IN VITRO SCREENING FOR NATURAL QUORUM SENSING INHIBITOR MOLECULES

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This thesis is dedicated to my family for love and support throughout my life...

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ABSTRACT

IN VITRO SCREENING FOR NATURAL QUORUM SENSING INHIBITOR MOLECULES

Increasing antimicrobial resistance in pathogenic bacteria has created the need for the development of novel therapeutic agents. Having a role in various mechanisms of gene regulation such as biofilm formation and production of virulence factors; quorum sensing (QS), cell-density dependent bacterial intercellular signaling mechanism, has become a target for developing next generation antimicrobials. Recent studies have shown that disrupting the communication between bacteria reduces their pathogenicity and, therefore, attenuate the possible infections. Both Gram negative and positive bacteria use this mechanism to regulate such physiological processes. Among all of them, *Pseudomonas aeruginosa*, being an opportunistic human pathogen, attracted the greatest focus on QS system investigations.

In this study, we aimed to screen some natural compounds (rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine) for their inhibitory effects on *Pseudomonas aeruginosa* QS system. Therefore, we initially detected these molecules whether they have QS inhibition activity or not. The results showed that all of these compounds were potential QS inhibitors. To test them further, the molecules were probed with a QS reporter system that allow gathering a dose-response relationship. According to our data; rose absolute was found to be the most effective compound on the inhibition of QS. This is the first study demonstrated that rose absolute has inhibitory effects on QS and it can be potential candidate to be used as a therapeutic agent to fight with bacterial infections in the future.

ÖZET

BAKTERİYEL İLETİŞİMİN ENGELLENMESİNİ SAĞLAYAN DOĞAL KAYNAKLI MOLEKÜLLERİN *İN VİTRO* İNCELENMESİ

Patojenik bakterilerin antibiotiklere karşı dirençlerinin artması yeni terapi yöntemlerinin geliştirilmesine duyulan ihtiyacı arttırmıştır. Biyofilm oluşumu ve virulans faktörlerinin kontrolü gibi bir çok mekanizmada önemli role sahip olan bakteriler arası "çevreyi algılama sistemi" (Quorum Sensing, QS) yeni nesil antimikrobiyal maddelerin geliştirilmesi için hedef haline gelmiştir. Son zamanlarda yapılan çalışmalar bakteriler arası iletişimin engellenmesinin patojenliği azalttığını, böylelikler olası enfeksiyon risklerini azalttığını göstermiştir. Çevreyi algılama sistemi Gram negatif ve Gram pozitif pek çok bakteri tarafından çeşitli fizyolojik işlemleri düzenlemek için kullanılmaktadır. Bunlar arasından fırsatçı patojen *Pseudomonas aeruginosa* en çok dikkati toplayan organizma olmuştur.

Bu çalışmanın amacı on *Pseudomonas aeruginosa* QS sistemlerini engelleme aktivitesine sahip çeşitli doğal molekülleri (gül absolütü, gül yağı, karanfil yağı, tarçın yağı ve çam terebentini) araştırmaktır. Bunun için öncelikle bu moleküllerin QS'i engelleme potensiyellerinin olup olmadığı belirlenmiştir. Sonuçlar moleküllerin hepsinin bu özelliğer sahip olduğunu göstermiştir. Bunun üzerine araştırmaya moleküllerin doza tepki ilişkilerinin tespit edilmesiyle devam edilmiştir. Sonuçlara göre diğer moleküller arasından gül absolütü en etkili QS engelleyicisi olarak belirlenmiştir. Bu çalışmada, ilk kez, gül absolütünün QS'i engelleme aktivitesine sahip olduğu ve bakteriyel hastalıkların tedavisinde kullanılabilecek potansiyel terapötik ajan olabileceği saptanmıştır.

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LIST OF SYMBOLS / ABBREVIATIONS

ABC	ATP-binding cassette
ACP	Acyl carrier protein
AHL	Acyl homoserine lactones
AI2	The autoinducer-2
AIP	Auto-inducing polypeptides
C4-HSL	*N*-butanoylhomoserine lactone
C6-HSL	*N*-hexanoyl-homoserine lactone
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylene-diamineteraacetic acid
FBS	Fetal bovine serum
GC-MS	Gas Chromatography equipped with Mass Spectrometry
GFP	Green fluorescence protein
IPTG	Isopropyl Thiogalactoside
OD	Optical Density
PA	Penicillic acid
PBS	Phosphate buffered saline
QS	Quorum sensing
QSI	Quorum sensing inhibitor
QSIS	Quorum sensing inhibitor selector
SAM	S-adenosyl methionine
3-oxo-C_6-HSL	*N*-(3-oxo-hexanoyl)-homoserine lactone
3-oxo-C_{12}-HSL	*N*-(3-oxododecanoyl) homoserine lactone
4-NPO	4-nitro-pyridine-N-oxide

1. INTRODUCTION

1.1. QUORUM SENSING: AN OVERVIEW

The adaptation of bacteria to changing environmental conditions and their ability to sense these surroundings are important for their survival. Various mechanisms allow bacteria to sense environmental conditions such as pH, nutrient availability and population density [1-3]. Quorum sensing (QS) is the mechanism by which bacteria can monitor the surrounding population density and coordinately respond to that information by altering different gene expressions through production of signal molecules [4-5].

Some of the signal molecules used in QS is able to diffuse through bacterial membranes, whereas others are actively transported [6]. Signal molecules, also named as autoinducers, are constantly produced and received at a basal level by bacteria. A particular threshold must be accomplished in the surrounding of bacteria before cell signaling starts. Usually this threshold level is achieved between late log to stationary phase [7]. When the bacteria reach a certain population density and the signal molecules accumulate to a particular threshold, a transcriptional regulator is activated. That, in turn, regulates the expression of various genes including virulence factor and biofilm forming genes [8].

Since termed by EP Greenberg and colleagues, QS became a popular subject for scientists from all over the world and many studies have been carried out to understand this system [9]. The results of the studies performed to understand the QS mechanism have shown that QS is an evolutionary advantage for the microorganisms that use this system. As shown by several transcriptomic and proteomic studies, QS systems have been found to regulate diverse bacterial processes including luminescence, conjugation, sporulation, antibiotic synthesis, swarming and most importantly biofilm formation and virulence factor production such as toxins, enzymes and adhesion molecules [1-4].

Many human pathogens including *Pseudomonas aeruginosa, Burkholderia cepacia, Salmonella typhimurium* and *Yersinia enterocolitica* regulate expression of virulence factors required for pathogenicity via QS. Among these bacterial species, *P. aeruginosa* is

the best studied one since it is an emerging opportunistic human pathogen [10]. QS also enables bacteria to escape host defense mechanism. In order to establish into a host organism, bacteria should sense the environment and should adapt to environmental conditions. Since QS allows bacteria to monitor the surrounding and therefore enable the adaptation, it increases the pathogenicity of the bacteria. A small amount of virulence factors would not be enough to cause an infection. However, a coordinated expression of virulence genes by a high density bacterial population would allow bacteria to produce virulence factors at high enough levels to overcome the host defense and to start an infection [11].

Coordinating the functions accomplished by QS systems is not limited to bacteria within a same species. There are two kinds of QS systems: species specific (intraspecific) and interspecific. Intraspecific mechanism is important in behavior regulation and adaptation to environment. Although the signal molecules used in QS systems have the same basic chemical composition, slight modifications in the fatty acid structure can result in species specificity. Contrarily, many different bacteria can use the same signal molecule and this brings out interspecific QS systems. Interspecificity enables bacterial species to recognize a universal language that allows interspecific signaling between them. The communication of bacteria across species by using signal molecules is termed cross-talk [12].

Several different kinds of QS signal molecules have been identified so far [13]. Acyl homoserine lactones (AHL), diketopiperazines, γ-butyrolactones and post-translationally modified peptides are some of these signal molecules [14-15]. Mainly there are three groups of autoinducers: AHLs which are used by Gram negative bacteria, oligopeptides which are used by Gram positive bacteria and another group of autoinducer used by both Gram positive and Gram negative bacteria. AHLs are diffusible molecules whereas oligopeptides are not diffusible. Therefore, they are produced as precursor peptides, modified and exported outside by protein transport machinery [16]. Being used by over 70 Gram negative bacterial species, AHLs are the best characterized QS signal molecules [17]. The autoinducer-2 (AI2) has been found both in Gram positive and Gram negative bacteria and is therefore thought to be a universal interspecies signaling molecule signaling molecule that allows bacteria to sense other bacterial populations [1-3]. These molecules, identified over 55 pathogens, have an important role in many physiological processes such

as multispecific biofilm formation and controlling of virulence genes [18]. Figure 1.1 shows some autoinducers used in different QS systems.

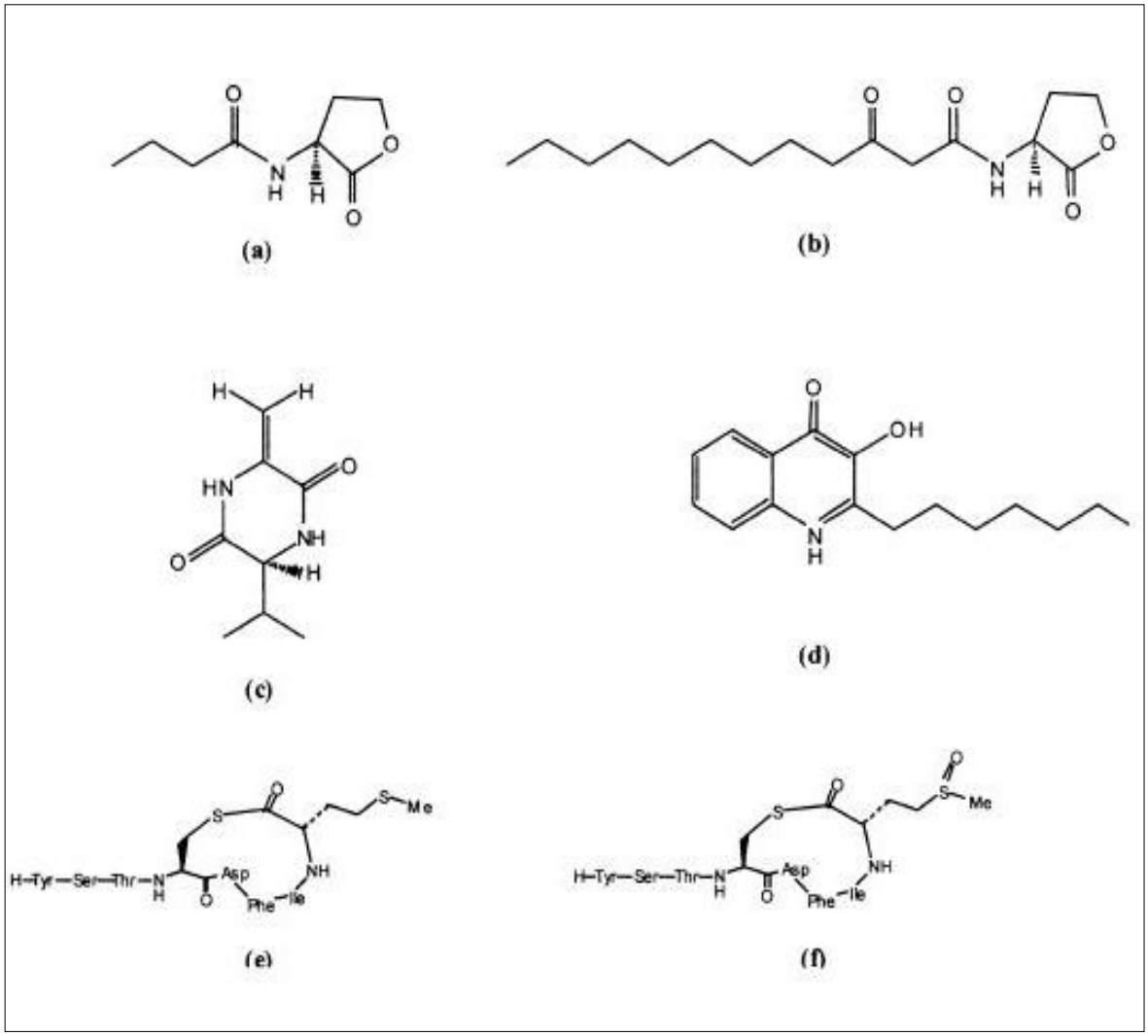

Figure 1.1. Structures of some representative QS signal molecules [19]
a. C4-HSL, b. 3-oxo-C_{12}-HSL, c. cyclo(ΔAla-L-Val), d. 2-heptyl-3- hydroxy-4-quinolone, e. group I *Staphylococcus aureus* cyclic peptide thiolactone, f. the corresponding group I methionyl sulphoxide peptide

1.1.1. First Discovery of Quorum Sensing and *Vibrio fisheri*

The concept of QS was first demonstrated in the marine luminescent bacterium *V. fisheri* in the early 1990s [8, 20]. This bacterium is present on the open sea but also grows in the light organs of certain fish and squid species. It was discovered that these bacteria express

genes controlling light emission only when associated with the host organism. Luciferase enzyme, responsible for light emission, is not produced when the bacterium lives in the ocean freely. When *V. fisheri* was grown *in vitro,* luciferase expression was rapidly induced coordinatedly at the late log phase of cell growth. In order to understand the mechanism of this gene regulation a series of experiment have been performed. In an experiment, scientists added conditioned, cell-free culture media from bacteria growing at high population density to a low-density culture. Bacteria at low population density expressed luciferase enzyme in the presence of the conditioned media. This led the scientists to the consequence that a molecule is secreted by bacteria as their density increases and this molecule induces expression of the luciferase-encoding genes. This was the first experiment that elucidates QS mechanism [20-21].

Since *V. fisheri* can be cultivated *in vitro a*nd *in vivo*, this bacterium is a well studied example of QS systems. So, it has become a model organism to understand QS. *V. fisheri* was the first species where AHL (N-(3-oxo-hexanoyl)-homoserine lactone) and the *luxI* and *luxR* genes were identified [17, 22]. Figure 1.2 illustrates the QS mechanism in *V. fisheri.*

After colonizing at the host organism, *V. fisheri* undergoes some morphological changes. After 12 hours entering the host, bacteria gain a persistent state. The flagella are gone, the size of cells is decreased, growth rate are reduced and bioluminescence is induced. Induction of bioluminescence prevents production of toxic oxygen radicals by host enzymes. When the population density exceeds 10^{11} cells per milliliter, luminescent genes are activated resulting in luminescence as a consequence of symbiotic corporation of bacteria and the host organism [23]. The genes directing the luminescence system (*luxICDABE* and *luxR*) are found in the *lux* operon. The proteins LuxA and LuxB form the light generating enzyme luciferase [24]. The products of *luxI* and *luxR* regulate the operon. These genes are the main regulators for the luminescence, at least at the late bacterial colonization state where they reach high density [25]. *luxI* encodes for an autoinducer synthase that synthesizes the signal molecule *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C_6-HSL). This molecule is synthesized at basal levels at low population density. When the population density reaches a certain threshold level, the molecule binds to activator protein LuxR. The bound complex activates transcription of *luxICDABE* genes enabling

transcription of all the necessary components of the luminescence system resulting in exponential increase in luxI synthesis and light production. LuxR also binds to the *luxR* promoter in a positive feedback inhibition loop [2, 23].

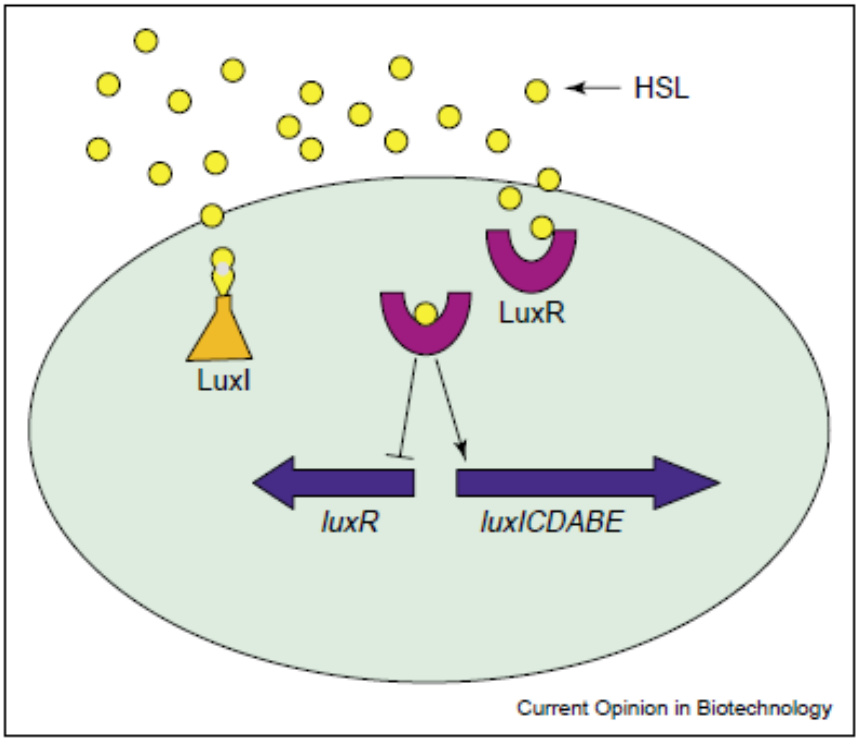

Figure 1.2. QS in *V. fischeri* [9]

1.1.2. Role of Quorum Sensing in Bacterial Biofilms

Bacterial survival in most environments depends on surface attachment and biofilm development. Several studies have been made in order to understand the mechanism of biofilm formation and the physiological characteristics of biofilms. A biofilm is a microbial community composed of cells attached to a surface where they aggregate to release extracellular polysaccharides that form a polymeric matrix or glycocalyx. Being embedded in the polymeric matrix, microorganisms display a modified phenotype, concerning the growth rate and gene transcription [26-27]. Figure 1.3.a illustrates the development of a bacterial biofilm. Biofilms are considered as the most successful expression of the prokaryotic genome since the cells forming the biofilm are metabolically more efficient and have resistance to environmental stresses. Biofilms are also considered as the primitive

form of cellular differentiation, with a “primitive circulatory system”, “homeostazy”, “integrality”, similar to eukaryotic tissues in their intercellular cooperation [28].

Biofilms carry medical importance because they can be found on catheters, artificial joints and stints. These biofilms are extremely difficult to remove since they are protected from host defenses like phagocytosis and antibodies. They also develop resistance to antibiotics. Moreover, biofilms can cause chronic infections. Biofilms developed by *P. aeruginosa* are considered as the main cause of persistent infections in the lungs of cystic fibrosis patients as well as ocular infections in people using contact lenses. Figure 1.3.b. shows confocal microscope images of a biofilm developed by *P. aeruginosa* [27].

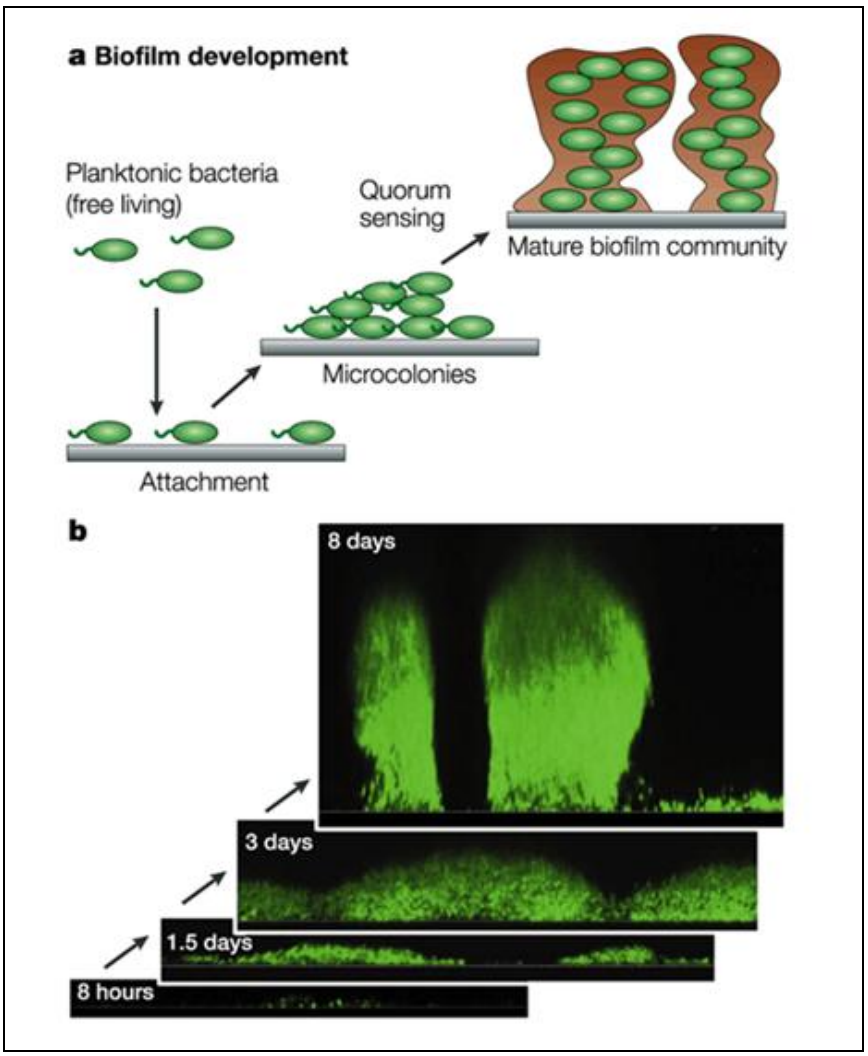

Figure 1.3. a. Development of a biofilm, b. Confocal microscope images of a biofilm developed by *P. aeruginosa* [29]

Biofilms are architecturally complex structures where a series of physical, chemical and biological processes direct their formation. Biofilms develop in three stages: bacterial attachment to surface, proliferation resulting in the formation of microcolonies and differentiation of the microcolonies into distinct structures. Considering that high population density is required for QS, it cannot take place in the attachment or proliferation stages. It has been thought that QS may have role in differentiation of a biofilm [30]. It has been shown that without QS signal molecules, biofilms are thin and uniform. Exceeding a critical threshold, the expression of some related genes are regulated. As a result of this regulation biofilm's cell become different from their counterpart and gain resistance to all kind of environmental stress conditions. This is why biofilms are not affected by host defenses or antibiotics [31].

1.1.3. Acyl Homoserine Lactones (AHLs)

So far, several signaling molecules used in QS system have been identified [13]. Among them, the most intensively studied family of signal molecules is the acyl-homoserine lactones (AHLs) in Gram-negative bacteria [17]. AHLs are produced by over 70 species of Gram-negative bacteria varying in the length and oxidation state of the acyl side chain. Since the discovery in *V. fischeri*, AHL dependent QS has been shown to play a role in diverse processes such as bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer and the production of virulence determinants in animal, fish and plant pathogens [32-34].

AHLs are highly conserved molecules. They have the same homoserine lactone functional group, but different acyl side chains and substitution at the C3 carbon [29]. AHLs are usually synthesised by enzymes of the LuxI family, which use the appropriately charged acyl carrier protein (acyl-ACP) as the major acyl chain donor while S-adenosyl methionine (SAM) maintains the homoserine lactone moiety. AHLs are diffusible molecules that can pass the cellular envelope. After accumulating in the extracellular matrix, they activate a transcriptional regulator of the LuxR family once a critical threshold has been exceeded [4, 32]

AHL-mediated QS systems have been characterized in bacteria causing human diseases

such as *P. aeruginosa* [35], *Yersinia pseudotuberculosis* [36], *Clostridium difficile* [37], *B. cepacia* [38], and *Escherichia coli* [39]. Figure 1.4 shows some AHLs in different bacteria. AHL mediated QS system involves two major components: an AHL synthase gene that belongs to the LuxI protein family and a modular transcriptional response regulator that belongs to the LuxR protein family [40].

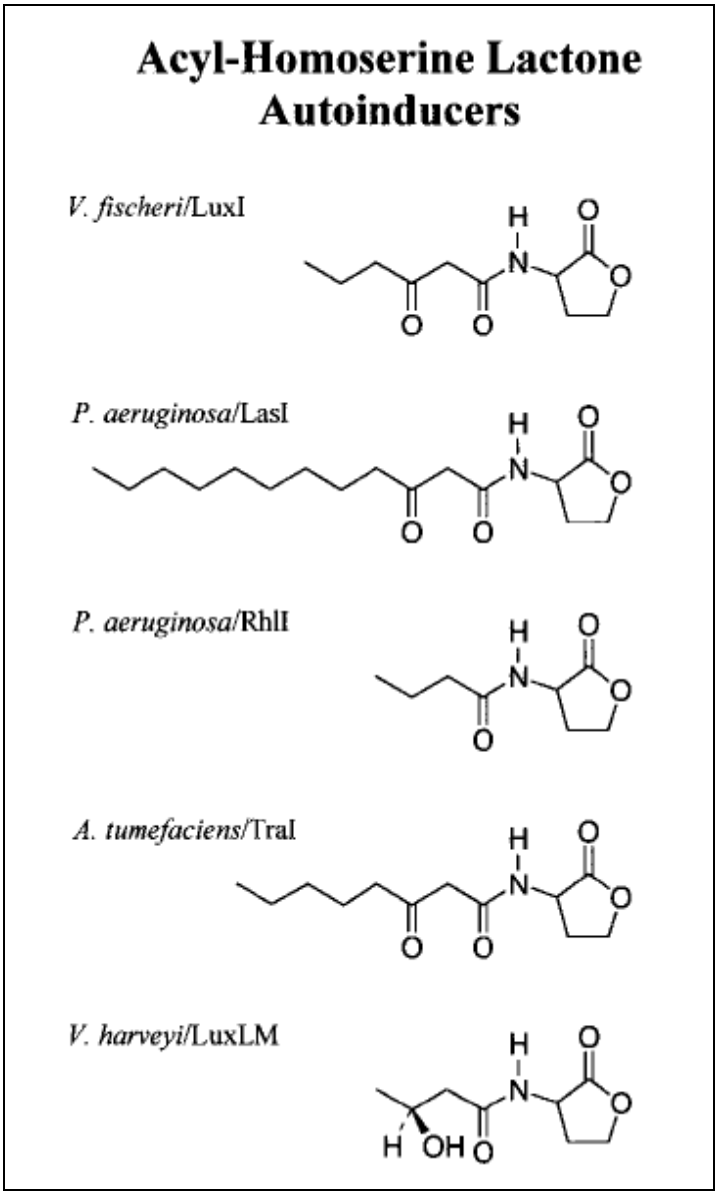

Figure 1.4. AHLs used by Gram-negative bacteria [41]

Studies that have been conducted to understand the AHL mediated QS mechanism used bacterial biosensors that enable to detect the presence of exogenous AHLs. These

biosensors do not produce AHLs and contain a LuxR-family protein that regulates a target gene promoter directing transcription of a reporter gene [42].

Studying the biosynthesis, reactivity, and degradation of AHLs is important not only for understanding of QS mechanisms, but also important for medicinal, environmental, agricultural, and industrial applications such as treating cystic fibrosis or developing transgenic plants with improved disease resistance [19, 27, 43].

1.2. QUORUM SENSING IN DIFFERENT BACTERIA

QS was first elucidated in the marine bacterium *V. fisheri* and was thought to be restricted to only a limited series of species. Since then extensive studies have been conducted and it was found out that similar QS system is present in many other Gram positive and Gram negative bacteria [44-45]. Having role in biofilm formation [46], virulence adaptation [47-48] and production of antimicrobial substances [49], many non-pathogenic and especially pathogenic bacteria have been studied for their QS mechanisms [50]. While the AHL mediated QS in Gram negative bacteria is the most intensively studied mechanism, similar other processes in Gram positive bacteria have also been investigated. The QS systems of *Streptococcus pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus*, for instance, have been extensively studied [2, 45].

1.2.1. Quorum Sensing in Gram Positive Bacteria

In gram positive bacteria, there are several processes that are governed by QS. These processes involve competence for DNA uptake in *B. subtilis* and *S. pneumoniae*, virulence in *S. aureus*, conjugation in *Enterococcus faecalis*, sporulation in *B. subtilis* and bacteriocin production in lactic acid bacteria [51-52].

Many Gram positive bacteria use post-translationally modified peptides created from larger precursors as QS signal molecules. These auto-inducing polypeptides (AIPs) are made up of 5–17 amino acids with side chain modifications consisting, for example, of isoprenyl groups *(Bacillus* spp.) or thiolactone rings (*Staphylococcus* spp.) [53-54]. Figure 1.5 shows some Gram positive autoinducers, their designations, and the organisms that produce them.

The AIPs are usually secreted by ATP-binding cassette (ABC) transporters. They are synthesized in the cytoplasm as precursor peptides, and then cleaved, modified and transported to the extracellular environment. Some of them interact with membrane-bound sensor kinases which converted into a signal across the membrane. The others are transported into the cell by oligopeptide permeases. There, they interact with intracellular receptors. In the surroundings of the producing bacterial cell, the AIPs can be detected by membrane-bound two-component detection systems. Detection of the AIPs by the sensorial part of the two component systems leads to phosphorylation and therefore activation of the response regulator protein [54]. This response protein interacts with the target promoter DNA and the expression of the QS regulated genes is switched on.

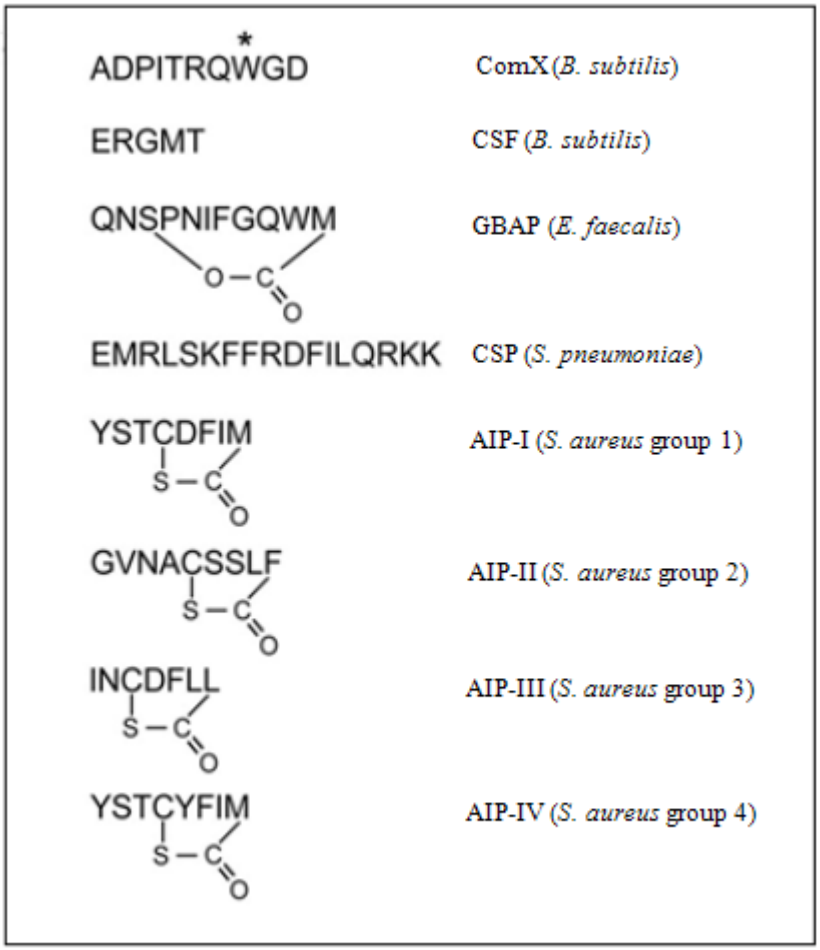

Figure 1.5. Autoinducers used by Gram positive bacteria [58]

Being post-translationally modified peptides created from larger precursors, AIPS shows

great variety between different Gram-positive species. Chemical structure of the AIPs affects the binding efficiency on the receptors, so it is generally accepted that mainly Gram positive, but also some Gram negative bacteria use these molecules as part of their QS mechanisms. Therefore, QS mediated by AIPs may be both intraspecific and interspecific [55-57].

1.2.2. Quorum Sensing in Gram Negative Bacteria

After described by Engebrecht and Silverman, the simple signal-response mechanism has now been shown to be used by over 30 species of Gram-negative bacteria for the control of different processes accomplished by QS [8, 59]. The use of AHLs is common in these systems. AHLs are composed of a homoserine lactone ring that is connected to an acyl side chain of variable length and variable extra modification. AHLs with a short acyl side chain can pass passively through the bacterial membrane, whereas AHLs with a long acyl side chain need to be actively transported [57, 60-61].

The use of AHLs depends on a *luxI* homologue and a *luxR* homologue that encodes a transcriptional activator protein. This protein enables to detect the cognate AHL and therefore induce the expression of related genes. Several studies have shown that there are more complex systems worked by *LuxI* and *LuxR*. For instance, in *P. aeruginosa*, there are two LuxI/R pairs (LasI/R, RhlI/R) and they control the production of virulence factors [62-64]. In *Ralstonia solanacearum,* QS controls the expression of virulence factors including plant cell-wall-degrading enzymes. In *R. solanacearum*, LuxI/LuxR-like autoinduction system (SolI/SolR) is regulated by a LysR-like transcriptional regulator called PhcA that responds to 3-hydroxy-palmitic acid methyl ester. The SolI/SolR system is also controlled by RpoS, the stationary phase sigma factor [65-66]. There exist many other examples like mentioned above; almost all of them rely on the LuxI/LuxR system [67-68]. Table 1.1. shows some QS systems in Gram-negative bacteria.

Table 1.1. Some QS systems in gram negative bacteria [71]

Organism	**Major AHL(s)**	**LuxR**	**LuxI**	**Phenotypes**
Aeromonas hydrophila	C4-HSL	AhyR	AhyI	biofilms, exoproteases
Aeromonas sabnonicida	C4-HSL	AsaR	AsaI	exoprotease
Agrobacterium tumefaciens	3-oxo-C8-HSL	TraR	TraI	plasmid conjugation
Agrobacterium vitiae	C14:1 HSL, 3-oxo-C16:1-HSL	AvsR	AvsI	virulence
Burkholderia cenocepacia	C6-HSL, C8-HSL	CepR, CciR	CepI, Ccil	exoenzymes, biofilm formation, swarming motility, virulence, siderophore
Burkholderia mallei	C8-HSL, C10-HSL	BmaR1, BmaR3 BmaR4, BmaR5	BmaI1, BmaI3	virulence
Chromobacterium violaceum	C6-HSL	CviR	CviI	exoenymes, cyanide, pigment
Erwinia carotovara spp. carotovara	3-oxo-C6-HSL	ExpR/CarR	CarI	carbapenem, exoenzymes, virulence
Pantoea (Erwinia) stewartii	3-oxo-C6-HSL	EsaR	EsaI	exopolysaccharide
Pseudomonas aeruginosa	C4-HSL, 3-oxo-C12-HSL	LasR, RhlR, QscR, VqsR	LasI, RhlI	exoenzymes, secretion, HCN, biofilms
Pseudomonas aureofaciens	C6-HSL	PhzR, CsaR	PhzI, CsaI	phenazines, proteases, colony morphology, aggregation
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C12-HSL	PpuR	PpuI	biofilm formation
Pseudomonas chlororaphis	C6-HSL	PhzR	PhzI	phenazine-1-caboxamide
Pseudomonas syringae	3-oxo-C6-HSL	AhlR	AhlI	exopolysaccharide, swimming motility, virulence
Rhodobacter sphaeroides	7-*cis*-C14-HSL	CerR	CerI	aggregation
Serratia spp. ATTC 39006	C4-HSL	SmaR	SmaI	antibiotic, pigment, exoenzymes
Serratia liquefaciens MG1	C4-HSL	SwrR	SwrI	swarming motility, exoproteases, biofilm development
Serratia proteamaculans B5a	3-oxo-C6-HSL	SprR	SprI	exoenzymes
Sinorhizobium meliloti	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL	SinR, ExpR, TraR	SinI	nodulation/symbiosis
Vibrio fisheri	3-oxo-C6-HSL	LuxR	LuxI	bioluminescence
Yersinia enterocolitica	3-oxo-C6-HSL	YenR, YenR2	YenI	swimming, swarming

QS system of *P. aeruginosa* is the best characterized one among other Gram negative bacteria. In the early 1990s, a homologous system to *V. fisheri lux* QS sytem was discovered in *P. aeruginosa* and since then extensive studies have been made to Figure out the processes governed by QS in these organisms [69-70].

1.2.2.1. Pseudomonas aeruginosa: an opportunistic human pathogen

*P. aeruginos*a, commonly found in soil and water, is an increasingly prevalent opportunistic human pathogen that leads to a variety of infections. This organism causes respiratory track infections in cystic fibrosis patients and also causes blood, skin, eye and genitourinary tract infections in patients with defective immune system function, such as those immunocomprised by surgery, cytotoxic drugs, burns and HIV [72-73]. High rates of morbidity and mortality in patients with cystic fibrosis is due to *P. aeruginosa* infections of pulmonary airways [10]. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 8 % of surgical wound infections [74], 12 % of hospital-acquired urinary tract infections and 10 % of bloodstream infections [75].

The variety of diseases *P. aeruginosa* causes is due to an impressive array of both cell-associated and extracellular virulence factors produced by this organism. *P. aeruginosa* produces a great variety of virulence factors that can cause extensive tissue damage and promote bloodstream invasion [73]. Figure 1.6 illustrates *P. aeruginosa* producing some virulence factors.

Many cell-associated factors such as flagellum, pilus and lipopolysaccharide are important in *P. aeruginosa* virulence. In addition to that, a variety of extracellular virulence factors including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease, and alkaline protease), and hemolysins (phospholipase and rhamnolipid) is the cause of different infections caused by *P. aeruginosa.* These factors have been found to contribute to the virulence of *P. aeruginosa* in animal models [76] *in vitro* studies [77-78] and clinical studies [79-80].

Many virulence factors are expressed as a consequence of an environmental stimulus such as nitrogen availability, temperature or osmolarity, but generally, virulence factor expression does not occur until high cell density is achieved [77-78]. The mechanism, QS, regulates the expression of many virulence factors.

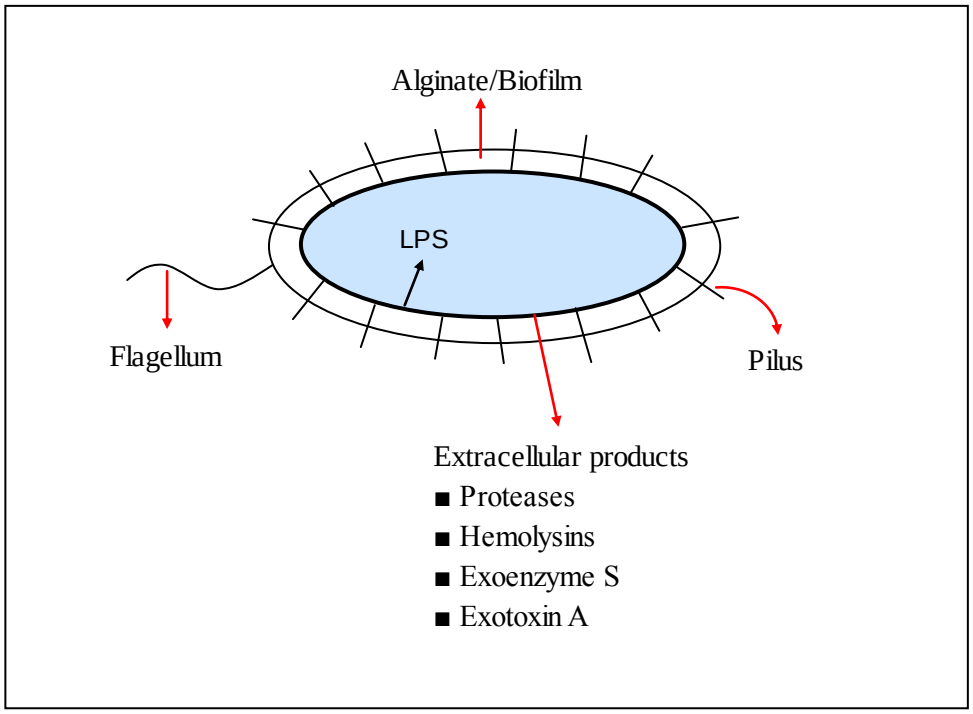

Figure 1.6. Virulence factors of *P. aeruginosa* [72]

1.2.2.2. Quorum Sensing Systems of Pseudomonas aeruginosa

P. aeruginosa possess two well-investigated QS systems, the *las* and *rhl* systems. At least six percent of *P. aeruginosa* genome is regulated by AHL via *las* and *rhl* QS systems [11, 81]. The first discovered QS system was shown to regulate elastase genes (*lasB* and *lasA*) and was therefore named as "*las* system" [70]. The second QS system, discovered in the mid-nineties was named as "*rhl* system" because of its ability to regulate rhamnolipid biosynthesis [82-83]. These two systems are organized in a hierarchy where *las* is dominant over *rhl* [84]. Continued studies on *P. aeruginosa* have revealed that many other genes are controlled by QS. In addition to elastase and rhamnolipid genes, the genes for alkaline protease (*aprA*), exotoxin A (*toxA*), pyocyanin, pyoverdine, the Xcp translocation machinery, cyanide, lipase, *rpoS*, twitching motility, azurin (*azu*), alginate and chitinase, as well as catalase and superoxide dismutase (*katA*, *sodA* and *sodB*) were found to be controlled by QS [69, 85].

las and *rhl* QS systems are comprised of *lasI*, *lasR* and *rhlI* and *rhlR*, respectively [62, 69-70]. The major signal molecules synthesized by *lasI* and *rhlI* are *N*-(3-oxododecanoyl)

homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively. In Figure 1.1 the structure of 3-oxo-C_{12}-HSL and C4-HSL was shown. In addition to synthesis of 3-oxo-C_{12}-HSL and C4-HSL, *lasI* also directs *N*-(3-oxohexanoyl) homoserine lactone (3O-C_6-HSL) synthesis, and *rhlI* directs *N*-hexanoyl-homoserine lactone (C6-HSL) synthesis [82]. 3-oxo-C_{12}-HSL is secreted to the extracellular environment via the efflux pumps encoded by the mexAB-OprM operon, whereas C4-HSL is a freely diffusible molecule [6].

In the *las* QS system, formation of 3-oxo-C_{12}-HSL via the direction of *lasI* gene product causes an increase in the extracellular concentration of this molecule. 3-oxo-C_{12}-HSL molecules then interact with the transcriptional activator LasR and LasR-3-oxo-C_{12}-HSL complex activates the expression of several genes including those that direct the production of virulence factors [69-70].

In the *rhl* QS system, as the freely diffusible signal molecule C4-HSL synthesized by *rhlI* attains adequate levels, it binds and activates the transcriptional activator RhlR. RhlR-C4-HSL complex then regulates the rhamnolipid biosynthesis operon *rhlAB*, alkaline protease, pyocyanin, the *lasB*-encoded elastase, and rhlI itself [85].

las and *rhl* QS systems are arranged in a hierarchy where *las* autoinducer 3-oxo-C_{12}-HSL has an inhibitory affect on *rhl* system. Unbound 3-oxo-C_{12}-HSL prevents C4-HSL from binding to the transcriptional activator RhlR. As a result, *lasR* transcription is upregulated and the concentration of LasR-3-oxo-C_{12}-HSL complex is increased. Afterward the complex activates the *rhl* system. Figure 1.7 illustrates *las* and *rhl* QS systems and the hierarchy between them.

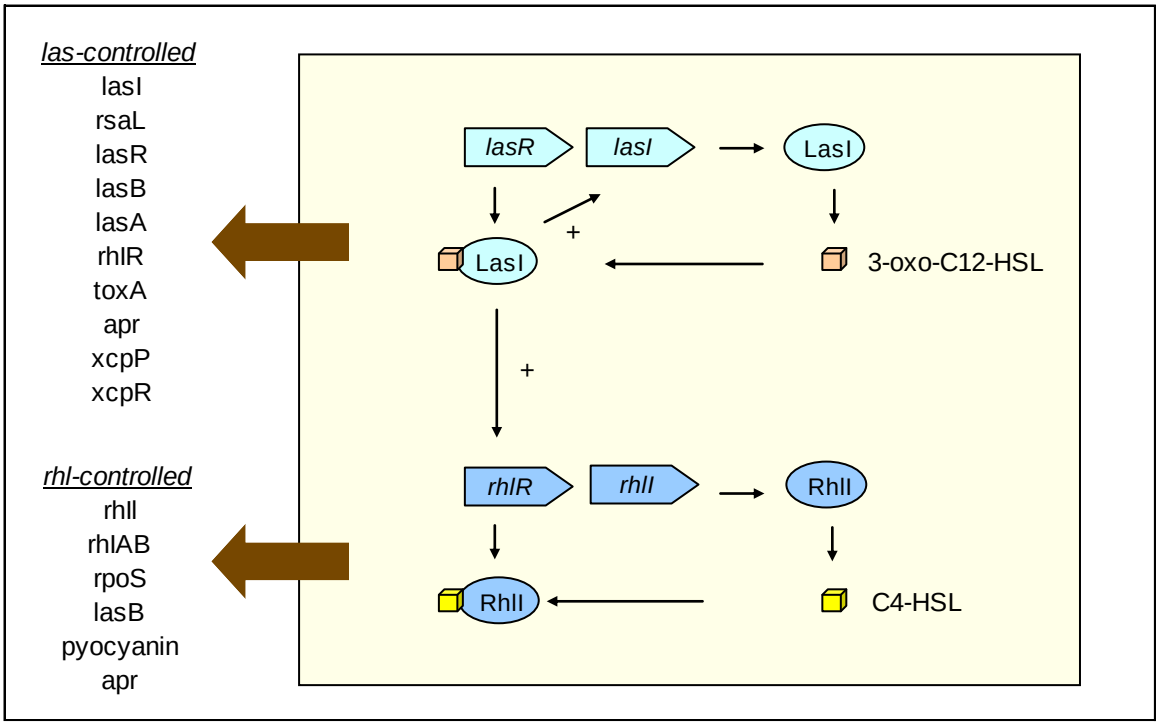

Figure 1.7. *las* and *rhl* QS systems and the hierarchy between them [63]

1.3. INHIBITION OF QUORUM SENSING

Antimicrobial treatments for bacterial infections have been aimed to eradicate infections by inhibiting microbial growth. Various antibiotics such as penicillin and sulphonamides are either toxic or inhibitory to bacterial growth. Now, pharmaceutical companies attempt to overcome a challenging problem. Indiscriminate use of antibiotics has created antimicrobial resistance in pathogenic bacteria. Increasing numbers of bacteria have gained higher tolerance against conventional antibacterial agents including broad-spectrum antibiotics [86-87]. Resistance is achieved by reduced outer membrane permeability or multidrug efflux pumps (tetracycline, imipenem, fluoroquinolones, aminoglycosides), or production of antibiotic modifying enzymes (aminoglycosides, ß-lactams). Figure 1.8 illustrates the increase in resistance against different antibiotics since the first use of antibiotics. Repeated exposures to same antibiotics also increase bacterial resistance [88-89]. Therefore, the development of novel therapeutics to fight against infections has been a tough issue. Attempts have been done to find an innovative solution.

Biofilm formation, swarming and secretion of virulence factors regulated by QS

mechanisms facilitate bacteria to generate an accomplished infection and even a recurrent one. As these processes weaken the host defense, invasion of blood vessels, dissemination and systemic inflammatory response syndrome may occur. It may also lead to death. Since antibiotics barely stop these processes, they have to be prevented before the related QS gene expressions are turned on. Recent researches have focused on the development of therapeutic agents that prevent bacterial pathogenesis by targeting QS systems. Molecules inhibiting QS mechanisms do not have any direct effect on bacterial growth, they reduce the pathogenicity making bacteria vulnerable to host defense [90]. Another reason why QS inhibiting molecules are needed to overcome infections is the difficulty to destroy biofilms by conventional antimicrobial therapies. Most bacteria live in microbial communities known as biofilms [91]. Bacteria living in the biofilm are about 1000-fold more tolerant to antibiotics, biocides and heavy metals than planktonic-growing cells [92]. Various factors including limited penetration of antimicrobial agents into the biofilm, binding of the compounds to the exopolysaccharide matrix surrounding the biofilm and genes expressed differently are thought to increase the resistance. The gene *pvrR* (phenotypic variant regulator) is an example to these genes. *pvrR* has a role in the conversion of wild-type *P. aeruginosa* into a different form that grow in rough colonies and has high tolerance to antibiotics [93]. *ndvB* is another gene that has importance in biofilms. The gene *ndvB* provides higher resistance to the antibiotic tobramycin in a *P. aeruginosa* strain, PA14 [94]. Nevertheless, this gene is not considered as a general mechanism for tobramycin resistance since it is not expressed differently in other strains such as *P. aeruginosa* PAO1 strain [95].

Considering all the factors that require novel approaches for the treatment of bacterial infections, attempts to inhibit QS mechanism carries great importance in the sense of being a promising and innovative scope. QS inhibitory molecules may also be combined by antibiotics. The combination might improve the effectiveness of antibiotics, decrease possible tolerance of bacteria and might even increase the self-life of antibiotics. Destroying biofilms renders bacteria vulnerable to the immune system of the host. After dissolving the biofilms by QS inhibitory molecules, the bacteria released from the biofilm can be killed by conventional antibiotics. Some QS inhibitory molecules including furanone, patulin and penicillic acid were tested in mouse models having *P. aeruginosa* biofilms. It was observed that these molecules increased the sensibility of *P. aeruginosa* to tobramycin [11, 96].

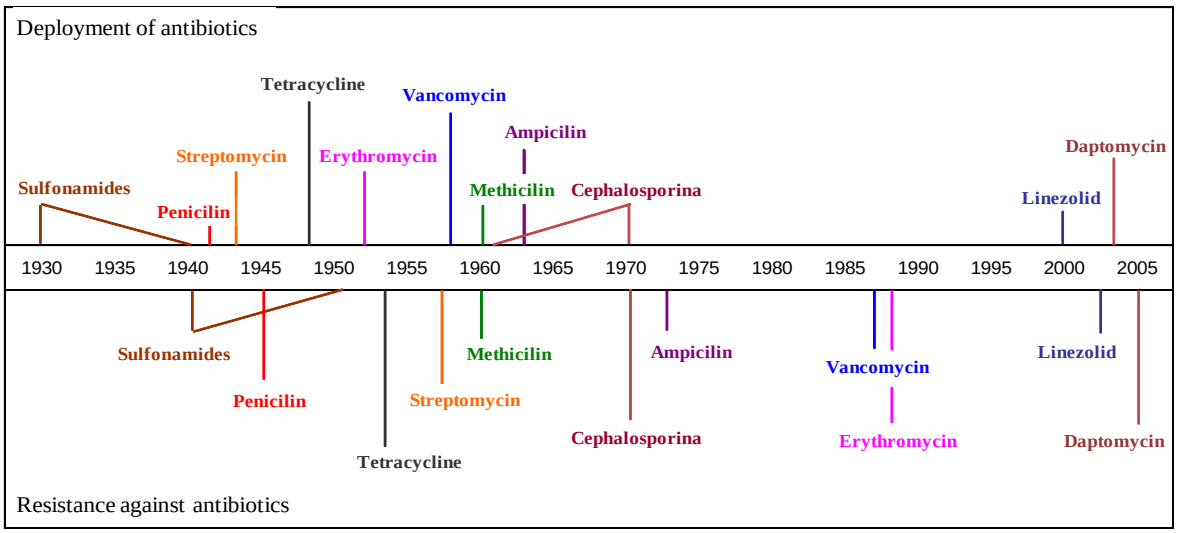

Figure 1.8. Timeline of first antibiotic use and date of first recorded antibiotic resistance [97]

1.4. INHIBITION STRATEGIES

As the importance of QS in occurrence of infections and several diseases has been understood, several attempts have been carried out to develop new strategies that could inhibit QS systems of pathogens. The strategies used in disrupting QS could be classified in three groups: Inhibition of signal molecule biosynthesis, inactivation and degradation of signal molecule and blocking signal transduction [98].

1.4.1. Inhibition of signal molecule biosynthesis

Blocking the production of signal molecules is the least investigated and preferred strategy to inhibit QS systems. Blockage of AHL production has been studied by a group of researches and a few substrate analogs have been found to inhibit AHL biosynthesis. These analogs including L/D-S-adenosylhomocysteine, holo-ACP, sinefungin and butyryl-S-adenosylmethionine (butyryl-SAM) were tested *in vitro;* none of them have been tested on bacteria in *vivo*. It was found that S-adenosylcysteine is the most effective analogue that could lower the activity of *P. aeruginosa* LuxI homolgue RHLI by 97% [64].

In spite of the presence of substrate analogs inhibiting AHL biosynthesis, further studies

are needed because how these analogs would affect other cellular functions and whether they will be useful or not in fighting with infections are still not known [99].

1.4.2. Inactivation and degradation of signal molecule

An extensively studied strategy for QS inhibition is inactivation or complete degradation of signal molecules. Various mechanisms including chemical and enzymatic methods have been suggested to inactivate or degrade AHL molecules.

Inactivation of AHL can simply achieved by increasing the pH above 7 since AHLs are unstable at those pH levels. Alkaline pH causes AHLs to undergo lactonolysis - ring opening- and to lose biological activity [100-101]. Elevated pH levels were observed in unbuffered media in which *Erwinia carotovora, P. aeruginosa* and *Y. pseudotuberculosis* stationary-phase culture were grown. The observed active AHL levels were also very low. Even supplied from outside AHLs cannot activate QS systems. Several higher organisms also make use of this property in defense against pathogenic bacteria. Plants which are infected with the tissue-macerating pathogen *E. carotovora* increase pH at the site of attack [101]. The kinetics of the ring opening is affected by several factors. Increased temperature accelerates the rate of opening. Increased length of side chains decreases the rate of lactonolysis. These properties indicate that in order to be active under physiological pHs, an AHL signal molecule have to possess a side chain length of at least four carbon atoms [100-101].

Lactonolysis of AHLs can also be achieved by an AHL degrading enzyme, AiiA. This enzyme has been found in several *Bacillu*s species including *B. thuringiensis, B. cereus* and *B. mycoides.* This enzyme decreases the amount of bioactive AHLs by catalaysing the lactonolysis [102-103]. When a plasmid carrying *AiiA* gene is inserted in *E. carotovora,* its virulence towards potato, egg plant, Chinese cabbage, carrot, celery, cauliflower, and tobacco could be weakened. Several other bacteria including *P. aeruginosa* PAI-A, *Arthrobacter spp., Klebsiella pneumoniae, Ag. tumefaciens* and *Rhodococcus spp.*, have been found to produce AiiA homologues. Other bacteria such as *Comamonas spp.* have been found to degrade AHLs [104-106].

Besides the lactone ring, the oxidized AHL signal molecules are another strategy for inhibiting AHL signal molecules. The oxidized AHL signal molecules such as 3-oxo-C_{12} HSL can react with oxidized halogen compounds including hypobromous and hypochlorous acids. This property is used by some organisms to fight with pathogens which use QS to control expression of virulence factors. An example to these organisms is the marine algae *Laminaria digitata* that produces oxidized halogen compounds [107].

Another method for the inactivation of AHL signal molecules is to metabolize it. *Variovorax paradoxus* and *P. aeruginosa* are known to grow on AHLs as sole source of carbon, nitrogen and energy. An aminoacylase produced by bacteria cleaves the peptide bond of signal molecule resulting in a fatty acid and homoserine lactone. The acid goes through ß-oxidation and is used as energy and carbon source. The nitrogen is obtained by the action of lactonases that release ammonium from the amide bond [106, 108].

Not only bacteria, but also humans have the ability to degrade AHLs. Differentiated human airway epithelial cells have been found to breakdown certain AHL signal molecules. The cells are able to inactivate 3-oxo-C_{12}-HSL and C6-HSL but not 3-oxo-C_6-HSL and C4-HSL. This suggests that the state of degradation is affected by both the length of the side chain and the oxidation state [109].

1.4.3. Blocking signal transduction

The most widely investigated QS inhibition strategy is the blockage of signal reception. Inhibition of AHL receptor site with an AHL analogue has been carried out by various competitive molecules either being natural or synthetic [18]. Halogenated furanones from Australian macroalgae, *Delisa pulchra*, were the first discovered natural analogue and since then several molecules having QS inhibition property have been tested and found. In order to access this kind of molecules easier, scientists started to synthesize synthetic molecules [110].

1.4.3.1. Natural Inhibitors

Natural QS inhibitor (QSI) molecules could be isolated from plants or fungi. As these organisms are invaded by QS bacteria for millions of years, some of them developed

mechanisms to disrupt QS in order to reduce pathogenicity. The red marine algae *D. pulchra* produces the most investigated QSI; halogenated furanones that is antagonist for AHL mediated QS [111]. Due to the similarity with AHLs (shown in Figure 1.9), halogenated furanones are thought to bind to LuxR type proteins without activating them [110, 112]. Halogenated furanones have been shown to have the ability to inhibit the QS controlled swarming phenotype of *Serratia liquefaciens* [111, 113]. They can also suppress the expression of bioluminescence genes located on a reporter plasmid in *S. liquefaciens,* having no effect on the growth of bacteria. Halogenated furanones affect not only *S. liquefaciens* but also some other bacteria including *V. fisheri, Vibrio harveyi* and *Serratia ficaria.* However, natural furanones have no effect on *P. aeruginosa.* To overcome this problem some synthetic derivatives of furanones without acyl side chain have been developed and found to be effective against the two QS systems found in *P. aeruginosa* [11, 114].

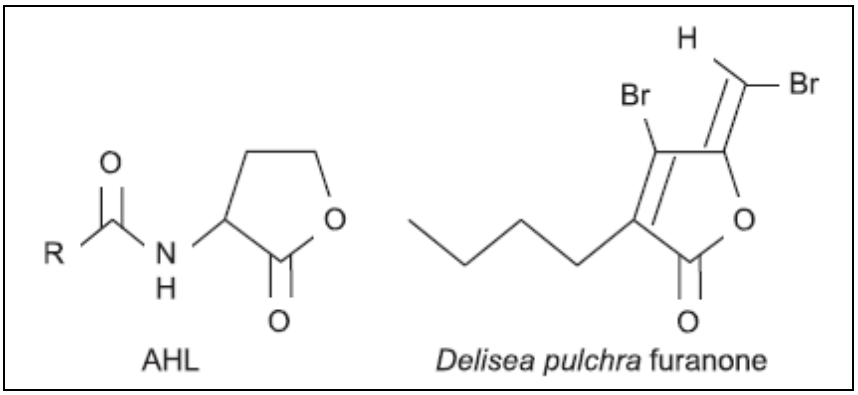

Figure 1.9. Structural similarity between AHL and halogenated furanone [98]

Various plant species have been found to have anti-QS activity. Crown vetch, carrot, soybean, water lily, tomato, pea seedlings (*Pisum sativum*), habanero (chilli) and garlic (*Allium sativum*) produce compounds that interfere with QS systems. Investigations have showed that garlic extract contain a minimum of three different QS inhibitors. One of them was found to be a cyclic disulphur compound [115-117].

A number of secondary metabolites have been also shown to have QSI activity. Among them penicillic acid (PA) and patulin (Figure 1.10) produced by *Penicillium Radicicola* and *Penicillium coprobium*, respectively, have strong effects on QS. According to a target

validation analysis performed by DNA microarray-based transcriptomics, patulin was found to affect 45% of the QS genes in *P. aeruginos*a and PA affects 60 % of them. This finding suggested that these compounds target the LasR and RhlR QS regulators. Western blot analysis with antibodies against the LuxR protein revealed that these compounds increased the turnover rate of LuxR [96]. A plant metabolite curcumin, citrus flavonoids and flavanone naringenin have also been found to have anti-QS activity [118-119].

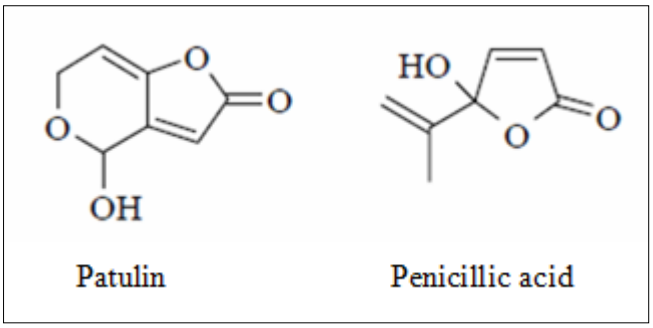

Figure 1.10. Structure of patulin and penicillic acid [120]

1.4.3.2. Synthetic Inhibitors

To prevent the QS signal molecules from being received by the bacteria, synthesis of synthetic QSI molecules have become an attractive technique. When developing synthetic QSIs, researches inspired from natural QSIs, from the structure of AHL itself and from precursors of AHL. Three basic techniques have been suggested to generate a synthetic analogue: introducing substitutions in the acyl side chain without changing the lactone ring, introducing substitutions to the lactone ring without changing the acyl side chain and modifying both the acyl side chain and the lactone ring [99]. Studies revealed that AHL derivatives differentiated in the number of carbon atoms in the side chain did not provide QSI activity. Conversely, many of those molecules were shown to have agonistic effect [121]. In a study the C-3 atom in the acyl side chain was replaced with sulphur and analogues that can inhibit expression in both LuxR- and LasR-controlled QS reporters were developed [117].

To create more QSI molecules, aryl substitutes could be placed at the end of the side chain. However, the size of the substitutes should not be more than that of a phenyl group; otherwise the antagonistic effect will be lost. It is thought that if the cyclic alkyls and the

aryl substituents have the lowest size difference, the aryl compounds could interact with aromatic amino acid residues in the LuxR protein and that will prevent the normal activity [122]. To increase the QSI activity of the aryl AHLs, the C-1 carbonyl group of the side chain could be replaced by a sulphonyl group [123].

Many different synthetic furanone analogs have been investigated for their anti-QS activity. A synthetic derivative of the *D. pulchra* halogenated furanones, (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone has been determined as a highly active AHL antagonist. This compound used in a concentration of 10 μM considerable reduces the virulence factor expression in pure cultures of *P. aeruginosa* PAO1 [11]. In a mouse pulmonary infection model the synthetic furanones reduced the virulence of *P. aeruginosa* by destroying the biofilm. The compounds increased the clearance of *P. aeruginosa* by the mouse immune system and diminished the severity of lung pathology, resulting in an increase in the mice survival rate.

Several other compounds have been tested for their QS inhibition ability. Compounds having different structure from the signal molecules including 4-nitro-pyridine-N-oxide (4-NPO), indole, p-benzoquinone, 2,4,5-tribromoimidazole and 3-nitrobenzene sulphone amide were found as QSI [115].

1.4.3.3. Natural Inhibitors versus Synthetic Inhibitors

Discovery of natural QSIs lead scientists to produce synthetic analogues in order to ease the access to these kinds of molecules. Several molecules have been tested for their inhibition activities. Some found as effective whereas some not. When looking in general, most of these compounds have been found to possess less QS inhibition ability than natural molecules. Some of them including halogenated furanone derivatives are toxic for humans [120]. Synthetic molecules, in general, could be harmful to the nature since they cause environmental pollution. On the other hand, natural molecules are always safer and healthier. It is easier and cheaper to obtain them in compare to the synthetic ones. Considering these favorable properties, searching natural QSI molecules is more sensible than developing synthetic molecules.

Rosa damascena Mill. (Damask rose) being the most important rose species producing

high-value essential oil is a member of Rosaceae family and endemic to Europe and Middle East countries, Iran and Turkey [124]. Turkey has a great importance in growing industrial *R. damascena* producing from 6000 to 8000 tones of rose flowers annually. The endemic *R. damascene* from Isparta meets this huge production. Commercial cultivation focuses on the essential oil of rose. Along with essential oil, hydrosol and absolute are abundantly obtained from *R. damascena* [125]. In addition to the usage in perfumery and cosmetic industry, rose essential oil is also used for its biochemical activities, such as analgesic, hypnotic, and anti-inflammatory effects [126-127]. These properties allow rose extracts to have use in medicine, food and cosmetic industries [128].

Several studies have revealed both the antioxidant and antibacterial activities and chemical composition of rose essential oil. The total phenolic content of *R. damascena* hydrosol and absolute has also been investigated. According to GC-MS analysis of rose products, citrenellol was found to be the major compound of rose essential oil followed by eraniol, nonadecane and nerol [129-132]. GC–MS analyses of rose absolute determined the major compounds as phenylethyl alcohol, citrenellol, nonadecane and geraniol. Table 1.2 shows the results of GC-MS indicating the percentage of major constituents of rose extracts [131].

Clove oil, obtained by distillation of the flowers, stems and leaves of the clove tree (*Eugenia aromatica* or *Eugenia caryophyllata*, Fam. Myrtaceae), has been studied for its antibacterial, antimicrobial, and antifungal properties and shown to be environmentally safe and nontoxic to humans for use in medicine, perfume, and food flavoring [133]. In addition to its worldwide use as a food flavoring agent, it has also been employed for centuries as a topical analgesic in dentistry. Humans have used clove oil for centuries, as an anaesthetic for toothaches, headaches and joint pain [134].

Table 1.2. GC–MS analysis of rose extracts: percentage of major constituents and their retention times [131]

Compound	rt (min)	Rose oil (%)	Rose absolute (%)	Hydrosol (%)
Alpha pinen	7,2	0,8	*	*
Linalool	35	0,53	*	*
Citrenellol acetate	42,3	0,7	*	*
Heptadecane	44,3	0,9	*	*
Germacrene-D	45	0,45	*	*
Geranyl acetate	47,9	2	*	*
Citrenellol	48,2	35,23	9,91	29,44
Nerol	50,4	10,26	1,43	16,12
Geraniol	53	22,19	3,71	30,74
Nonadecane	55,9	13,85	4,35	*
9-Nonedecane	56,8	2,79	*	*
Phenylethyl alcohol	57,1	2,3	78,38	23,7
Metyhl eugenol	62,5	1,97	0,69	*
Heneicosane	66,7	4,85	*	*
Eugenol	70,5	1,18	1,52	*
* Not detected				

Cinnamon, belonging to the genus *Cinnamomum,* has been found to possess anti inflammatory, antioxidant, anticancer and antibacterial properties [135]. Cinnamon has been used to treat dyspepsia, gastritis, blood circulation disturbance and inflammatory diseases in many countries since ancient age. Currently, cinnamon and its essential oils are widely used in pharmaceutical preparations, seasonings, cosmetics, foods, drinks, commodity essences and chemical industries [136]. The essential oils of cinnaomon also have been studied for QSI potential and found to have an inhibitory effect on QS [137].

Turpentine is a fluid obtained by the distillation of resin obtained from live trees, mainly pines. It is composed of terpenes, mainly the monoterpenes alpha-pinene and beta-pinene. Besides employed for fragrance and flavor use, turpentine has also use in pharmaceutical industry. It is also used in disinfectants, cleaning agents and other products having a "pine" odor [138].

2. AIM OF THE STUDY

In this study, it was aimed to detect the QS inhibition ability of rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine on *P. aeruginosa rhl* QS system. By testing the anti-QS activity of these compounds it is expected to develop a new technique to fight with infections caused by *P. aeruginosa.* Obtained from natural sources, these compounds offer a safe strategy for the inhibition of QS. This study also, for the first time, demonstrates the strong potential QSI properties of rose absolute.

3. MATERIALS

3.1. BACTERIAL STRAINS

QSIS1 strain (recombinant *E. coli*) obtained from Dr. Michael Givskov (Denmark) and *P. aeruginosa* (PAO1- *rhl*) obtained from Dr. Thomas Bjarnsholt (Denmark) were used in QSIS 1 and dose response assays, respectively.

3.2. REAGENTS

For QSIS 1 and dose response assays; glucose calcium chloride and 3-oxo-C_6-HSL were purchased from Sigma-Aldrich (Seelze, Germany). Bacto agar was purchased from DIFCO (Detroit, USA). X-gal and IPTG were purchased from Apollo (Cheshire, UK). Casamino acid, thiamine, ferric chloride and ammonium sulphate were purchased from Fluka (Steinheim, Germany). Sodium chloride was purchased from PRS Panreac (Barcelona, Spain). Disodium hydrogen phosphate, potassium dihydrogen phosphate and magnesium chloride were purchased from AppliChem (Darmstadt, Germany).

In order to use in toxicity experiments, Dulbecco's modified essential medium, fetal bovine serum, PSA and Trypsin-EDTA were purchased from Invitrogen, Gibco (Paisley, UK). MTS assay (CellTiter96 Aqueous One Solution) was purchased from Promega (Madison, USA). PBS was purchased from Thermoscientific (Utah; USA).

3.3. LABORATORY EQUIPMENTS

During the whole experiment; ELISA plate reader (Biotek), 30°C incubator with shaker (Edmund Bühler), 30°C incubator without shaker (Memmert), 50 ml falcon centrifuge (Beckman), laminar flow (Heal Force), Fluorescence plate reader (Beckman-Coulter), 15 mL falcon centrifuge (Sigma), UV spectrophotometer (Thermo), 37°C incubator (Thermo) were used.

As other equipments, elisa plates for toxicity assay (BIOFIL), elisa plates for dose response assay (Nunc), sterile petri dishes (Isolab), 15 and 50 mL falcon tubes (Sigma), T-75 flasks (Zelkultur Flaschen) were used.

Pipettes, micropipettes, pipette tips, plastic and glass erlenmeyers, beakers, eppendorfs, spectrophotometer cuvettes, tweezers and lancets were also used in different parts of the experiment.

4. METHODS

4.1. PREPARATION OF CULTURE MEDIA

Culture media used in QSIS1 assay and dose response assay were ABT media and ABT agar which were prepared as described in literature [139]. ABT medium was prepared by adding 10 % A-10, 0.025 % thiamine, 0.5 % glucose and 0.5 % casamino acid to B-medium. Ingredients of A-10 and B-medium were listed in table 4.1 and 4.2, respectively. For preparation of A-10, after adding ingredients the pH was adjusted to 6.4 by means of adding 0.1 % HCl or 0.1% NaOH. A-10 solution and B-medium were sterilized by autoclaving to prevent contamination.

ABT agar was prepared by adding Bacto agar to ABT medium to a final concentration of 2% (wt/vol).

Table 4.1. Ingredients of A-10

Ingredients	**Amount**
Ammonium sulphate	20 g
Disodium hydrogen phosphate	60 g
Potassium dihydrogen phosphate	30 g
Sodium chloride	30 g
Distilled water	1 L

Table 4.2. Ingredients of B-medium

Ingredients	Concentration
Magnesium chloride	1 mM
Calcium chloride	0.1 mM
Ferric chloride	0.01 mM
Distilled water	1 L

4.2. QSIS 1 ASSAY

QSIS 1 assay was done to screen the possible QSI compounds. The method, developed by Rasmussen and his coworkers [115], is based on *V. fisheri luxR* QS system that is cloned in an *E. coli* strain. The bacteria will die in the presence of phospholipase A that is encoded by the gene inserted under the control of the *luxI* promoter. If there any QSI molecule is added to culture medium the bacteria will grow since no phospholipase A was produced. Figure 4.1 shows the construct of recombinant *E. coli*, the monitor strain.

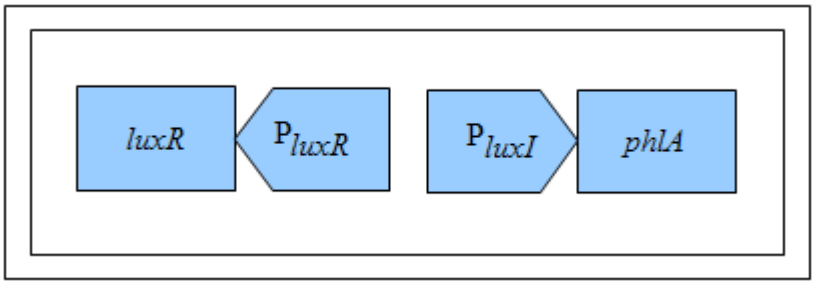

Figure 4.1. Construct of recombinant *E. coli* strain (80)

4.2.1. Preparation of overnight culture

The monitor strain, recombinant *E. coli* harboring *V. fisheri luxR* QS system, was inoculated from stock culture in 10 ml ABT media in a 15 mL falcon tube. The culture was incubated for 14 hours in a shaker at 180 r.p.m. at 30° C.

4.2.2. Preparation of QSIS 1 plates

B-agar medium was prepared by adding Bacto agar to B-Medium to a final concentration of 2% (wt/vol). After autoclaving, 250 mL of B-agar medium was cooled to 45° C. The ingredients shown in Table 4.3 were added in given amounts and the mixture was gently shaken. 1 mL of monitor strain was added to the medium when it is about 43° C and the flask was mixed. The temperature had to be between 40-43° C because higher temperature could kill the monitor bacteria and at lower temperature the medium could solidify. 25 mL of the mixture was pour into petri dishes using serological pipettes. The media was let to cool down and solidify. By using the back tip of a 200 µL test tube, wells were formed in the solidified media. 50 µL of 0.1 M of rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine were added to the wells. As positive control, patulin was used. The plates were let at room temperature for 1 hour to enable the compounds evenly diffuse on the medium's surface. The plates were incubated overnight at 30° C. A day after, the plates were observed for the formation of blue circular ring around the wells. The presence of a ring meant that the compound was a possible QSI.

Table 4.3. Ingredients added to B-Agar medium

Ingredients	Concentration
X-gal	80 µg/mL
IPTG	100 µM

Ampicillin	100 µg/mL
Glucose	4 mg/mL
Casamino acid	1 mg/mL
Thiamine	25 µg/mL
A10	100 mg/mL
3-oxo-C_6-HSL	200 nM

4.3. DOSE RESPONSE ASSAY

QSI molecules selected in QSIS 1 assay were tested further with dose response assay that allowed quantitative measurement. The assay measures the response of bacteria to different concentrations of QSIs [87, 115]. The monitor strain to test QSIs was chosen as a *P. aeruginosa* strain (PAO1) which produced green fluorescent protein (GFP). The strain included a *rhlA-gfp* or *lasB-gfp* fusion together with P_{lac}-lasR-mini-Tn5 inserted upstream to enhance the sensitivity. When *rhlA* was induced, the inserted *gfp* gave rise to a burst of fluorescence. In the case where a QSI was present, the *gfp* signal was reduced. Figure 4.2 shows the construct of recombinant *P. aeruginosa* strain.

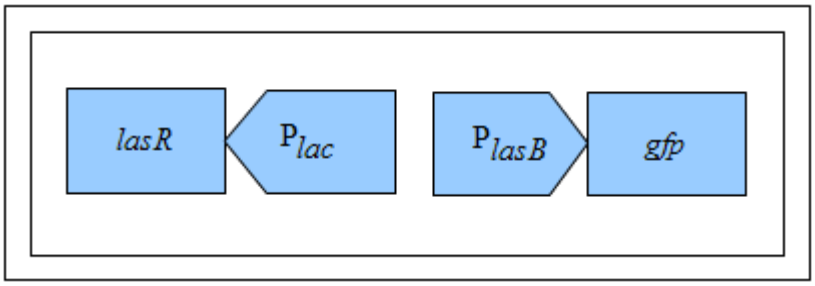

Figure 4.2. Construct of recombinant *P. aeruginosa* strain [115]

4.3.1. Preparation of overnight culture

The monitor strain, *P. aeruginosa* harbouring an *rhl-gfp* fusion was inoculated from stock culture in 10 ml ABT media in a 15 mL falcon tube. The culture was incubated overnight in a shaker at 180 r.p.m. at 30° C.

4.3.2. Preparation of microtiter dish

Before starting to prepare the microtiter dish, overnight bacterial culture was diluted in 0.9% NaCl to an OD_{450} = 0.2. 150 µL of ABT media was put into all rows in the six columns of a 96-well microtiter dish. 150 µL of 16 mM rose absolute was added to wells in the first column. Using a multipipette, wells of first column were mixed and 150 µL from those wells was transferred to the the wells in the second column. In this manner, twofold serial dilution is made from column 1 to column 5. 150 µL of 0.9% NaCl in which rose absolute was dissolved was added to the sixth column. Any rose absolute was added to sixth column since it was the negative control. 150 µL of diluted bacterial culture was put into each well of all columns and mixed to make a total volume of 300 µL. So, the concentration of first column fell down to 4 mM. The microtiter dish was put in Beckman-Coulter DTX-880 plate reader. Bacterial growth was measured at 450 nm every 30 minutes for 15 hours. The temperature was set at 34° C during the measurement process. A day after, a new microtiter dish was prepared in the same way and GFP expression as fluorescence was measured with an excitation and emission wavelength at 485 nm and 535 nm, respectively.

To prepare the microtiter dish for rose oil, clove oil, cinnamon oil and pine turpentine the same procedure was followed. The concentration of pine turpentine in the first column was 12.5 mM. Five dilutions were made as in the rose absolute. The concentrations of rose oil, clove oil and cinnamon oil in the first column were 25 mM. That time, six dilutions were made. The last columns were negative control as in the rose absolute.

4.4. TOXICITY ASSAY

L929s at passage 12 were seeded on 96 well-plates at concentration of 3000 cells per well. A day after, three concentrations of rose absolute (4, 2 and 1 mM) was prepared. After diluted with 0.9% NaCl, solutions were prepared in DMEM with 10% (v/v) fetal bovine serum (FBS) and 1 % (v/v) PSA. Solutions were added on wells. The cell viability was measured by the MTS assay according to the manufacturer's instructions. MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, inner salt) is a colorimetric assay for measuring the activity of mithocondrial dehydrogenase enzyme that reduces MTS to formazan dyes giving a purple colour. Coloured formazan product can be directly read at 490 nm. MTS assay is widely used because of its accuracy, ease of use and quick detection of toxicity [140-141]. After cells were incubated with rose absolute for 24, 48 and 72 hours, 10 µl MTS reagent with 100 µl growth medium was added to each well. Following three hours incubation, absorbance was read at 490 nm with an ELISA plate reader.

5. RESULTS

5.1. INITIAL DETECTION OF QSI ACTIVITY

Rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine were detected for their QSI activity by using QSIS1 assay which made use of *V. fisheri luxRI* QS system established in *E. coli.* Formation of blue circular rings around the wells showed that all of these molecules have anti QS activity. The results were shown in Figure 5.1.a and b.

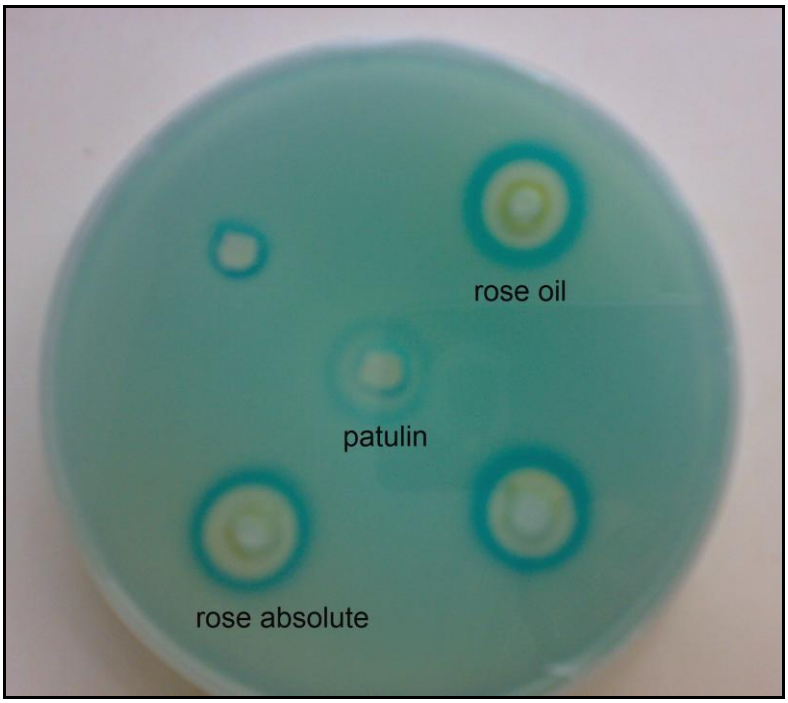

Figure 5.1.a. QSIS1 assay result of rose absolute and rose oil in the presence of QSIS1 selector strain

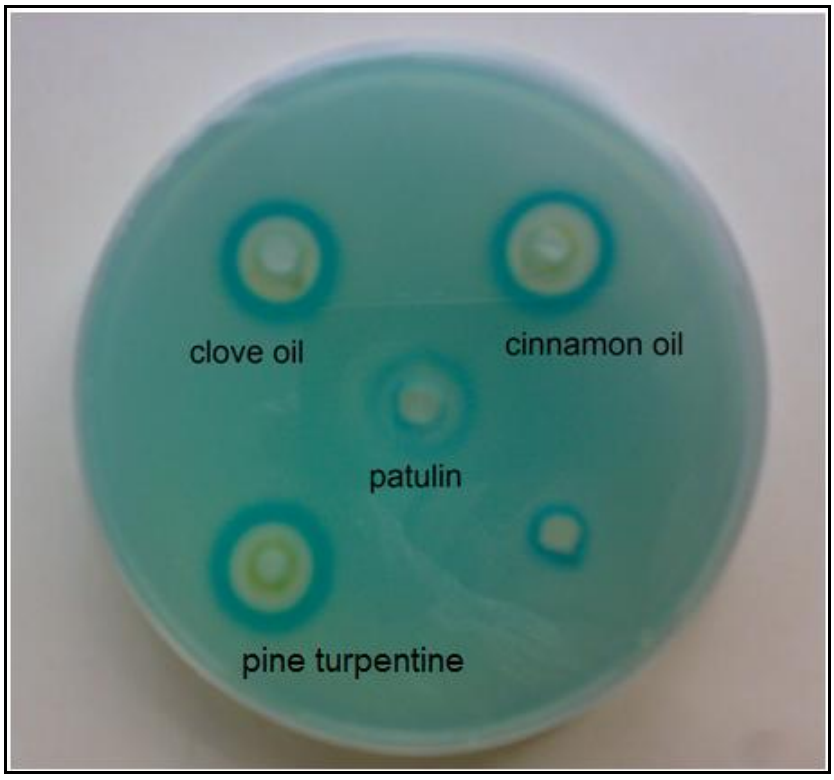

Figure 5.1.b. QSIS1 assay result of clove oil, cinnamon oil and pine turpentine oil in the presence of QSIS1 selector strain

5.2. DETERMINATION OF DOSE RESPONSE RELATIONSHIP

The dose response relationship of rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine were determined at different concentrations of these molecules by using dose response assay as described in the literature. GFP expression and growth were recorded every 30 minutes for 15 hours.

Results of dose response assay showed that rose absolute had no significant effect on the growth of bacteria. GFP expression was reduced at 2 mM and 4 mM in compare to the control group. The result was shown in Figure 5.2.a and b.

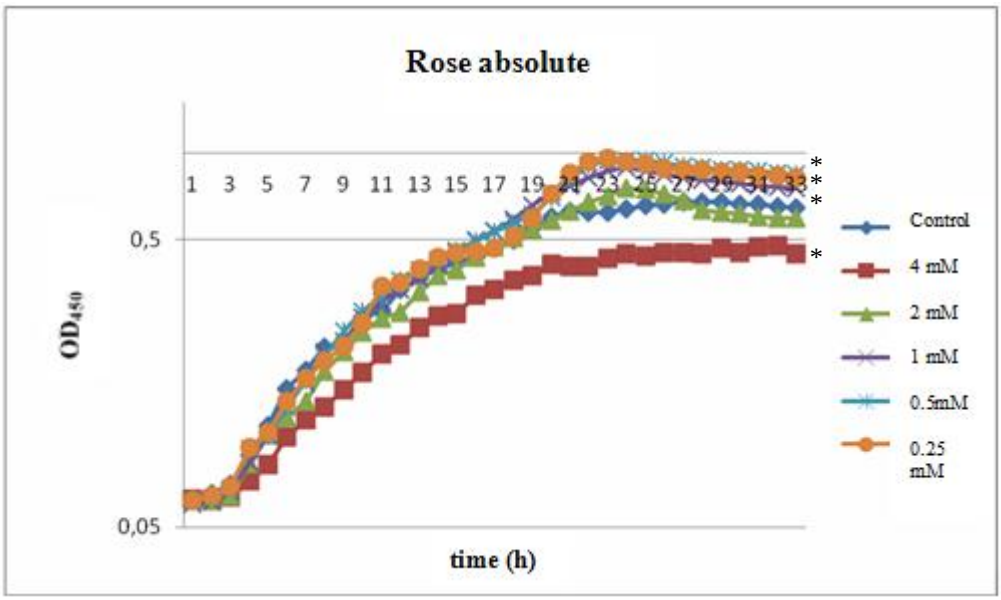

Figure 5.2.a. Growth curve of *P. aeruginosa* PA01 in the presence of rose absolute (*P< 0.05)

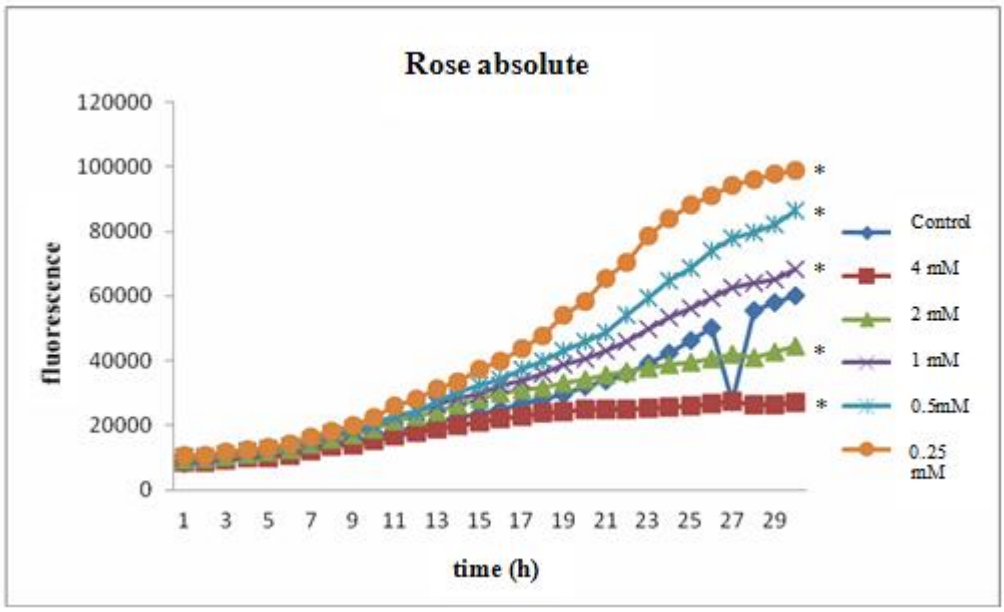

Figure 5.2.b. GFP expression of *P. aeruginosa* PA01 in the presence of rose absolute (*P< 0.05)

According to the results, rose oil was found to be very toxic at 25 mM. At 12 and 25 mM, GFP expression fell down. The results for rose oil were shown in Figure 5.3.a and b.

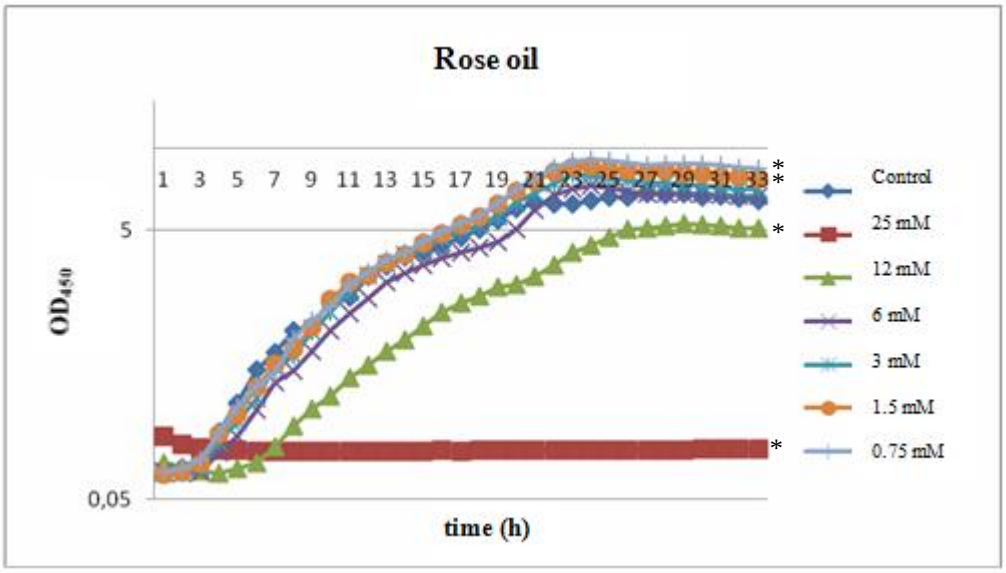

Figure 5.3.a. Growth curve of *P. aeruginosa* PA01 in the presence of rose oil (*P< 0.05)

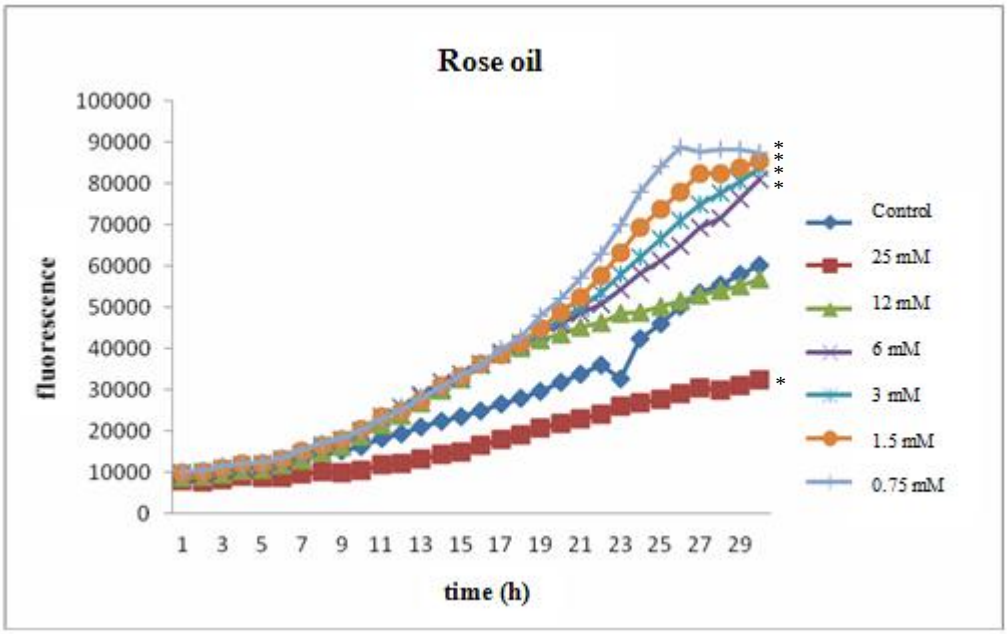

Figure 5.3.b. GFP expression *P. aeruginosa* PA01 in the presence of rose oil (*P< 0.05)

As shown in Figure 5.4.a clove oil at 25 mM had a slight toxic effect on bacterial growth. GFP expression was less at that concentration (Figure 5.4.b).

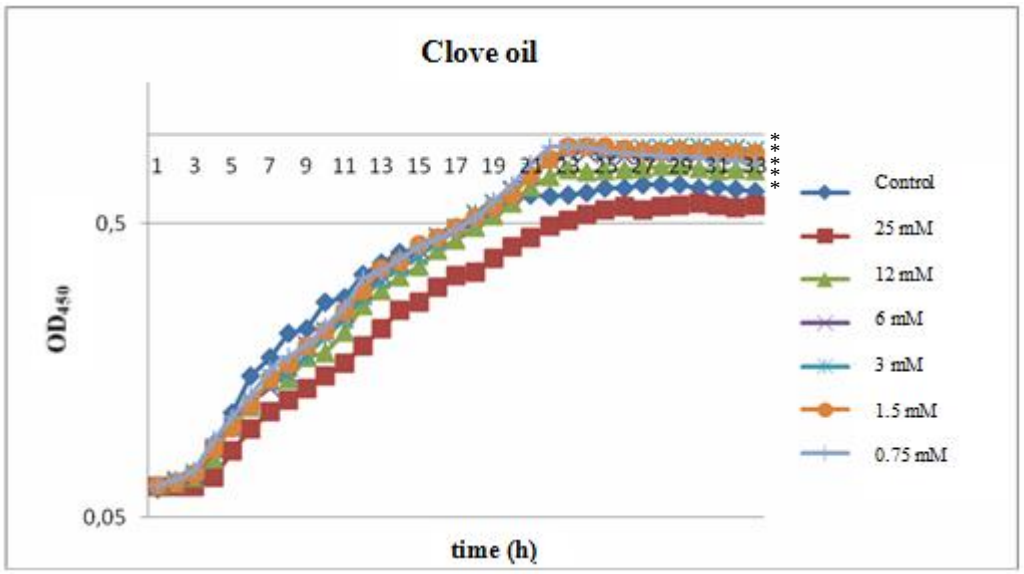

Figure 5.4.a. Growth curve of *P. aeruginosa* PA01 in the presence of clove oil (*P< 0.05)

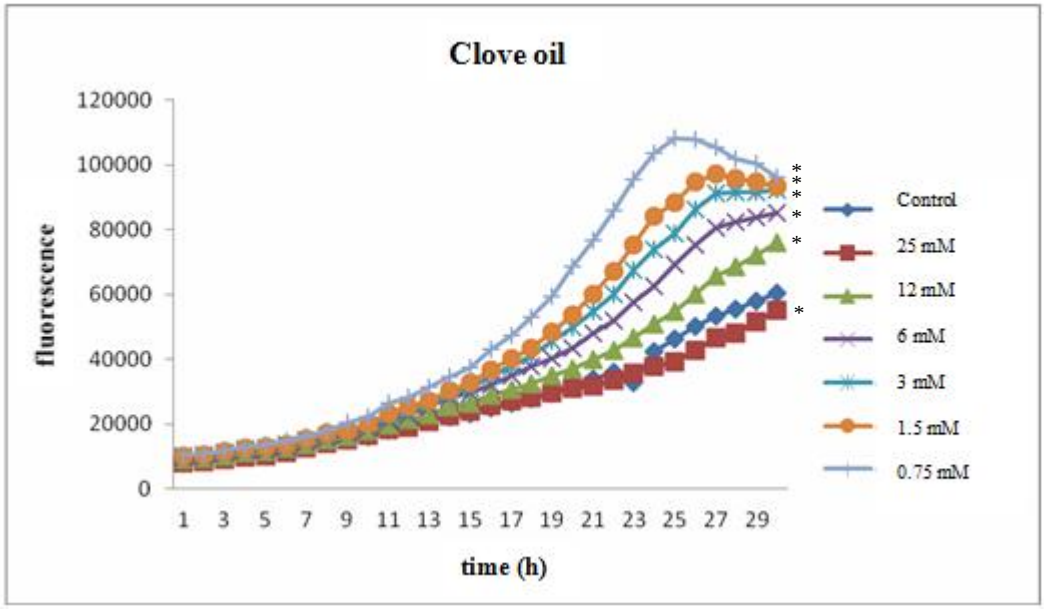

Figure 5.4.b. GFP expression *P. aeruginosa* PA01 in the presence of clove oil (*P< 0.05)

Pine turpentine did not have any inhibitory effect on bacterial growth and caused no reduction in GFP expression as shown in Figure 5.5.a and b. Cinnamon oil was found to be toxic at 12 and 25 mM. At 6, 12 and 25 mM, GFP expression was reduced. The results were shown in Figure 5.6.a and b.

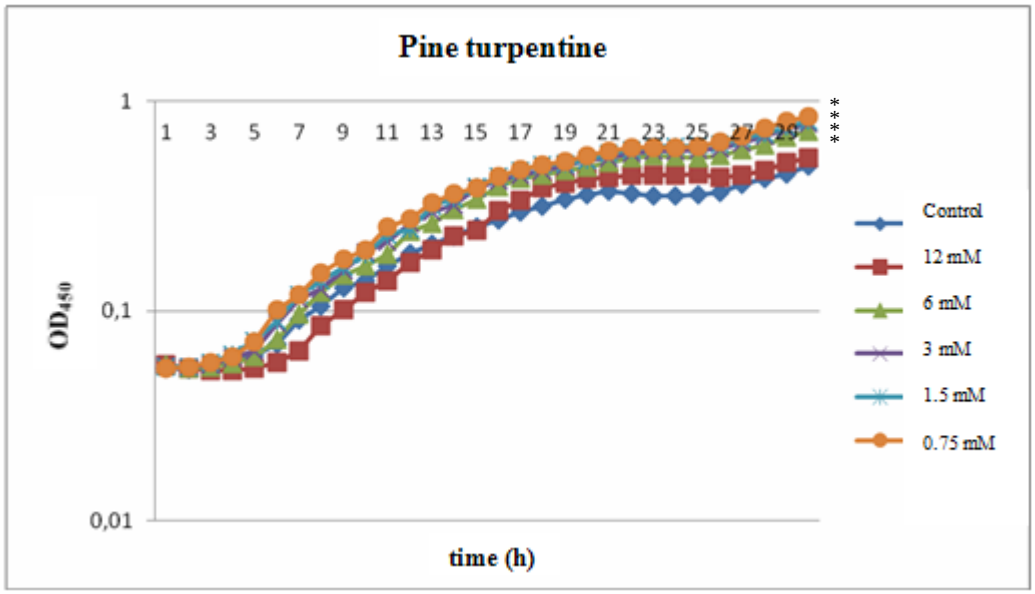

Figure 5.5.a. Growth curve of *P. aeruginosa* PA01 in the presence of pine turpentine (*P< 0.05)

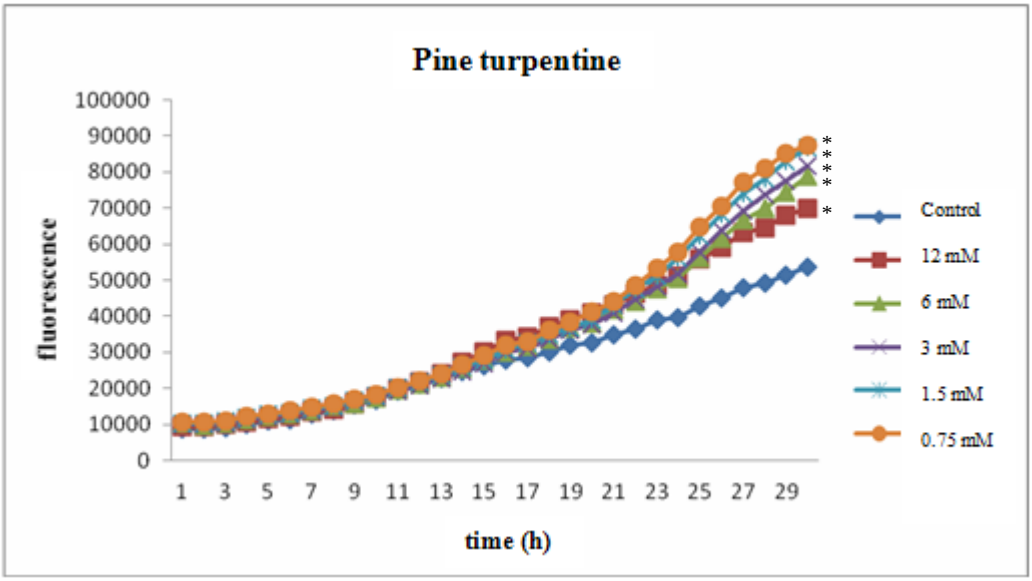

Figure 5.5.b. GFP expression *P. aeruginosa* PA01 in the presence of pine turpentine (*P< 0.05)

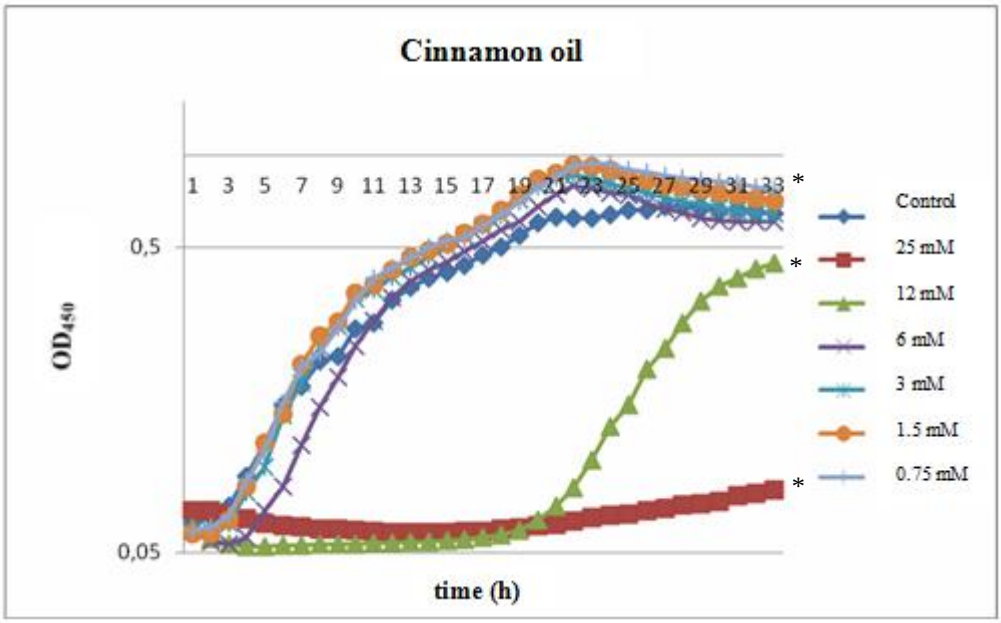

Figure 5.6.a. Growth curve of *P. aeruginosa* PA01 in the presence of cinnamon oil (*P< 0.05)

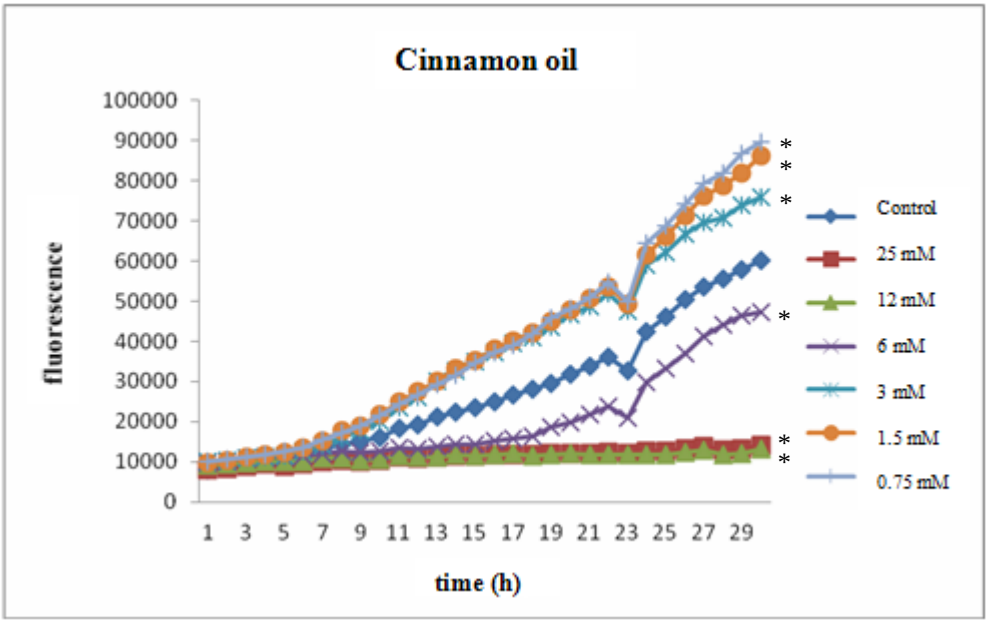

Figure 5.6.b. GFP expression *P. aeruginosa* PA01 in the presence of cinnamon oil (*P< 0.05)

5.3. TOXICITY OF ROSE ABSOLUTE

Depending on the literature, toxicity assay was performed at three concentrations of rose absolute for three days. The results showed that the molecule was not toxic for the cells. It was shown in Figure 5.7.

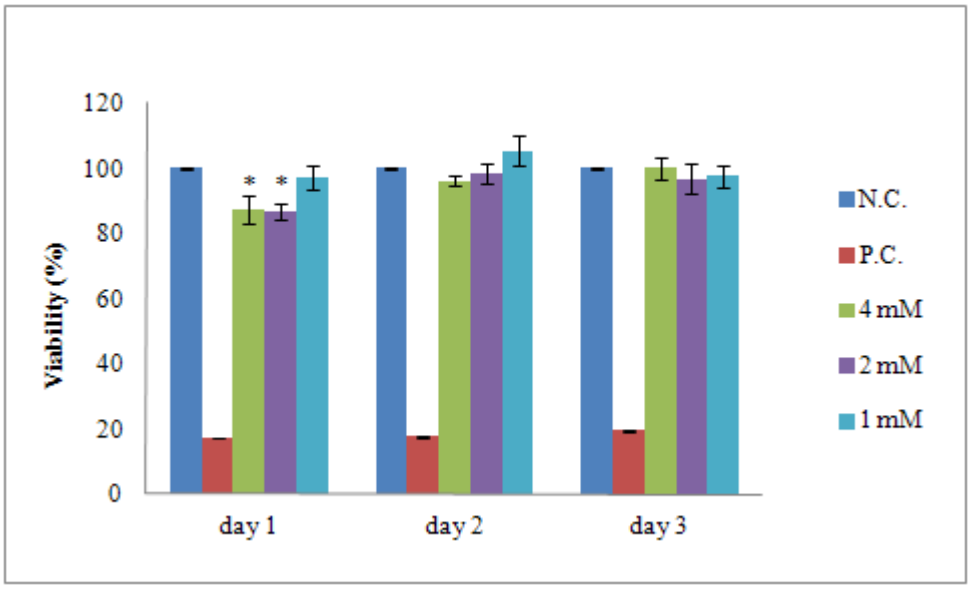

Figure 5.7. Toxicity results of three concentrations of rose absolute (*P< 0.05)

6. DISCUSSION

The development of novel therapeutics to fight against infections is a major challenge as increasing numbers of bacteria have gained resistance against conventional antibacterial agents including broad-spectrum antibiotics [86]. This disconcerting fact is common to many bacterial species including various strains of the opportunistic pathogen *P. aeruginosa*. Being a gram negative bacterium, *P. aeruginosa* causes blood, skin, eye and genitourinary tract infections particularly in patients who immunocomprised by surgery, cytotoxic drugs, burns and HIV. The variety of diseases *P. aeruginosa* causes is due to an impressive array of both cell-associated and extracellular virulence factors including toxins, proteases and hemolysins produced by this organism [72-73].

The resistance against antibiotics is achieved by multidrug efflux pump system, impermeability of the outer membrane, adaptive mutations or production of antibiotic modifying enzymes. Repeated exposures to same antibiotics also increase bacterial resistance [88-89]. Besides causing resistance development, antibiotics are also inefficient to destroy bacterial biofilms which can cause chronic infections as well as persistent infections in the lungs of cystic fibrosis patients. Biofilms are considered as the most successful expression of the prokaryotic genome since the cells forming the biofilm are metabolically more efficient and have resistance to environmental stresses. The bacteria in the biofilm can withstand host immune response and are more tolerant to various antibiotics. The reasons of this tolerance is due to restricted penetration, heterogeneous metabolic activity of biofilm forming bacteria and expression of certain genes related to enhanced tolerance against antibiotics. The level that an antibiotic can destroy a biofilm exceeds the highest deliverable doses and this makes the treatment impossible [28, 92].

Considering all the drawbacks of antibiotics, novel approaches are required to control bacterial infections. The discovery that bacterial intercellular communication, namely QS, regulates virulence of bacteria and biofilm formation opens up new ways to treat infections. Due to QS, bacteria can monitor the surrounding population density and coordinately respond to that information by altering different gene expressions through production of signal molecules. In addition to the regulation of virulence factor and biofilm

formation, QS also control various bacterial processes including luminescence, conjugation, sporulation and swarming [1-2].

The signal molecules used in QS are mainly divided into three groups as AHL which are used by Gram negative bacteria, oligopeptides which are used by Gram positive bacteria and another group of autoinducer used by both Gram positive and Gram negative bacteria. AHL mediated QS in Gram negative bacteria has been the most intensively studied mechanism. The system functions by means of *luxI* and *luxR* genes. *luxI* encodes for AHL molecule which binds to activator protein LuxR when the population density reaches a certain threshold level. The bound complex, in turn, activates the transcription of target genes [2].

Most researches on QS have focused on *P. aeruginosa* which possess two well-investigated QS systems, the *las* and *rhl* systems that sense 3-oxo-C_{12}-HSL and C4-HSL signal molecules, respectively. The system basically resembles to LuxR-LuxI homologous system that mentioned above [11]. Since *P. aeruginosa* is the main cause of many infections, investigating this organism and finding solutions to combat it have been a great issue. In this research we used a *P. aeruginosa* strain, PAO1, and focused on inhibiting its *rhl* QS system.

Several studies have shown that QS has a significant role in biofilm formation. When treated with biocides, the biofilm formed by a *lasI* mutant of *P. aeruginosa* was easily detached and dispersed in compare to its wild-type counterpart [30]. Another research revealed that bacteria in a biofilm formed by a *lasRrhlR* double mutant could be killed by 20 μg/ml tobramycin which did not harm a wild type biofilm. Bjarnsholt and coworkers showed that a biofilm formed by QS deficient mutant is much more prone to killing than its wild-type counterpart [142]. QS carries great importance also in the production of virulence factors which are required for bacterial pathogenicty. Many virulence factors are expressed as a consequence of an environmental stimulus, but generally, virulence factor expression does not occur until high cell density is achieved which is controlled by QS [77-78]. A coordinated expression of virulence genes by a high density bacterial population would allow bacteria to produce virulence factors at high enough levels to overcome the host defense and to start an infection [11].

Since development of bacterial infections are supported by QS controlled mechanisms like biofilm formation and virulence factor production, inhibition of QS is a promising approach to combat bacteria. These mechanisms facilitate bacteria to cause infections. As these processes weaken the host defense, invasion of blood vessels, dissemination and systemic inflammatory response syndrome may occur. Since antibiotics barely stop these processes, they have to be prevented before the related QS gene expressions are turned on. There have been several studies that have focused on the attenuation of bacterial pathogenesis by inhibiting QS systems. Although QS inhibiting molecules reduce pathogenicity, they do not affect bacterial growth [90]. In our study, we aimed to explore such molecules that did not reduce the growth of *P. aeruginosa* while inhibiting its QS systems.

So far, several natural and synthetic molecules have been investigated for their ability to inhibit QS. Halogenated furanones from *Delisea pulchra* were the first discovered one. Manefield and coworkers showed that these molecules structurally resemble AHLs and display inhibitory activity at ecologically realistic concentration in AHL bioassays [110]. Although natural furanones have inhibitory effect on many bacterial strains, they have no effect on *P. aeruginosa.* To overcome this problem various synthetic derivaties of furanones have been developed and found to be effective against QS systems found in *P. aeruginosa* [11, 114]. Besides natural and synthetic furanones, various plant species have been shown to have anti-QS activity including crown vetch, carrot, soybean, water lily, tomato, pea seedlings (*Pisum sativum*), habanero (chilli) garlic (*Allium sativum*) and *Scorzonera sandrasica* [115-117]. In a study, Tinaz et. al. showed that the chloroform-soluble compounds extracted from *Scorzonera sandrasica* inhibits the production of violacein in *C. violaceum* and carbapenem antibiotic in *E. Carotovora* [150]. Various secondary metabolites have been also shown to have QSI activity. Among them penicillic acid (PA) and patulin have been found to inhibit QS strongly. Since patulin was found to affect 45% of the QS genes in *P. aeruginos*a, it has been used as positive control in many studies [96]. In our study, when testing QSI activity of various molecules we used patulin as positive control.

All these findings showed that several molecules have potential to inhibit QS. Some further investigations demonstrated that QSI compounds can make biofilms more vulnerable to

antimicrobial agents. Hentzer and coworkers showed that a *P. aeruginos*a PA01 biofilm treated with the halogenated furanone compounds 30 and 56 was easily destroyed by tobramycin treatment in compare to untreated biofilm. The treated biofilm was also more susceptible to dispersal by SDS [11]. In their researches, Rasmussen et. al. demonstrated that biofilms treated with either garlic extract, patulin or penicillic acid were easily destroyed by tobramycin contrary to the untreated biofilms [96, 115].

Although various synthetic molecules have been tested for QSI activity and found to be effective, most of these compounds possess less QS inhibition ability than natural molecules. In addition to that, some of these molecules including halogenated furanone derivatives are toxic for humans [120]. Considering the fact that synthetic molecules could be harmful to the human and nature, discovering natural molecules that inhibit QS is more sensible and healthier. However, this does not mean that all natural QSI molecules are safe, healthy and applicable to treatment of human patients. For example, patulin and penicillic acid have strong QS inhibition activity but they are mycotoxins. Garlic, being a strong QSI, has to be consumed by humans in a huge amount if wanted to be used as treatment and that quantity would cause severe secondary effects [99]. So, in our study we aimed to search novel QSI molecules from natural sources that are nontoxic and safe for both human beings and nature. In our previous studies, we screened several natural compounds and determined five of them (rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine) to have potential to inhibit QS. Therefore, we focused on these compounds and investigated their effects on *P. aeruginos*a *rhl* QS system.

Obtained from *Rosa damascena* Mill.; rose essential oil and absolute are widely used for various industrial purposes [126]. Because of its analgesic, hypnotic, and anti-inflammatory effects; rose extracts have a wide usage in medicine, food and cosmetic industries [128]. *R. damascene* is produced in extremely large amounts in Isparta. In our investigations we focused on *R. damascene* from Isparta, because it is safe, healthy, overly produced and endemic. Since it is endemic, it is easily accessible and cheap, as well. Furthermore, there is no research on its effects on QS systems. *R. damascena* from Isparta has not been explored for its ability to inhibit QS. Especially, there is not any study that investigates the anti-QS properties of rose absolute in the literature. GC–MS analyses of rose absolute determined its major constituent as phenylethyl alcohol (78.38%) [131].

Phenylethyl alcohol has been approved by the U.S. Food and Drug Administration (FDA) for usage in foods as a flavoring substance. Phenylethyl alcohol is naturally found in more than 200 foods. It is considered as ”generally recognized as safe” (GRAS) by the Flavor and Extract Manufacturer’s Association (FEMA) Expert Panel [143].

Besides rose essential oil and absolute, we screened cinnamon oil, clove oil and pine turpentine for their anti-QS properties. Cinnamon was found to possess anti inflammatory, antioxidant, anticancer and antibacterial properties [135]. Proven to have no toxic and adverse effects on humans, essential oils of cinnamon are widely used in pharmaceutical preparations, seasonings, cosmetics, foods, drinks, commodity essences and chemical industries [136]. There have been some investigations indicating that cinnamon oil has QSI properties [137]. Therefore, we used it as a positive control in our study and compared our results to the findings in the literature. Clove oil has been studied for its antibacterial, antimicrobial, and antifungal properties and shown to be environmentally safe and nontoxic to humans for use in medicine, perfume, and food flavoring [133]. Therefore, we included clove oil in our research and explored its effects on QS. Being natural molecule, pine turpentine is employed for fragrance and flavor use and has also use in pharmaceutical industry [138]. There are not any investigations screening it as a QSI.

In order to detect the anti-QS effects of rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine, we first tested them with QSIS 1 assay. This assay, developed by Rasmussen et. al., is based on *V. fisheri luxR* QS system that is cloned in an *E. coli* strain. Phospholipase A encoded by the gene inserted under the control of the *luxI* promoter causes bacterial death. However, in the presence of QSI compounds the bacteria will grow since no phospholipase A is produced. If a QSI is present, on the B-agar medium, a blues ring forms around the well containing the molecule to be tested [115]. In their research, Rasmussen et. al. screened 54 different molecules including food sources, herbal medicines and chemical compounds. 13 of them were found to have QSI activity. In our study, blue circular ring formed around all the wells indicating that all of our compounds had QSI activity. We used patulin as positive control. The rings formed around the other compounds were more distinctive in compare to the ring formed around patulin. This demonstrated that our molecules had strong QSI properties. After getting this result, we continued our experiments with another assay, dose response, which enabled us to get quantitative

measurements.

For quantitative detection we made use of the system that was developed by Hentzer et. al. The *gfp*-based analysis measures the response of bacteria to different concentrations of QSIs. [87]. *P. aeruginosa* PAO1 harboring *rhlA-gfp* was used as monitor strain. In this system, when *rhlA* is induced, the inserted *gfp* give rise to a burst of fluorescence. In the case where a QSI is present, the *gfp* signal is reduced. From QSIS 1 assay we obtained crude estimation about QSI potential of our compounds. In this assay, we tested several different concentrations of the compounds to determine the maximal concentration at which bacterial growth is not affected but maximum reduction in *gfp* production is obtained. Therefore, we made two-fold dilution series of each molecule and measured bacterial growth at 450 every 30 minutes for 15 hours. We measured *gfp* expression as fluorescence with an excitation and emission wavelength at 485 nm and 535 nm, respectively, every 30 minutes for 15 hours.

Measurement of bacterial growth in the presence of rose absolute showed that this compound at all concentrations did not significantly affect bacterial growth. It was seen that at 2 and 4 mM, *gfp* expression was decreased. At 4 mM, there was about 55% reduction in the expression of *gfp* in compare to the control group. At 2 mM, about 26% reduction was observed in *gfp* expression. These findings indicate that rose absolute at 2 and 4 mM interferes with the inhibition of *rhl* QS system of *P. aeruginosa.* In their research, Hentzer et. al., investigated the QSI potential of furanone 56. The results showed that 5 μg furanone ml^{-1} caused a 40% reduction in *gfp* expression. 10 μg furanone ml^{-1} caused a 60% reduction.

Dose response assay for rose oil indicated that 25 mM was highly toxic. 12 mM and lower concentrations did not affect bacterial growth. At 12 and 25 mM, *gfp* expression fell down. Reduction at 25 mM was due to the bacterial death. Reduction at 12 mM demonstrated that this concentration inhibits QS without killing bacteria. Measurement of bacterial growth in the presence of clove oil showed that concentrations below 25 mM did not have any significant effect on growth. At 25 mM *gfp* expression fell down indicating a QS inhibition effect. Dose response assay for pine turpentine showed that this compound had no toxic effect on bacterial growth. However, any reduction in *gfp* expression was observed. This

might be explained as it possessed no inhibitory effect on *rhl* QS systems and might have inhibitory effect on *lasB* QS system. Measurement of bacterial growth in the presence of cinnamon oil indicated that it was toxic at 12 and 25 mM. At 6, 12 and 25 mM, the expression of *gfp* was reduced. The decrease at 12 and 25 mM was due to the reduction in the growth. The decrease at 6 mM interferes with the inhibition of QS. In a research, Niu et. al. investigated the anti-QS effects of cinnamaldehyde which is the major constituent of cinnamon oil. They found out that cinnamaldehyde at concentrations of 100 μmol l^{-1} or less significantly reduced 3-hydroxy-C4-HSL and 3-oxo-C_6-HSL mediated signaling indicating its QSI potential [137]. This finding strengthened our result that cinnamon oil was a potential QSI.

Using the same protocol, Rasmussen et. al. investigated the dose response relationship of several compounds. Among them, they found garlic extract and 4-NPO as the most active. At a concentration of 2% (vol/vol), garlic extract was found to significantly reduce the expression of *gfp* without affecting the bacterial growth. 4-NPO at concentration of 100 μM was determined as the optimum inhibition concentration that did not affect growth.

Looking at the remarkable and promising results of dose response assay for rose absolute and considering the advantageous features of this molecule such as being safe, cheap and easily accessible we decided to continue our study with rose absolute. Bearing in mind that there is no investigations on anti-QS effects of rose absolute, we focused on this compound. Therefore, we investigated the toxicity of rose absolute on mouse fibroblast cells. In order to continue with *in vivo* experiments in the future part of this study we explored the possible toxic effects of rose absolute on fibroblasts. We measured the cell viability by MTS assay. After we incubated fibroblast cells with 4, 2 and 1 mM rose absolute for 24, 48 and 72 hours, we added MTS reagents to each sample. Following three hours incubation, we measured absorbance at 490 nm. The results showed that rose absolute did not have any toxic effects on cells. Even after, three days incubation, the viability did not significantly change.

In addition to the methods that we used to detect QS inhibition activity, several other methods have been employed for the detection of a number of natural and synthetic QSI molecules. When screening furanone 30, Hentzer et. al. investigated the effect of this

molecule on the production of some QS controlled extracellular virulence factors such as protease, pyoverdin and chitinase. They found out that the production of these factors was suppressed in *P. aeruginos*a cultures that were grown in the presence of furanone 30 [11]. In another study, to investigate the effect of azithromycin on QS, the change in the production of elastase and rhamnolipid was monitored in the presence of azithromycin. To monitor the elastase production, elastin Congo red assay was used and to determine the rhamnolipid production, azithromycin gradient incorporated M9-based agar plates were used. Results showed that azithromycin inhibited QS of *P. aeruginos*a PAO1 strain without affecting the bacterial growth [144]. McLean et. al. developed a simple soft agar overlay method to rapidly screen potential QSI molecules. The system was based on pigmentation inhibition caused by either *P. aureofaciens* or *C. violaceum* which were used as indicator cultures [145]. Using this system, Vattem et. al. screened dietary phytochemicals for their QS inhibition activity. All the phytochemical extracts they used were found to inhibit QS [146]. Another novel method to screen QSI molecules was developed by Yang and coworkers. They used structure-based virtual screening to search QSIs from a database consisting of approved drugs and natural compounds. They found out that salicylic acid, nifuroxazide and chlorzoxazone significantly inhibited QS. The results showed that this computer aided identification system was an efficient tool to screen novel QSIs [147].

Although many compounds that inhibit different QS systems have been found so far, there are still some limitations for the inhibition of QS. The first limitations might be resistance development. In a research, Zhu et. al. demonstrated that bacteria could simply escape from QS inhibition by overexpressing QS related genes. They found out that many synthetic AHL analogues inhibited QS in wildtype *A. tumefaciens*, whereas any inhibition occurred in a transformed strain that overexpressed the *A. tumefaciens* LuxR homologue TraR [148]. Another limitation to the use of QSIs might be due to the lack of specificity. Different pathogens have different QS systems, but most of the QSIs found so far do not specifically inhibit the QS of one or more pathogens. Therefore, QSIs are needed to be much more pathogen-specific in compare to conventional wide-spectrum antibiotics. These molecules might also require some diagnostic systems since their use is not depend on the identity of organism but depend on the QS system they possess [149].

7. CONCLUSION and FUTURE WORK

In conclusion this study revealed that rose absolute, rose oil, clove oil, cinnamon oil and pine turpentine are potential QSIs. At particular concentrations, these molecules do not have any toxic effects on bacterial growth and have inhibitory effects on *rhl* QS system of *P. aeruginosa.* Among these molecules, we focused on rose absolute, because so far there is no study on its potential to inhibit QS. Other reasons why we focused on rose absolute are that this molecule obtained from *R. damascena* from Isparta is endemic to Turkey and therefore easy to obtain. Furthermore, in compare to the rose essential oil it is much cheaper.

The results of this study showed that rose absolute has strong inhibitory effects on *rhl* QS. At 2 and 4 mM, rose absolute inhibits QS without killing bacteria. For the future, it might be possible to use this compound as an antipathogenic agent in the treatment of bacterial infections. In order to have such kind of usage, it first has to be tested *in vivo.* In our further investigations, we tend to investigate OS inhibitory effects of rose absolute *in vivo.* In the case of obtaining positive results, rose absolute might be formulated to be used as an antipathogenic agent. Combinations with antibiotics might increase its effect. The combination might decrease the adverse effects of antibiotics and also increase their shelf-life.

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