INHIBITION OF QUORUM SENSING IN *Pseudomonas aeruginosa* PAO1 BY *Phyllanthus amarus* EXTRACT AND BAICALEIN

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ABSTRACT

Quorum sensing (QS) is a sophisticated signalling system employed by bacteria to regulate a population wide gene expression with the utilization of diffusible chemical signalling molecules known as autoinducers. This system is widely exploited by both Gram-negative and Gram-positive bacteria to control a wide array of physiological functions. *Pseudomonas aeruginosa* is one of the infamous bacteria that employ the QS system to regulate the expression of virulence factors such as biofilm formation, antibiotic resistance, and the production of toxins, proteases, and haemolysins. Hence, this study aimed to determine the mode of action of QS inhibitors against *P. aeruginosa* using crude plant extracts and synthetic compound. A total of 15 Malaysian endemic plants were selected and screened for their anti-quorum sensing properties using *Chromobacterium violaceum* CVO26. Subsequently, only *Phyllanthus amarus* was chosen for further screenings. The methanolic extract of *P. amarus* successfully inhibited the short and long chain AHL activities when screening using *Escherichia coli* bioluminescence biosensors. The extract also attenuated *P. aeruginosa* QS-controlled virulence factors, namely, swarming, pyocyanin production, and the expression of the cytotoxic lectin. This is the first report of anti-quorum sensing activity of *P. amarus* against several QS-controlled virulence factors of *P. aeruginosa*. Our work also focussed on synthetic compound that are of plant origin. Baicalein, a flavone was chosen for the study. Baicalein exhibited promising anti-quorum sensing activities during preliminary screenings using *E. coli* biosensors by inhibiting bioluminescence produced. Baicalein, at low concentrations, also significantly inhibited several virulence factors of *P. aeruginosa* without impairing its' growth. These significant findings have prompted us to divulge the global gene expression of *P. aeruginosa* when exposed to these two compounds by using RNA-sequencing technology. Bioinformatics analysis has given us insights into the mechanism of QS-inhibition by baicalein. It is

hoped that the findings from this research will be of use in a pharmacological perspective especially in the current crisis of the emergence of new multidrug resistant organisms (MDRO).

ABSTRAK

Penderiaan kuorum adalah satu sistem isyarat istimewa yang digunakan oleh bakteria untuk mengawal pelbagai ekspresi gen dengan menggunakan signal resap yang dikenali sebagai "pencetus-auto" dalam keadaan populasi ambang. *Pseudomonas aeruginosa* ialah satu bakteria yang terkenal sebagai bakteria yang menggunakan sistem penderiaan kuorum untuk mengawal pembentukan biofilm dan ekspresi faktor kebisaan seperti pengeluaran toksin, protease dan haemolysin. Oleh demikian, kajian ini bertujuan untuk penentuan cara tindakan kompaun anti-penderiaan kuorum terhadap *P. aeruginosa* PAO1. Kompaun anti-penderiaan kuorum yang digunakan ialah ekstrak mentah dari tumbuhan dan kompaun sintetik. Ekstrak dari sejumlah 14 tumbuhan endemik dari Malaysia dipiih dan ditapis untuk mengetahui ciri-ciri anti-penderiaan kuorum dengan biosensor bakteria, *Chromobacterium violaceum* CV026. Selepas itu, ekstrak *Phyllanthus amarus* dipilih untuk kajian seterusnya kerana ia adalah satu-satunya sampel yang menunjukkan keputusan positif. Ekstrak methanolik daripada P. amarus melarang bioluminisasi biosensor *Escherichia coli* yang diuji terhadap *P. aeruginosa* PAO1 yang mengeluarkan kedua-dua rantaian pencetus-auto yang pendek dan panjang. Ekstrak ini juga menghentikan faktor pembisaan yang dikawal oleh penderiaan kuorum pada *P. aeruginosa* PAO1 seperti, perayapan, produksi pyocyanin dan ekspresi cyto-toksik lectin. Sehingga kini, ini adalah laporan pertama mengenai kesan anti-penderiaan kuorum oleh ekstrak *P. amarus* terhadap faktor kebisaan yang dikawal penderiaan kuorum pada *P. aeruginosa* PAO1. Selain daripada kompaun natural, kajian ini fokus kepada kompaun sintetik yang berasal dari tumbuhan. Kompaun sintetik iaitu baicalein telah dipilih. Baicalein mempamerkan ciri-ciri anti-penderiaan kuorum yang memberangsangkan dengan menghalang bioluminisasi biosensor *E. coli*. Baicalein tidak menjejaskan pertumbuhan *P. aeruginosa* PAO1. Penemuan yang menarik ini telah membawa kepada kajian ekspresi gen global dalam *P. aeruginosa* PAO1. Teknologi

penjujukan RNA digabungkan dengan analisis bioinformatik telah mendedahkan mekanisma anti-penderiaan kuorum oleh kompaun sintetik (baicalein). Kajian ini diharap dapat memberikan faedah kepada bidang pharmokologi dalam mengawal kemunculan bakteria yang tahan-dadah.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
×	:	Times
× *g*	:	Gravity
°C	:	Degree Celsius
µM	:	Micromolar
3-hydroxy-C4-HSL	:	*N*-(3-hydroxybutanoyl)-L-homoserine lactone
3-hydroxy-C8-HSL	:	*N*-(3-hydroxyoctanoyl)-L-homoserine lactone
3-hydroxy-C10-HSL	:	*N*-(3-hydroxydecanoyl)-L-homoserine lactone
3-hydroxy-C12-HSL	:	*N*-(3-hydroxydodecanoyl)-L-homoserine lactone
3-oxo-C6-HSL	:	*N*-(3-oxo-hexanoyl)-L-homoserine lactone
3-oxo-C8-HSL	:	*N*-(3-oxo-octanoyl)-L-homoserine lactone
3-oxo-C10-HSL	:	*N*-(3-oxo-decanoyl)-L-homoserine lactone
3-oxo-C12-HSL	:	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
3-oxo-C14-HSL	:	*N*-(3-oxo-tetradecanoyl)-L-homoserine lactone
ACN	:	Acetonitrile
AGE	:	Agarose gel electrophoresis
AHL	:	*N*-acyl homoserine lactone
AI-2	:	Autoinducer-2
AIP	:	Autoinducer peptide
bp	:	Basepair
C4-HSL	:	*N*-butanoyl-L-homoserine lactone
C6-HSL	:	*N*-hexanoyl-L-homoserine lactone
C7-HSL	:	*N*-heptanoyl-L-homoserine lactone
C8-HSL	:	*N*-octanoyl-L-homoserine lactone
C10-HSL	:	*N*-decanoyl-L-homoserine lactone

C12-HSL	:	*N*-dodecanoyl-L-homoserine lactone
dH_2O	:	Distilled water
dsDNA	:	Double-stranded deoxyribonucleic acid
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
g	:	Gram
HAQ	:	4-hydroxy-2-alkylquinolines
HHQ	:	2-heptyl-4-hydroxyquinolone
h	:	Hour
HS	:	High sensitivity
Kb	:	Kilobase pair
L	:	Litre
LB	:	Luria-Bertani
M	:	Molarity
min	:	Minute
mg	:	Milligram
mL	:	Mililitre
mm	:	Milimetre
mM	:	Milimolar
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
NCBI	:	National Center for Biotechnology Information
n	:	Number
ng	:	Nanogram
nM	:	Nanomolar
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction

PGAP	:	Prokaryotic Genome Annotation Pipeline
PQS	:	*Pseudomonas* quinolone signal
qRT-PCR	:	Quantitative real-time polymerase chain reaction
QQ	:	Quorum quenching
QS	:	Quorum sensing
QSI	:	Quorum sensing inhibitors
RNA	:	Ribonucleic acid
s	:	Second
SAM	:	*S*-adenosyl-L-methionine
ssDNA	:	Single-stranded deoxyribonucleic acid
TBE	:	Tris-boric acid ethylenediaminetetraacetic acid
VRE	:	Vancomycin resistance enterococci
v/v	:	Volume per volume
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Study Background

Pseudomonas aeruginosa is a persistent opportunistic pathogen that is responsible for severe and fatal infections in cystic fibrosis (CF) patients and immuno-compromised patients such burned victims and patients with acquired immune deficiency syndrome (AIDS) (Goldberg & Pier, 2000; Pesci *et al.*,1997). *P. aeruginosa* is also one of the most common Gram negative bacteria in nosocomial infections (Van Delden & Iglewski, 1998). The rapid evolution of *P. aeruginosa* in its' natural defences against antibiotics as well as the continuous abuse in the prescription of antibiotics in the healthcare industry has led to the emergence of resistant strains of this bacterium (Koh *et al.*,2013). This alarming crisis necessitates the discovery and development of anti-pathogenic therapies to treat *P. aeruginosa* infections.

Research in the early years have shown that bacteria use signalling molecules known as "autoinducers" to communicate within a population and to relay signals for collective gene expressions. This process is known as quorum sensing,"QS" (Williams, Winzer, Chan, & Cámara, 2007). In *P. aeruginosa,* the QS system plays a crucial role in their adaptation in various environmental niches and *in vivo* conditions as well as activating or repressing numerous physiological pathways such as the expression of virulence factors (Williams & Cámara, 2009). The discovery of QS systems in *P. aeruginosa*, provided researchers new insights towards attenuating pathogenicity with a reduced risk in developing resistance. Many researchers have turned towards natural plants and compounds as means of intercepting the QS systems of pathogens. This study highlights the potential of *Phyllanthus amarus* crude extracts and baicalein as QS inhibitors of *P. aeruginosa* PAO1.

1.2 Hypothesis

P. aeruginosa PAO1 are dependent on their intricate QS system in the expression of virulence factors. *P. amarus* and baicalein may function as potent inhibitors of QS-regulated virulence determinants of *P. aeruginosa* PAO1.

1.3 Objectives

The objectives of this study were as follows:

1. To study *Phyllanthus amarus* and a synthetic plant-based compound, baicalein, for their anti-QS properties.
2. To investigate the ability of the *Phyllanthus amarus* crude extracts and synthetic compound, baicalein, against a spectrum of signalling molecules (AHLs) by conducting anti-QS based bioassays.
3. To access the effects of the selected plant crude extracts and baicalein against the QS-regulated virulence determinants of *P. aeruginosa* PAO1.
4. Transcriptomics analyses by RNA-sequencing to enable accurate gene expression study on the effects of baicalein on *P. aeruginosa* PAO1.

CHAPTER 2: LITERATURE REVIEW

2.1 Quorum Sensing (QS)

Earlier researchers have uncovered bacteria to have their own language of communication, known as, quorum sensing or QS. The term "quorum sensing" was devised by Fuqua *et al*. (1994) describing bacteria cell-to-cell communication process in which bacteria use small signalling molecules known as "autoinducers" (Fuqua, Winans, & Greenberg, 1994). These autoinducers are produced by bacteria population as a mean of determining their population density in a growth condition or environment as well as to regulate the expression of QS-dependent behaviours. These bacterial behaviours are deemed unproductive if executed as a single individual cell but becomes effective when expressed collectively as a group, for example, the secretion of virulence factors and biofilm formation (Bassler, 2002).The circuitry of QS involves the synthesis and release of autoinducers, followed by its detection by receptor molecules. The extracellular concentration of autoinducers increases with the growth in the population of the autoinducer-producing bacteria. When the concentration of autoinducers reaches a threshold level, the bacterial cells responds with a population wide gene expression, resulting in specific alterations in their behaviour. This phenomenon provides leverage to bacterial cells to only express certain behaviours when surviving as a population and not as a single bacterium (Henke & Bassler, 2004).

The research in QS was sparked by the discovery of bacterial cell-density dependent expression of bioluminescence in the marine organism, *Vibrio fischeri.* Sufficient autoinducer concentration in the external environment of *V. fischeri* initiates a signal transduction cascade that ultimately leads to the production of luciferase (Engebrecht,

Nealson, & Silverman, 1983). The findings of this research were the benchmark for the subsequent studies conducted in the aims of understanding QS in various bacteria genera (Bassler, 1999).

2.1.1 Acyl-homoserine lactone (AHL) Dependent QS System of Gram negative Bacteria

A large consortium of bacterial species possesses the mechanism for autoinducer synthesis and response to regulate a population dependent behaviour, as described by Engebrecht *et al.* (1983). A diverse bacteria genera belonging to the Gram-negative proteobacteria utilizes the acyl-homorserine lactone (AHL) regulated QS system. Proteobacteria subdivision that employs this QS system includes alphaproteobacteria (α), betaproteobacteria (β), and gamma proteobacteria (γ). However, to date, no AHL-producing Gram-positive bacteria have been identified or reported (Williams *et al.*,2007). The AHL-dependent QS system possesses 2 main components which are the LuxI and LuxR protein families (Fuqua *et al.*,1994). The AHLs produced are released across the bacterial cells and into the extracellular environment. These AHLs are synthesized by the components LuxI protein families, also referred as AHL synthase. The continuous production and release of AHLs results in its accumulation. Once the concentrations of AHLs produced have reached the threshold, the AHLs bind to the LuxR transcriptional regulator protein family, forming a complex, the LuxR/AHL complex. This complex plays an important role in expression or repression of numerous target genes (Swift *et al.*,2001). Figure 2.1 shows the graphical representation of the synthesis of AHLs by the LuxI synthase and formation of the LuxR/AHL complex.

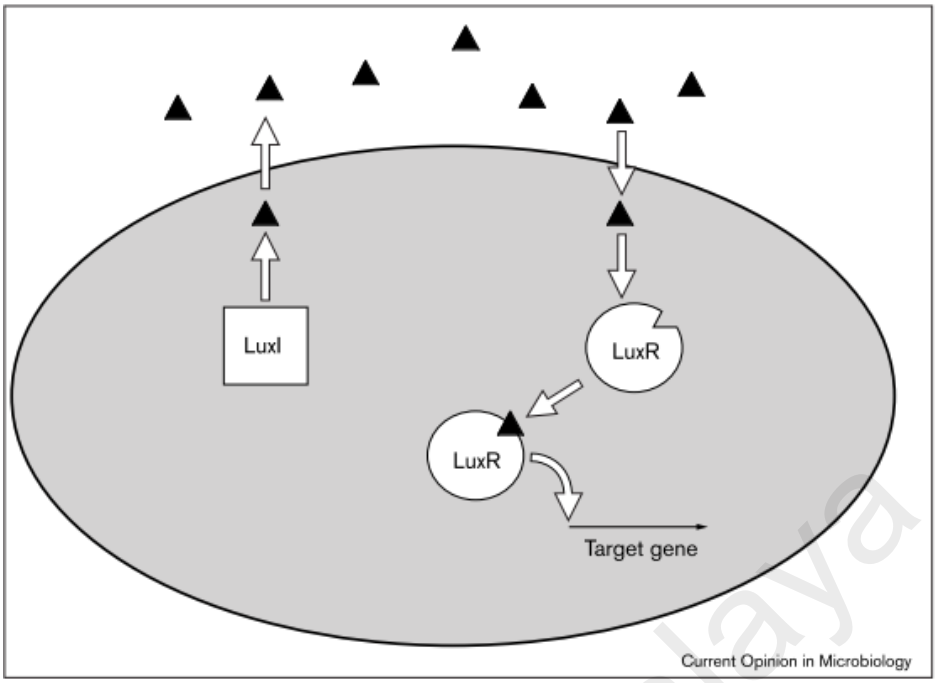


Figure 2.1: The LuxI/LuxR QS system. In numerous proteobacteria, LuxI-like AHL synthases (squares) produces specific AHLs (triangles) into the extracellular environment across the cell membrane. Once a thereshold concentration is reached, the AHLs bound to its cognate receptor, LuxR-like proteins (circle), and subsequently forming the LuxR/AHL complex. This complex activates the transcription of target genes (Bassler, 1999).

However, it is almost often that the regulation of the LuxI protein is in direct target of the LuxR/AHL complex. This is known as positive autoinduction whereby the AHLs produced controls its own synthesis and release. In depth researches in many types of proteobacteria have shown that these organisms possess more than one LuxI/LuxR system which often works in synchrony (Schuster & Greenberg, 2006). Table 2.1 highlights some examples of AHL-dependent QS systems in proteobacteria.

Table 2.1: Examples of AHL-dependent QS systems in Gram negative bacteria.

Organism	Major AHLs	LuxI/LuxR Homologue	Phenotypes	References
Cedecea neteri SSMD04	C4-HSL	*cneI/cneR*	Lipase activity	Tan, Tan, Yin, & Chan, 2015
Pandoraea pnomenusa RB38	C8-HSL	*ppnI/ppnR1, ppnR2*	Lignin degradation, sulphur oxidation	Lim *et al.*,2015
Enterobacter asburiae L1	C4-HSL, C6-HSL	*easI/easR*	Intracellular spreading	Lau, Yin, & Chan, 2014; Lau, Sulaiman, Chen, Yin, & Chan, 2013
Aeromonas caviae YL12	C4-HSL; C6-HSL	*acaI/acaR*	Chitinolytic activity	Lim, Robson, Yin, & Chan, 2014
Aeromonas hydrophila strain 187	C4-HSL	*ahyI/ahyR*	Exoprotease, Biofilm Formation, Hemolysin protein	Chan, Chua, Yin, & Puthucheary, 2014
Pantea stewartii	3-OC6-HSL	*esaI/esaR*	Adhesion, host colonization, exopolysaccharide	Koutsoudis, Tsaltas, Minogue, & Von Bodman, 2006
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C12-HSL	*ppuI/ppuR*	Biofilm formation	Dubern, Lugtenberg, & Bloemberg, 2006

Table 2.1, continued

Organism	Major AHLs	LuxI/LuxR Homologue	Phenotypes	References
Vibrio fischeri	3-oxo-C6-HSL	*luxI/luxR*	Bioluminescence	Kaplan & Greenberg, 1985
Aeromonas hydrophila	C4-HSL	*ahyI/ahyR*	Biofilm and exopolysaccharide (EPS) production	Garde *et al.*,2010
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C10-HSL	*ppuI/ppuR*	Biofilm formation	Arevalo-Ferro, Reil, Görg, Eberl, & Riedel, 2005; Dubern *et al.*,2006
Pseudomonas syringae	3-oxo-C6-HSL	*ahlI/ahlR*	Swimming motility, EPS production	Quinones, Dulla, & Lindow, 2005
Serratia marscescens SS-1	C6-HSL, 3-oxo-C6-HSL	*spnI/spnR*	Pigment and nuclease production, sliding motility	Wei, Lai, Chen, Yeh, & Chang, 2004
Yersinia pseudotuberculosis	C6-HSL, 3-oxo-C6-HSL, C8-HSL	*ypsI/ypsR* and *ytbI/ypsR*	Motility	Atkinson, Throup, Stewart, & Williams, 2002

2.1.2 AHL Molecules

The structures of AHLs discovered so date differs in terms of the carbon number and substitutions at the acyl side chains. The AHL molecule consists of a conserved homoserine lactone (HSL) ring and a fatty acid acyl chain (Whitehead, Barnard, Slater, Simpson, & Salmond, 2001). Figure 2.2 depicts the general structure of an AHL molecule.

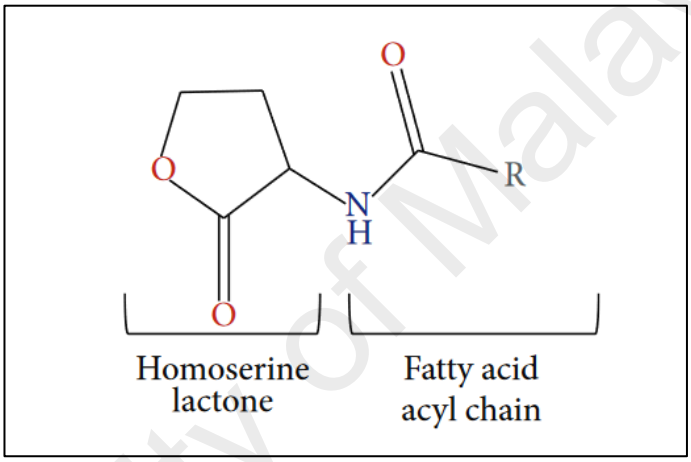


Figure 2.2: General structure of an AHL molecule. "R" represents the various length and substitutions at the acyl-side chains (Ngeow, Cheng, Chen, Yin, & Chan, 2013).

The AHLs contain an acyl side chain length of four to 18 carbons (C4-C18) and often so may contain an addition of an -oxo or -hydroxyl group at the 3rd carbon (Goh *et al.*,2016). The majority of the AHLs identified in Gram negative bacteria consist of an even number of carbons on their acyl side chains. However, productions of odd number carbon lengths, C5 and C7, have been reported (Horng *et al.*,2002; Lithgow *et al.*,2000). Many of the Gram negative bacterial species studied have been found to produce more than one type of AHL molecule.

AHLs are amphipathic (containing both hydrophilic and hydrophobic parts) molecules. The amphipathic nature of the molecules appears to facilitate the diffusion of AHLs freely from the intracellular to extracellular regions of the bacterial cells (Boyer & Wisniewski-Dyé, 2009). However, this only appears to apply only for short chain AHLs as hydrophobicity is affected by the complexity of the AHL molecule itself, for example, length of the acyl chain, and -oxo or -hydroxyl substitutions at C3 (Boyer & Wisniewski-Dyé, 2009; Kaplan & Greenberg, 1985). In cases of long chain AHL molecules, efflux pumps are required to actively transport AHLs out of the cells. An active efflux pump system was identified *Pseudomonas aeruginosa* for the translocation of a long chain AHL molecule with 12 carbons (C12-HSL) in its acyl side chain (Pearson, Van Delden, & Iglewski, 1999). These findings indicate that AHLs molecules with less complexity (i.e. short carbon chain length) may diffuse freely across the phospholipid bilayers of bacterial cells whereas more complex AHL molecules may require an active transport system (Whitehead *et al.*,2001).

2.1.3 QS System in Gram positive Bacteria

Similar to Gram negative bacteria, Gram positive bacteria are also known to regulate several behaviours in a cell density dependent manner. Gram positive bacteria utilize post-translationally modified peptides as their QS signalling molecule. These peptides are known as autoinducing peptides or AIPs (Williams *et al.*,2007). Three types of AIPs classes have been identified: (a) the oligopeptide lantibiotics (Quadri, 2002), (b) 16-membered thiolactone peptides (Chan, Coyle, & Williams, 2004), and (c) the isoprenylated tryptophan peptides (Okada *et al.*,2005). Similar to the LuxI/LuxR QS system, once the extracellular concentrations of the AIPs reaches the threshold, the AIPs then binds to a two component sensor kinase which subsequently results in the

phosphorylation of a response regulator protein. This ultimately results in the transcription of target genes (Rutherford & Bassler, 2012). Among the well-studied QS system in Gram positive bacteria includes *Staphylococcus aureus* (Yarwood & Schlievert, 2003) and *Bacillus subtilis* (Omer, Pollak, Hizi, & Eldar, 2015).

2.1.4 Interspecies Signalling: Communication beyond Boundaries

Similar to AI-1 that is used widely in proteobacteria in species-specific QS, it seemed plausible to researchers that bacteria would require a system to detect the population of other bacterial species colonizing a niche. The hypothesis was conceivable as bacteria would require a mechanism to determine its ratio in a diverse population and initiate a suitable behaviour (Federle & Bassler, 2003). The evidences for the fore mentioned hypothesis was observed in the bioluminescence producing Gram negative *Vibrio harveyi. V. harveyi* responded by producing bioluminescence to cell-free supernatants of containing AI-2 produced by the tested Gram negative and Gram positive bacteria (Bassler, Greenberg, & Stevens, 1997). The AI-2 QS system was also found to be the key regulator in mixed-species biofilm formation between *Streptococcus gordonii* and *Porphyromonas gingivalis,* bacterial species known for their association in periodontitis. The expression of *luxS* in either one of the two mentioned species was sufficient to induce mixed-species biofilm formation (Federle & Bassler, 2003; McNab *et al.,*2003). In addition, AI-2 synthase, LuxS, is widely distributed in the various genera suggesting its role in interspecies communication (De Keersmaecker, Sonck, & Vanderleyden, 2006). These findings highlight the importance of interspecies communication in colonization.

2.2 Detection of AHLs by QS Biosensors

A large number of QS systems of numerous bacterial genera were able to be identified and studied extensively via the use of biosensors or signal molecule reporter strains which are able to detect AHLs produced by other bacteria. These biosensors do not produce AHLs. However, they carry a functional LuxR protein which is cloned together with a target promoter. Presence of exogenous AHLs induces the transcription of the reporter genes, for example, bioluminescence and the production of violacein pigment (Steindler & Venturi, 2007). Furthermore, the development of biosensors has rendered the study of anti-quorum sensing (anti-QS) or quorum quenching to be possible. The inhibition of certain phenotypic response to exogenously supplied AHLs in the presence of a compound would indicate anti-QS activity. *Chromobacterium violaceum* CVO26 is one the biosensors that are extensively used in preliminary screening of anti-QS activity of plant crude extracts (Krishnan, Yin, & Chan, 2012; Priya, Yin, & Chan, 2013; Tan, Yin, & Chan, 2013). Wild-type *C. violaceum* produces the antibacterial purple pigment, violacein, via the CviI/R QS system by producing and responding to C6-HSL. *C. violaceum* CVO26 is a AHL-negative double mini-Tn*5* mutant that does not produce violacein (McClean *et al.*,1997). *C. violaceum* CVO26 best responds to C6-HSL but is able to detect short chain AHLs (C4-HSL – C8-HSL) and their -oxo derivatives. Formation of a halo zone or a colourless turbid zone on a purple background indicates anti-QS properties of the tested compound or plant crude extract (Priya *et al.*,2013). The development of biosensors has assisted in numerous researches in search of a potential QS inhibitor.

2.3 Transcriptomics: The Power of RNA-Sequencing

Transcriptome refers to the complete set of transcripts produced in a cell and their quantity at a specified growth stage or physiological condition (Z. Wang, Gerstein, & Snyder, 2010). Several technologies, such as hybridization or sequenced-based techniques, have been developed over the years to identify and quantify transcripts produced in a cell. One of the popular techniques of transcriptomics used until today is DNA microarrays. The ability of DNA microarrays to study thousands of genes simultaneously has led to significant findings in various biological fields, for example, identification of genes that are differentially expressed in healthy and diseased tissues (Zhang *et al.*,2015). Although DNA microarray is still popular among researchers, the technology has questionable limitations. Probe-transcript hybridization of DNA microarrays are only limited to those genes for which the probes are designed for. Furthermore, the hybridization technique limits the accuracy for the quantification of the number of transcripts especially for low abundance transcripts (Zhao, Fung-Leung, Bittner, Ngo, & Liu, 2014).

Sequencing-based technology has the potential to overcome the limitations of DNA microarray. RNA-sequencing (RNA-seq) uses next-generation sequencing (NGS) technology that generates thousands of megabases of DNA per experiment in a single run in a matter of days. The data generated can be directly analysed to measure the gene expression levels (Trapnell, Pachter, & Salzberg, 2009). RNA-seq technology is able to overcome the limitations of the conventional DNA microarray. At a comparable cost, RNA-seq provides a much better resolution in gene expression quantitation. Furthermore, RNA-seq enables the identification of novel transcripts and provides the

abundance of the transcripts in expressed in the experiment (Marioni, Mason, Mane, Stephens, & Gilad, 2008).

To date, only a few QS inhibitors (QSIs) have been tested for their effects against certain pathogenic bacteria by means of transcriptomics or RNA-seq. The analysis of the effects of QSIs on the global gene expression of *P. aeruginosa* revealed that 40% - 60% of QS-regulated genes are the target of a single QSI (Rasmussen *et al.,* 2005a; Rasmussen *et al.,* 2005b). The outcome of a RNA-seq experiment largely depends on the overall experimental paradigm and objectives, especially in terms of number of samples and the time-point of which RNA is harvested (Rasmussen & Givskov, 2006a).

2.4 Role of QS in Bacterial Pathogenicity and Infections

Bacterial pathogens such as *P. aeruginosa*, *S. aureus*, and *Burkholderia cepacea* (*B. cepacea*) use QS system to regulate and express virulence factors and coordinate population-dependent behaviour such as biofilm formation (Li & Tian, 2012). In pathogenic bacteria, QS-controlled genes often encodes for virulence factors/exoproducts such as siderophores, toxins, proteases, and cell adhesins (Antunes, Ferreira, Buckner, & Finlay, 2010). The expressions of virulence factors are at the expense of the bacterial cells and is only energy saving if expressed in a larger population density (Pai, Tanouchi, & You, 2012). Thus, in terms of establishing an infection, the expression of QS-regulated virulence factors is delayed until adequate bacterial densities have been achieved. This coordinated attack in a host enables a maximal probability of establishing an infection and spreading throughout the host (Castillo-Juárez *et al.*,2015). In cohort with QS, the infectiousness of pathogens is also

determined by immune subversion. It is the ability of the pathogen to evade and to a greater extend, killing immune cells such as phagocytes (Gama, Abby, Vieira-Silva, Dionisio, & Rocha, 2012; Lachmann, 2002). The interaction between pathogenic bacteria and the host is greatly affected by the population density of the bacteria. Therefore, the QS system employed by these bacteria and the manner of which it regulates the genes encoding virulence determinants became a great interest in the field of QS (Winzer & Williams, 2001).

2.5 QS System in *Pseudomonas aeruginosa*

P. aeruginosa is a Gram negative opportunistic pathogen often found to be the leading cause of nosocomial infections. It is the most common pathogen predominantly found causing fatal infections in immunocompromised (i.e. burned victims and acquired immune deficiency syndrome (AIDS) patients) and cystic fibrosis patients (Van Delden & Iglewski, 1998). In addition, *P. aeruginosa* is a versatile organism with unique cell metabolism which enables them to thrive in diverse environments. Environmental strains of *P. aeruginosa* are able to transit from their habitats to become opportunistic pathogens in humans, animals, as well as plants (Lyczak, Cannon, & Pier, 2000). The resistance of *P. aeruginosa* to antibiotic treatments over the past decade has added to the pre-existing crisis of infection severity and high incidence (Obritsch, Fish, MacLaren, & Jung, 2004). These problems have prompted researchers to venture into various therapeutic strategies to address these issues. Targeting the pathogenic and virulence factors of *P. aeruginosa* without stressing the growth bacterial cells were a promising alternative. The dependency of *P. aeruginosa* to QS system to regulate and express hundreds of genes has provided opportunities for exploitation (Kipnis, Sawa, & Wiener-Kronish, 2006). The next sections describes in detail the AHL-dependent QS systems of *P. aeruginosa* and the QS-regulated virulence factors.

2.5.1 AHL-dependent QS systems of *P. aeruginosa* and QS-Regulated Virulence Factors

The regulation and expression of multiple virulence factors of *P. aeruginosa* is dependent on the two sets of LuxR/LuxI homologues. The first QS system discovered in *P. aeruginosa* was the LasI/LasR system. The Las QS system produces and responds to *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL). The AHL, 3-oxo-C12-

HSL, is produced by the LasI synthase and recognized by the transcriptional regulator, LasR (Jimenez *et al.*,2012; Williams *et al.*,2007). The LasR/I QS system regulates the expression of a myriad of virulence factors associated with host cell damage and severe infections. Among the virulence factors regulated by the LasR/I QS system includes; the production of elastases, LasA and LasB (Pearson, Pesci, & Iglewski, 1997; Toder, Gambello, & Iglewski, 1991), synthesis of exotoxin A and alkaline proteases (Gambello, Kaye, & Iglewski, 1993).

The second AHL-dependent QS system is the RhlR/RhlI system which produces and responds to *N*-butanoyl-homoseine lactone (C4-HSL). The AHL is synthesized and sensed by RhlI and RhlR, respectively (Jimenez *et al.*,2012; Williams *et al.*,2007). This system was shown to be responsible in the expression of *rhlAB,* genes involving the synthesis of rhamnolipids biosurfactants (Schmidberger, Henkel, Hausmann, & Schwartz, 2013). Furthermore, the RhlR/RhlI system also regulates the expression of cytotoxic lectins, LecA and LecB (Winzer *et al.*,2000), pyocyanin (Dietrich, Price-Whelan, Petersen, Whiteley, & Newman, 2006; El-Fouly, Sharaf, Shahin, El-Bialy, & Omara, 2015), and hydrogen cyanide (Pessi & Haas, 2000). Interestingly, further work into understanding the intricate QS systems of *P. aeruginosa* revealed a hierarchical relationship between the Las and Rhl systems. The LasR/I system controls the transcription of the RhlR/RhlI system whereby 3-oxo-C12-HSL/LasR ligand-receptor complex up-regulates the transcription of *rhlR* and *rhlI.* Therefore, the activation of LasR/I system gears the subsequent activation of the RhlR/I system (Latifi, Foglino, Tanaka, Williams, & Lazdunski, 1996). The *lasI* and *rhlI* genes are among the genes that are up-regulated by 3-oxo-C12-HSL/LasR AHL-receptor complex. This is known as an autoinduction effect which allows for the rapid increase in AHLs signal production and dissemination throughout the environment (Jimenez *et al.*,2012;

Schuster & Greenberg, 2006; Williams *et al.*,2007). Figure 2.3 illustrates the regulation of virulence factors by Las/Rhl QS system and the hierarchical interactions between the systems.

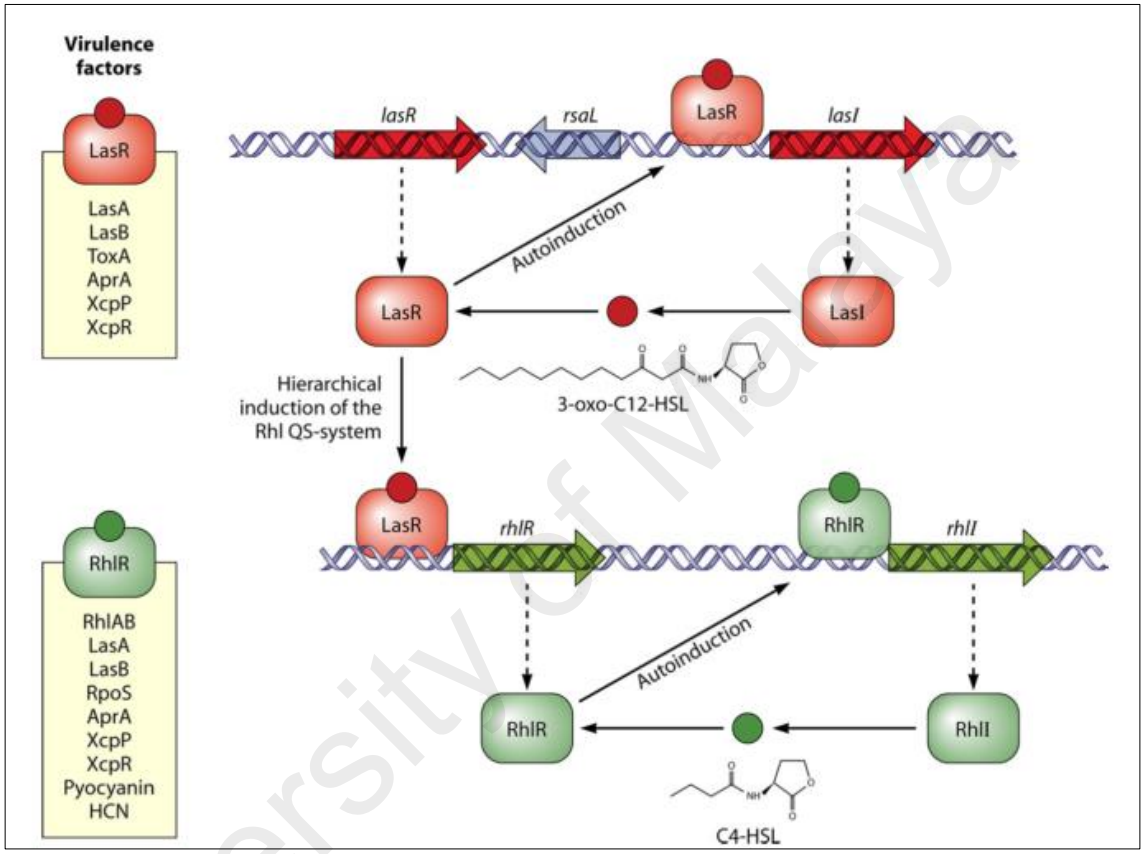


Figure 2.3: The regulation of virulence factors by Las and Rhl QS systems and their interaction in *P. aeruginosa*. Once the extracellular concentration of 3-oxo-C12-HSL has reached the threshold, the AHL-receptor complex then binds to multiple promoter regions, thus inducing or repressing the transcription of genes involved. Figure source: (Jimenez *et al.*,2012).

Transcriptome study on the QS regulation in *P. aeruginosa* revealed that the QS-regulated genes are scattered throughout the bacterial genome. This revolutionary

finding supports the notion that *P. aeruginosa* intricate and complex QS circuit constitutes a global regulatory system (Schuster, Lostroh, Ogi, & Greenberg, 2003; Wagner, Gillis, & Iglewski, 2004).

2.5.2 Pseudomonas Quinolone Signal (PQS)

The complexity of *P. aeruginosa* further increases with another QS system known as the Pseudomonas quinolone system (PQS) which employs a distinctive signalling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (HHQ). HHQ was first discovered in 1940s and studied for their antibacterial properties (Lee & Zhang, 2014). The work of Pesci and colleagues leads to the identification of HHQ as a signalling molecule in *P. aeruginosa* (Pesci *et al.*,1999). Similar to AHLs, PQS regulates the expression of several virulence factors in *P. aeruginosa* such as pyocyanin, cytotoxic lectins, elastase, rhamnolipids, and biofilm (Diggle *et al.*,2007; Pesci *et al.*,1999). However, PQS are able to surpass the requirement for cell density dependent-production of virulence factors but produces the factors in a growth-phase dependent manner. HHQ is required for the expression of *rhl*-dependent exoproducts at the beginning of stationary phase (Diggle *et al.*,2003). Furthermore, mutations in genes encoding for the efflux protein, MexI, and porin, OpmD, (multi-drug efflux pump MexGHI-OpmD) resulted in complete inhibition of 3-oxo-C12-HSL and PQS production by *P. aeruginosa*. The levels of C4-HSL were significantly reduced as well. The supply of exogenous PQS restored AHL, virulence factors production, and antibiotic susceptibility (Aendekerk *et al.*,2005). The findings of this study highlight the synchronisation between the AHL and PQS QS systems in *P. aeruginosa* in coordinating the regulation and expression of virulence factors and antibiotics resistance in *P. aeruginosa*.

The QS systems in other pathogenic bacteria such as *S. aureus, B. cepacea,* and, *Vibrio cholera*, have been extensively studied throughout the years. The virulence factors regulated by these pathogens are summarised in Table 2.2. These examples highlight the importance of a QS system in regulating virulence factors and hence, eliciting infection and dissemination throughout the host.

Table 2.2: Summary of AHL-mediated QS systems of pathogenic bacteria and their QS-regulated virulence factors.

Organism	AHL-mediated QS system	QS-regulated virulence factors	Reference(s)
Staphylococcus aureus	AgrB(S)/AgrC(R), and AIP(s)	Biofilm formation, production of haemolysins and exoenzymes. Expression of cell surface adhesins.	Novick & Geisinger, 2008; Yarwood & Schlievert, 2003
Burkholderia cepacea	CepI(S)/CepR(R), and C8/C6-HSL(s)	Swarming motility. Biofilm formation. Production of proteases, siderophores, and toxins.	Suppiger, Schmid, Aguilar, Pessi, & Eberl, 2013
Acinetobacter baumannii	AbaI(S)/AbaR(R), and 3HOC12-HSL(s)	Antibiotic resistance and biofilm formation.	Castillo-Juárez *et al.*,2015
Vibrio cholera	LuxI(S)/LuxR(R), AI-2(s), and CqsA(S)/CqsS(R), CAI-1(s)	Biofilm formation and production of exopolysaccharide (EPS).	Castillo-Juárez *et al.*,2015
Pseudomonas aeruginosa	LasI(S)/LasR(R), RhlI(S)/RhlR(R), and PQS	Biofilm formation and swarming motility. Production of pyocyanin, rhamnolipids, elastase, and proteases.	Winzer & Williams, 2001

Note: (S) denotes the AHL synthase; (R) denotes the AHL receptor, while (s) denotes the signalling molecule.

2.5.3 Integrated QS Signalling System (IQS)

More recently, a new QS signal was identified in *P. aeruginosa* which enables the bacteria to accommodate the environmental stresses along with the regulation of the QS network. Lee and colleagues identified and described a novel QS signal known as IQS, [2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde], which is the product of the *ambBCDE* genes (Lee *et al.*,2013). The study showed that the disruption in the IQS signal resulted in decreased PQS and C4-HSL production. The work also highlights the significant reduction in the expression of virulence factors such as pyocyanin, elastase, and rhamnolipds. These phenotypes regained their functionality once IQS was supplemented to the growth medium, thus indicating that IQS plays a significant role in intercellular communication of *P. aeruginosa*. In addition, IQS possesses the ability to some extent gain the function of the *las* signalling system under phosphate limiting conditions which is often a situation encountered by the pathogen in establishing infection in a host. Furthermore, the disruption in the activity of the synthases, *lasI* and *lasR*, completely abolished the expression of *ambBCDE* genes as well as IQS synthesis (Lee *et al.*,2013; Lee & Zhang, 2014). The outcome of that study was pivotal finding as it helped researchers understand the regulation of QS systems in *P. aeruginosa* that harbours a mutant *lasI* or *lasR* gene (Ciofu, Mandsberg, Bjarnsholt, Wassermann, & Høiby, 2017). The discovery of the additional QS signalling molecules shows the complexity of the QS systems in *P. aeruginosa.*

2.6 QS Inhibitors (QSIs) as an Anti-pathogenic Drug

The continuous emergence of multidrug resistant organisms over the recent years is a consequence of excessive and irresponsible use of antibiotics as a treatment in bacterial infections (Alanis, 2005). The increased frequency in bacterial evolution has further exacerbated the crisis. Many opportunistic pathogens such as *P. aeruginosa* are dependent on their QS systems to regulate the expression of virulence factors and ultimately causing a myriad of infections in the host. The QS-dependent expressions enable evasion of host innate immune system as well as resistance to antibiotics. Thus, inhibition of the QS system is an attractive target in the search of a novel class of anti-pathogenic drugs which would efficiently interfere with the QS signals *in vivo*. As the QS system does not directly regulate the growth of bacteria, the inhibition of the QS system by QSIs does not cause selective pressure which leads to the development of resistance (Rasmussen & Givskov, 2006a).

For a compound, may it be of natural or synthetic source, to be considered as an effective QSI, it needs to fulfil several criteria: (a) a small molecular weight compound capable of reducing the expression of QS-regulated genes, (b) to be highly specific to the studied QS system with minimal or no toxicity effects towards the bacteria or hosts (c) chemically and structurally stable towards possible enzymatic degradation by host metabolic systems, and (d) structurally longer than the bacteria's native AHL(s) (Hentzer & Givskov, 2003; Kalia, 2013; Rasmussen & Givskov, 2006b).

Over the years as the search of novel anti-pathogenic drugs became one of the major focuses in the field of QS, numerous anti-QS compounds of natural and synthetic origins have been identified and extensively studied (Rasmussen & Givskov, 2006a).

2.6.1 Potential Targets of QSIs in AHL-dependent QS Systems

The three core components that are potentially the targets of QSIs are: (a) the LuxI-homologues which synthesize the AHL molecules, (b) the AHL molecule itself, and (c) the LuxR-homologues or the receptor protein which binds to the AHL molecules produced (Kalia, 2013). Intervening in either one of the components of the QS system would ultimately cause a "communication breakdown" within the bacterial community (Geske, Neill, & Blackwell, 2008).

Inhibition in the synthesis of AHLs signalling molecules by the LuxI-type synthases would be a straightforward approach to obstruct QS as cell-to-cell signalling is impossible without a signal (Galloway, Hodgkinson, Bowden, Welch, & Spring, 2012). Remarkably, however, only a handful of studies that reports synthetic molecules that are able to thwart activity of the LuxI synthase protein (Geske *et al.*,2008). Most research targeting the LuxI-type synthase focuses on the development of AHL analogues. The 3D structures of the LuxI-type synthases are determined and used as a basis in designing AHL analogues (Galloway, Hodgkinson, Bowden, Welch, & Spring, 2011; Geske *et al.*,2008).

Another strategy in QS system inactivation is the degradation of the signalling molecule itself. Degradation of the AHL molecule can be achieved by methods of (a) chemical degradation, (b) enzymatic degradation, and (c) metabolism of the AHL molecules (Rasmussen & Givskov, 2006a). AHL molecules that carry the oxo-substitution at C3, such as 3-oxo-C8-HSL, are reactive with oxidised halogenated compounds, such as hypochlorous and hypobromous acids. Marine algae, *Laminaria digtata*, produce natural haloperoxidases that were capable of deactivating 3-oxo-C6-HSL, and thus, intervening with the regulation of QS-controlled gene involved in biofilm formation (Borchardt *et al.*,2001).

The third strategy in interfering QS system is by obstructing the reception of the AHL signals from being recognised by the bacteria. This strategy can be achieved either by blocking or degrading the receptor protein, LuxR-type protein (Kalia, 2013; Rasmussen & Givskov, 2006a). Synthetic QSIs are very often designed as an AHL analogue to block the LuxR-type receptor molecule (Galloway *et al.*,2012). QSIs of plant source have been reported to produce compounds that structurally mimic AHLs. This approach creates competitive binding between the AHLs and plant compounds which reduces the chances of AHL-receptor complex formation (Koh *et al.*,2013; Teplitski, Robinson, & Bauer, 2000).

2.7 Natural QSIs: Anti-QS Activities of Plant Sources

Natural products or plant sources are usually used in conventional and traditional medical practices due to their numerous therapeutic values. However, the advances in science in the study of plant phytochemicals have increased the interest of researchers in the biological functions and pharmacological potentials of these phytochemicals. Unlike humans and animals, plants do not possess an innate or adaptive immune response to defend against invading pathogens. This has led to the hypothesis that plants produce biologically active compounds as a defence mechanism (Koh *et al.*,2013).

To date, thousands of plant extracts have been screened for their anti-QS activity. This has led to the discovery of novel phytochemicals as anti-pathogenic drugs. One of the significant findings was the discovery of halogenated furanones from the red marine algae, *Delisea pulchra* (Hentzer *et al.*,2003). The halogenated furanones produced were structurally similar to bacterial AHLs, thus, inhibiting QS-mediated communication by competing with AHLs with the receptor binding site (LuxR). The binding of the halogenated furanones does not activate the receptor sites and eventually causing a rapid turnover of the LuxR receptors (Manefield *et al.*,1999; Manefield, Harris, Rice, de Nys, & Kjelleberg, 2000).

The studies of anti-QS activity of plant crude extracts have been made plausible by the development of QS biosensors. QS biosensors such as *C. violaceum* CVO26, *E. coli* [pSB401], and *E. coli* [pSB1075] does not produce AHLs on their own but responds by exhibiting traits such as bioluminescence and violacein production with the supply of synthetic AHLs. Any significant inhibition in the mentioned traits indicates anti-QS activity of the plant extract (Koh *et al.*,2013). Table 2.3 highlights some of the recent anti-QS activities of plant extracts.

Table 2.3: Quorum sensing inhibitors (QSIs) of Plant Origin.

Plant Source	Effective against:		Reference
	Organisms/Pathogens	QS Activity	
Rubus rosaefolius	*C. violaceum* CVO26 *P. aeruginosa*	Violacein production Swarming motility, biofilm formation	Oliveira *et al.*,2016
Eucalyptus globulus (essential oil) *Eucalyptus radiata* (essential oil)	*C. violaceum* CVO26	Violacein production	Luís, Duarte, Gominho, Domingues, & Duarte, 2016
Berberis aristata *Camellia sinensis* *Holarrhena antidysenterica*	*E. coli* (Carbapenem resistant)	Biofilm formation, cell adhesion , superoxide scavenging activity	Thakur *et al.*,2016
Eugenia brasiliensis (pulp extract)	*C. violaceum* CVO26 *Aeromonas hyrophila* *Serratia marscecens*	Violacein production Swarming motility	Rodrigues *et al.*,2016
Piper betle (leaf extract)	*P. aeruginosa*	Swarming, swimming, and twitching motility, biofilm, pyocyanin	Datta, Jana, Maity, Samanta, & Banerjee, 2016
Acer monspessulanum	*C. violaceum* CVO26 *C. violaceum* ATCC 112472 *P. aeruginosa* PAO1	Violacein production Swarming motility	Ceylan, Sahin, & Akdamar, 2016

Table 2.3, continued

Plant Source	Effective against:		Reference
	Organisms/Pathogens	QS Activity	
Euodia ruticarpa	*Campylobacter jejuni* *Vibrio harveyi*	Cell adhesion, biofilm formation Bioluminescence	Bezek *et al.*,2016
Amomum tsaoko	*C. violaceum* CVO26 *S. aureus* *Salmonella thyphimurium* *P. aeruginosa*	Violacein production Swarming motility Biofilm formation	Rahman, Lou, Yu, Wang, & Wang, 2015
Punica granatum L. (peel extract)	*C. violaceum* CVO26 *Yersinia enterocolitica*	Violacein production Swarming motility, biofilm formation, AHL synthesis	Oh, Chang, Chun, Kim, & Lee, 2015
Melicope lunu-ankenda (leaf extract)	*E. coli* [pSB401] *E. coli* [pSB1075] *C. violaceum* CVO26 *P. aeruginosa* PAO1	Bioluminescence Violacein production Swarming motility, pyocyanin	Tan, Yin, & Chan, 2012
Syzygium aromaticum	*E. coli* [pSB401], [pSB1075] *C. violaceum* CVO26 *P. aeruginosa* PAO1	Bioluminescence Violacein production Swarming motility, pyocyanin	Krishnan *et al.*,2012

Table 2.3, continued

Plant Source	Effective against:		Reference
	Organisms/Pathogens	QS Activity	
Capparis spinosa	*C. violaceum* CVO26 *P. aeruginosa* PAO1 *E. coli* *Proteus mirabilis* *Serratia marcescens*	Violacein production Biofilm formation, EPS production, swimming and swarming motility	Issac Abraham, Palani, Ramaswamy, Shunmugiah, & Arumugam, 2011
Ananas comosus *Ocimum sanctum* *Musa paradiciaca* *Manilkara zapota*	*C. violaceum* CVO26 *C. violaceum* ATCC 12472 *P. aeruginosa* PAO1	Violacein production Pyocyanin, staphylolytic protease, elastase, and biofilm formation	Musthafa, Ravi, Annapoorani, Packiavathy, & Pandian, 2010
Myristica cinnamomea	*C. violaceum* CVO26 *P. aeruginosa* PAO1	Violacein production Pyocyanin production, biofilm formation	Chong *et al.*,2011
Pisum savitum (exudates)	*C. violaceum* CVO26 *C. violaceum* wild-type *Serratia liquefaciens* MG44	Violacein production Extracellular exoprotease, exo-chitinase Swarming	Teplitski *et al.*,2000
Combretum albiflorum	*P. aeruginosa* PAO1	Pyocyanin, biofilm, elastase production	Vandeputte *et al.*,2010

2.8 *Phyllanthus amarus* Schumach. & Thonn.

Phyllanthus amarus Schumach. & Thonn. belongs to the family of Euphorbiaceae and approximately 800 species has been identified (Verma, Sharma, & Garg, 2014). *P. amarus* is a wide spread tropical herb often found growing as weeds in uncultivated or wastelands in tropical and subtropical regions (Priya *et al.*,2013). The name *"Phyllanthus"* defines as "leaf and flower" due to the appearance of the plant where the flower, fruit, and leaf appears to be fused together **(**Kumar, Choudhary, & Seniya, 2011). *P. amarus* is a small woody shrub which could grow until 30 – 60 cm in height with thin, leaf-bearing branches. The leaves are elliptical-oblong with a rounded base. The flowers are often yellow, white, or greenish in hue. The fruits are found beneath the leaves as small, soft round capsules (Verma *et al.*,2014). Figure 2.3 depicts a *P. amarus* plant. *P. amarus* is used widely in Indian Ayurvedic system of medicine as well as Traditional Chinese Medicine (TCM) (Patel, Tripathi, Sharma, Chauhan, & Dixit, 2011; Qi *et al.*,2013).

Figure 2.4: *Phyllanthus amarus* Schumach. & Thonn. (This study)

In the Ayurvedic system of medicine, *P. amarus* was used to treat illnesses related to the stomach, liver, spleen, kidney, and genitourinary system. Furthermore, the whole plant is used as treatment in diseases such as gonorrhoea, menorrhagia, dysentery, gastropathy, fevers, genital infections, and wounds (Foo, 1993; Yeap & Wong, 1992). The leaves of *P. amarus* were used as traditional medication to cure rash and itchiness by the people of China and Thailand (Inta, Shengji, Balslev, Wangpakapattanawong, & Trisonthi, 2008).

2.8.1 Phytochemical Studies

P. amarus is rich in numerous secondary metabolites which includes flavonoids, alkaloids, tannins (ellagitannins) lignans, sterols, polyphenols, triterpenes, and volatile oils. The main secondary metabolites of *P. amarus* are lignans. Several other secondary metabolites and their respective phytochemicals that were isolated from *P. amarus* are enlisted in Table 2.4.

Table 2.4: List of several phytochemicals reported in *P. amarus.*

Secondary metabolites	Phytochemicals
Lignans	Niranthin, nirtetralin, phyltetralin, hypophyllanthin, phyllanthin, hinokinin, isonirtetralin, lintetralin, isolintetralin.
Flavanoids	Rutin, kaempferol, astragalin
Tannins (ellagitannins)	Gallic acid, ellagic acid, gallocatechin, geraniin, corilagin, 1,6-digalloyglucopyranoside rutin, quercetin-3-*O*-glucopyranoside, amarulone, phallanthusiin A-D, amariin, and melatonin.
Alkaloids	Securinine, securinol, phyllanthine, isobubbialine, epibubbialine, allo-securine, tetrahydrosecurinine, phenazine and phenazine derivatives
Triterpenes	Farnesil, farnesol, lupeol, ursolic acid, oleanolic acid, phyllanthenol
Volatile oils	Phytol, linalool
Sterols	Amarosterol A, B

Note: The list of phytochemicals were obtained from Foo, 1993; Houghton, Woldemariam, O'Shea, & Thyagarajan, 1996; Moronkola *et al.*,2009; Patel *et al.*,2011; Verma *et al.*,2014; Yeap Foo & Wong, 1992.

2.8.2 Pharmacological Activities of *P. amarus*

Due to its numerous phytochemical constituents, *P. amarus* has been the main subject of several researchers to explore its potential in the field of pharmacology. Among the pharmacological potential of *P. amarus* includes antiamnesic (Hanumanthachar & Milind, 2007), antibacterial (Eldeen, Seow, Abdullah, & Sulaiman, 2011; S. Kumar *et al.*,2011), anticancer activity (Krithika *et al.*,2009; Parvathaneni, Battu, Gray, & Gummalla, 2014), antifungal activity (Sahni *et al.*,2005), anti-diarrhoeal, anti-ulcer, and gastroprotective activity (Raphael & Kuttan, 2003), antioxidant activity (Karuna, Reddy, Baskar, & Saralakumari, 2009; Krithika *et al.*,2009), antiviral activity (Kolodziej *et al.*,2005; Ravikumar *et al.*,2011), hepatoprotective activity (Chirdchupunseree & Pramyothin, 2010; Surya Narayanan, Latha, & Rukkumani, 2011), nephroprotective activity (Adeneye & Benebo, 2008), and immunomodulatory properties (Jantan, Harun, Septama, Murad, & Mesaik, 2011; Kumar & Kuttan, 2005). However, no anti-QS activities of *P. amarus* have been reported.

2.9 Flavonoids as Potential QSIs

Flavonoids, a group of plant secondary metabolites, are extensively produced by plants and possess important therapeutic functions. Interestingly, a large number of flavonoid compounds exhibited anti-QS activities against QS-mediated pathogens. Flavonoid-rich fractions of *Centella asiatica* L. reduced violacein production by *C. violaceum* ATCC12472 and significantly inhibited several QS-regulated virulence factors of *P. aeruginosa* such as biofilm formation, pyocyanin production, elastolytic and proteolytic activities, and swarming motility (Vasavi, Arun, & Rekha, 2016). Furthermore, flavonoids obtained from *Piper delineatum* showed remarkable anti-QS activity at micromolar concentrations. The flavonoid fractions reduced bioluminescence and biofilm formation in *V. harveyi* (Martín-Rodríguez *et al.*,2015). Naringenin, a flavanone (sub-group of flavonoids), isolated from *Combretum albiflorum*, inhibited the production of pyocyanin and elastase in *P. aeruginosa.* In addition, naringenin also down regulated the expression of several QS-regulated genes of *P. aeruginosa*; *rhlA, rhlI, rhlR, lasA, lasB, phzA1, lasR,* and *lasI*. Significant reduction in the AHLs, 3-oxo-C12-HSL and C4-HSL (produced by *lasI* and *rhlI*, respectively), shows naringenin interferes with the synthesis of AHL molecules by *P. aeruginosa* (Vandeputte *et al.*,2011). Flavon-3-ol catechin from the bark extract of *Combretum albiflorum* reduced biofilm formation, pyocyanin and elastase production by *P. aeruginosa* PAO1 (Vandeputte *et al.*,2010). These examples elucidate the potential of flavonoids as QS inhibitors against a wide range of AHL-mediated pathogenic bacteria.

2.9.1 Baicalein

Baicalein is one of the major flavonoid that is found in the roots dried roots of *Scutellaria baicalensis*. Also known as "Huangqin" in China (Chinese skullcap), the dried roots are widely used in traditional Chinese medicine (TCM) to relieve disease-related symptoms such as sore throat, fever, and insomnia (Perez, Wei, & Guo, 2009). In TCM, baicalein is prescribed in Baicalein is also the major flavonoid found in American skull cap, *Scutellaria lateriflora,* suggesting the notion that baicalein is present in various species of the genus *Scutellaria* (Brock, Whitehouse, Tewfik, & Towell, 2013). Baicalein demonstrates a wide range of pharmacological potential. Baicalein exhibited extraordinary anticancer effects against several cancer cell lines, such as MDA-MB-231 human breast cancer cells (L. Wang *et al.,* 2010), A549 lung cancer cells (Shi *et al.,* 2016), HepG2 and Bel7042 hepatocellular carcinoma cells (Han *et al.,* 2015). Baicalein also demonstates anti-inflammatory activities in diabetic retinopathy (Yang *et al.,* 2009). The pharmacology potential of baicalein also extends towards synergism with anti-fungal drugs such as fluconazole and amphotericin B against *Candida albicans* (Cao *et al.,* 2008; Dai *et al.,* 2009).

Several studies conducted showed the remarkable synergic effects of baicalein with antibiotics. The synergistic effects of baicalein with tetracycline as well as β-lactams have shown to have effects significant against methicillin-resistant *Staphylococcus aurues* (MRSA). The study focussed on the effects of baicalein on the efflux pump of tetracycline by an *E. coli* strain harbouring the *tetK* gene, a gene that encodes for tetracycline efflux pump. Baicalein significantly inhibited the transport of tetracycline from the bacterial cells (Fujita *et al.,* 2005). In another *in vitro* study, the synergism of baicalein and gentamicin were found to be effective against vancomycin-resistant

Enterococcus (VRE). The combination of gentamin and baicalein resulted in significant reduction in the growth of VRE (Chang *et al.,* 2007). Furthermore, baicalein significantly inhibited biofilm formation by *P. aeruginosa*, incited proteolysis of TraR QS-signal receptor protein of *E. coli* as well as having synergistic activity with ampicillin against *P. aeruginosa.* The results from this study demonstrated that baicalein carries the potential as a QSI against *P. aeruginosa* (Zeng *et al.,* 2008). However, the mechanism of which baicalein interferes with QS regulations of *P. aeruginosa* at a global gene expression level have yet to be reported.

CHAPTER 3: METHODOLOGY

3.1 Materials

3.1.1 Equipment and Instruments

Equipment and instruments used in this study were industrial grade blender, incubators (Memmert GmbH + Co. KG, Germany), shaking incubator (N-Biotek, Korea), rotary evaporator (EYELA, Tokyo, Japan), fume hood, Tecan Microplate Reader (Infinite M200, Mannerdorf, Switzerland), spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent), Leica Inverted Laboratory Microscope DM IL LED (Leica Microsystems, Illinois, U.S.A), UV transilluminator (UV Products,Inc.), laminar flow and Class II biological safety cabinets (Esco), weighing machines (Sartorious AG, Germany), centrifuges, thermomixers, and pipettes (Eppendorf, Hamburg, Germany), Agilent Bioanalyzer (Agilent Technologies, CA, USA), autoclave machine (Hirayama, Japan), NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA), Qubit® fluorometer (Life Technologies, CA, USA), Illumina Eco™ Real-Time PCR system (Illumina, CA, USA), NextSeq 500 Sequencing System (Illumina, CA, USA), MiSeq Sequencing System (Illumina, CA, USA), thermal cycler (Bio-Rad, CA, USA), and Bio-Rad CFX96 real-time system (Bio-Rad, CA, USA).

3.1.2 Chemical, Reagents, and Solvents

Chemicals and reagents used in this study were purchased from the following companies, solvents; hexane, chloroform, methanol, dimethyl sulfoxide (DMSO), and sodium hydroxide (Merck KGaA, Darmstadt, Germany). Chemicals and reagents were purchased from BD Difco™ Laboratories (New Jersey, USA), Sigma-Aldrich (Merck KGaA, MO, USA), Fisher Scientific (Thermo Fisher Scientific, USA), Amresco® (OH, USA), BDH Ltd. (England), Cayman Chemicals (MI, USA), and Cambrex Bio-Science Rockland Inc. (ME, USA).

3.1.3 Commercial Kits

Commercial kits used in this study are listed in Table 3.1.

Table 3.1: Commercial Kits

Commercial Kits	Manufacturer	Origin
NucleoSpin® RNA Extraction Kit	Macherey-Nagel (MN)	Düren, Germany
QIAamp DNA Mini Kit	Qiagen	Hilden, Germany
Agilent Bioanalyzer-RNA 6000 Pico/Nano/High Sensitivity DNA Kit	Agilent Technologies	California, USA
Ribo-Zero™ rRNA Removal Kits (Bacteria)	Epicentre	Wisconsin, USA
ScriptSeq™ v2 RNA-seq Library Preparation Kit	Epicentre	Wisconsin, USA
ScriptSeq™ – Index PCR Primers	Epicentre	Wisconsin, USA
Qubit® dsDNA/RNA High Sensitivity (HS) Assay Kit	Life Technologies	California, USA
KAPA Library Quantification Kit	KAPA Biosystems	Massachusetts, USA
QuantiTect Reverse Transcription Kit	Qiagen	Hilden, Germany
KAPA SYBR® FAST qPCR Kit Master Mix Universal	KAPA Biosystems	Massachusetts, USA
Agencourt AMPure XP/ RNAClean XP	Beckman Coulter	California, USA

3.1.4 Growth Media

All growth and culture media were prepared in distilled water and autoclaved at 121°C for 15 min with a pressure of 15 psi.

3.1.4.1 Lysogeny Broth (LB) and Agar

LB culture medium used throughout this study. The LB medium consistently used in this study consists of 5.0 g yeast extract, 10.0 g tryptone, 10.0 g sodium chloride (NaCl), and 15.0 g bacto agar. The compositions were prepared in 1 L distilled water and autoclaved as stated in Section 3.1.4.

3.1.5 Antibiotics

Antibiotics were added to culture media in necessary bioassays at the following concentrations, tetracycline at 20 µg/mL and ampicillin at 100 µg/mL. Antibiotics were purchased from Sigma-Aldrich (Merck KGaA, MO, USA). The stocks and dilutions were kept at -20°C.

3.1.6 Synthetic acyl-homoserine lactones (AHLs)

Synthetic AHLs were purchased from Sigma-Aldrich (Merck KGaA, MO, USA) and Cayman Chemicals (MI, USA). The synthetic AHLs were resuspended with acetonitrile (ACN) to the desired stock concentration and stored at -20°C. Further dilutions were made prior to usage in assays to required concentrations using sterile ultrapure water.

3.1.7 Agarose Gel Electrophoresis (AGE) and Buffers

The buffers, agarose gel composition, and reagents used to conduct AGE are listed as follows:

i. 10× Tris-Borate-EDTA (TBE) buffer used consisted of 10.8 g Tris base, 5.5 g boric acid, and 7.44 g $Na_2EDTA.2H_2O$. The buffers were diluted to 1× using sterile autoclaved distilled water and its' pH value adjusted to pH 8. The buffer was stored at room temperature.

ii. Agarose gel with the final percentage of 2% (w/v) was prepared by dissolving 1.0 g of agarose powder in 50 mL 1× TBE. The mixture was boiled using a microwave until the agarose completely dissolved. The cooled agarose gel was pre-stained with 1 μL of GelStar™ Nucleic Acid Gel Stain (Lonza, Basel, Switzerland) prior to gel casting.

Other buffers and reagents used in this study also include:

i. Phosphate buffered saline or PBS was used in the biofilm assay. PBS (10×) prepared consisted of 80.0 g sodium chloride (NaCl), 2.0 g potassium chloride (KCl), 14.4 potassium phosphate dibasic (Na_2HPO_4), and 2.4 g of monopotassium phosphate (KH_2PO_4) in 1 L distilled water. The pH of the solution was adjusted to pH 6.0 and stored at room temperature. The PBS buffer was diluted to 1× prior to usage in the assay.

ii. Hydrochloric acid, HCl, with a final concentration of 0.2 M was used in the pyocyanin assay. Glacial HCl (Sigma-Aldrich) with an approximate acid strength of 32.7% and molarity of 12.1 M were diluted to 0.2 M using sterile ultrapure water. Dilutions were prepared in a fume hood.

3.1.7.1 DNA Ladder

The ladders used in gel electrophoresis (AGE) include GeneRuler™ 100 bp DNA ladder and GeneRuler™ 1 kb DNA ladder. The ladders were purchased from Fermentas International Inc. (Fermentas, MA, USA).

3.2 Plant Sample Collection and Processing

A total of 15 plant samples (Table 3.2) were collected from various sites and identified at Rimba Ilmu, University of Malaya. The plant samples collected were primarily leaves as there were numerous findings indicating their medicinal potential. Collected plant samples were washed with sterile distilled water and rinsed with 70% (v/v) ethanol. Samples were then dried at 40°C for three days in an incubator (Memmert GmbH + Co. KG, Germany) prior to processing to ensure complete desiccation. Samples were blended finely using an industrial grade blender and then soaked sequentially for 3 days while shaking at 220 rpm in extraction solvents which were hexane, chloroform, and methanol (in a ratio of 1:10 w/v). Solvents used were in increasing polarity to enable extraction of non-polar to more polar compounds. Plant-solvent mixtures were the filtered with Whatman No.1 filter paper twice to ensure complete removal of plant powder residues. Solvents were then removed using a rotary evaporator (EYELA, Tokyo, Japan), leaving only the separated compounds behind. Plant crude extracts were then further dried in fume hood to ensure complete removal of solvents and later, placed in desiccators for moisture removal.

Table 3.2: Plant Samples Collected and Processed

No.	Plant	Scientific Name	Part of Plant
1.	Rambutan	*Nephelium lappaceum*	Leaves
2.	Great Morinda (Mengkudu)	*Morinda citrifolia*	Leaves
3.	Stonebreaker (Dukung Anak)	*Phyllanthus amarus*	Leaves
4.	Five-leaved Chaste Tree	*Vitex negundo*	Leaves
5.	Lotus Plant	*Nelumbo nucifera*	Leaves
6.	Lotus Plant	*Nelumbo nucifera*	Flower
7.	Castor Oil Plant	*Ricinus communis*	Leaves
8.	Honey guava	*Syzygium samarangense*	Leaves
9.	Common Fig	*Ficus carica*	Leaves
10.	Cempedak tree	*Artocarpus integer*	Leaves
11.	Jackfruit tree	*Artocarpus heterophyllus*	Leaves
12.	Tamarind plant	*Tamarindus indica*	Leaves
13.	Mulberry plant	*Morus alba*	Leaves
14.	Hummingbird tree	*Sesbania grandiflora*	Leaves
15.	Indian Bael	*Aegle marmelos*	Leaves

3.3 Bacterial Strains, Biosensors, and Culture Conditions

All strains and biosensors stated in Table 3.3 were routinely cultured in fresh lysogeny broth (LB) or agar (LBA) (Scharlab, Barcelona, Spain). The strains were grown aerobically at 37°C with shaking at 220 rpm with the exception of *Chromobacterium violaceum* CVO26 which were grown at 28°C in LB medium. The LB medium for the growth of *Escherichia coli lux*-based biosensor strains were supplemented with tetracycline at 20 µg/mL.

Table 3.3: Bacterial strains and biosensors used in this study.

Strains/Biosensors	Description	Source/Reference
Pseudomonas aeruginosa PAO1	Wild type	K. G. Chan *et al.*,2011
Pseudomonas aeruginosa PAO1 *lecA::lux*	Genomic reporter fusion, *lecA::luxCDABE,* in PAO1	Winzer *et al.*,2000
Chromobacterium violaceum CVO26	Produces a purple pigment, violacein, in the presence of exogenously supplied short chain AHL. Best responds to 3-oxo-C6-HSL.	McClean *et al.*,1997
Escherichia coli [pSB401]	Produces bioluminescence in the presence of short chain AHL. A *luxCDABE*-based plasmid sensors. Responds best to 3-oxo-C6-HSL.	Winson *et al.*,1998
Escherichia coli [pSB1075]	Produces bioluminescence in the presence of long chain AHL. A *luxCDABE*-based plasmid sensors. Responds best to 3-oxo-C12-HSL.	Winson *et al.*,1998

3.4 Stock Solution and Preparation of Dilutions

3.4.1 Plant Crude Extracts

Stock solutions of plant crude extracts of 10 mg/mL were made using 100% DMSO (Merck KGaA, Darmstadt, Germany) and then diluted to 1-5 mg/mL using sterile ultrapure water (Mili-Q, Merck, KGaA, Darmstadt, Germany). Remaining crude extracts were kept at -20°C for long term storage. Stock solutions and dilutions of the crude extracts were prepared fresh each time an assay was being carried out. The crude extracts were dissolved completely in DMSO before dilutions were made. DMSO of percentages 10% - 50% were used as a negative control that corresponded to the concentration of plant crude extracts used. It was to ensure that the QS inhibition activity observed in the assays was not caused by the effects of the solvent.

3.4.2 Synthetic Compound

Stock solutions of Baicalein (obtained from Sigma-Aldrich) were prepared at a concentration of 20 mM in 100% DMSO. Dilution to concentrations of 0.2 µM, 2 µM, 20 µM, and 200 µM were prepared fresh prior to usage in assays using sterile ultrapure water. The concentrations were used in all the bioassays conducted in this study. Catechin and gallic acid (Sigma-Aldrich), well-known anti-QS compounds, served as the positive controls in this study (Pimenta *et al.*,2013; Vandeputte *et al.*,2010). DMSO of percentages 0.001% - 1% (v/v) corresponded to the concentrations of baicalein used in this study.

3.5 Bacterial Growth

A 24 h bacterial growth curve study was carried out to rule out the possibility of antibacterial activity of the crude extracts and the tested synthetic compounds towards the bacterial strains, *C. violaceum* CVO26 and *P. aeruginosa*, which may result in false anti-QS results. The optical densities of the overnight bacterial culture were adjusted to OD 600 of 0.1. A volume of 230 µL of the bacterial culture and 20 µL of the crude extract at concentrations 1 mg/mL – 5 mg/mL, were dispensed into each well of a 96-well mirotitre plate. As for baicalein, a volume of 180 µL of bacterial culture and 20 µL of the compound were dispensed into each well to a final concentration of 0.2 µM, 2 µM, 20 µM, and 200 µM. The microtitre plates were incubated the optimum temperature of each bacteria and the growth were measured every 30 min for 24 h using a Tecan Microplate Reader (Infinite M200, Mannerdorf, Switzerland) (L. Y. Tan *et al.*,2013).

3.6 Screening for QS Inhibition using *Chromobacterium violaceum* CVO26

Plant crude extracts with negative anti-bacterial activity were tested for its anti-QS activity by preliminary screening using *C. violaceum* CVO26. A volume of 15 mL of *C. violaceum* CVO26 overnight culture was seeded into 200mL of warm molten LB agar supplemented with 3-oxo-C6-HSL to a final concentration of 0.25 µg/mL. Small wells were made on the agar using sterile 1 µL pipette tips. A volume of 20 µL of the plant extracts of concentrations ranging from 1 mg/mL – 5 mg/mL were dispensed into the wells made. DMSO (10%-50% (v/v)) served as negative control that corresponded of each plant extract concentration used. The plates were incubated for 24 h at 28°C in an upright position. Formation of a halo zone or a colourless turbid ring on a purple background indicated anti-QS activity of the crude extract against *C. violaceum* CVO26. Similar methods were used to screen for anti-QS activity of baicalein with concentrations 0.2 µM, 2 µM, 20 µM, and 200 µM.

3.7 Bioluminescence Quantification

Bioluminescence was quantified using biosensor *E.coli* [pSB401], *E.coli* [pSB1075], and inhibition of *P. aeruginosa* lecA was performed using *P. aeruginosa* PAO1 *lecA::lux*. Overnight cultures of *E.coli* [pSB401], *E.coli* [pSB1075], and *P. aeruginosa* PAO1 *lecA::lux* were diluted to OD_{600} of 0.1 using freshly prepared LB broth. *E.coli* [pSB401] and *E.coli* [pSB1075] produces bioluminescence as a QS-respond towards the exogenously supplied AHLs which are 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively. A reduction in the luminescence produced by *E.coli* biosensors indicates QS-inhibition activity of the crude extract/baicalein against the short and long chain AHL supplied, suggesting a broad range of AHL inhibition activity. As for *P. aeruginosa lecA::lux,* the expression of the LecA protein was coupled with

luminescence by the insertion of *luxCDABE* from *Photorhabdus luminescence* into *lecA* region of *P. aeruginosa* PAO1 (Winzer *et al.*,2000). Thus, inhibition of bioluminescence of *P. aeruginosa lecA::lux* is in direct correlation with the expression of *lecA*. The cultures of *E.coli* [pSB401] and *E.coli* [pSB1075] were supplemented with 0.00005 µg/mL 3-oxo-C6-HSL and 0.5 µg/mL 3-oxo-C12-HSL, respectively. No synthetic AHLs were supplemented for *P. aeruginosa lecA::lux*. A total volume of 230 µL of the diluted bacterial culture was loaded into each well of a 96-well plate, followed by 20 µL of plant crude extract/baicalein, separately. The bioluminescence produced by the biosensors and its growth (optical density at 495 nm) were measured every 30 min for a duration of 24 h Tecan luminometer (Infinite M200). Luminescence produced was calculated as relative light units (RLU) per unit of optical density at 495 nm.

3.8 Attenuation of Virulence Factors of *Pseudomonas aeruginosa* PAO1

3.8.1 Pyocyanin Quantification Assay

The overnight culture of *P. aeruginosa* PAO1 was diluted and the absorbance was adjusted to OD_{600} of 0.05. A volume of 4.75 µL of the diluted culture was mixed thoroughly with 250 µL of plant crude extract/baicalein, separately, to the desired final concentration. The cultures were then incubated at 37°C for 18 h with shaking at 220 rpm. After 18 h incubation, the treated cultures were centrifuged at 14, 000 rpm for 10 min and the supernatants were transferred into new 50 mL polypropylene tubes. Pyocyanin from the 5 mL supernatants was extracted with 3 mL chloroform (Merck KGaA, Darmstadt, Germany). The supernatant-chloroform mixtures were vortexed vigorously and centrifuged at 14,000 rpm for 10 min to allow complete separation between the two layers. The chloroform layers were then transferred to new 15 mL polypropylene tubes and pyocyanin was re-extracted with 1 mL of 0.2 M HCl. The

chloroform-HCl mixtures were vortexed vigorously and centrifuged at 14, 000 rpm for 5 min. The topmost later consisting of HCl was removed and its absorbance was measured 520 nm UV-visible spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent).

3.8.2 Swarming Assay

P. aeruginosa PAO1 swarming agar was prepared with the following composition: glucose (1% w/v), Bacto agar (0.5% w/v), Bacto peptone (0.5% w/v), and yeast extract (0.2% w/v); the agar was prepared fresh prior to usage. A total volume of 10 mL of swarming agar was poured into petri dishes and allowed to solidify for 45 min in a laminar-flow biosafety cabinet. The solidified agar layer was overlaid with 5 mL of swarming agar seeded with 250 µL of plant crude extract/baicalein, separately, to the desired final concentrations. The agar plates were once again dried for 45 min under laminar air flow conditions. A volume of 0.5 µL of *P. aeruginosa* PAO1 culture with OD_{600} = 0.500 was inoculated in the middle of the agar and incubated at 37°C for 18 h in an upright position.

3.8.3 Biofilm Inhibition Assay

Biofilm inhibition assay was conducted using 6-well plate. The absorbance of the overnight culture of *P. aeruginosa* PAO1 was adjusted to OD_{600} = 0.05 using fresh, sterile LB broth. A total volume of 2.5 mL of diluted culture were added into each well followed by 0.5 mL of baicalein and catechin, separately, to a final concentration of 0.2 µM – 200 µM. DMSO of 0.1% - 1% (v/v) served as the negative control. The plates were then statically incubated for 24 h at 37°C. Planktonic bacterial cultures were

carefully removed from each well without disturbing the biofilm layers. The biofilm layers were then washed twice with 1× phosphate buffer saline (PBS) and air dried for 30 min. Next, the biofilm layers were stained with 1 mL of 0.1% (v/v) crystal violet for 45 min. Excess stain was removed and the stained biofilm was washed once with PBS (W. S. Tan *et al.*,2014; Yunos *et al.*,2014). The plates were air-dried for 15 min and de-stained using 1 mL of 95% (v/v) ethanol. The resulting crystal violet solution was transferred to a new, sterile 96-well plate and the absorbance was determined at OD_{590}.

3.9 Statistical Analysis

All the results presented in this study were means of standard deviations (SD) of independent experiments conducted in triplicates. The graphical representation and significance of the data sets were calculated by Student's *t*-test using GraphPad Prism Software v7.

3.10 Transcriptome Analysis of Baicalein against *P. aeruginosa* PAO1

Baicalein was used in the transcriptomics study (RNA-sequencing) on the effects of synthetic compounds on *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 cells from -80°C stock culture were streaked on LB agar to obtain pure colonies. A single colony was then picked from the agar and inoculated into 50 mL fresh, sterile LB broth. The culture was grown for 18 h in 37°C with shaking at 220 rpm. Pre-warmed to 37°C, three flasks of LB broths were supplemented with 20 µM baicalein, another 3 flasks with 0.1% DMSO (solvent control), and additional 3 flasks of LB broths served as untreated *P. aeruginosa* PAO1 which were not supplemented with solvents or compounds. Each treatment was conducted separately and in triplicates. A total volume of 500 µL of the

18 h overnight culture was then sub-cultured into the abovementioned flasks and grown to mid-exponential phase (OD_{600} = 0.5) at 37°C (K. G. Chan *et al.*,2016). A total volume of 1 mL of *P. aeruginosa* PAO1 cultures subjected to the treatment paradigms were transferred to new and sterile 1.5 mL microcentrifuge tubes. The cells were immediately subjected to RNA extraction.

3.11 RNA Extraction and Quality Check

Total RNA was extracted using NucleoSpin® RNA Extraction Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol and instructions. The extracted RNA was eluted in 40 µL of sterile, RNAse-free water. The qualities of the extracted RNA samples were assessed using NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). RNA samples with A_{260}/A_{280} and A_{260}/A_{230} values of 2.0 and above were selected for further work. The integrity and degradation levels of the RNA samples were determined by obtaining the RNA Integrity Number (RIN) by using Agilent Bioanalyzer-RNA 6000 Nano Kit (Agilent Technologies, CA, USA). RNA samples with RIN values of more than 8.0 were selected for rRNA depletion using Ribo-Zero™ rRNA Removal Kits (Bacteria) (Epicentre, WI, USA). RNA samples were assessed for the loss of intact rRNA using Agilent Bioanalyzer-RNA 6000 Pico Kit (Agilent Technologies, CA, USA) (K. G. Chan *et al.*,2016). The qualities of the extracted RNA were subjected to strict quality check to ensure optimal cDNA library preparations and sequencing quality.

3.12 cDNA Library Preparation and RNA-sequencing

RNA samples with its' rRNA depleted were proceeded with cDNA synthesis using ScriptSeq™ v2 RNA-seq Library Preparation Kit (Epicentre, WI, USA) as per manufacturer's instructions. The transcriptome library size and quality were determined using Agilent Bioanalyzer-High Sensitivity DNA Chip and Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, CA, USA), respectively. The optimal library size for each sample was fixed to be between 350 bp – 550 bp. The transcriptome libraries of each samples were then quantified using the KAPA Library Quantification Kit (KAPA Biosystems, MA, USA) coupled with the Illumina Eco™ Real-Time PCR system (Illumina, CA, USA). It was of outmost importance for accurate library quantification to ensure each sample is equally represented in a multiplexed sequencing. Samples that did not meet the quality requirements for sequencing were subjected to library preparation once again. Baicalein treated *P. aeruginosa* PAO1 transcriptome libraries were sequenced using NextSeq 500 Sequencing System (Illumina, CA, USA).

3.13 Trancriptomics Data Analyses and Gene Expression Profiling

P. aeruginosa PAO1 was used as the reference genome (GenBank accession number: AE004091) was obtained from National Centre for Biotechnology Information (NCBI) and functionally annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The raw transcriptomics data obtained from the sequencing run (fastq) were trimmed using CLC Genomics Workbench version 7.5 (CLC Bio, Denmark) with default parameters. Trimmed reads were aligned to the reference genome using TopHat v2.0.9 (Trapnell *et al.*,2009) and Bowtie v1.0.0 (Langmead, Trapnell, Pop, & Salzberg, 2009) using recommended default parameters. The resulting BAM files were subjected

to differential gene expression analysis using Partek® Genomics Suite® software, version 6.6 Copyright©; 1993-2012 Partek Inc., St. Louis, MO, USA. The reads per kilo-base of exon model per million mapped reads (RPKM) for each gene mapped to the reference genome were normalized using quantile normalization. Significant differentially expressed genes were determined using analysis of variance (ANOVA) with a filter parameters of ≥ 2 fold change, ≤ 2 fold change and p value <0.05. Principle component analysis (PCA), box plot, and volcano plot were generated using normalized values of the differentially expressed genes using CLC Genomics Workbench and Partek Genomics Suite. The sequences and functions of the genes in the gene list obtained upon analysis were confirmed at Pseudomonas Genome Database (Winsor *et al.*,2015). The DNA sequences obtained from baicalein transcriptomics analysis were made available in the public database, Sequence Read Archive (NCBI/SRA).

3.14 Gene Validation of Transcriptome Data

3.14.1 Gradient PCR

The affected gene obtained from the RNA-sequencing analysis of baicalein treated *P. aeruginosa* PAO1 were randomly selected for validation purposes. The primer sets for each of the selected genes were designed using Primer 3 version 0.4.0 (Koressaar & Remm, 2007; Untergasser *et al.*,2012). The validity of the designed primers was assessed using OligoAnalyzer version 3.1 which is an online freeware. Reverse and forward primer sequences for the reference genes and selected genes for baicalein are listed in Table 3.4.

Prior to RT-qPCR, the optimal temperatures for the annealing of designed primers to the template cDNA were determined using gradient PCR (Bio-Rad, CA, USA) with the following conditions; initial denaturation at 94°C for 30 sec, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing (gradient) at 55°C - 61°C for 30 sec, followed by extension/elongation at 68°C for 1 minute and the final extension step at 68°C for 5 min. The gradient PCR reagents master mix is listed in Table 3.5.

Table 3.4: Designed primers for transcriptomics gene validation.

Genes	Primer Sequences	Primer length	References
	Housekeeping Genes		
chez	F: 5' – GCG AAC TGG TGG ACT GTC T – 3' R: 5' – AAT GCG GGT CGA TCT GGA AA – 3'	F: 19 bp R: 20 bp	This study
oprL	F: 5' – CCA ACA GCG GTG CCG TTG A – 3' R: 5' – GCC ATA TTG TAC TCG CGG GT – 3'	F: 19 bp R: 20 bp	Matthijs *et al.*,2013
rpoB	F: 5' – GAT CAC CGA GAC CAC ACC – 3' R: 5' – GCT CCT GGA CGA CAA GTT – 3'	F: 18 bp R: 18 bp	Gomila, Pena, Mulet, Lalucat, & Garciaa-Valdes, 2015
Genes	**Gene validation for baicalein treated *P. aeruginosa* PAO1**	**Primer length**	**References**
flgM	F: 5'- CCC AGA ACA TGC AGA AAG TCA -3' R: 5'- AGC GCT GGG ATT CGA AGT C -3'	F: 21 bp R: 19 bp	This study
narG	F: 5'- CAA GGA CGG CAT GGT GAT -3' R: 5'- CAG TCG ACC TTG CTC ATC TTG -3'	F: 18 bp R: 21 bp	This study
narK1	F:5'- CAT CAC CAT GCT GTT CAC CTT -3' R:5'- GCT CGG GTA GTA GTC GTG GAT -3'	F: 21 bp R: 21 bp	This study
narK2	F:5'- CTC AGC AAG ACC CAG TTC -3' R: 5'- CGA AGA AGA ACA GCA GGA -3'	F: 18 bp R: 18 bp	This study

Table 3.4, continued

Genes	Primer Sequences	Primer length	References
pvdA	F: 5'- GCG AAT ACC ACA ACA CCA ACT -3' R: 5'- GAT CAC TGC GTC GTA GGT CTC -3'	F: 21 bp R: 21 bp	This study
fecI	F: 5'- TTC GTC AAG GTC CTG GTT TC -3' R: 5'- CTC TTC GCT GGG GAC TTC -3'	F: 20 bp R: 18 bp	This study
iscR	F: 5'- GGC ATT TCC CTG TCC TAT CTC -3' R: 5'- GTG GTG GGT CAG ACA GGT ATC -3'	F: 21 bp R: 21 bp	This study
tonB1	F: 5'- GAG TCC CTG CCA TGC GTG -3' R: 5'- ATG GTC TTC GGC AGT TCG -3'	F: 18 bp R: 18 bp	This study

Table 3.5: Gradient PCR reagents master mix

PCR Components	Final Concentration (25 µL reaction)
5× One*Taq* Standard Reaction Buffer	1×
10 mM dNTPs	200 µM
10 µM forward primer	0.2 µM
10 µM reverse primer	0.2 µM
One*Taq* DNA Polymerase	0.625 units
Template DNA	~1000 ng
Nuclease-free water	Variable (up to 25 µL)

3.14.2 Real Time-Polymerase Chain Reaction (RT-PCR)

Real-time polymerase chain reaction (RT-qPCR) was performed to quantify and validate the level of *P. aeruginosa* PAO1 genes expression that was affected upon exposure to 20 µM baicalein. The treatment paradigm and RNA extraction for baicalein treated *P. aeruginosa* PAO1 were repeated as an independent experiment (refer to Section 3.9) to determine the reproducibility of data obtained in the transcriptomics analysis. The extracted RNA reversed transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, USA) as per manufacturer's instructions. Selected genes for each experiment were amplified using KAPA SYBR® FAST qPCR Kit Master Mix Universal (Kapa Biosystems, USA) on the Bio-Rad CFX96 real-time system (Bio-Rad, CA, USA).

The RT-qPCR conditions for each of the target genes were as follows; enzyme activation at 95°C for 3 min, followed by a 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 57°C - 60°C for 30 s. The RT-qPCR components and its final concentrations are listed in Table 3.6. Data obtained were analysed and the graphical representation were generated using Bio-Rad CFX Manager™ Software version 2.1. Reference genes (primers listed in Table 3.4) with *M* value or expression stability values of less than 0.7 were selected for normalisation.

Table 3.6: RT-qPCR components master mix

PCR Components	Final Concentration (10 µL reaction)
2X KAPA SYBR® FAST qPCR Master Mix Universal	1X
10 µM forward primer	100 nM
10 µM reverse primer	100 nM
Template DNA	~10 ng
Nuclease-free water	Variable (up to 10 µL)

CHAPTER 4: RESULTS

4.1 Screening of Plant Extracts for anti-QS Properties using *C. violaceum* CVO26

A total of 15 plants were processed and assessed for their anti-QS properties. Crude extracts of each plant were obtained using hexane, chloroform, and methanol. Therefore, a total of 45 crude extracts were tested using *C. violaceum* CVO26. Table 4.1 summarizes the results of the preliminary screening.

Table 4.1: List of plant collected, processed, and screened for anti-QS activity.

No.	Plants	Hexane Extract	Chloroform Extract	Methanol Extract
1.	*Nephelium lappaceum*	×	×	×
2.	*Morinda citrifolia*	×	×	×
3.	*Phyllanthus amarus*	×	×	✓
4.	*Vitex negundo*	×	×	×
5.	*Nelumbo nucifera*	×	×	×
6.	*Nelumbo nucifera*	×	×	×
7.	*Ricinus communis*	×	×	×
8.	*Syzygium samarangense*	×	×	×
9.	*Ficus carica*	×	×	×
10.	*Artocarpus integer*	×	×	×
11.	*Artocarpus heterophyllus*	×	×	×
12.	*Tamarindus indica*	×	×	×
13.	*Morus alba*	×	×	×
14.	*Sesbania grandiflora*	×	×	×
15.	*Aegle marmelos*	×	×	×

Note: The symbol "×" signifies that the corresponding crude extract did not produced a significant halo zone formation or anti-QS activity when tested using *C. violaceum* CVO26 plate assay. The symbol "✓" indicates significant halo zone formation when tested using *C. violaceum* CVO26 plate assay.

4.1.1 Identification of *P. amarus* and Deposition of Plant Specimen

P. amarus were identified and a voucher specimen was deposited at Rimba Ilmu, University of Malaya, with the accession number KLU 47768.

Figure 4.1: *P. amarus* voucher specimen.

4.1.2 Anti-QS Activity of the Methanolic Extract of *P. amarus*

In the preliminary screening, only the methanolic extract of *Phyllanthus amarus* exhibited significant halo zone formation against the purple *C. violaceum* CVO26 lawn, as seen in Figure 4.2. The hexane and chloroform extracts of *P. amarus* did not produce significant halo zone formation. Figure 4.2 demonstrates that the formation of halo zone was observable at 1 mg/mL and increased in diameter as the concentration of the extract increased to 5 mg/mL. DMSO which served as the negative control did not exhibit any anti-QS or bactericidal effects. Thus, the formation of halo zone observed indicated anti-QS activity of the methanolic extract of *P. amarus.*

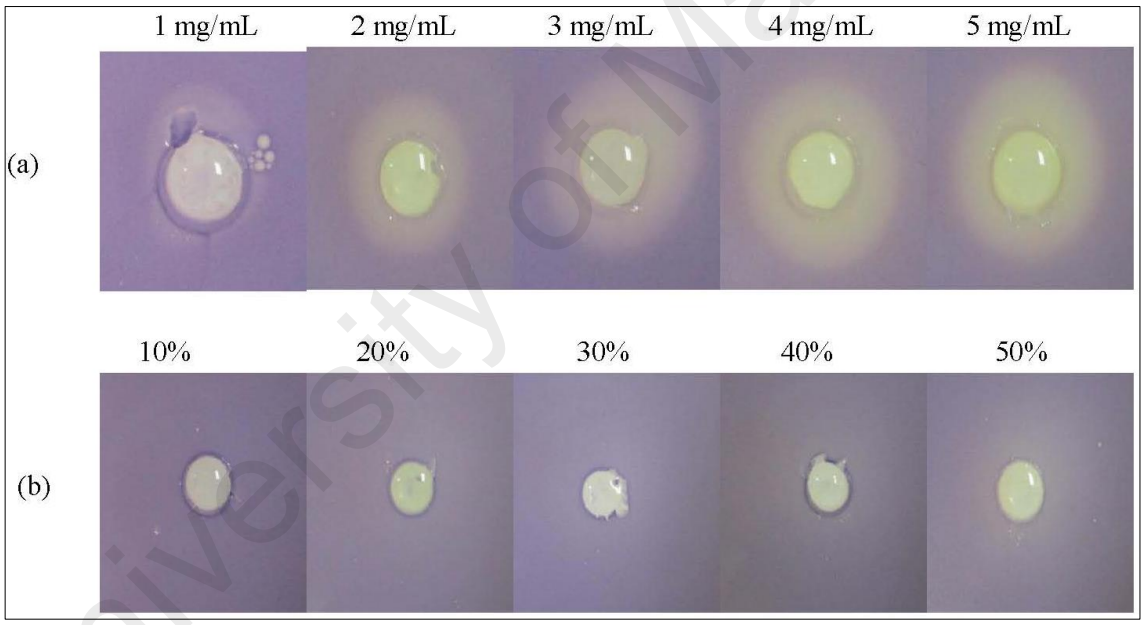
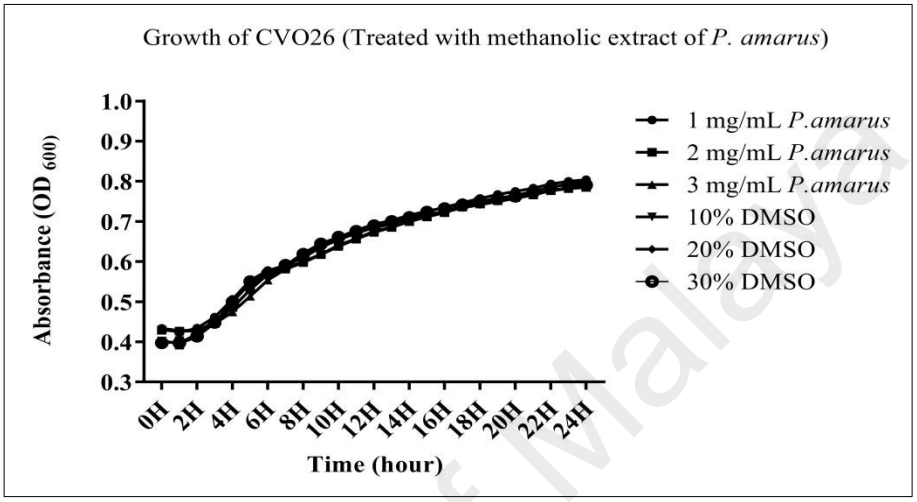


Figure 4.2: QS inhibition of methanolic extract at (a) 1, 2, 3, 4, and 5 mg/mL and negative control (b) DMSO at 10% - 50% (v/v) which corresponds to each concentration of the crude extract.

4.2 Growth of *P. aeruginosa* PAO1 and *C. violaceum* CVO26 in Methanolic Extract of *P. amarus*

Figure 4.3 (a) and (b) shows that the methanolic extract of *P. amarus* does not interfere in the growth of both the bacterial strains used in this study.

(a)



(b)

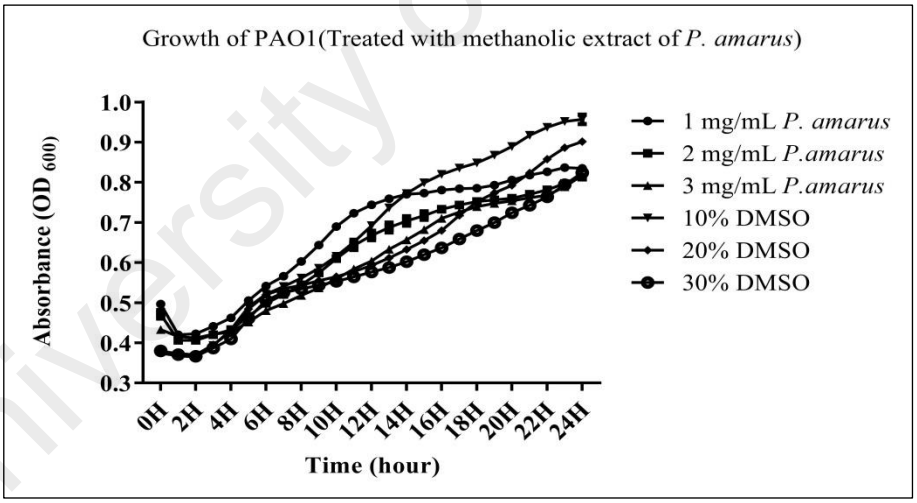
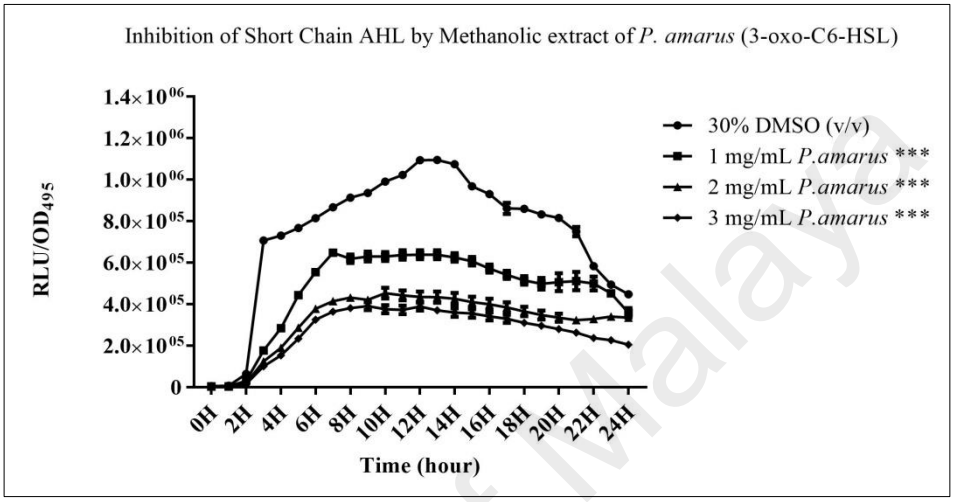


Figure 4.3: Growth of *P. aeruginosa* PAO1 (a) and *C. violaceum* CVO26 (b) supplemented with 1 – 3 mg/mL methanolic extract of *P. amarus.*

4.3 Bioluminescence Quantification: Inhibition of *lux*-based biosensors

Figure 4.4 shows luminescence produced by both *E. coli* biosensor strains were significantly reduced with increasing concentration of methanolic crude extract of *P. amarus*, *p*-value < 0.05, as compared to the negative control DMSO (30% v/v).

(a)



(b)

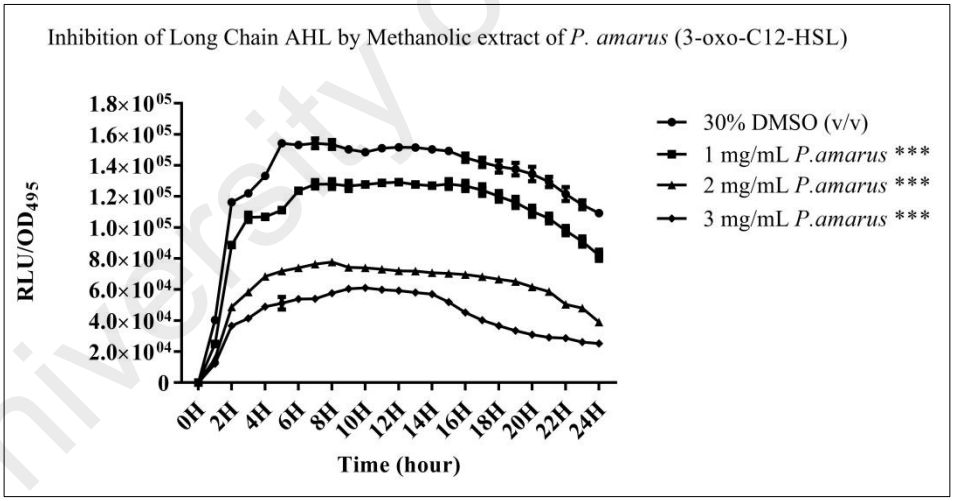


Figure 4.4: Inhibition in bioluminescence produced by (a) *E.coli* [pSB 401] and (b) *E.coli* [pSB 1075] by methanolic extract of *P. amarus*. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: 30% DMSO). **, $p < 0.005$ (versus the control: 30% DMSO), ***, $p < 0.0005$ (versus the control: 30% DMSO). Statistical analysis was conducted using *t-test.*

4.4 Quantification of *P. aeruginosa* PAO1 *lecA* Expression

Figure 4.5 show that the methanolic extract of *P. amarus* reduced the *lecA* expression of *P. aeruginosa* PAO1 with increasing concentration. However, the inhibition of *lecA* was only significant at 3mg/mL. Therefore, this indicates that a higher concentration of the methanolic extract was required to inhibit the expression of *lecA.*

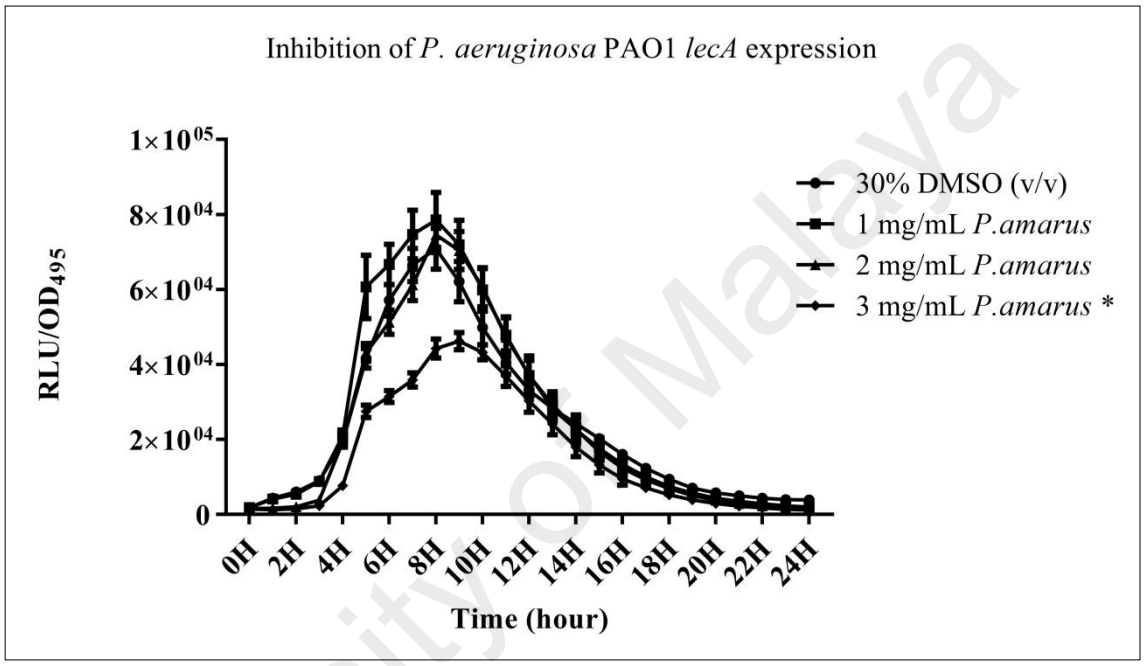


Figure 4.5: Inhibition of *P. aeruginosa* PAO1 *lecA* expression by methanolic extract of *P. amarus*. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: 30% DMSO). **, $p < 0.005$ (versus the control: 30% DMSO), ***, $p < 0.0005$ (versus the control: 30% DMSO). Statistical analysis was conducted using *t-test.*

4.5 Effects of Methanolic Extracts on QS-regulated Virulence Determinants of *P. aeruginosa* PAO1

4.5.1 Pyocyanin Production

Figure 4.6 shows that that pyocyanin produced by *P. aeruginosa* PAO1 reduces with increasing concentration of the extract as compared to 30% DMSO which served as the negative control.

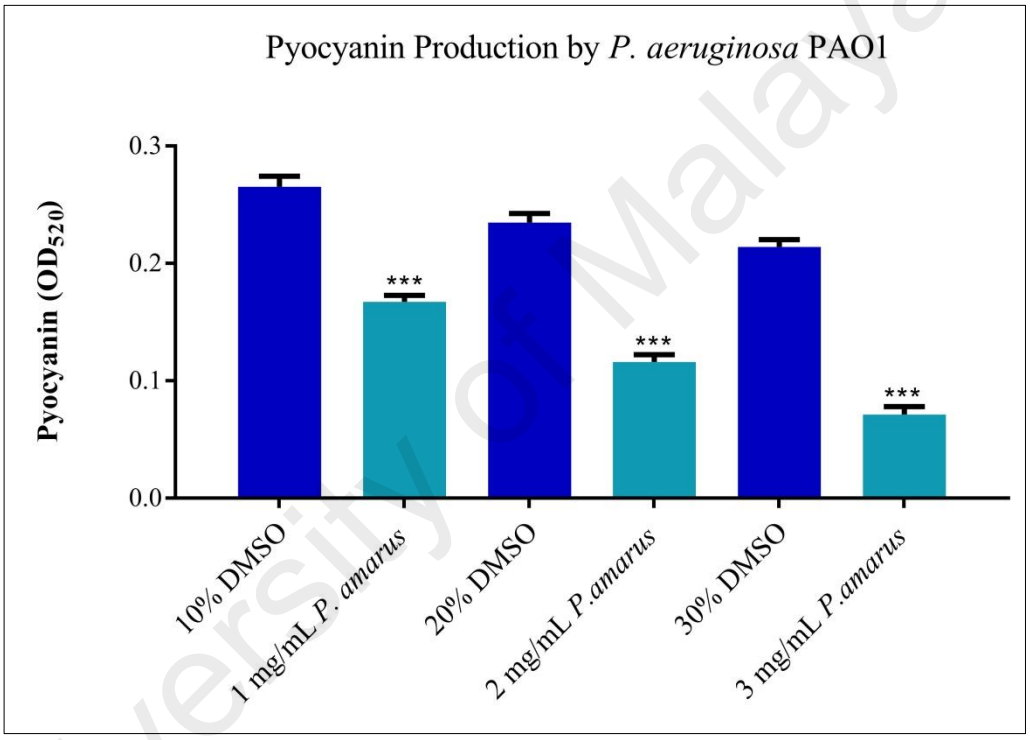
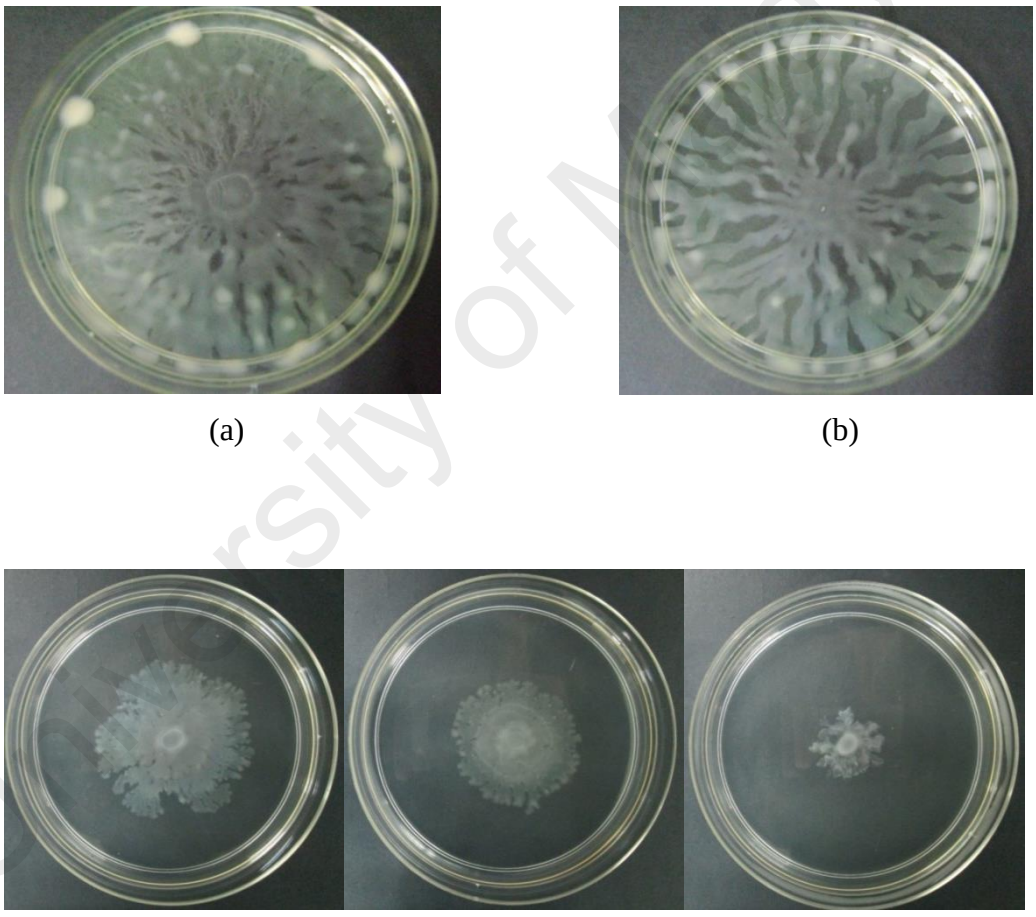


Figure 4.6: Inhibition of pyocyanin production by methanolic extract of *P. amarus.* Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: 30% DMSO). **, $p < 0.005$ (versus the control: 30% DMSO), ***, $p < 0.0005$ (versus the control: 30% DMSO). Statistical analysis was conducted using *t-test.*

4.5.2 Swarming Assay

Methanolic extract of *P. amarus* significantly inhibited swarming of *P. aeruginosa* PAO1 with increasing concentration. Inhibition was observable at the lowest concentration tested, 1 mg/mL, as seen in Figure 4.7 (c). No inhibition was observed in (a) unsupplemented swarming agar and (b) swarming agar supplemented with 30% DMSO.



(a) (b)

(c) (d) (e)

Figure 4.7: Swarming inhibition by methanolic extract of *P. amarus.* Swarming agar inoculated with (a) *P. aeruginosa* PAO1, and supplemented with (b) 30% DMSO, (c) 1 mg/mL, (d) 2 mg/mL, (e) 3 mg/mL of methanolic extract of *P. amarus.*

4.6 Swarming Assay as a Screening Method for anti-QS Activity of Baicalein

Baicalein did not exhibit halo zone formation when *C. violaceum* CVO26 plate assay was performed as a screening method (Figure 4.8). Only a slight halo zone formation was observable at 200 µM. Results observed indicated that *C. violaceum* CVO26 was not a suitable biosensor to be used for screening for anti-QS effect of baicalein. Swarming assay was used instead as a rapid screening method to determine QS inhibitory effects of baicalein against *P. aeruginosa* PAO1. Figure 4.9 shows that swarming of *P. aeruginosa* PAO1 was inhibited at 20 uM and completely inhibited at 200 uM. Baicalein of concentrations 0.2 uM, 2 uM, 20 uM, and 200 uM were then used in subsequent bioassays to determine their activities against quorum sensing.

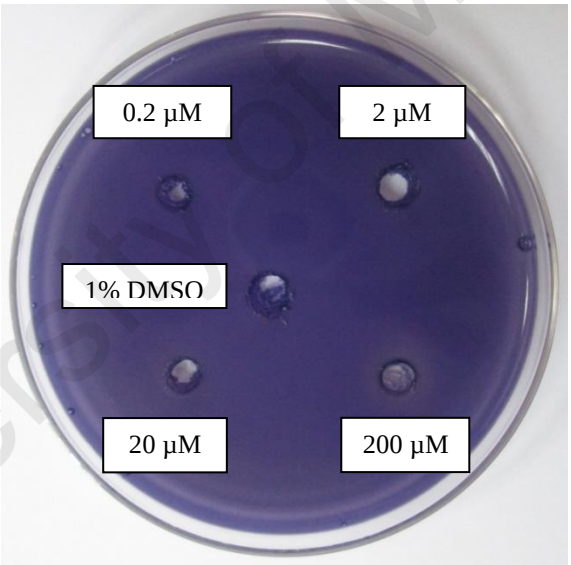


Figure 4.8: *C. violaceum* CVO26 plate assay. Baicalein of concentrations 0.2 µM, 2 µM, and 20 µM did not produce any notable inhibition. However, slight halo zone formation was observable at 200 µM.

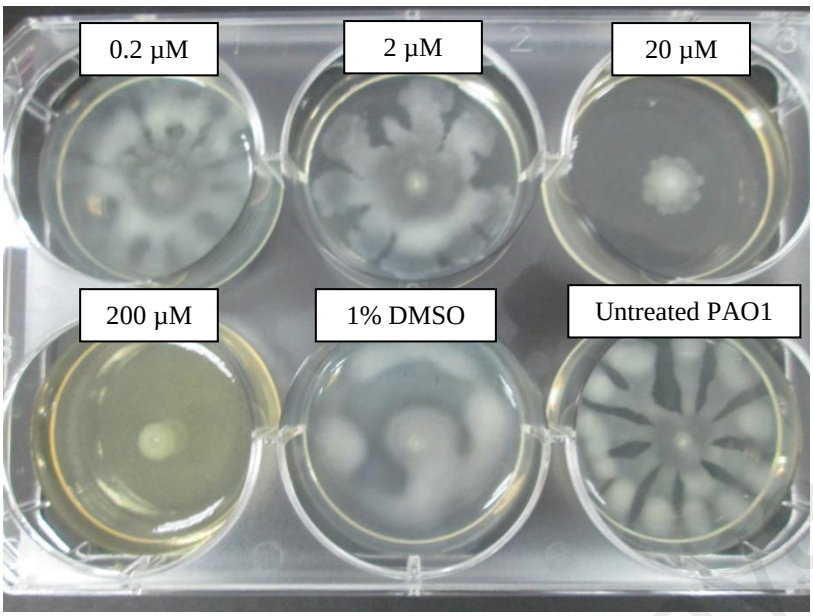


Figure 4.9: Swarming assay was performed in a 6-well plate in triplicates. Top row (left to right): 0.2 µM, 2 µM, and 20 µM baicalein; bottom row (left to right): 200 µM baicalein, 1% (v/v) DMSO, and untreat ned *P. aeruginosa* PAO1. Swarming inhibition was observable at 20 µM and 200 µM. DMSO (1% v/v) and untreated *P. aeruginosa* PAO1 served as controls.

4.7 Antibacterial Studies of Baicalein

Once the concentrations of baicalein were determined from the screening assays, the growth of *P. aeruginosa* PAO1 in the presence of baicalein was tested for a period of 24 hours to eliminate any possible anti-bacterial or bactericidal effects. Figure 4.10 shows that the concentrations of baicalein used, 0.2 µM, 2 µM, 20 µM, and 200 µM, does not inhibit the growth of *P. aeruginosa* PAO1. On similar note, catechin, gallic acid (at similar concentrations) and DMSO (corresponding to the concentrations of baicalein) which served as the positive and negative controls, respectively, were also tested for any anti-bacterial affect towards *P. aeruginosa* PAO1. Figures 4.11 (a-b) and 4.12

demonstrate that catechin, gallic acid and DMSO did not affect the growth of *P. aeruginosa* PAO1.

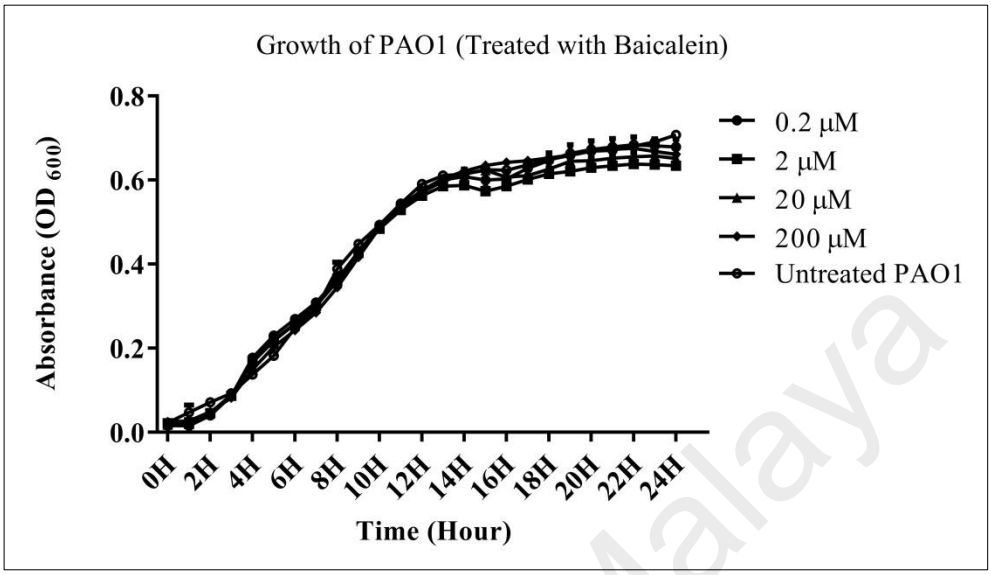


Figure 4.10: Growth of *P. aeruginosa* PAO1 in the presence of baicalein at concentrations 0.2 uM, 2 uM, 20 uM, and 200 uM. The concentration used did not affect the growth of *P. aeruginosa* PAO1.

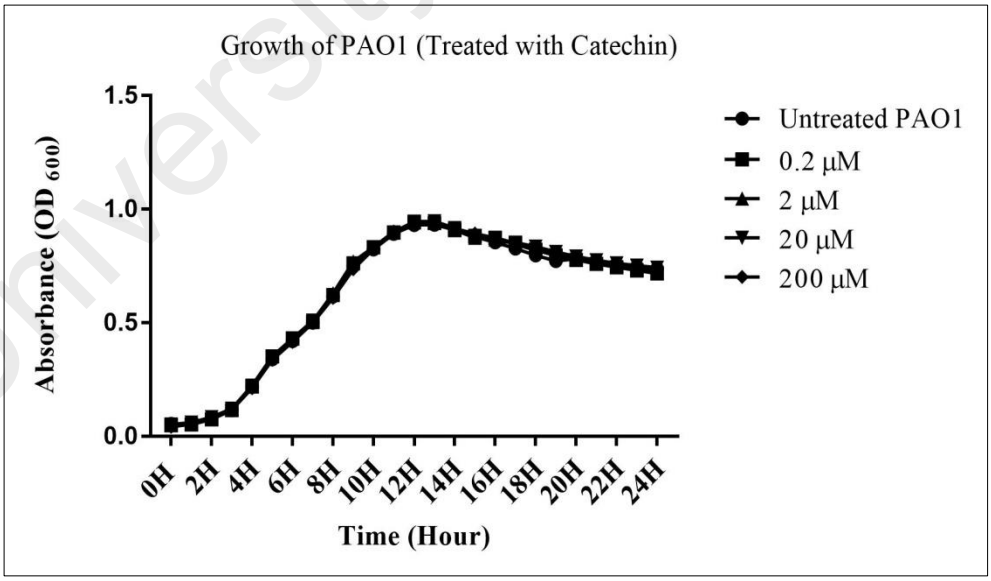


Figure 4.11 (a): Growth of *P. aeruginosa* PAO1 in the presence of catechin (positive control) at concentrations 0.2 uM, 2 uM, 20 uM, and 200 uM. The concentration used did not inhibit the growth of *P. aeruginosa* PAO1.

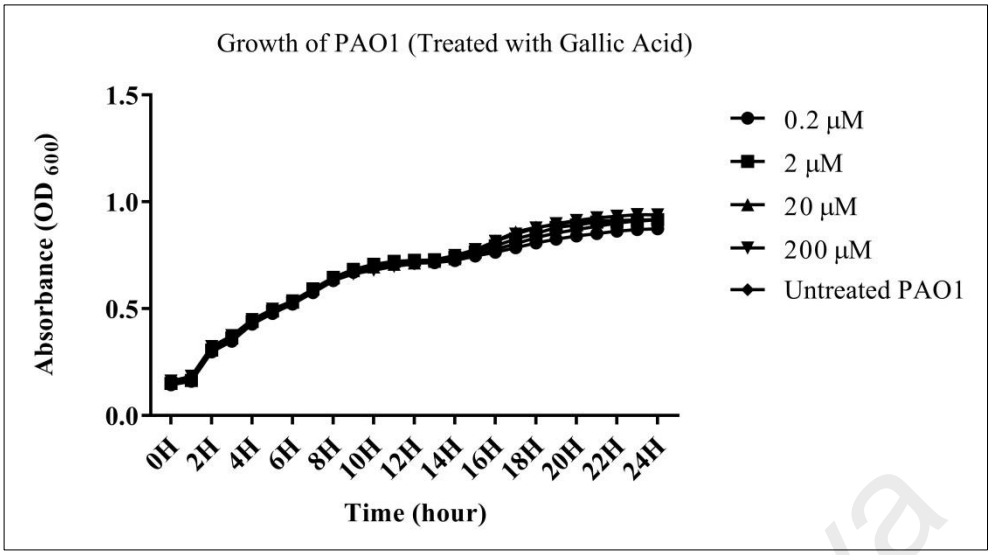


Figure 4.11 (b): Growth of *P. aeruginosa* PAO1 in the presence of gallic acid (positive control) at concentrations 0.2 µM, 2 µM, 20 µM, and 200 µM. The concentration used did not inhibit the growth of *P. aeruginosa* PAO1.

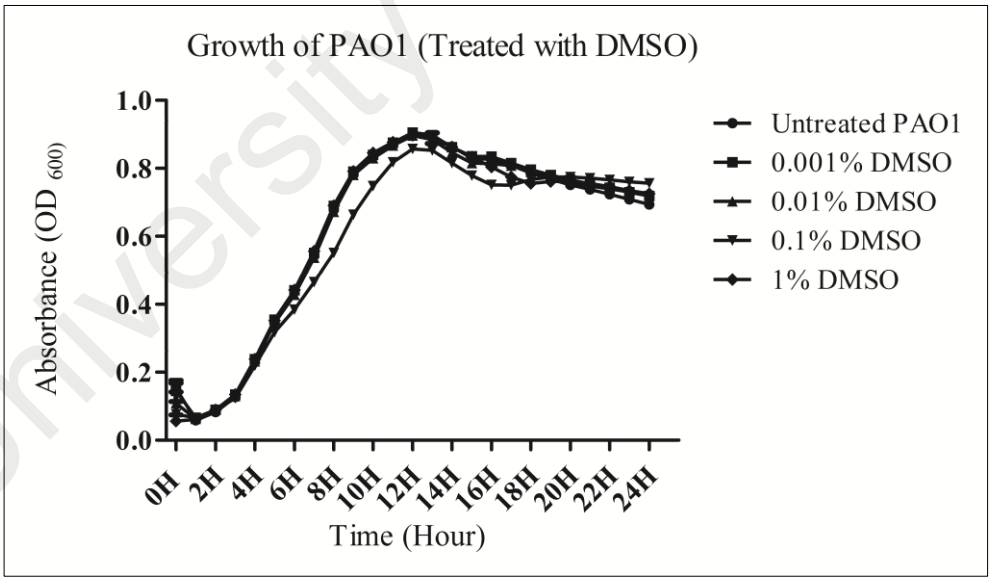
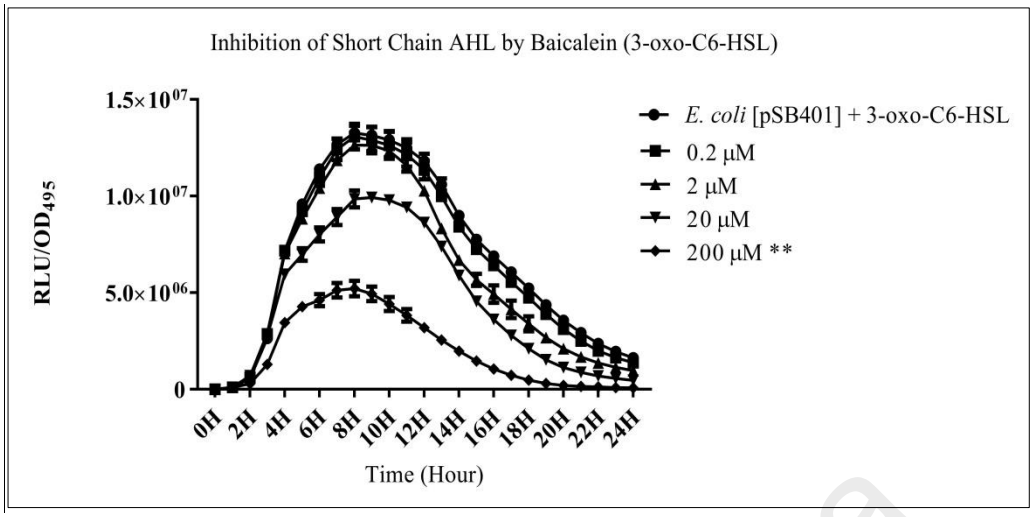


Figure 4.12: Growth of *P. aeruginosa* PAO1 in the presence of DMSO (control) at percentages 0.001%, 0.01%, 0.1%, and 1% (v/v) which corresponds to each concentration of baicalein tested.

4.8 Inhibition of QS mediated Bioluminescence

The *luxCDABE*-based biosensors, *E.coli* [pSB 401] and *E.coli* [pSB1075], were used to further test the anti-quorum sensing activity of baicalein against short and long chain AHLs, 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively. These biosensors respond optimally by producing luminescence when the culture conditions were supplied with AHLs. Reduction in the luminescence produced by the biosensors with increasing concentration of compounds would indicate inhibition in the activity on the *luxCDABE*-based QS system. Figures 4.13 (a) and (b) show significant inhibition at the concentration of 200 uM against both *E.coli* biosensors with $p < 0.005$ (versus the control: untreated *P. aeruginosa* PAO1). Baicalein interrupted the bioluminescence activity of both short and long chain AHLs, indicating potential broad range of anti-QS activity. On the hand, catechin significantly inhibited bioluminescence production in both *E. coli* biosensors at 2 µM with $p < 0.05$ (versus the control: untreated *P. aeruginosa* PAO1) and concentration of 200 µM with $p < 0.005$ (versus the control: untreated *P. aeruginosa* PAO1) as seen in Figures 4.14 (a) and (b). No inhibition was observable in the DMSO (control) as seen in Figures 4.15 (a) and (b) in both *E. coli* biosensors.

(a)



(b)

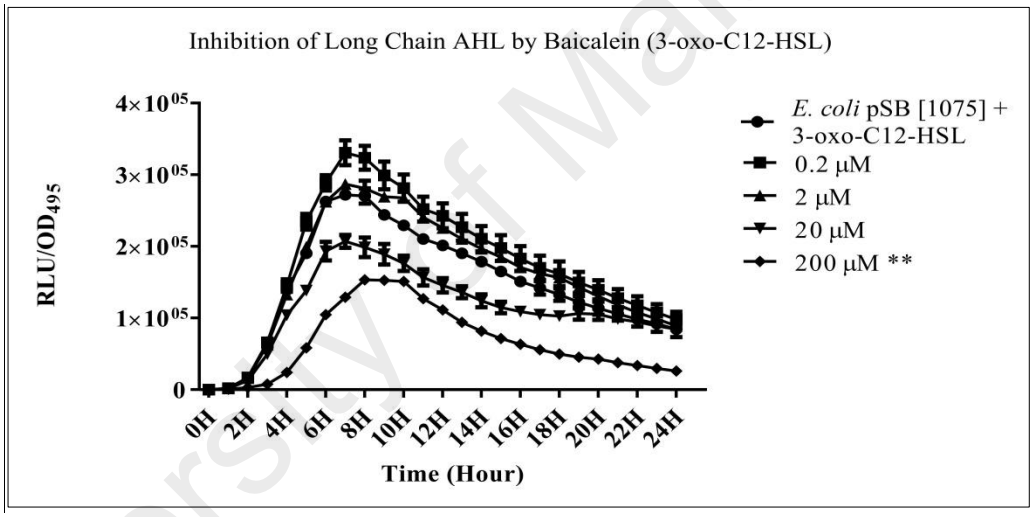
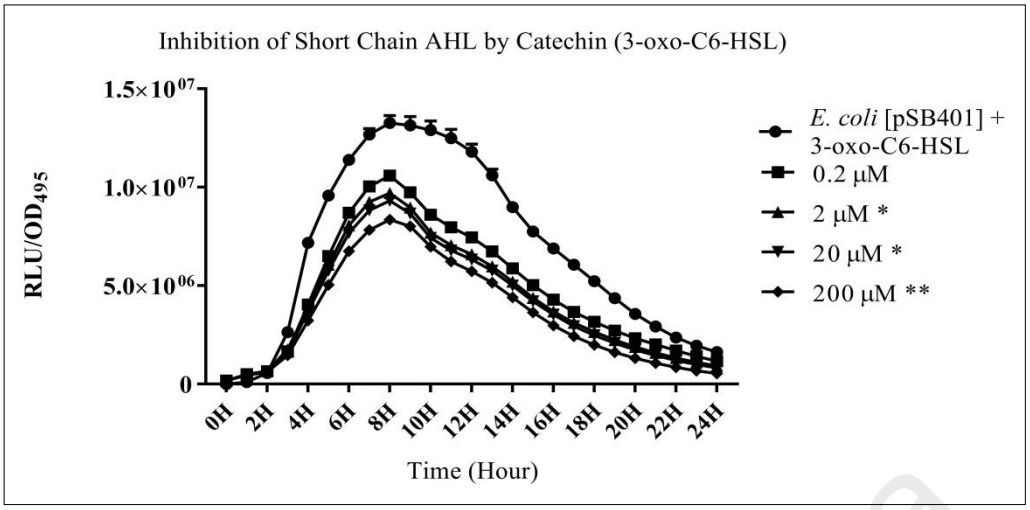


Figure 4.13: Inhibition of short chain AHL (a) (3-oxo-C6-HSL) and long chain AHL (b) (3-oxo-C12-HSL) by baicalein. Significant inhibition was observable at 200 µM in both *E.coil* biosensors. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: untreated PAO1). **, $p < 0.005$ (versus the control: untreated PAO1). Statistical analysis was conducted using *t-test.*

(a)



(b)

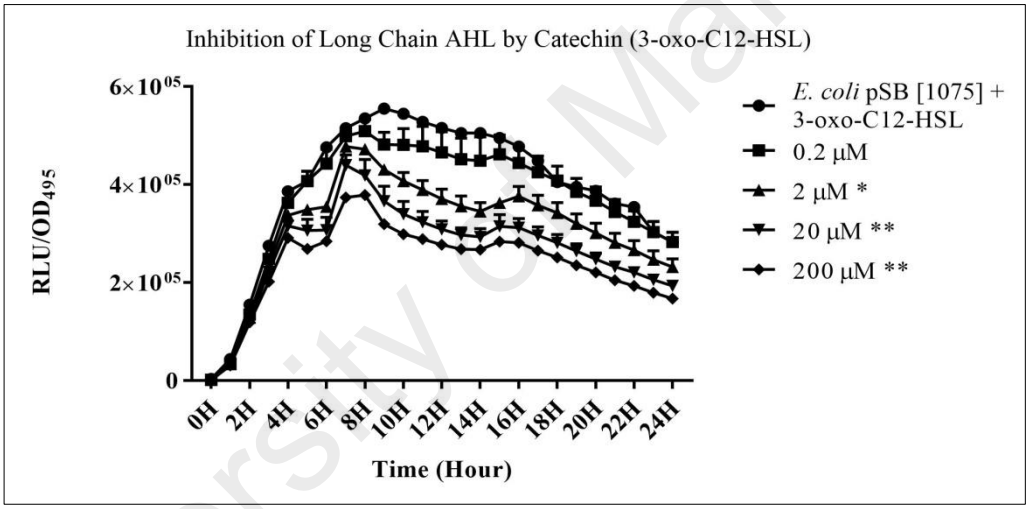
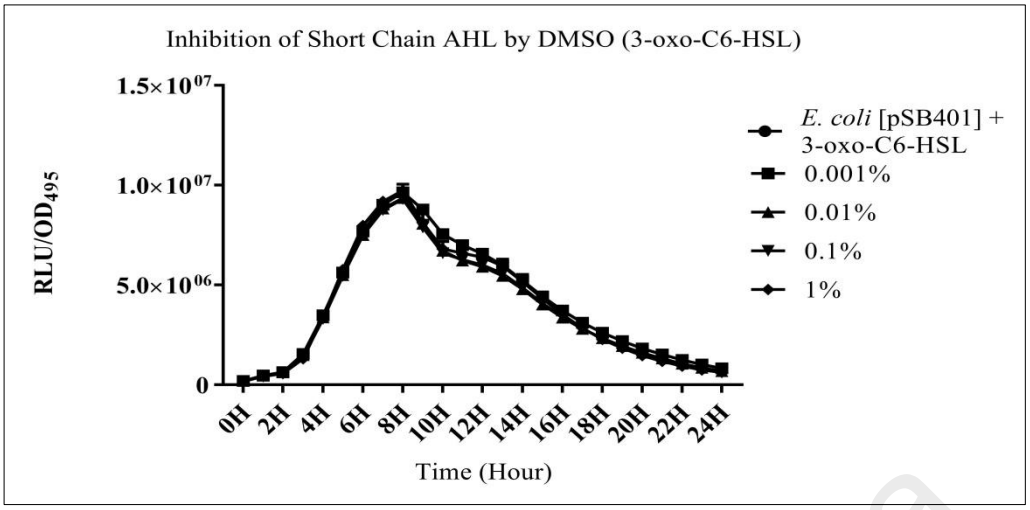


Figure 4.14: Inhibition of short chain AHL (a) (3-oxo-C6-HSL) and long chain AHL (b) (3-oxo-C12-HSL) by catechin (positive control). Significant inhibition was observable at 2 µM, 20 µM, and 200 µM in both *E.coil* biosensors. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: untreated PAO1). **, $p < 0.005$ (versus the control: untreated PAO1), ***, $p < 0.0005$ (versus the control: untreated PAO1). Statistical analysis was conducted using *t-test.*

(a)



(b)

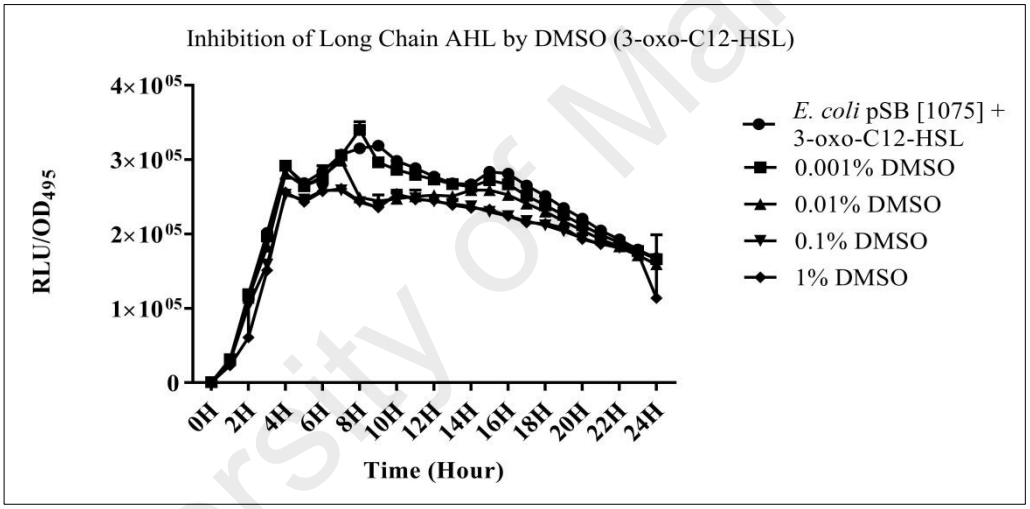


Figure 4.15: Inhibition of short chain AHL (a) (3-oxo-C6-HSL) and long chain AHL (b) (3-oxo-C12-HSL) by DMSO (control). No significant inhibition was observable in both *E.coil* biosensors. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control: untreated PAO1). **, $p < 0.005$ (versus the control: untreated PAO1), ***, $p < 0.0005$ (versus the control: untreated PAO1). Statistical analysis was conducted using *t-test.*

4.9 Attenuation of Virulence Factors of *P. aeruginosa* PAO1

4.9.1 Pyocyanin Quantification Assay

As compared to catechin (positive control), baicalein had significant effect towards the pyocyanin production in *P. aeruginosa* PAO1. Significant inhibition by catechin was observable only at 200 µM. Baicalein significantly inhibited pyocyanin production at concentration as low as 2 µM. No significant inhibition was caused by DMSO (control). It can be concluded that pyocyanin inhibition was exclusively the effect of baicalein against *P. aeruginosa* PAO1 and not affected by the percentage of DMSO.

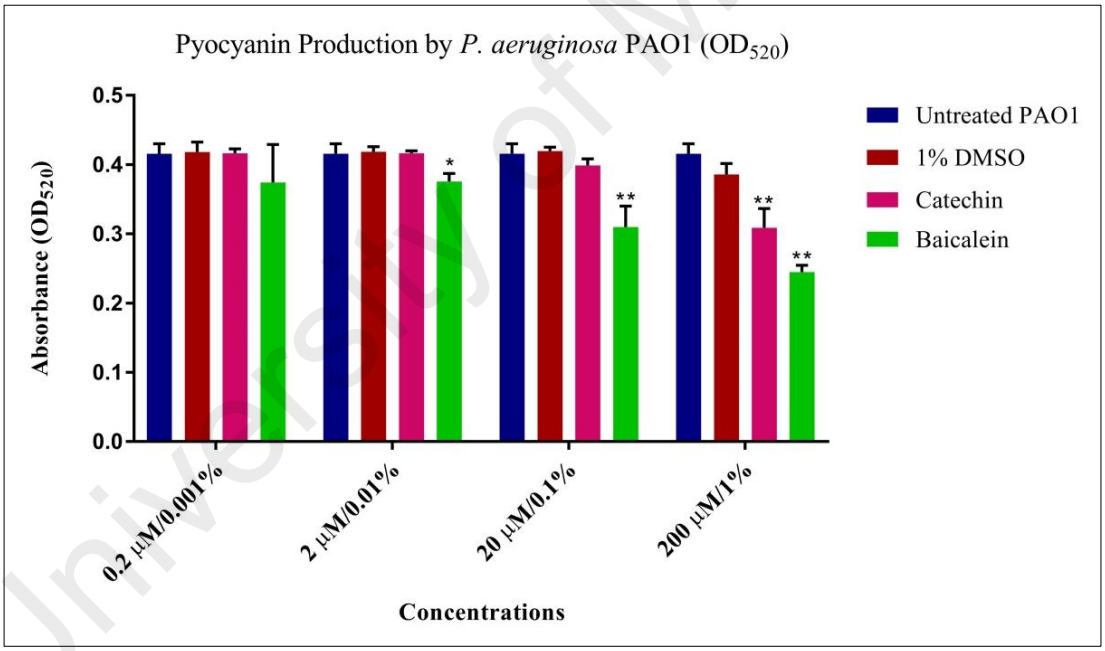


Figure 4.16: A decrease in pyocyanin production in *P. aeruginosa* PAO1. Baicalein significantly inhibited pyocyanin production at starting at 2 µM and very significantly at 20 µM and 200 µM. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control; untreated PAO1). **, $p < 0.005$ (versus the control; untreated PAO1). Statistical analysis was conducted using *t-test,* ***, $p < 0.0005$ (versus the control: untreated PAO1). Statistical analysis was conducted using *t-test.*

4.9.2 Quantitative Analysis of Biofilm Inhibition

Baicalein and catechin (positive control) significantly reduced the biofilm formed by *P. aeruginosa* PAO1. Biofilm formation was significantly inhibited by both compounds at 20 µM with $p < 0.0005$ (versus the control: untreated PAO1). DMSO of 1% (control) which corresponded to the final percentage of solvent in 200 µM baicalein did not inhibit biofilm formation by *P. aeruginosa* PAO1.

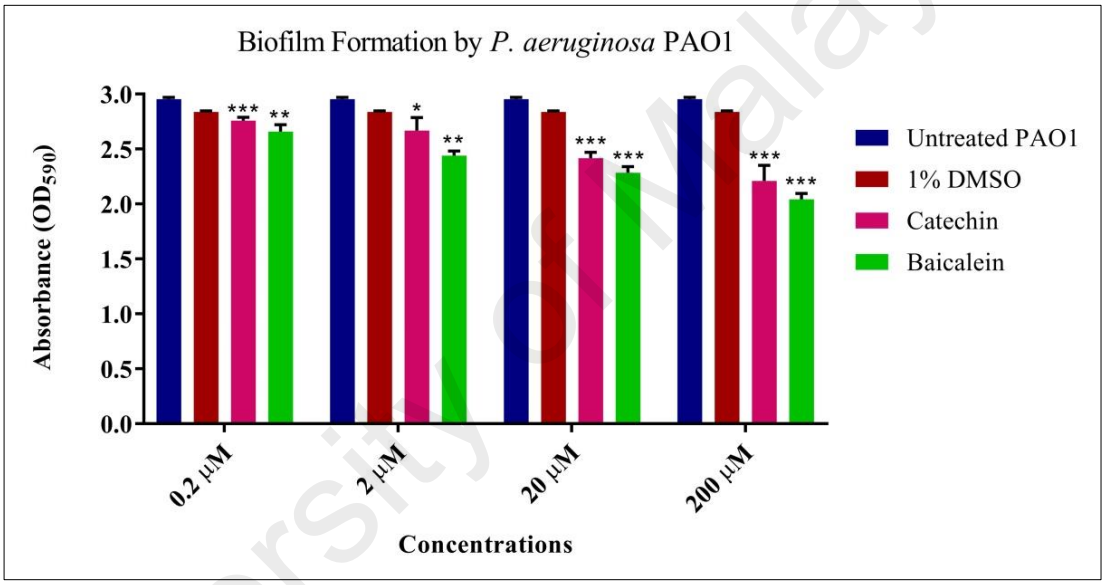
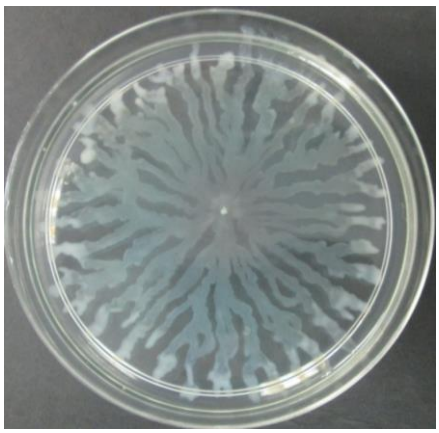


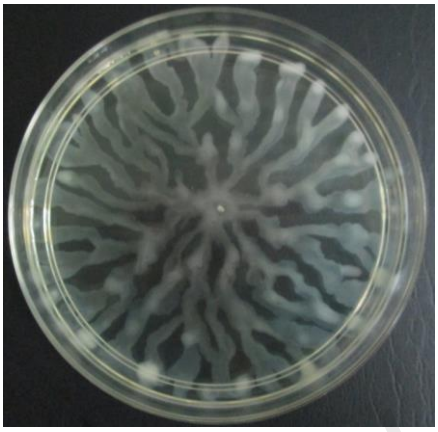
Figure 4.17: Biofilm inhibition in *P. aeruginosa* PAO1. Error bars indicate standard deviations (SD) of 3 measurements. *, $p < 0.05$ (versus the control; untreated PAO1). **, $p < 0.005$ (versus the control; untreated PAO1), ***, $p < 0.0005$ (versus the control; untreated PAO1). Statistical analysis was conducted using *t-test.*

4.9.3 Swarming Inhibition by Baicalein and Gallic Acid

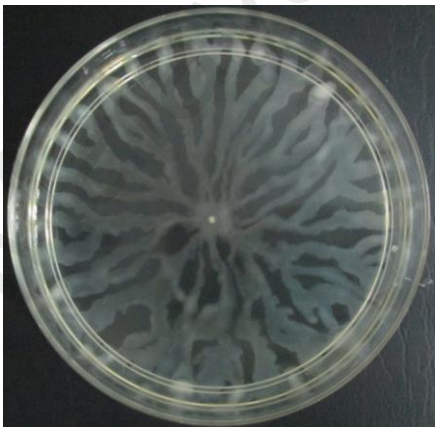
Figure 4.18 (a) shows swarming of *P. aeruginosa* PAO1 on a swarming agar that is not supplemented with any compound or solvent whereas Figures 4.18 (b), (c), (d), and (e) are swarming agars supplemented with 0.001%, 0.01%, 0.1%, 1% (v/v) DMSO, respectively. Percentages of DMSO corresponded to the final percentage of DMSO present in each final concentration of baicalein. No inhibition was observed in any of the percentage of DMSO. Figures 4.19 (a – d), exhibit swarming inhibition by baicalein. Significant inhibition was observed at 20 µM and completely diminished at 200 µM. Figures 4.20 (a – d) shows the swarming inhibition by gallic acid. Significant inhibition was observable at 200 µM.



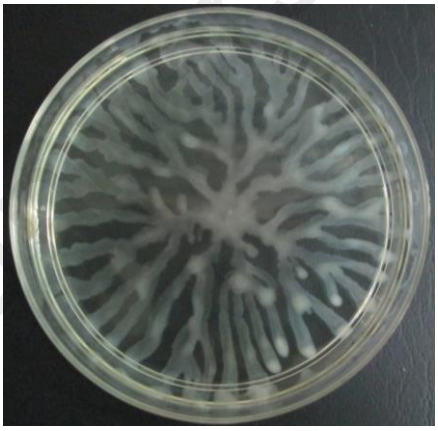
(a) Untreated swarming agar



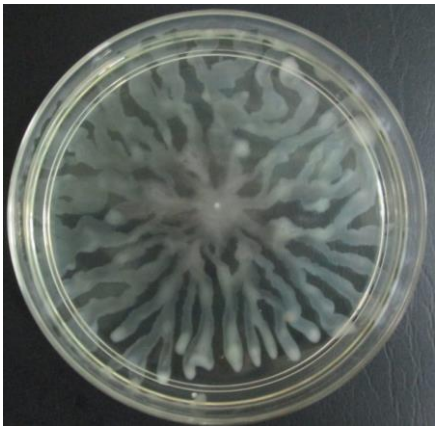
(b) 0.001% DMSO



(c) 0.01% DMSO

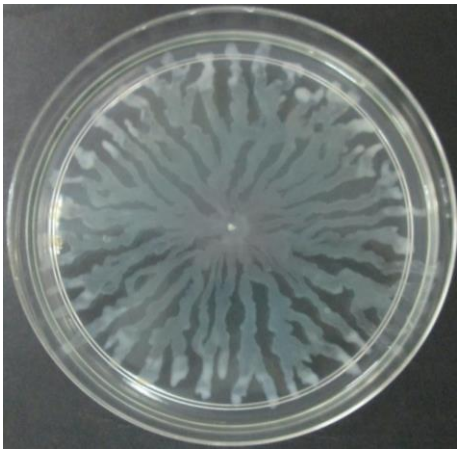


(d) 0.1% DMSO



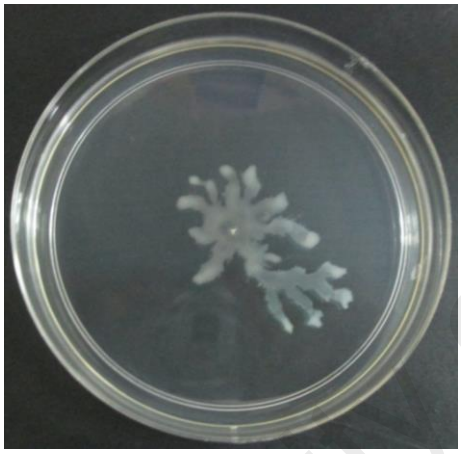
(e) 1% DMSO

Figure 4.18: Swarming of *P. aeruginosa* PAO1 on agar supplemented with (a) 0.001% DMSO, (c) 0.01% DMSO, (d) 0.1% DMSO, and (e) 1% DMSO while (a) is unsupplemented swarming agar.

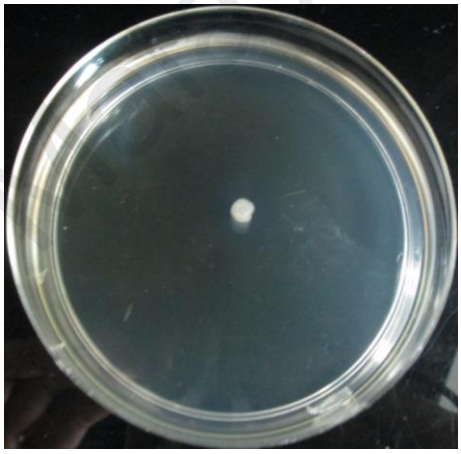


(a) 0.2 µM

(b) 2 µM

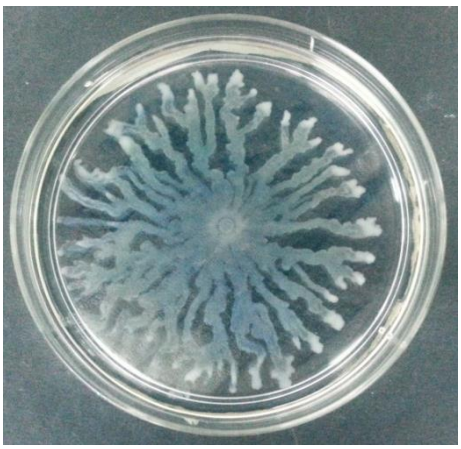


(c) 20 µM



(d) 200 µM

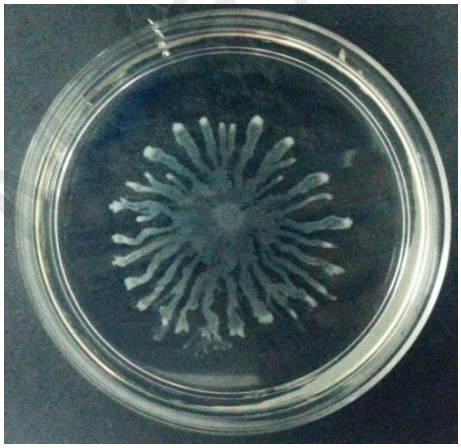
Figure 4.19: Swarming of *P. aeruginosa* PAO1 on agar supplemented with baicalein. Concentrations were as follows: (a) 0.2 µM, (b) 2 µM, (c) 20 µM, and (d) 200 µM.



(b) 0.2 µM

(b) 2 µM

(c) 20 µM



(d) 200 µM

Figure 4.20: Swarming of *P. aeruginosa* PAO1 on agar supplemented with gallic acid (positive control). Concentrations of gallic acid were as follows: 0.2 µM (a), 2 µM (b), 20 µM (c), and 200 µM (d).

4.10 Transcriptome Analysis of *P. aeruginosa* PAO1 Co-cultured with 20 µM Baicalein

4.10.1 RNA Extraction and Library Preparation

The quality and quantity of the extracted RNA and the cDNA library for sequencing of all samples were assessed prior to sequencing. The quality check results and quantification of extracted RNA, rRNA-depleted samples, and cDNA libraries were recorded (Appendix A, B, and C).

4.10.2 Transcriptome Data Analysis

RNA-sequencing was conducted to gain insights towards the global gene regulation of *P. aeruginosa* PAO1 upon exposure to 20 µM baicalein. Concentration of 20 µM was chosen as it was the lowest concentration of baicalein needed to cause a significant QS inhibition in *P. aeruginosa* PAO1. Furthermore, as the final concentration of DMSO present in baicalein would be 0.1%, it would reduce the solvent effect on the gene expression as compared to 200 µM baicalein where the final concentration of DMSO would be 1%. Figure 4.21 shows the box plot of normalised expression values of each sample of untreated and baicalein-treated *P. aeruginosa.* The box plot indicates the $\log_2$ transformed values of the differentially expressed genes in *P. aeruginosa* PAO1. Figure 4.22 shows the principle component analysis (PCA) plot of the triplicates of each treatment in the RNA-seq experiments. The PCA plot shows that triplicates of each sample are clustered closely together. A profile separation among sample group indicates little variation among the triplicate samples within a group. Figure 4.23 (volcano plot) gives the general view of the differentially expressed genes. Dots on the left signified the down-regulated genes while dots on the right signified the up-regulated

genes. The volcano plot represents the total of 151 differentially expressed genes with $p < 0.05$. Treatment of the cultures with baicalein resulted in 81 down-regulated genes (Table 4.2) and 70 up-regulated genes (Table 4.3) ($p \leq 0.05$ and fold-change of ≤ -2 or ≥ 2 as the cut-off value). Genes affected by DMSO were different compared to those affected by baicalein (Appendix D). A large portion of the up-regulated genes were found to be conserved hypothetical proteins or proteins with probable gene functions. Amongst the up-regulated genes with high fold-change values includes *exaB* (fold change: 4.39669), *narG* (fold change: 4.12891), *narK1* (fold change: 3.85046), and *narK2* (fold change: 4.12166). The most down-regulated genes were found to be the *xphA* gene (fold change: -9.44487). Following the *xphA* gene, 4 of the subsequent down regulated genes (PA0668.5, PA4280.1. PA4690.1, and PA5369.1) were genes that encodes for 5S ribosomal RNAs. The focus of the study was directed towards several gene clusters that were down-regulated. Several genes that belong to the iron-sulphur cluster ([Fe-S]) operon, *iscS*, *iscR*, and *iscU*, and pyoverdine synthesis, *pvdA*, *pvdQ*, *pvdO*, and *pvdN*, were significantly down-regulated.

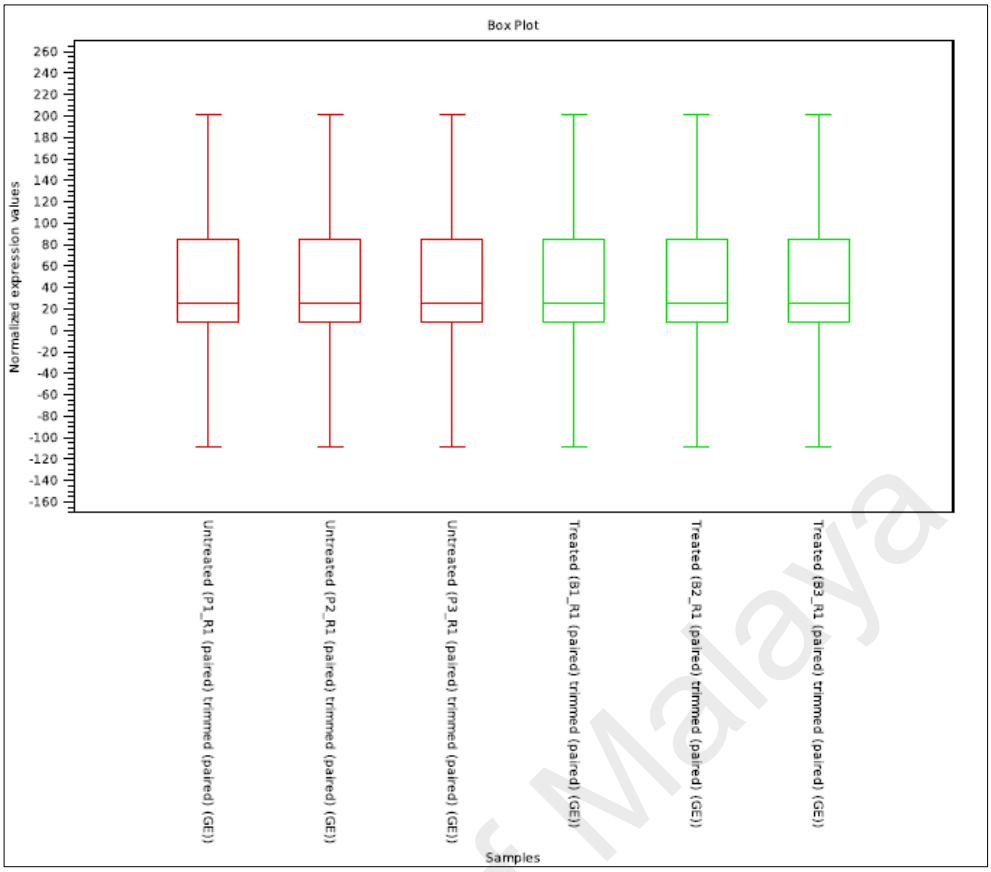


Figure 4.21: Boxplot of normalised values of untreated *P. aeruginosa* (untreated) and *P. aeruginosa* co-cultured with 20 µM baicalein (treated).

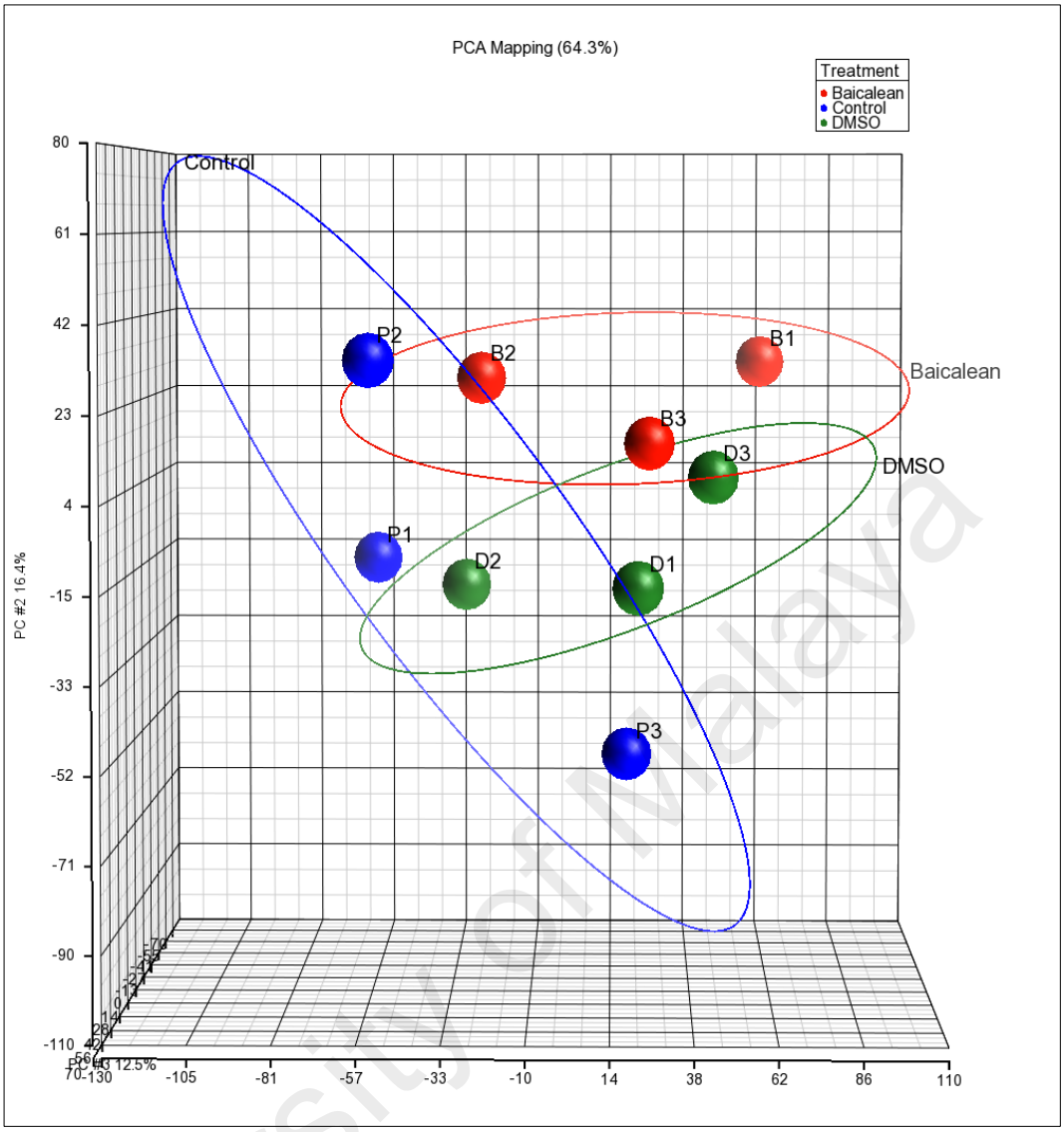


Figure 4.22: PCA plot of triplicates samples of each treatment of baicalein RNA-seq experiment. The profile separation among treatment categories were made based on figures generated with $p < 0.05$.

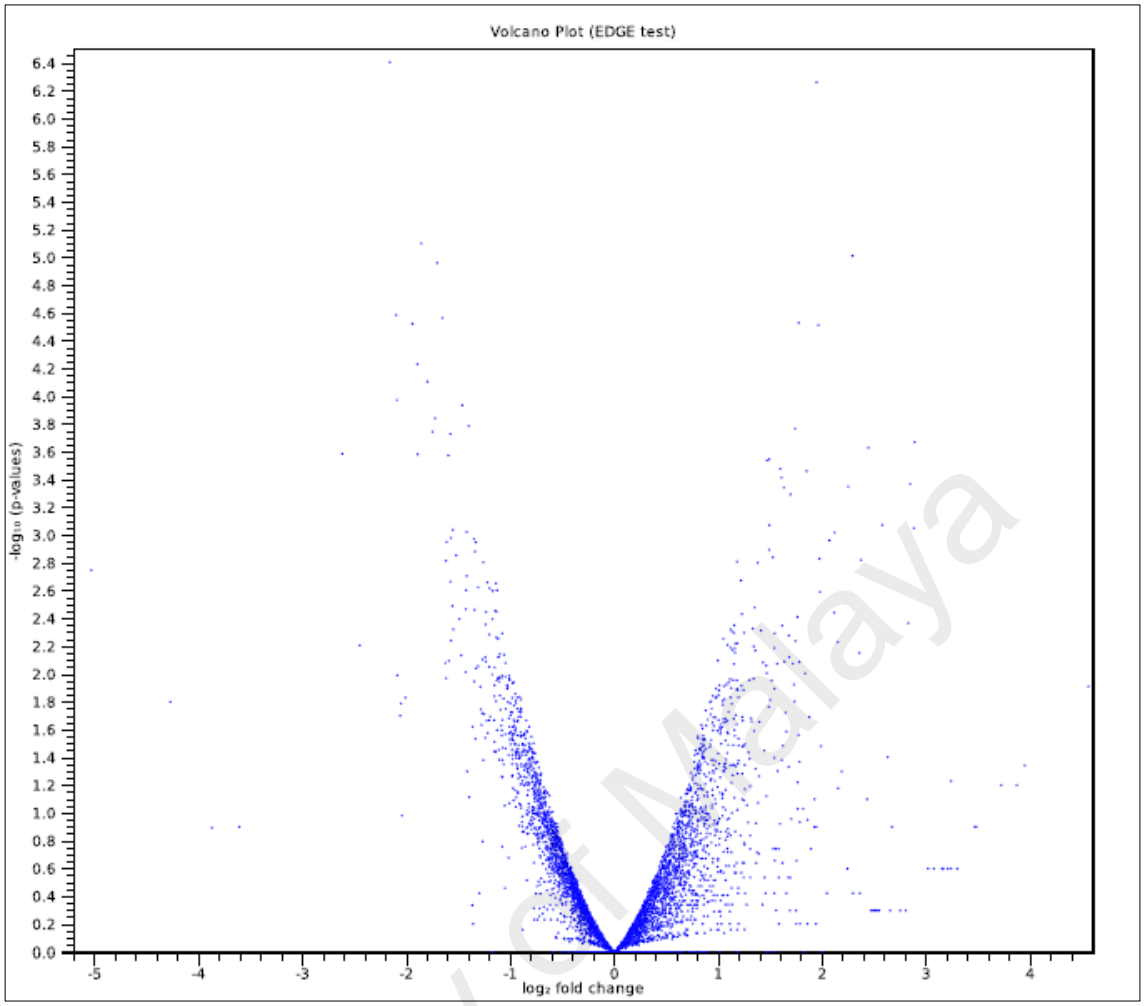


Figure 4.23: Volcano plot of genes expressed by untreated *P. aeruginosa* and *P. aeruginosa* co-cultured with 20 µM baicalein.

Table 4.2: Significantly down-regulated genes of *P. aeruginosa* PAO1 when co-cultured in 20 µM baicalein.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
xphA	PA1867	0.00886192	-9.44487	Type II protein secretion system complex
PA0668.5		0.0137461	-6.07565	5s Ribosomal RNA
PA4280.1		0.0137461	-6.07565	5s Ribosomal RNA
PA4690.1		0.0137461	-6.07565	5s Ribosomal RNA
PA5369.1		0.0137461	-6.07565	5s Ribosomal RNA
PA1781.1	*naIA*	0.0282726	-6.07565	Nitrogen assimilation leader A. Regulation of nitrate assimilation
PA2451		0.0399145	-5.98142	Hypothetical Protein. Probable esterase
PA1478		0.0303214	-5.6341	Hypothetical Protein
PA2155		0.0499802	-4.78213	Probable phospholipase
PA1953	*fapD*	0.0144213	-4.48827	Amyloid fibril formation
PA1313		0.00178414	-4.10917	Probable major facilitator superfamily (MFS) transporter
iscS	PA3814	0.000127628	-3.82705	L-cysteine desulfurase (pyridoxal phosphate-dependent)
iscR	PA3815	0.00931765	-3.79086	Iron-sulfur cluster assembly transcription factor
iscU	PA3813	0.0141798	-3.78096	Probable iron-binding protein
pvdA	PA2386	0.000972304	-3.61246	L-ornithine N5-oxygenase. Pyoverdine biosynthetic process
PA3920		0.0373875	-3.56087	Probable metal transporting P-type ATPase
PA2067		0.033912	-3.3986	Probable hydrolase
eraR	PA1980	0.0137383	-3.38319	Response regulator
PA5023		0.000150062	-3.16406	Conserved hypothetical protein
PA3900	*fecR*	0.0078708	-3.14761	Sigma factor antagonist activity
PA0046		0.024971	-3.12547	Hypothetical Protein
gloA2	PA0710	0.0294008	-3.03734	Lactoylglutathione lyase
PA3523		0.0247818	-3.0127	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor

Table 4.2: continued.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
PA0046		0.024971	-3.12547	Hypothetical Protein
gloA2	PA0710	0.0294008	-3.03734	Lactoylglutathione lyase
PA3523		0.0247818	-3.0127	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor
PA3899	*fecI*	0.0195859	-2.95847	Regulation of transcription
pvdQ	PA2385	0.0350764	-2.94755	3-oxo-C12 homoserine lactone acylase. Pyoverdine biosynthetic process
femI	PA1912	0.00333399	-2.86192	ECF sigma factor.
tonB1	PA5531	0.00651805	-2.81818	Bacterial-type flagellum-dependent swarming motility
cyoA	PA1317	0.00831651	-2.79462	Cytochrome o ubiquinol oxidase subunit II
pfeR	PA2686	0.0115702	-2.77336	Two-component response regulator
purB	PA2629	0.0298298	-2.74809	Adenylosuccinate lyase
fecA	PA3901	0.000654663	-2.67629	Fe(III) dicitrate transport protein
PA4645		0.0237439	-2.67082	Probable purine/pyrimidine phosphoribosyl transferase
pvdO	PA2395	0.0352079	-2.64752	Pyoverdine biosynthetic process
PA2770		0.00314882	-2.64177	Hypothetical Protein
PA2422		0.0489867	-2.63591	Hypothetical Protein
hscB	PA3811	0.0037999	-2.61675	Heat Shock Protein
pvcC	PA2256	0.00728645	-2.58883	Paerucumarin biosynthesis protein
PA4896		0.00677585	-2.57974	Probable sigma-70 factor, ECF subfamily
PA2692		0.00244607	-2.57057	Probable transcriptional regulator
phuR	PA4710	0.000748442	-2.56154	Heme/Hemoglobin Uptake Outer Membrane Receptor
PA4365		0.0318721	-2.55465	Probable transporter
hasE	PA3405	0.000924452	-2.53414	Metalloprotease secretion protein
PA2375		0.00289495	-2.51867	Hypothetical protein
PA2393		0.0140145	-2.51806	Putative dipeptidase

Table 4.2: continued.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
ligT	PA2861	0.0462183	-2.44214	2'-5' RNA Ligase
fptA	PA4221	0.0461119	-2.43755	Fe(III)-pyochelin outer membrane receptor precursor
pfeS	PA2687	0.00016202	-2.39437	Two-component sensor
PA0264		0.0337551	-2.38911	Hypothetical Protein
PA1302		0.00589955	-2.37084	Probable Heme Utilisation Protein Precursor
PA3592		0.0203867	-2.32321	Conserved hypothetical protein
PA0449		0.0488231	-2.31772	Hypothetical Protein
PA4928		0.0101353	-2.2832	Conserved hypothetical protein
PA1847	*nfuA*	0.00389466	-2.28291	Iron-sulfur cluster assembly. Protein maturation
opmQ	PA2391	0.0101154	-2.26685	Probable outer membrane protein precursor
hscA	PA3810	0.00184707	-2.25034	Probable heat shock protein
codB	PA0438	0.00654477	-2.24919	Cytosine permease
PA1879		0.00228963	-2.23184	Hypothetical Protein
PA5030		0.0111328	-2.22269	Probable major facilitator superfamily (MFS) transporter
PA2079		0.0470643	-2.17386	Probable amino acid permease
PA2776	*pauB3*	0.0113468	-2.16542	FAD-dependent oxidoreductase
PA3741		0.0367162	-2.165	Hypothetical Protein
PA0915		0.0286534	-2.15242	Conserved hypothetical protein
PA1782		0.0299015	-2.13578	Probable serine/threonine-protein kinase
fhp	PA2664	0.000109673	-2.11967	Flavohemoprotein
PA1406		0.0201889	-2.11292	Hypothetical Protein
PA2059		0.0246886	-2.11265	Probable permease of ABC transporter
PA3470		0.0060907	-2.10941	Hypothetical Protein
fis	PA4853	0.0243636	-2.10353	DNA-binding protein
PA4705	*phuW*	0.0220452	-2.07525	Hypothetical. Prokaryotic membrane lipoprotein lipid attachment site profile

Table 4.2: continued.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
PA2408		0.025421	-2.0521	Probable ATP-binding component of ABC transporter
PA0801		0.024755	-2.05067	Hypothetical Protein
pvdN	PA2394	0.0296056	-2.03933	Pyoverdine biosynthetic process
nth	PA3495	0.0304856	-2.03489	Endonuclease III/Base excision repair
PA2282		0.0085652	-2.03165	Hypothetical Protein
PA2691		0.00573376	-2.02985	Conserved hypothetical protein
PA1791		0.0483705	-2.0283	Conserved hypothetical protein
PA2695		0.0244022	-2.02829	Conserved hypothetical protein
potC	PA3609	0.00370151	-2.02709	Polyamine transport protein
PA3019		0.0284377	-2.02365	Probable ATP-binding component of ABC transporter
PA0858		0.0249157	-2.00673	Conserved hypothetical protein
PA3659		0.0287138	-2.00648	Probable aminotransferase

Note: Gene functions and predicted gene functions were obtained from Pseudomonas Genome Database at www.pseudomonas.com (Winsor *et al.*,2015). Grey highlighted box in the "locus tag/gene symbol" indicates that the respective gene only possess the locus tag but does not possess gene symbol due to uncharacterised gene function, hypothetical proteins, or are genes with probable gene functions.

Table 4.3: Significantly up-regulated genes of *P. aeruginosa* PAO1 when co-cultured in 20 µM baicalein.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
PA5480		0.01141	6.87714	Hypothetical Protein
PA2499		0.0481	5.91321	Probable deaminase
PA3287		0.02531	4.47307	Conserved Hypothetical Protein
exaB	PA1983	0.01751	4.39669	Cytochrome C550
PA3338		0.03508	4.39318	Hypothetical Protein
PA2051		0.01088	4.30434	Probable transmembrane sensor
narG	PA3875	0.01206	4.12891	Respiratory nitrate reductase alpha chain
narK2	PA3876	0.01131	4.12166	Nitrite extrusion protein 2
PA2031		0.00153	4.01106	Hypothetical Protein
PA1676		0.04008	3.9951	Hypothetical Protein
narK1	PA3877	0.00134	3.85046	Nitrite extrusion protein 1
PA0910		0.00573	3.75669	Hypothetical Protein
PA1844	*tse1*	0.04624	3.65317	Amidase activity
moaB1	PA3915	0.00393	3.43359	Molybdoprotein biosynthetic protein B1
capB	PA3266	0.0385	3.38679	Cold acclimation protein B
flgM	PA3351	0.01349	3.34407	Flagellar assembly
PA2805		0.04396	3.16387	Hypothetical Protein
PA1076		0.00486	3.09252	Hypothetical Protein
PA3376		0.01366	3.07609	Probable ATP-binding component of ABC transporter
PA3530	*bfd*	0.01598	3.0488	Bacterioferritin-associated ferredoxin
rplW	PA4261	0.04394	2.92838	50S ribosomal protein
PA1006		0.02035	2.92478	Protein binding
PA5303		0.01222	2.87217	Conserved Hypothetical Protein
ssrS	PA5227.1	0.01603	2.76713	6S RNA

Table 4.3: continued.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
PA1977		0.00423	2.76512	Hypothetical Protein
PA3309		0.02004	2.71352	Conserved Hypothetical Protein
rpsH	PA4249	0.013	2.70533	30S ribosomal protein S8
PA0589		0.02111	2.69316	Conserved Hypothetical Protein
nirC	PA0517	0.0441	2.6727	Probable c-type cytochrome precursor
hcnA	PA2193	0.04226	2.66656	Hydrogen cyanide synthase
PA1159		0.00486	2.66191	Probable cold-shock protein
PA3354	PA3354	0.00372	2.58174	Hypothetical Protein
PA1747		0.00066	2.55953	Hypothetical Protein
acpP	PA2966	0.00041	2.54065	Acyl carrier protein
PA4328		0.01536	2.44477	Hypothetical Protein
rbsA	PA1947	0.04675	2.43599	Ribose transport protein
hupB	PA1804	0.00676	2.42643	DNA-binding protein HU
PA2453		0.0152	2.38809	Hypothetical Protein
rpmC	PA4255	0.02957	2.37332	50S ribosomal proteinn L29
PA4346		0.02516	2.37087	Hypothetical Protein
rpmE	PA5049	0.03608	2.3683	50S ribosomal protein L31
PA2116		0.04154	2.30518	Conserved Hypothetical Protein
PA0805		0.02432	2.26541	Hypothetical Protein
PA1825		0.03277	2.26325	Hypothetical Protein
ccpR	PA4587	0.00894	2.26256	Cytochrome C551 Peroxidase Precursor
PA0623		0.04984	2.25866	Probable bacteriophage protein
PA1059	*shaF*	0.0455	2.25298	Monovalent inorganic cation transport
proC	`PA0393	0.02329	2.22078	Pyrroline-5-carboxylate reductase
PA5428		0.00681	2.19253	Probable transcriptional regulator

Table 4.3: continued.

Gene Symbol	Locus Tag/Gene Symbol	*p*-value	Fold Change	Gene Function
gcdH	PA0447	0.01598	2.19207	Glutaryl-CoA-dehydrogenase
PA3575		0.02558	2.19099	Hypothetical Protein
PA4502		0.01238	2.17928	Probable binding protein component of ABC transporter
glpM	PA3585	0.00793	2.16931	Membrane protein
PA4349		0.01813	2.15995	Hypothetical Protein
pstS	PA5369	0.03732	2.14368	Phosphate ABC transporter, periplasmic phosphate-binding protein
PA4350	*olsB*	0.00409	2.13883	Membrane lipid biosynthesis process
sigX	PA1776	0.00595	2.13667	ECF sigma factor
PA1168		0.04411	2.13591	Hypothetical Protein
PA2318		0.04284	2.12322	Hypothetical Protein
PA3389		0.01574	2.12154	Probable ring-cleaving dioxygenase
PA3940		0.04581	2.11566	Probable DNA binding protein
PA3845		0.04894	2.10424	Probable transcriptional regulator
PA1429		0.02111	2.0957	Probable cation-transporting P-type ATPase
PA2769		0.00997	2.08329	Hypothetical Protein
PA0321		0.03173	2.01943	Probable acetylpolyamine aminohydrolase
PA0131	*bauB*	0.03378	2.01365	Beta-alanine biosynthesis process
PA1328		0.00533	2.01263	Probable transcriptional regulator
PA3623		0.00447	2.00998	Conserved Hypothetical Protein
PA3336		0.04247	2.00623	Probable major facilitator superfamily (MFS) transporter
PA3847		0.047	2.0015	Conserved Hypothetical Protein

Note: Gene functions and predicted gene functions were obtained from Pseudomonas Genome Database at www.pseudomonas.com (Winsor *et al.*,2015). Grey highlighted box in the "locus tag/gene symbol" indicates that the respective gene only possess the locus tag but does not possess gene symbol due to uncharacterised gene function, hypothetical proteins, or are genes with probable gene functions.

4.11 Gene Validation using RT-qPCR

Several genes from the differentially expressed genes of baicalein treated *P. aeruginosa* PAO1 were selected for validation purposes. Up-regulated genes; (*narK1, narK2,* narG) and down-regulated genes; (*pvdA, tonB1, fecI,* and *iscR*) were selected for qPCR analysis. Figure 4.24 shows that the expression levels of the selected genes correlated with the expression values obtained in RNA-sequencing. Although the expression of the selected genes was concurrent with the findings of RNA-sequencing, the expression values differed slightly.

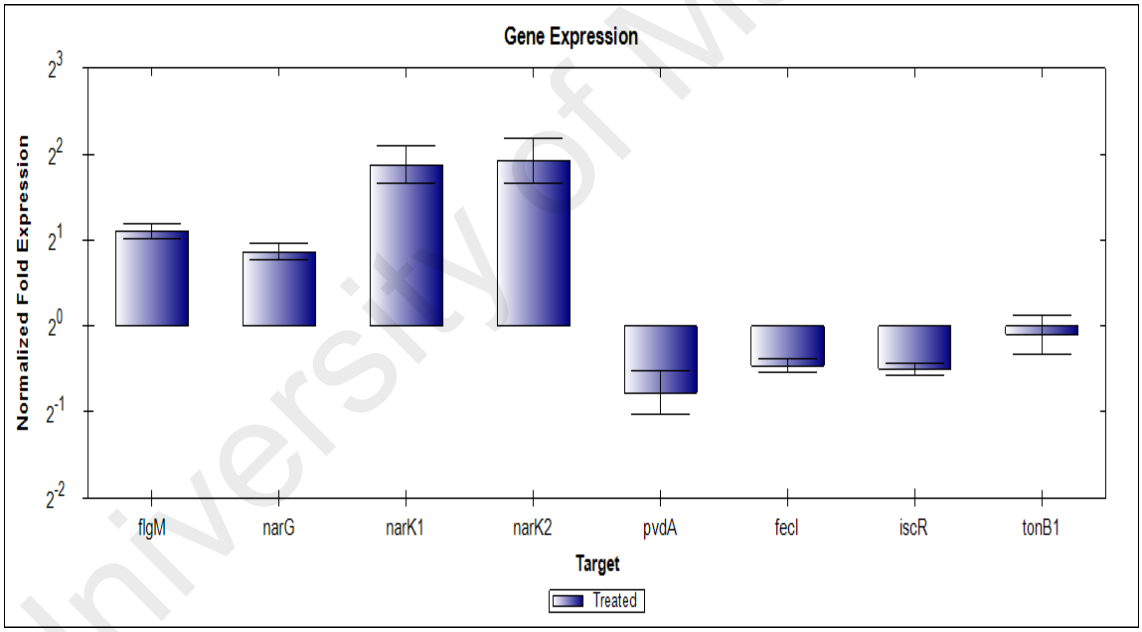


Figure 4.24: Validation of selected differentially expressed genes using qPCR. Expressions of selected genes were in accordance to the findings in RNA-sequencing.

CHAPTER 5: DISCUSSION

5.1 Identification of *Phyllanthus amarus* and Processing of Plant Samples

P. amarus plant samples that were collected from the surrounding compound of University of Malaya, Malaysia were identified by the curator of University of Malaya Herbarium (Rimba Ilmu). Voucher specimen of *P. amarus* was deposited in the herbarium with the specimen identification number of KLU 47768.

After the washing step of the plant processing steps, the selected temperature of 45°C allowed for the water content and moisture to dry completely and ensured complete desiccation of the plant materials. The temperature selected allows desiccation of the plant samples without causing denaturation of vital plant phytochemicals.

In this study, the method used in the processing of dried plant samples is known as maceration whereby whole or powered plant materials are soaked in extracting solvent and allowed to stand at room temperature for a designated period of time with agitation until all the soluble plant particulate dissolves in the solvent. The first step to this method is the reduction in the size of the plant material, preferably into powder form. In this study, the dried plant samples were ground to powder form using an industrial grade blender. The purpose of doing so was to rupture the plant cells and tissues to maximise the exposure of the plant phytochemicals or active materials into the extraction solvent. In addition, smaller size of plant particulates maximises the surface area exposed to the solvent. This enhances mass transfer of the active materials into the extraction solvent. This step was followed by soaking of the powdered plant into

extraction solvent and lastly, filtration to remove solid plant material (Kostova *et al.*,2010; Sasidharan, Chen, Saravanan, Sundram, & Yoga, 2011). The filtrate or the solvent containing the soluble plant phytochemicals were evaporated to dryness in a rotary evaporator. This step was to remove all the solvents, leaving only the crude extract. Collected crude extract was further dried in the fume hood to ensure complete evaporation of the extraction solvent. This step was conducted to reduce the effects of any residual solvents in the bioassays that might produce false positive results.

Three solvents with increasing polarity were used in this study, namely, hexane, chloroform, and methanol. These solvents were used to extract phytochemicals from collected plant samples (Table 4.1). This is known as the serial exhaustive extraction whereby extraction solvents used ranges from non-polar to more polar to ensure a wide range of compound with varying polarity is extracted (Tiwari, Kumar, Mandeep, Kaur, & Kaur, 2011). The most polar solvent used were methanol, followed by chloroform with intermediate polarity, and hexane which was the least polar. Hexane extracts volatile oils; chloroform extracts lipophilic compounds such as terpenoids and flavonoids; whereas methanol extracts more hydrophilic compounds anthocyanins, terpenoids, saponins, tannins, totarol, flavones, and phenones (Sasidharan *et al.*,2011; Tiwari *et al.*,2011). Hexane is also used in some cases to remove plant chlorophyll (Cos, Vlietinck, Berghe, & Maes, 2006).

In preparation of the plant crude extract stock solutions, the desired weight of the extracts were measured. DMSO was chosen as it possesses the ability to completely dissolve a wide range of compounds present in the crude extracts. Among the advantages of using 100% DMSO in the preparation of stock solutions are: (a) reducing

the chances of microbial contamination, therefore, eliminating the need for methods sterilisation of plant samples which may affect the integrity of the phytochemicals, (b) assuring good solubility of crude extracts in dilution steps (Cos *et al.*,2006). Since DMSO interferes with the normal physiology of bacterial cells, it is imperative to include DMSO as a solvent control or negative control in the bioassays conducted. Furthermore, final concentration of DMSO in bioassays and tests should not exceed 1% (Cos *et al.*,2006).

5.2 Anti-QS activity of Methanolic extract of *P. amarus*

Malaysia is well known for its biodiversity and abundance in medicinal plants which are still broadly used by various ethnics for treating diseases and illnesses. These medicinal plants have yet to be tested for their anti-QS properties and may possess remarkable novel anti-pathogenic compounds. The plant samples collected in this study were obtained locally. The collected plants (Table 4.1) were chosen as there were no prior publications or reports on their anti-QS activities. In local terms, *P. amarus* is commonly known as “dukung anak”. Among the Malay ethnic, the decoction of the plant roots are often drank as a tonic after childbirth (Ong & Norzalina, 1999). Meanwhile, in the “orang asli” communities, decoction of the whole plant is traditionally used to treat jaundice (Samuel *et al.*,2010). *P. amarus* is also traditionally used in the treatment of heart diseases and diabetes (Sekar *et al.*,2014).

The methanolic extract of *P. amarus* showed significant anti-QS activity as well as reducing the phenotypic expression of *P. aeruginosa* PAO1. To the best of my knowledge, this is the first report of anti-QS activity of *P. amarus*. Preliminary

screening using *C. violaceum* CVO26 showed significant violacein inhibition at concentration as low as 2 mg/mL. A wild-type *C. violaceum* produces the purple pigment, violacein, which is a QS-regulated trait of the bacterium (Stauff & Bassler, 2011). On the other hand, *C. violaceum* CVO26 is a mutant strain with a transposon insertion in its AHL synthase, *cviI* that causes the mutant to be incapable of producing C6-HSL. However, the synthesis of violacein can be restored with supply of synthetic C6-HSL exogenously (McClean *et al.*,1997). In the *C. violaceum* plate assay, halo zone formation by the methanolic crude extract indicated that the compounds present in the extract may have acted as an antagonist against the cytoplasmic receptor, CviR, thus inhibiting the binding of C6-HSL to its cognate receptor (Swem *et al.*,2010). Furthermore, the active compound in the extract may have also induced the enzymatic degradation of the AHL or promoted the transport of C6-HSL out from the bacterial cells (Kalia, 2013). The halo zone formed was turbid and cloudy in appearance indicating that the inhibition zone formed was due to QS inhibition and was not caused by inhibition in the growth of *C. violaceum* CVO26. This was further supported by *C. violaceum* CVO26 growth study whereby by the 24 h growth was not affected by the concentrations of tested methanolic extract.

Two *lux*-based biosensors used in this study, *E. coli* [pSB401] and *E. coli* [pSB1075], responds by producing bioluminescence in the presence of short chain and long chain AHLs, respectively (Winson *et al.*,1998). Methanolic extract of *P. amarus* significantly reduced luminescence increasing concentration. This indicated that the active metabolite present in the crude extract were capable of intervening with the QS activity of both short and long chain AHLs, suggesting a broad range of anti-QS activity of the methanolic crude extract.

In order to survive the host immune responses and maintaining its virulence, *P. aeruginosa* produces a myriad of cytotoxic exoproducts. *P. aeruginosa* produces soluble cytotoxic carbohydrate-binding proteins or lectins known as LecA and LecB. LecA galactophilic lectin functions as adhesins that binds to the hydrophobic galactosides of host cells with high affinity and specificity. LecA was also found expressed in biofilm cells suggesting its role in biofilm formation and maturation (Diggle *et al.*,2006). The virulence of this protein is further enhanced by its cytotoxic effects towards lung epithelial cells causing detrimental damages to lung tissues, thus contributing to the persistent infection of *P. aeruginosa* (Bajolet-Laudinat *et al.*,1994). To study the expression of *lecA*, the *luxCDABE* gene region from *Photorhabdus luminescence* was cloned into the functional *lecA* gene region of *P. aeruginosa* PAO1 (Winzer *et al.*,2000). In this study, the methanolic extract of *P. amarus* was able to significantly reduce *lecA* expression at 3 mg/mL. Works of Winzer and colleagues revealed that the expression of *lecA* is tightly regulated by RhlR/I-C4-HSL QS system of *P. aeruginosa*. Furthermore, *P. aeruginosa* PAO1 mutant of *rpoS* sigma factor resulted in abolished LecA synthesis in *P. aeruginosa.* The findings of the study demonstrated that both the RhlR/I-C4-HSL QS system and RpoS sigma factor are required for lectin synthesis (Winzer *et al.*,2000). Thus, it is hypothesized that the inhibition of *lecA* by the methanolic crude extract may have been caused by its effects on the RhlR/I-C4-HSL QS system or on the expression of RpoS sigma factor.

Swarming motility of *P. aeruginosa* is divided into three general stages; (1) bacterial cells differentiate into swarmer cells with the characteristics of elongated and hyperflagellated cells; (2) migration of the swarmer cell colonies and lastly; (3) consolidation (end of cell migration) (Fraser & Hughes, 1999). Swarming motility is a bacterial cell surface translocation which requires flagella and pili as well as a QS-

regulated phenotype of *P. aeruginosa* (Daniels, Vanderleyden, & Michiels, 2004). Swarming often occurs on semi-solid mediums and moist surfaces. This cell translocation motion enables bacterial cells to migrate and colonise surrounding environments and niches (Sharma & Anand, 2002). In this study, swarming inhibition was observable at 1 mg/mL and increases with higher concentration of the methanolic crude extract. Swarming of *P. aeruginosa* was found be tightly regulated by the QS system. *P. aeruginosa* that were *lasI/lasR* mutant reduced and delayed swarming progression. In the same study, *P. aeruginosa rhlI/rhlR* mutants completely abolished swarming ability of *P. aeruginosa* (Kohler, Curty, Barja, Van Delden, & Pechere, 2000). Furthermore, rhamnolipid, a lipopeptide biosurfactant, is an important component for optimal swarming motility of *P. aeruginosa*. A study conducted showed that *P. aeruginosa* that harbours mutant *rhlB* and *rhlC*, genes that encodes for monorhamnolipid and dirhamnolipid production, respectively, resulted in abnormally shaped tendrils and altered swarming patterns (Shanks, 2016). In a separate study, *P. aeruginosa rhlI* mutant strains were unable to synthesize rhamnolipids and exhibited reduced rhamnosyltransferase activity. Rhamnolipid production in the mutant strains were restored when cell-free spent supernatant of the wild-type strains or synthetic AHLs were added into the culture condition (Ochsner & Reiser, 1995). These findings indicate that rhamnolipid production by *P. aeruginosa* is also tightly regulated by QS (Brint & Ohman, 1995). Therefore, the bioactive compounds present in the methanolic crude extract may have interfered with the QS systems of *P. aeruginosa* or interfered in the synthesis of rhamnolipids but more work is required to confirm this hypothesis.

Pyocyanin, a blue redox reactive toxic phenazine produced by *P. aeruginosa* PAO1 was extracted and quantified after co-cultured with methanolic extract of *P. amarus* for 18 h. The results obtained showed that the crude extracts significantly reduced

pyocyanin produced at the lowest tested concentration (1 mg/mL). *P. aeruginosa* is the predominant pathogen often found to colonize the airways of cystic fibrosis (CF) patients. Pyocyanin produced contributes to the persistent infection of *P. aeruginosa* by mediating damages to the lung epithelial cells and causing lung tissue necrosis (G. W. Lau, Ran, Kong, Hassett, & Mavrodi, 2004). Furthermore, pyocyanin also contributes to persistent infection by inducing human neutrophil apoptosis, thus ingeniously evading host immune response (Usher *et al.*,2002). Mutations in the QS systems; *lasR/lasI*, *rhlR/rhlI*, and *mvfR-haq*, resulted in the loss of pyocyanin production. These findings revealed that pyocyanin synthesis is mediated by the complex synchrony of all three QS systems (Brint & Ohman, 1995; H. Cao *et al.*,2001; Gallagher, McKnight, Kuznetsova, Pesci, & Manoil, 2002). The plant metabolites may have intervened with the mentioned QS systems or acted as an antagonist of *P. aeruginosa* QS systems. The QS inhibition activities exhibited by the methanolic extract of *P. amarus* were very much comparable to other plant extracts that were studied for its anti-QS properties against various other QS-regulated pathogens. Some reports of QS inhibition by plant crude extracts shows that the inhibition activity only occurs in QS biosensors but no significant inhibition was observed when QS regulated pathogen was used. A narrow action antagonistic effect may have limitations for its clinical value (Koh *et al.*,2013). The results demonstrated by *P. amarus* shows otherwise. The extract was capable to interfere with QS regulation by both the biosensors and QS regulated *P. aeruginosa* PAO1.

The significant results obtained from the methanolic extract of *P. amarus* prompted the search for the compound responsible in the QS inhibition observed in *P. aeruginosa* PAO1. However, the fractions obtained from the fractionation process of the crude extract did not produce reliable and replicable results in the QS bioassays conducted. It was hypothesized that the compound(s) was not stable once fractionated out from the

crude extract. Furthermore, the compounds may be extremely temperature sensitive and this factor may have caused the degradation of the compounds during the fractionation step as well as the temperature of which the fractions were stored. Therefore, baicalein was selected to assess the QS inhibition in *P. aeruginosa* PAO1 by a pure compound. The significant QS inhibitory effects produced by baicalein prompted a more in depth gene expression study by using the transcriptomics approach.

5.3 Baicalein as QS antagonists

Plant-derived compounds which are able to intervene with QS activities of pathogens at minimal concentrations with low toxicity towards the bacterial cells makes an enticing therapeutic agent (Sadlon & Lamson, 2010). Baicalein possesses these imperative criteria against *P. aeruginosa* PAO1.

In this study, baicalein exhibited excellent anti-QS activity and successfully attenuated several virulence factors of *P. aeruginosa* PAO1 which were; pyocyanin production, swarming, and biofilm formation. These virulence factors were significantly reduced by baicalein at concentrations as low as 20 µM. Baicalein also reduced bioluminescence activity in both *E. coli* biosensors. The fore mentioned QS inhibition exhibited by baicalein was achieved at low concentration without affecting the growth and survival of *P. aeruginosa* PAO1. Baicalein as a single compound may intervene the QS of *P. aeruginosa* PAO1. Results obtained in this study show the promising potential of baicalein as an anti-pathogenic compound.

A study conducted by Zhirui and colleagues showed that baicalein interfered with *P. aeruginosa* biofilm attachment and maturation on a surface of a glass slide at a concentration of 200 µM (Zeng *et al.*,2008). The results obtained in this work differed from those observed in the fore mentioned research. In this study, biofilm produced by *P. aeruginosa* PAO1 was significantly reduced at a lower concentration of 20 µM after 24 hours of static incubation. In another study, baicalein attenuated QS-controlled virulence factors of *P. aeruginosa* at concentrations ranging from 32 µg/mL – 128 µg/mL (Luo *et al.*,2016). In terms of molarity, the concentrations correspond to 118.41 µM – 473.65 µM. However, in this study, the results obtained highlights that significant inhibition by baicalein on the QS-controlled virulence factors of *P. aeruginosa* PAO1 was achieved at a much lower concentrations of 20 µM. The work of Luo *et al.* (2006), solvent used is DMSO, which may have effect on the production of virulence factors and the expression levels of QS genes (*lasI, lasR, rhlI,* and *rhlR*). The researches in the study mentioned, failed to incorporate a solvent as control that corresponded to the concentration of baicalein used in the study. At high concentrations of 118.41 µM – 473.65 µM, the residual solvent may have an effect on the global gene expression of *P. aeruginosa* PAO1. The study also showed that baicalein suppressed the expression of several QS genes of *P. aeruginosa* PAO1 (*lasI, lasR, rhlI,* and *rhlR*). In comparison to the transcriptome of baicalein-treated *P. aeruginosa* PAO1 conducted in this study, the following genes (*lasI, lasR, rhlI,* and *rhlR*) were not found to be up or down regulated upon exposure to baicalein. Rather, the expression levels of great number QS-regulated genes were found to be affected. Transcriptomics analysis shows the genes expression of all the genes in the genome. The genes found to be suppressed in Luo *et al*., (2006) did not fall into p-value < 0.05, thus considered not significant. The findings indicated that baicalein was able to effect the global gene expression of *P. aeruginosa* PAO1, by

not only affecting the QS genes. Therefore, baicalein has the ability to target QS-regulated genes that are found scattered thorough out *P. aeruginosa* PAO1 genome.

5.4 Transcriptome of baicalein treated *P. aeruginosa* PAO1

The most down regulated gene this transcriptomics study is the *xphA* gene (fold change: -9.44487). The XphA/XqhA protein subunit is a novel protein subunit of the *P. aeruginosa* type II secretion system (T2SS) (Michel, Durand, & Filloux, 2007). The T2SS is divided into the Xcp system and the Hxc system. The XphA/XqhA protein plays an important role in the secretion of multiple hydrolytic enzymes such as exotoxin A, and elastase (LasB) (Filloux, 2004). Genes involved in the Xcp system, known as the *xcp* genes, are positively regulated by QS (Chapon-Hervé *et al.*,1997; Wagner, Bushnell, Passador, Brooks, & Iglewski, 2003). *P. aeruginosa xcpP* mutant strain which carries a deletion in the *xphA* gene abolished the remaining protein secretion of the mutant strain. Findings of study indicated that XphA protein subunit encoded by the *xphA* gene plays a significant role in the functionality of T2SS (Michel *et al.*,2007). The study also showed that *xphA/xqhA* genes were transcribed and expressed at early stages of bacterial growth. The authors hypothesized that transcription of these genes during an early growth phase is necessary to enable *P. aeruginosa* to secrete hydrolytic enzymes such as exotoxin A, and elastase, to establish an infection in a host. The suppression of *xphA* gene by baicalein is a significant finding as it possesses the potential to intervene with the secretion of virulence factors at early growth phase as required by *P. aeruginosa* to establish an infection and exert its pathogenicity.

Following the expression of *xphA* genes, five ribosomal RNA genes (PA0668.5, PA4280.1, PA4690.1, and PA5369.1) were found to be down regulated in *P.*

aeruginosa PAO1. This could be resulted by incomplete rRNA depletion that was performed prior to cDNA library synthesis. Extra caution would be required in future RNA sequencing studies to ensure complete elimination of these ribosomal RNAs.

The transcriptome analysis also showed that baicalein suppressed the expression of two different gene clusters that are responsible for iron acquisition, transport, and regulation in *P. aeruginosa*. Baicalein down regulated 4 *pvd* genes that are required during the synthesis of pyoverdine. *P. aeruginosa* produces fluorescent yellow-green siderophores known as pyoverdine to facilitate in the acquisition of free iron from the environment especially during iron limiting conditions such as that in a host (Elliott, 1958). Similar to other bacteria, iron is necessary for optimal functioning and growth of bacterial cells. Pyoverdine is a strong iron (III), (Fe^{3+}) chelating siderophore ingeniously produced by *P. aeruginosa* as a virulence mechanism in the establishment of infections in a host. Free irons are not freely available in a host as they possess a strong affinity to other host proteins such as metalloproteins and haemoproteins (Visca, Imperi, & Lamont, 2007). Due to the strong Fe^{3+} affinity of pyoverdine, it is able to displace iron that were originally bound to human iron-binding protein, transferrin (Meyer, Neely, Stintzi, Georges, & Holder, 1996).

Furthermore, a cytoplasmic membrane protein known as TonB is required in the uptake of iron complexes of Fe^{3+}/pyoverdine. In this study, the expression of *tonB1* gene was found to be significantly down regulated (fold change: -2.81818). Therefore, baicalein not only sequestered the expression of *pvd* genes that is required for iron scavenging but also impaired the system required for the reuptake of iron complexes into the bacterial cells. In addition, pyoverdine-mediated uptake of iron is required in

biofilm formation. Studies have shown that *P. aeruginosa* mutants that were incapable of producing the iron scavenging pyoverdine affected biofilm formation (Patriquin *et al.*,2008). In another study, only thin layers of biofilm were formed by *P. aeruginosa* mutants of impaired pyoverdine iron acquisition system, although the strain was grown in an iron-rich medium. Similar biofilm characteristics were observed in parent *P. aeruginosa* grown in iron-limiting conditions (Banin, Vasil, & Greenberg, 2005). The ability of baicalein to disrupt biofilm formation and reduce the expression of genes required for iron acquisition are significant findings as these features are amongst the causes of persistent infection by *P. aeruginosa* in cystic fibrosis patients (Hoiby, Ciofu, & Bjarnsholt, 2010; Moreau-Marquis, Stanton, & O'Toole, 2008).

The presence of multiple iron uptake systems in *P. aeruginosa* signifies the importance iron acquisition and its regulation in the bacterium. Iron acquisition genes enable *P. aeruginosa* to easily acquire a wide range of iron chelates from the environments (Visca, Leoni, Wilson, & Lamont, 2002). Transcriptome analysis also revealed that baicalein repressed the expression of several other iron acquisition and regulation genes of *P. aeruginosa.* Genes that regulates the iron-chelate (ferric (III) citrate) reuptake and transport across the phospholipid bilayer of *P. aeruginosa*, *fecI* (fold change: -2.95847), *fecR* (fold change: -3.14761), and *fecA* (fold change: -2.67629), were down regulated. The transport of ferric citrate into *P. aeruginosa* cells occurs through a process known as "surface signalling". The process starts with the interaction of ferric citrate with the outer membrane receptor protein, FecA (encoded by *fecA* gene). The resulting signal produced from this interaction then transmits from the periplasmic portion of FecA towards FecR (encoded by *fecR* gene) which spans in the cytoplasmic portion of the cell membrane. The signalling continues towards the extracytoplasmic function (ECF) sigma factor, FecI (encoded by *fecI*). FecI sigma factor

then induces and regulates the expression of *fecABCDE* operon which is responsible for ferric citrate transport (Visca *et al.*,2002).

Furthermore, baicalein also down-regulated the expression of genes involved in the iron-sulphur cluster biogenesis system, *iscR* (fold change: -3.79086), *iscU* (fold change: -3.78096), and *iscS* (fold change: -3.82705). Gene *iscR*, encodes for a transcriptional regulator, which contributes to the resistance towards oxidative stress and intracellular iron homeostasis (Romsang *et al.*,2014). This gene also contributes to peroxide resistance as well as virulence in *P. aeruginosa* PA14 (Kim, Lee, Lau, & Cho, 2009).

The outcome of down-regulated genes in *P. aerugnosa* PAO1 shows that baicalein possesses the competency in attenuating virulence and pathogenicity of the bacteria by affecting several iron acquisition, transport, and homeostasis genes, which are required for optimal physiological functions. The findings obtained from transcriptome correlated with the results of the virulence assays conducted. The diverse pathogenicity target of baicalein makes it an attractive anti-pathogenic drug.

Interestingly, transcriptome analysis showed that baicalein up regulated the expression of *narG* (fold change: 4.12891), *narK1* (fold change: 3.85046), and *narK2* (fold change: 4.12166). These genes play an important role in the nitrate/nitrite transport in *P. aeruginosa* in denitrification process (Sharma, Noriega, & Rowe, 2006). Denitrification is a process that reduces nitrate to dinitrogen (Zumft, 1997). Nitrate is transported into the bacterial cells by transport proteins where denitrification process occurs. In the cytoplasm, nitrate is reduced to nitrite. It has been demonstrated that in *P.*

aeruginosa, the resulting nitrites that are produced are immediately excreted to the external environments (Dias, Ventullo, & Rowe, 1990; Hernandez & Rowe, 1987). Nitrites are toxic to the bacterial cells as these anions possess the ability to bind to heme groups and ultimately obstructing the flow of electrons (Rowe, Yarbrough, Rake, & Eagon, 1979).Gene *narK* plays a role in nitrite excretion (Sharma *et al.*,2006). It is hypothesized that *P. aeruginosa* up regulated the expression of these genes in presence of baicalein as a protective measure to ensure continuous normal physiological functions of the bacteria. Nevertheless, the genes that were found to be up and down regulated when baicalein was co-cultured with *P. aeruginosa* could be caused by an indirect effect of the compound.

The gene expressions of selected genes that were validated by means of conducting qPCR were in agreement to the findings of RNA-seq. However, the expression values obtained in qPCR differed slightly to those in RNA-seq. This could be caused by usage of gene specific primers in qPCR compared to RNA-seq, which uses universal primers during the library amplification step. Furthermore, samples used in qPCR were from an independent experiment of baicalein-treated *P. aeruginosa* PAO1, which may explain the variation in the gene expression values (Chan *et al.*,2016).

5.5 Future Work

In the initial part of this study, the methanolic extract of *P. amarus* attenuated several QS-regulated virulence factors of *P. aeruginosa* PAO1. Bio-assay guided fractionation of this extract is being carried out to elucidate the active fractions or compounds responsible for the anti-QS activity observed. Furthermore, more in depth studies on the mechanism of baicalein as a QS antagonist would be carried out such as possible binding towards the AHL signal synthase or receptor via methods such as surface plasmon resonance (SPR) and *in silico* analysis. Baicalein could also be tested on other QS-regulated Gram negative and Gram positive bacteria.

CHAPTER 6: CONCLUSION

The rates of which multidrug resistant bacteria or superbug are emerging pose a threat towards human health on a global scale. The continuation of the alarming crisis could lead the human race towards a post-antibiotic era where simple infections could lead to mortality (Alanis, 2005). To address this crises, numerous studies conducted on anti-QS activity of natural compounds have shown promising results and a novel strategy known as anti-pathogenic drugs (Chong *et al.*,2011; Kalia, 2013; Koh *et al.*,2013; Tan, Yin, & Chan, 2013; Yap, Krishnan, Chan, & Lim, 2014).

Findings obtained in this investigation indicated the potential antagonists in *P. amarus* and baicalein as an alternative strategy against the opportunistic pathogen, *P. aeruginosa* PAO1. It is of utmost essentiality to further investigate and study the natures of these QS inhibitors and the mechanism by which QS is inhibited as well as the efficacy in a pharmacological perspective. Most importantly, the discovery of more anti-QS compounds assists other researchers in conducting a more focussed and directed studies in the quest of understanding these potent inhibitors of QS.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of Publications:

1. **Priya, K**., Yin, W.F., & Chan, K. G. (2013). Anti-Quorum Sensing Activity of Traditional Chinese Herb, *Phyllanthus amarus. Sensors, 13*(11), 14558-14569.
2. Chan, K. G., **Priya, K**., Chang, C. Y., Ahmad, Y. A. R., Tee, K. K., & Yin, W. F. (2016). Transcriptome analysis of *Pseudomonas aeruginosa* PAO1 grown at both body and elevated temperatures. *PeerJ, 4,* 10.7717/peerj.2223.
3. Chan, K. G., Yin, W. F., Tee, K. K., Chang, C. Y., & **Priya, K.** (2015). *Pandoraea* sp, Strain E26: Discovery of Its Quorum Sensing Properties via Whole-Genome Sequence Analysis. *Genome Announcement. 3*(3), 26-27.
4. Chan, K. G., Sulaiman, J., Yong, D. A., Tee, K. K., Yin, W. F., & **Priya, K.** (2015). Draft Genome Perspective of *Staphylococcus saprophyticus* Strain SU8, an *N*-Acyl Homoserine Lactone Degrading Bacterium. *Genome Announcement. 3*(5), 10.1128/genomeA.01097-15.

Papers Presented:

1. Title: Anti-quorum sensing properties of *Phyllanthus amarus.*

UTAR National Postgraduate Fundamental and Applied Sciences Seminar 2014 (NPFASS 2014). (Poster Presenter)