of the given test. Scores for MDCK test, >2 x 10-6cm/s represents excellent otherwise poor absorption.

Table 4.16 Compound scores for absorption.

Compound	**1r**	**2r**	**1a**	**2a**
HIA	0.004	0.22	0.011	0.002
Caco-2	-4.473	-4.597	-4.906	-4.96
MDCK	2.03E-05	1.73E-05	2.33E-05	9.97E-06

4.6 Molecular Dynamic Simulation assessment.

MD simulation is an approach to understand macromolecular structure to function relationship. The server used CABS-flex 2.0 gave the multimodal superimposed simulated structures along with fluctuation plots as a result. It calculates the RMSF value. The higher the value, the more flexible the complex.

Table 4.17 Multimodal superimposed simulated structures for rhlA.

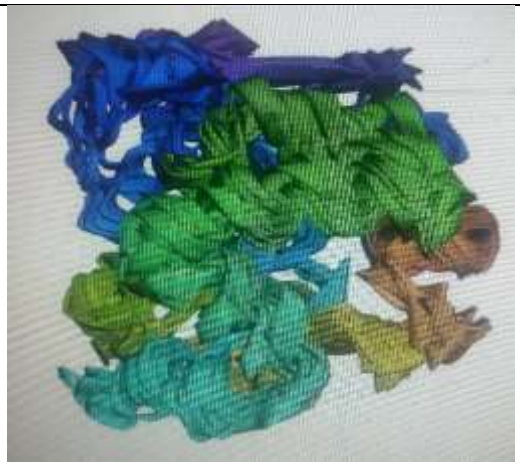
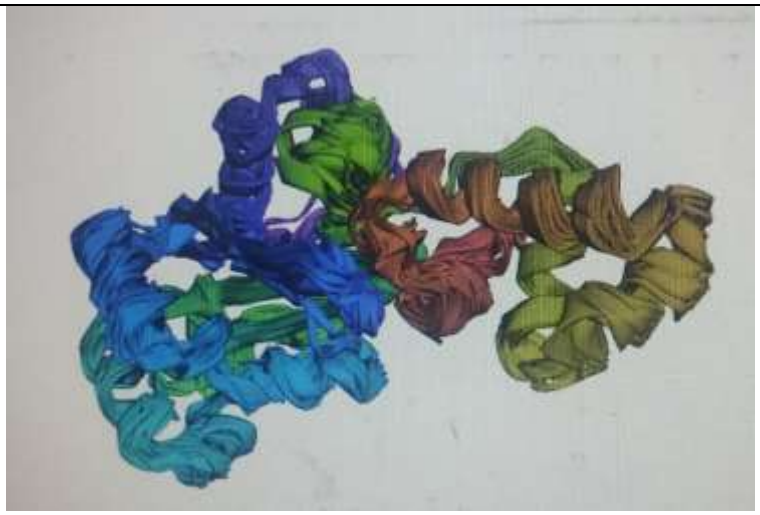
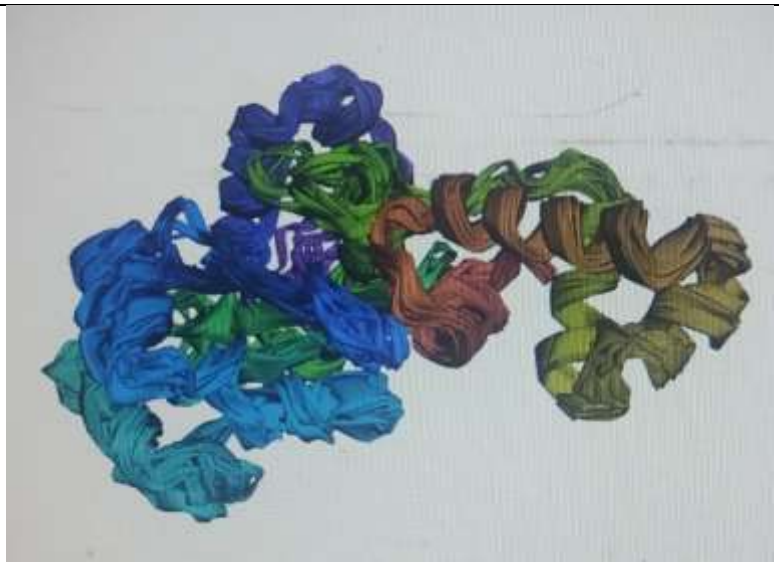
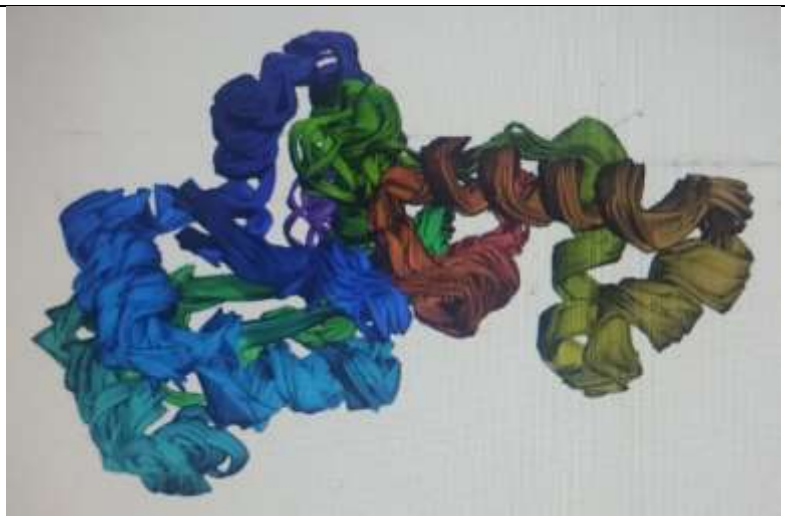
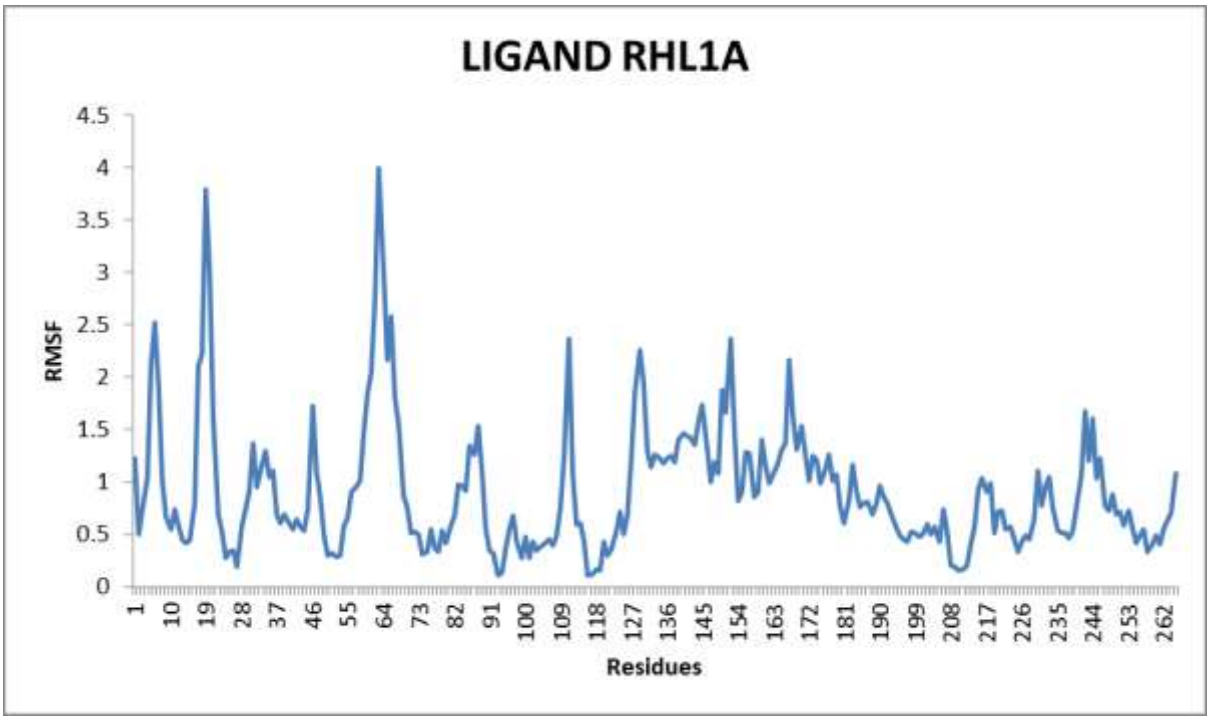
Protein rhlA	
1a	
2a	

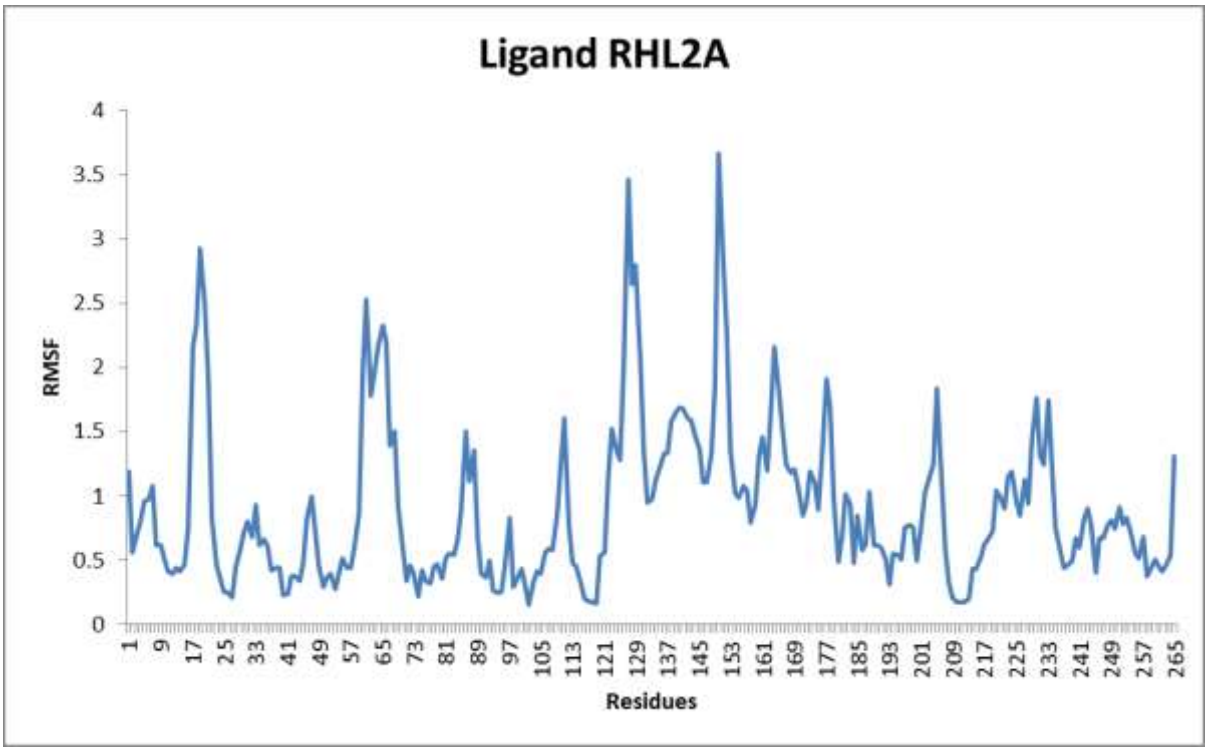
Table 4.18: Multimodal superimposed simulated structures for rhlR.

Protein rhlR	
1r	
2r	

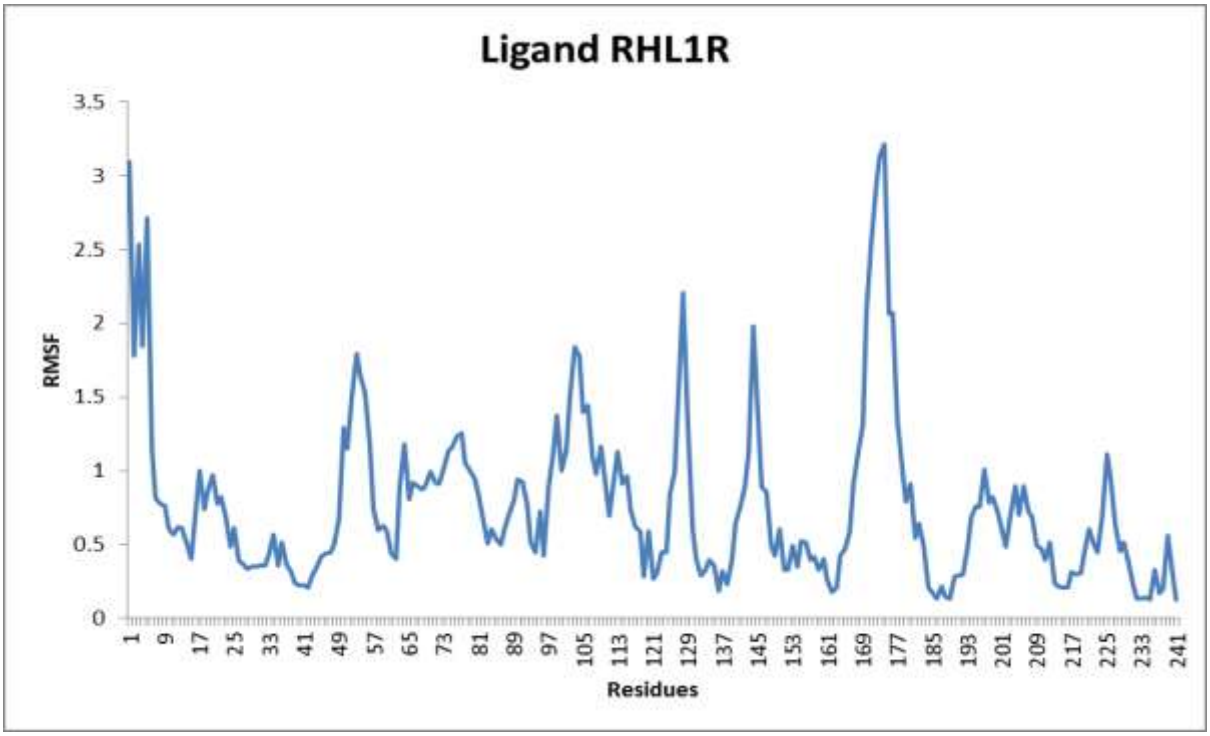
4.7 RMSF profiles of the ligands.



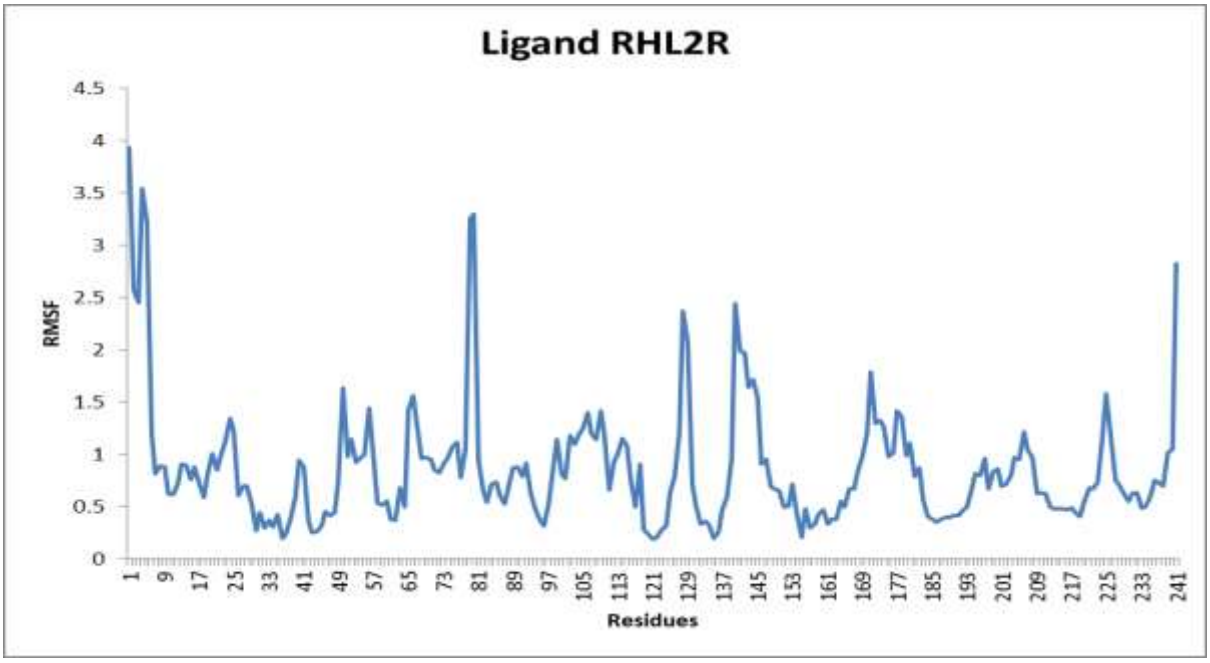
a) Ligand rhl1A



b) Ligand rhl2A



c) Ligand rhl1R



d) Ligand rhl2R

Figure 4.12: RMSF profiles obtained via simulations.

The results show an overall flexible movement with stable complex.

Table 4.19 Range for RMSF values for ligands.

Ligands	Ranges/ Å
rhl1A	0.114 to 3.793
rhl2A	0.153 to 3.660
rhl1R	0.122 to 3.134
rhl2R	0.191 to 3.937

CHAPTER 5

DISCUSSION

5. Discussion

Multidrug-resistant Pseudomonas *aeruginosa* has greatly hampered conventional antibiotics and therapeutics resulting in limited efficacy and severe adverse effects. It is crucial to identify prospective treatment targets as there are currently few targets accessible for antibiotics development, and drug development pipelines are beginning to dry up despite an increased public demand. Pseudomonas *aeruginosa* is of critical importance as it is enlisted in the organisms that show drug resistance. It causes life long and life threatening acute and chronic infections. Pseudomonas *aeruginosa* infections have become challenging to treat. It has been observed that some of the strains show resistance for many antibiotics that are widely used (Hancock and Speert, 2000). According to studies, biofilm formation is responsible for up to 65 to 80% of all infections including a wide range of illnesses and chronic infections (Jamal et al., 2018).

After attachment, Quorum sensing shows major part in existence and the colonisation during pathogenesis. Expression of genes regulated by QS plays a crucial role in the transition from acute to chronic infection. 10% of the genes in Pseudomonas *aeruginosa* are controlled by QS. They are responsible for the synthesis of virulence factors, movement, mechanisms for antibiotic resistance, biofilm formation and modification of stress response pathways (Moradali, Ghods & Rehm, 2017). A study carried out in Pakistan showed 84.1% of antibiotic resistance against Pseudomonas *aeruginosa* (Hope et al., 2006). The current scenario calls for the development of new therapies and treatment mechanisms that are more effective and efficient than the conventional ones. The development of drugs is extensive, interdisciplinary and tough. The convenience is that it makes research and development more affordable. Drugs help in preventing late stage clinical failures by selecting only a potent lead molecule. This reduces the research cost significantly. Also, in-silico drug designing offers a well-developed

foundation for the generation of ligands and inhibitors with preferred selectivity because of the readily available numerous technologies (Wadood et al., 2013).

Due to Pseudomonas *aeruginosa's* core ability to form biofilm which resulted in antibiotic resistance, it felt like the need of the hour to combat this pathogen. Therefore, we devised this research to halt QS in Pseudomonas *aeruginosa* by outlining an inhibitory molecule. The goal of this study was to filter out the best ligand which can target the biofilm formation and synthesis of rhamnolipids in Pseudomonas *aeruginosa*. It is well understood and promiscuous that in this post golden age of antibiotics, the resistance to antibiotics is increasing on an alarming rate. This calls for urgent alternative treatments against infections. "Pathoblockers" is an emerging concept that disarms the pathogen of its virulence mechanisms instead of directly killing them. These compounds reduce the selection pressure that leads to antibiotic resistance. Therefore, deep understanding of the virulence mechanisms is essential to unveil anti pathogenic compounds. One such promising strategy is to interfere with virulence factors and biofilm formation (Kamal et al., 2017). Quorum sensing is a major mechanism in formation of biofilm, hence, interfering with QS pathways can result in a non-antibiotic strategy to combat bacterial resistance.

In this study, we have combined a several steps in-silico drug design approach such as molecular docking, virtual screening and molecular dynamics to design and identify potential inhibitors for rhlA and rhlR. We identified promising compounds with favorable ADME properties and strong binding affinity to the target proteins. Two compounds against each protein were distinguished with strongest binding affinity. Obtaining the 3D structure of the target protein is a key initial step in rational drug designing. This structure serves as a base for understanding the detail of protein's structure and its molecular behavior. It also enables the identification of potent inhibitors that specifically target the enzyme of interest (Kopec et al., 2005). As the structure for both the proteins was not available, we performed comparative

modelling using Phyre2 server. To assess the structure's quality, Ramachandran plot analysis was implemented. 90.8% of rhlA residues were categorized in the favorable area whereas only 0.8% of the residues lie in the disallowed region, which refers to a good structure quality. Also for rhlR, 92.6% of the residues were categorized as in the most favored region and 0.9%. of the residues were placed in unfavorable region, hence, indicating a better structure quality.

ERRAT is a method that is developed to find incorrect regions within the structure of protein. (Al-Khayyat & Al-Dabbagh et al., 2016). It is used in our study to verify protein quality factor. The result of overall quality factor in ERRAT server for both the proteins Rhamnosyltransferase subunit A and Regulatory protein RHLR are 91.837% and 89.565% respectively. Binding site's presence indicates target protein and ligand interaction. Exploring these binding sites helps in removal of poor or nonexistent ligand binding abilities. Binding site characterizes how proteins function and offers important information creating antagonists and inhibitors. The size of cavities and voids on the protein surface are examined measuring using the CASTp method. Finding potential binding sites involves an understanding of the size, adjustment and flexibility of the site (Liao et al., 2022).

Also, site specific molecular docking is preferred over blind docking. Identifying the ligand binding domains help in site specific docking. By taking into account the unique characteristics and restrictions of the binding site, site specific docking improves the accuracy of docking predictions (Korb et al., 2009). Secondly, compared to global docking techniques, it requires less computing work. This effectiveness enables large-scale virtual screening or the investigation of various ligand binding mechanisms (Brylinski & Skolnick, 2008). A more targeted sampling of ligand conformations and protein-ligand orientations within the binding site is made possible by site-specific docking. The precision of the scoring systems used to assess ligand binding affinity is improved by this focused exploration (Plewczynski et al., 2010). Additionally, it is very helpful for investigating ligand selectivity, comprehending

binding mechanisms, and developing site-specific inhibitors. In-general, site-specific docking techniques offer a useful tool for investigating ligand binding. In this study, we performed site specific docking using PyRx. We ran seventeen ligands for rhlA and eight ligands for rhlR.

The more the negative binding affinity is, the tighter the binding and affinity. Two ligands against each protein were used with the most negative binding affinity. 1r has the binding affinity of -6.9 kcal/mol, 2r has -6.4 kcal/mol, 1a has a value of -8.2 and 2a has -7.3 kcal/mol. The correctness of docking lies on RMSD outcomes of the position of docked heavy atoms of ligand compared to the ones in crystal structure (Kufareva & Abagyan, 2015). The acceptable RMSD of the ligand is less than 2.0 Å. RMSD analyzes protein's firmness and predict conformational changes of protein. Lowest RMSD values are of the optimized proteins. RMSDs of the docked ligand 1a and 2a was 0.000Å. RMSDs of the docked ligand 1r and 2r was 0.000Å. This shows that the pose of ligand attachment and the structure of enzyme has no significant change. The little fluctuations and a low RMSD value were signs of system stability (Kuzmanic and Zagrovic, 2010). Usually compounds that contain sulfur scaffolds, for instance, enzyme inhibitors, antifungals, antitumor agents and antibiotics, are generally regarded to have distinct bioactivities (Feng et al., 2016). The selected twocompounds show a strong binding affinity and conserved residues in the binding groove. There is presence of sulfur group in both compound's structure.

The application and accessibility of a drug is of key importance in designing it and bioavailability helps in doing so. It tells about a drug's pharmacophoric properties. All the four selected candidates fulfilled Lipinski's rule of five, the pharmacophoric and toxicological properties for being a good drug. Lipinski's rule of 5 are four parameters that are predictors of permeability and absorption. If a drug breaks one or more rule, it does not mean that this drug is ineffective. The drugs that are beyond the rule of five need non-traditional administration techniques at the site of target as they have reduced bioavailability and reduced water solubility

(Benet et al.,2016). Selected compounds against rhlR show no violation whereas compounds against rhlA show one violation. The bioavailability score of all four compounds show that they are physiologically active. Also the synthetic accessibility of the compounds is low which means they are easy to synthesize. TPSA is a characteristic when observing the drug transport mechanism such as BBB penetration and the intestinal absorption (Prasanna et al.,2009). All the selected compounds fall in an ideal range of TPSA. The toxicity end points such as carcinogenicity, cytotoxicity and mutagenicity were all inactive with high confidence score for all four compounds. The anticipated toxicity of the substance is more likely to be true because a higher confidence value denotes a more reliable prediction. It is of key importance to check for the absorption, distribution, metabolism, excretion and toxicity in early designing of drug to avoid the failure of drug as undesirable pharmacokinetics and the toxicity of a compound leads to a drug being failed. ADMETlab 2.0 is an in silico tool that helps the researchers in designing and optimization of a drug (Xiong et al.,2021). Human hepatotoxicity, AMES toxicity and carcinogenicity were three aspects regarding toxicity that we determined in this study. For all the three toxicity tests, the ligands resulted in the least toxic category except for 1a which has a slightly high toxicity in Human hepatotoxicity test and ligand 1r which has a slightly high toxicity in carcinogenicity and Ames test. The results of these tests can help us further deduce the best drug among both such as 2a and 2r against their respective target proteins. For assessing the absorption, three parameters tested in this study are HIA, Caco-2 permeability and MDCK permeability. The results of all the ligands reflect excellent permeability. Therefore, this ensures that our proposed ligands can be easily absorbed into the intestines and pass through the intestinal membranes making it a good drug candidate. These attempts at experimental evaluations are suitable enough in predicting the physicochemical properties of the compounds. Hence, such compounds could be upcoming candidates for the effective treatment of infections caused by PAO1.

MD simulation has been used to explore the structure-function relationships of specific drug-target complexes. Also, it identifies the essential areas that have been highlighted by MD simulations, such as the stability of the complex, ligand binding kinetics and exploration of allosteric sites (Bera & Payghan, 2019). Flexibility is a key factor to evaluate the interaction of molecules with substrates or protein- protein interactions (Ghosh et al.,2021). RMSF value represents the conformational flexibility of the complex. Another key component for determining the flexible and rigid areas of the protein structure is the analysis of RMSF. Moreover, it is used to evaluate the backbone flexibility of both the protein and the ligand. The mean displacement of each atom in the protein structure and ligand was shown by the RMSF (Surti et al., 2020). The RMSF data allows us to determine whether a specific target is stable during the course of simulation (Fatariansyah et al., 2021). The RMSF value was seen to a minimum of 0.11Å for rhl1A, 0.15Å for rhl2A, 0.12Å for rhl1R and 0.19Å for rhl2R whereas maximum RMSFs of 3.79Å, 3.66Å, 3.13Å and 3.93Å were observed for rhl1A, rhl2A, rhl1R and rhl2R respectively. The ligand target generally appears to be stable enough and the conformation has little flexibility according to the RMSF values. The overall RMSF values are lower but when complexes are compared to the unbound protein, some of the ligand binding residues in the complex have a higher RMSF than the unbound protein due to reasons such as, flexible side chains, charged particle, large side chains and presence of the residue in the loop region of the protein. This outcome demonstrates overall flexible movements with stable complexes.

6. Conclusion

Novel and effective strategies are required for the development of efficacious, specific, and robust therapeutic agents to cope with multi drug resistant Pseudomonas *aeruginosa.* This study makes use of several in silico tools to design a rational drug that targets the virulence factors such as rhamnolipids production and biofilm synthesis. The selected specie PAO1 is prevalent in Pakistan and worldwide. The selected ligands prove to be a putative candidate for the elimination of pathogen. Four drug targets identified in this study have effective bioavailability score and exhibit stable docking results in terms of most negative binding affinity. These compounds have decreased cytotoxicity, mutagenicity and carcinogenicity. Moreover, they show good intestinal absorption and permeation results. The results obtained from MD simulation show overall flexible movement with stable complexes.

7. Future prospects

Despite relying on wet laboratory experimentation for validation, the results obtained from dry laboratory studies have proven to be invaluable in providing guidance and establishing a structured plan for various investigations. These investigations have identified potential targets worthy of being considered as strong candidates for further in vivo studies. By employing virtual screening techniques, new drug formulations can be developed and subjected to in vivo evaluation, leading to more promising outcomes. Additionally, this approach enables the repurposing of existing antibiotics and other drugs, harnessing their potential for novel therapeutic applications. Combination therapies and drug repurposing can be effective and can lead to compounds that target multiple pathways involved in biofilm formation. Consequently, the integration of dry laboratory findings with wet laboratory experimentation offers a synergistic approach that maximizes the efficiency and success of drug discovery and development endeavors. Incorporating pharmacodynamics can streamline the drug development process, reducing time and energy, reducing animal testing and leading to optimization of drug candidates. Integration of Artificial Intelligence & Machine Learning into drug design. Pharmacogenomics is a promising avenue leading to the design of personalized drug therapies, resulting in optimized treatment outcomes.

8. References

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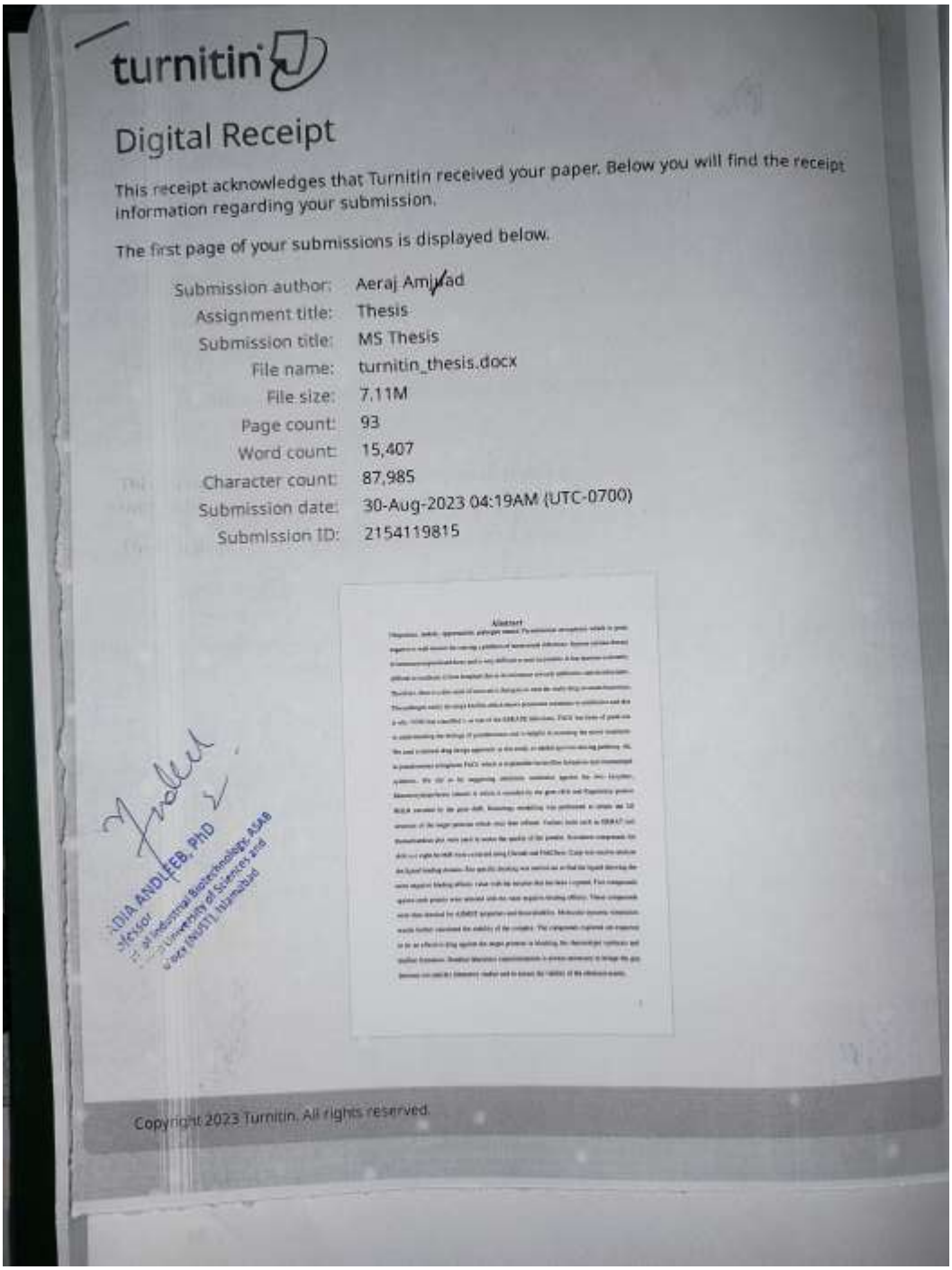

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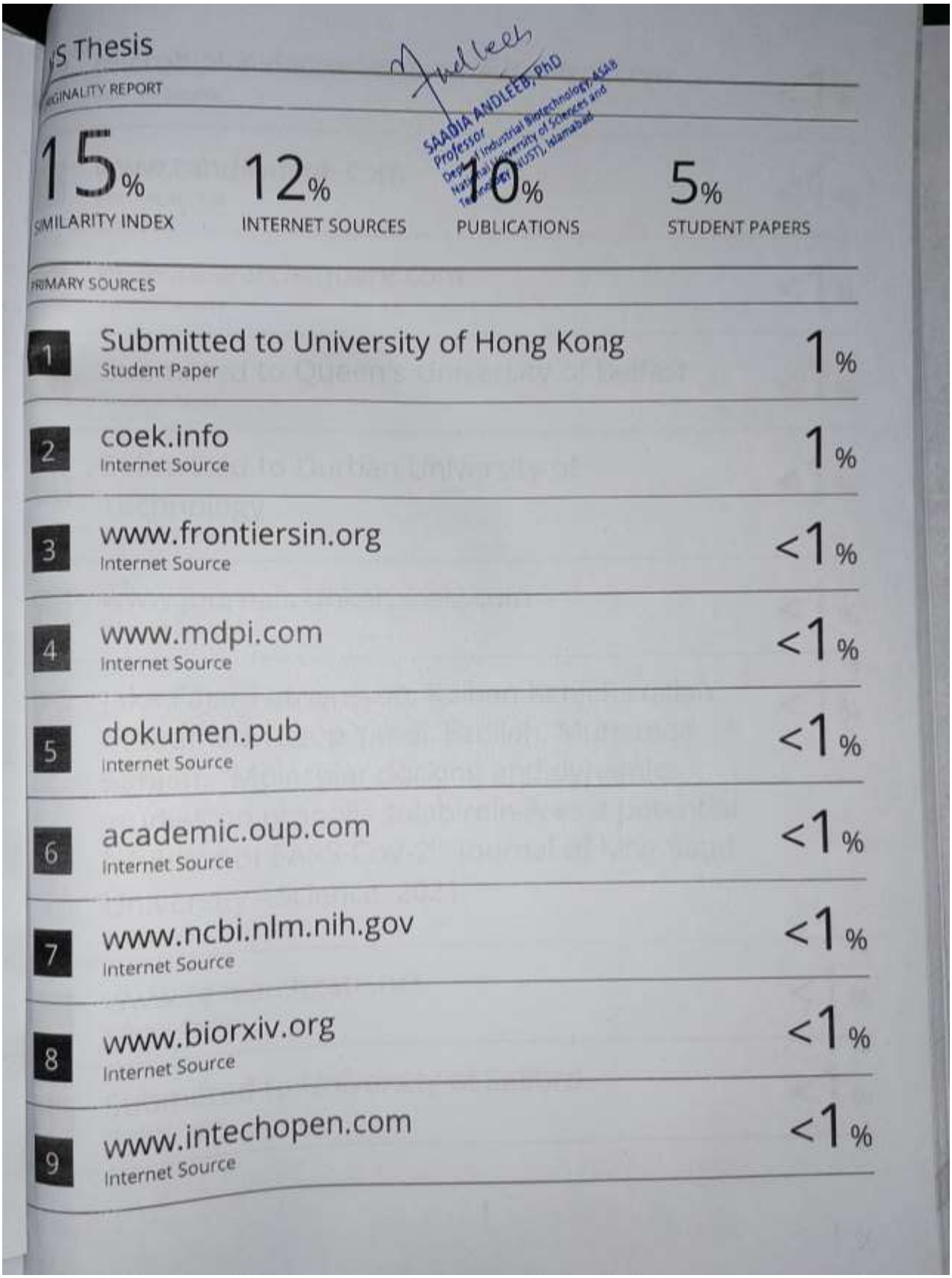

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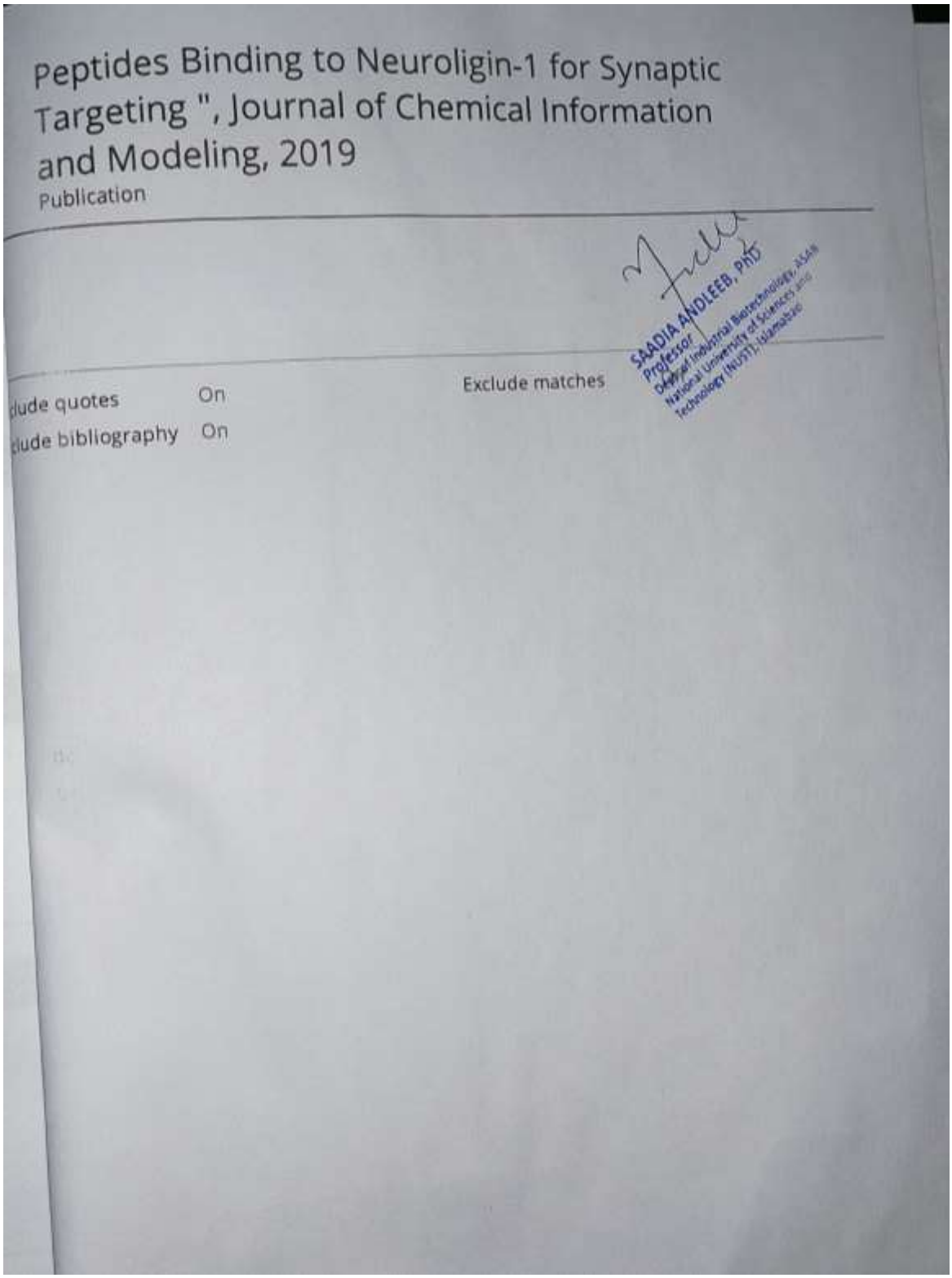
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