DISCOVERY OF NATURAL PRODUCTS EXHIBITING ANTI-QUORUM SENSING ACTIVITIES AGAINST
Pseudomonas aeruginosa PAO1

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2018

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
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KUALA LUMPUR

2018

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: CHONG YEE MENG

Matric No: SHC110041

Name of Degree: DOCTOR OF PHILOSOPHY

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

DISCOVERY OF NATURAL PRODUCTS EXHIBITING ANTI-QUORUM

SENSING ACTIVITIES AGAINST *Pseudomonas aeruginosa* PAO1

Field of Study: GENETIC MICROBIOLOGY

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DISCOVERY OF NATURAL PRODUCTS EXHIBITING ANTI-QUORUM SENSING ACTIVITIES AGAINST *Pseudomonas aeruginosa* PAO1

ABSTRACT

Quorum sensing (QS) generally refers to a phenomenon where the bacteria communicate and coordinates their phenotypes based on the population density by secretion and sensing of small and self-generated signaling molecules known as the autoinducers. *Pseudomonas aeruginosa* PAO1 is an opportunistic pathogen that employs the QS system to regulate its virulence factors, among others. This thesis aimed to study the effects of the plant extracts against the *P. aeruginosa* PAO1 QS-regulated virulence factors which includes LecA, pyocyanin, swarming, elastase, staphylolytic activity and biofilm. Out of the 15 plants extracts that were screened for anti-QS activities, *Garcinia mangostana* was chosen for purification using column chromatography with silica gel and catechin was isolated as the bioactive molecule which acts as QS inhibitor. Besides that, a total of 50 purified single compounds of plant origins obtained from Department of Pharmacology, Faculty of Medicine, University of Malaya (collaboration work) were also assessed for their potential antagonistic action on *P. aeruginosa* PAO1 QS system. By using the bioassay guided isolation, malabaricone C which was purified from *Mystrica cinnamomea* with preparative HPLC was found to inhibit the selected virulence determinants of *P. aeruginosa* PAO1 such as pyocyanin, swarming as well as biofilm formation. Both catechin and malabaricone C was shown to target the signal receptor of the *P. aeruginosa* PAO1 QS system. The transcriptomic analysis with RNA-sequencing technology was carried out in order to study the gene expression of *P. aeruginosa* PAO1 treated with catechin and malabaricone C. The sequence analysis revealed that

catechin down-regulated some QS-regulated genes including *pchC*, *pchG*, *pchR*, *pchD*, *pchF*, *pchE*, *pchA*, *fptA*, *ampO*, *ampP*, *yieF*, *pscO*, *eraR*, *lipH*, *nasS* and *napB* while malabaricone C down-regulated genes like *lhpM*, *yieF* and *nasS*. The data obtained in this study validate the idea that explore the higher plants as rich source of anti-QS compounds that can attenuate the bacteria virulence and may be valuable source for novel drug discovery especially with the emergence of multidrug resistant (MDR) bacteria that render the antibiotic treatments less efficient.

Keywords: Quorum sensing (QS), *Pseudomonas aeruginosa* PAO1, natural products, *Garcinia mangostana*, *Mystrica cinnamomea*

PENEMUAN TUMBUHAN SEMULAJADI MEMPAMER ANTI-PENDERIAN QUORUM (QS) AKTIVITI TERHADAP *Pseudomonas aeruginosa* PAO1

ABSTRAK

Penderian quorum (QS) merujuk kepada satu fenomena di mana bakteria boleh berkomunikasi dan menyelaras tingkah laku yang berbeza berdasarkan kepadatan bakteria menghasilkan isyarat molekul, dikenali sebagai auto-perangsang. *Pseudomonas aeruginosa* PAO1 adalah patogen oportunistik yang menggunakan sistem QS untuk mengawal ekspresi faktor kebisaan dan menyebabkan penyakit seperti jangkitan sistem pernafasan, jangkitan saluran kencing, dermatitis serta jangkitan tisu lembut. Kajian ini bertujuan untuk mengkaji kesan-kesan ekstrak tumbuhan terhadap ekspresi faktor kebisaan yang dikawal oleh QS di *P. aeruginosa* PAO1 termasuk bioluminasi, ekspresi cytotoksik lectin, produksi "pyocyanin", "swarming", "elastase", "staphylolytic" dan biofilem. Daripada 15 tumbuhan yang diplih dan ditapis untuk aktiviti anti-QS, *Garcinia mangostana* dipilih untuk difraksionasi dengan menggunakan kromatografi lajur dengan gel silika dan catechin diasingkan sebagai bioaktif molekul yang berfungsi sebagai perencat QS. Di samping itu, sejumlah 50 kompaun tunggal yang diekstrak daripada tumbuhan telah diperoleh dari Jabatan Farmakologi, Fakulti Perubatan, Universiti Malaya (kerja kolaborasi) juga dinilai untuk tindakan antagonistik potensinya terhadap system *P. aeruginosa* PAO1 QS. Dengan menggunakan bioesei berpandu, malabaricone C yang diekstrak dengan HPLC Preparatif didapati untuk menghalang ekspresi faktor kebisaan bagi *P. aeruginosa* PAO1. Kedua-dua catechin dan malabaricone C ditunjukkan untuk mensasarkan penerima isyarat sistem *P. aeruginosa* PAO1 QS. Penemuan yang penting telah membawa kepada kajian analisis transkriptik dengan teknologi penjujukan RNA untuk mengkaji ekspresi gen *P. aeruginosa* PAO1

yang dirawat dengan catechin dan malabaricone C. Analisis bioinformatik menunjukkan bahawa catechin mempengaruhi beberapa gen yang dikawal oleh QS termasuk *pchC*, *pchG*, *pchR*, *pchD*, *pchF*, *pchE*, *pchA*, *fptA*, *ampO*, *ampP*, *yieF*, *pscO*, *eraR*, *li*pH, *nasS* dan *napB* manakala malabaricone C yang mempengaruhi gen seperti *lhpM*, *yieF* dan *nasS*. Data yang diperolehi mengesahkan bahawa tumbuhan semulajadi mempunyai sumber yang kaya dengan sebatian anti-QS yang boleh melemahkan bakteria dan ini boleh menjadi sumber berharga untuk penemuan ubat baru terutama sekali dengan kemunculan bakteria yang tahan rintangan terhadap antibiotik.

Katakunci: Penderian quorum (QS), *Pseudomonas aeruginosa* PAO1, tumbuhan semulajadi, *Garcinia mangostana*, *Mystrica cinnamomea*

ACKNOWLEDGEMENTS

I would like to express my greatest appreciation to Associate Prof. Dr. Chan Kok Gan, for his invaluable guidance and supervision during the course of this project. His unwavering enthusiasm and constructive critiques helps to motivate me to strive harder in order to achieve my goals. I would also like to thank Prof. Dr. Soizic Prado from Muséum National d'Histoire Naturelle, Paris, for her input and assistance with the isolation and purification of bioactive molecule. And not to forget, Prof. Emeritus Dato' Dr. Lam Sai Kit and Prof. Emeritus Dr. Yong Hoi Sen for their invaluable advices throughout my PhD period.

I am truly blessed to have a group of lab mates that are forever helpful and are willing to share their technical expertise with me. I enjoyed the discussions and the time spent together, not to mention sharing the moments of joy and frustration over this period of time. I am also thankful to have Miss Yin Wai Fong as our lab manager and her efficiency allows the lab to operate smoothly, thus, makes me more productive and creative with my work.

I would like to acknowledge the staffs from High Impact Research (HIR) that provide all the necessary support and assistance during this research period. Not to forget, the Senates and staffs from Institute of Biological Sciences especially Puan Hapizah as well as the Faculty of Science and Thesis Unit (Institute of Graduate Studies) that helps to ensure the smooth process related to my thesis submission.

Finally, I would like to express my deepest gratitude to my dearest parents for their unconditional support, care and love especially when I faced with difficulty.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	degree Celsius
µg/mL	:	microgram per milliliter
µL	:	microliter
µm	:	micromolar
%	:	percentage
% (v/v)	:	percentage volume per volume
% (w/v)	:	percentage weight per volume
3-hydroxy-C8-HSL	:	N-(3-hydroxy octanoyl)-L-homoserine lactone
3-hydroxy-C10-HSL	:	N-(3-hydroxy decanoyl)-L-homoserine lactone
3-hydroxy-C12-HSL	:	N-(3-hydroxy dodecanoyl)-L-homoserine lactone
3-hydroxy-C16 HSL	:	N-(3-hydroxy hexadecanoyl)-L-homoserine lactone
3-hydroxy-C14-HSL	:	N-(3-hydroxy tetradecanoyl)-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
7-cis-C14-HSL	:	N-tetradec-7(Z)-enoyl-L-homoserine lactone
ACN	:	acetonitrile
AHLs	:	acyl homoserine lactones
AI-2	:	autoinducer-2
AIPs	:	autoinducer peptides
ATP	:	adenosine triphosphate
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
C14-HSL	:	*N*-(tetradecanoyl)-L-homoserine lactone
C18-HSL	:	*N*-(octadecanoyl)-L-homoserine lactone
$CaCl_2$	:	calcium chloride
Da	:	deka
DKPs	:	diketopiperazines
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucleic acid
DSF	:	diffusible extracellular factor
$dyne/cm^2$	:	dyne per square centimeter
ECR	:	elastin-Congo red
et al.	:	*et alia*
g	:	gram
HCl	:	hydrochloride
HHQ	:	2-heptyl-4-hydroxyquinolone
hrs	:	hours
HSLs	:	homoserine lactones
IQS	:	2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde
L	:	Liter
LB	:	Lysogeny broth
LBA	:	Lysogeny agar
lncRNA	:	long non-coding ribonucleic acid

M	:	molar
MDR	:	multidrug resistant
mg	:	milligram
mg/mL	:	milligram per milliliter
min	:	minutes
mL	:	milliliter
mL/min	:	milliliter per minute
mm	:	millimeter
mM	:	millimolar
MOPS	:	3-[N-morpholino] propanesulfonic acid
mRNA	:	messenger ribonucleic acid
Na_2EDTA	:	disodium salt of ethylenediaminetetraacetic acid
ng/µL	:	nanogram per microliter
NGS	:	next generation sequencing
nm	:	nanometers
OD	:	optical density
PCR	:	polymerase chain reaction
pH	:	potential of hydrogen
PQS	:	*Pseudomonas* quinolone signal
pri-miRNA	:	primary-micro ribonucleic acid
psi	:	pounds per square inch
QQ	:	quorum quenching
QS	:	quorum sensing
QSI	:	quorum sensing inhibitors
RIN	:	RNA Integrity Number
RLU	:	relative light units

RNA	:	ribonucleic acid
rRNA	:	ribosomal ribonucleic acid
rpm	:	revolutions per minute
SNPs	:	single nucleotide polymorphisms
TLC	:	thin layer chromatography
tRNA	:	transfer ribonucleic acid
UV	:	ultraviolet

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1   Study Background

Bacteria are said to be an individual cell that can only proliferate and multiply without being able to interact with one another (Bjarnsholt & Givskov, 2008). However, the discovery of the intercellular communication among the bacteria has led to the understanding that bacteria can basically coordinated their activities that was once believed to be only restricted to the multicellular organisms (Kievit & Iglewski, 2000). The "language" that is being used for the cell-to-cell communication or quorum sensing (QS) involves the release of the autoinducers into the surrounding environment followed by the regulation of certain specific genes in response to the bacteria population density (Miller & Bassler, 2001; Podbielski & Kreikemeyer, 2004; Williams, 2007).

Pseudomonas aeruginosa is an opportunistic pathogen that can cause infections in the respiratory system, urinary tract, soft tissue as well as the bone and joints. The bacteria can be found mainly in the gastrointestinal area and is a significant reservoir especially in the hospital (Sleigh & Timbury, 1994; Rubin *et al.*, 2008). Like any other bacteria, *P. aeruginosa* has also become intrinsically resistant to a wide series of antibiotics due to the presence of several drug efflux system and porins in addition to the formation of the biofilms by the bacteria itself (Rubin *et al.*, 2008). Besides that, *P. aeruginosa* also employed the phenomenon of QS in order to regulate various virulence factors such as motility, bioluminescence, biofilm maturation and production of antibiotics (Miller & Bassler, 2001; Henke & Bassler, 2004; Williams, 2007).

The continuous emergence of the multidrug resistant (MDR) bacteria strains has caused the prokaryotes and eukaryotes to come up with strategies in order to interfere with the bacteria QS system (Dong *et al.,* 2002). By blocking this cell-to-cell signalling mechanism, the pathogenic organisms that use QS to control the virulence could potentially be controlled (Kievit & Iglewski, 2000). Recently, there are a few QS inhibition mechanisms that have been identified and this process is known as quorum quenching (QQ) (Zhang & Dong, 2004). This serves as an advantage as it can help to minimize the possibility of the bacteria from becoming resistant mutants that rapidly (Hentzer & Givskov, 2003).

Natural products play a dominant role for treating and preventing infectious diseases among the humans (Koehn & Carter, 2005). Natural products can originate from different source of materials including the terrestrial plants, terrestrial microorganisms, marine organisms as well as the terrestrial vertebrates and invertebrates (Newman *et al*., 2000). As plants have been living in an environment with a very high bacteria cell density, they have long been shown to have protective mechanisms against the bacterial infections. Previous research has shown that higher plants produce compounds that can act as QS inhibitors (Givskov *et al*., 1996; Kjelleberg *et al*., 1997; Teplitski *et al*., 2000; Rasmussen *et al*., 2005; Chong *et al*., 2011; Koh *et al*., 2013). This study highlights the potential of 15 plant samples as well as purified compounds such as catechin and malabaricone C in inhibiting *P. aeruginosa* PAO1 QS-regulated activities.

1.2   Research Objectives

The objective of this research was to study the quorum quenching (QQ) effects of the natural products, catechin and malabaricone C against the *P. aeruginosa* PAO1 quorum sensing (QS) system. The specific objectives were:

1) To screen for anti-QS activities of 15 crude plant extracts against *P. aeruginosa* PAO1.

2) To isolate and characterize the bioactive molecules found in the leaves of *Garcinia mangostana* that attenuate virulence in *P. aeruginosa* PAO1.

3) To investigate the effects of catechin and malabaricone C on *P. aeruginosa* PAO1 QS system.

4) To determine the mode of action of catechin and malabaricone C against the *P. aeruginosa* PAO1 QS system.

5) To study the effects of catechin and malabaricone C on *P. aeruginosa* PAO1 genes expression through RNA-sequencing.

CHAPTER 2: LITERATURE REVIEW

2.1   Quorum Sensing (QS)

For decades, microbiologists believe that bacterial cells were asocial organisms that do not depend on other members of the population in the synchronization of bacterial gene expression. Bacteria were believed to exist as individual cells that mainly find nutrients and multiply without showing any of their virulence behaviour (Wagner *et al.*, 2006; Bjarnsholt & Givskov, 2008). In addition, bacteria were said to be incapable to interact with one another as well as collectively respond to environmental stimuli (Schauder & Bassler, 2001). However, this view has started to change two decades ago when scientists discovered the cooperative regulation of the luminescence in the Gram-negative marine bacteria *Vibrio fischeri* (Nealson *et al.*, 1970) and regulation of the genetic competence in the Gram-positive bacteria *Streptococcus pneumonia* (Claverys & Håvarstein, 2002). Bacteria were also found to take advantage in coordinating activities as a group such as ability to migrate to a more suitable environment with better nutrient supply as well as to adopt new modes of growth that includes sporulation or biofilm formation (de Kievit & Iglewski, 2000).

Bacteria can coordinate their behaviour through the secretion of specific signalling molecules known as autoinducer in a population density-dependent manner (Winzer *et al.*, 2002). During the growth, bacteria will secrete the signalling molecules and accumulate around the surrounding environment. Once the population density has reach the critical threshold concentration or "quorum size", certain sets of genes will then be regulated and expressed. This signalling network will allow the bacteria to do a quick survey of their surrounding and thus, help the bacteria to develop a strategy to

counter any threats. This type of cell-to-cell communication was termed as quorum sensing (QS) (Yang *et al*., 2006; Williams, 2007). Previous research in *Pseudomonas aeruginosa* has revealed that every QS-regulated gene has its own quorum size and this indicates that different genes are activated at different population densities (Schuster *et al*., 2003; Wagner *et al*., 2003). QS can be divided into two main parts which is the LuxI/LuxR-type of systems in the Gram-negative bacteria and oligopeptide/two component-type of systems in the Gram-positive bacteria (Federle & Bassler, 2003).

The way the bacteria uses the QS system to regulate the genes that encodes virulence determinants have sparked great interest as the interaction between the pathogenic bacteria and the host is highly affected by the bacteria population density (Winzer & Williams, 2001). As the expression of the virulence factors requires energy, the pathogenic bacteria prefer to only attack the host and spread the infection when the population densities have been attained (Pai *et al*., 2012). Both Gram-negative and Gram-positive bacteria make good use of the QS system as it can facilitate the regulation of a variety of virulence phenotypes such as biofilm maturation, spore formation, toxin production, exopolysaccharide production, competence, symbiosis, bioluminescence, plasmid transfer, conjugation, production of antibiotics, virulence factor production as well as motility (Miller & Bassler, 2001; Chevrot *et al*., 2006; Williams, 2007; Dunny *et al*., 2008).

2.1.1   Gram-negative Bacteria QS System

QS in Gram-negative bacteria was first studied in *V. fischeri* where it produces autoinducer that allows the induction of luciferase synthesis once the concentration reaches a critical level (Nealson *et al.*, 1970). *V. fischeri* interacts symbiotically with the Hawaiian squid, *Eupryma scolopes*, and provides the host with light emission that act as a lure for the host's prey. In exchange, the bacteria will live in nutrient rich environment, allowing proliferation in numbers unachievable in seawater (Visick & Ruby, 2006). The QS system in *V. fischeri* serves as a basic model for other QS studies in Gram-negative bacteria where it requires the contribution of two regulatory components, namely the autoinducer synthase (LuxI) and transcriptional activator protein (LuxR). As the accumulation of autoinducers increase to a certain threshold level, it will bind to and activate the R protein that leads to the inducement of gene expression. The R protein consists of two domains (a) N terminus which interacts with the autoinducer and (b) C terminus which is involved in DNA binding (de Kievit & Iglewski, 2000).

LuxI is principally responsible for the synthesis of AHLs in most of the bacteria such as *Rhizobium, Agrobacterium, Pseudomonas, Serratia, Erwinia* as well as the *Burkholderia* where their products share conserved residues that are required for the activity (Costa & Loper, 1997; Lewenza *et al.*, 1999). The LuxI synthase catalyzes specifically on the amide bond formation between the *S*-adenosylmethionine (SAM) and a fatty acyl-acyl carrier protein of a specific chain length. In addition to that, LuxI also catalyzes the formation of the acyl homoserine lactone form the acyl-SAM intermediate (Hanzelka & Greenberg, 1996; More *et al.*, 1996). The specificity of the AHL synthase to a particular chain length varies as it depends on the bacterial strain (Watson *et al.*, 2002). The same bacteria that produce several AHLs usually have

multiple synthases with each synthase being in charged for the synthesis of a limited range of AHLs (Schaefer *et al.*, 1996).

LuxR, on the other hand, is a transcriptional regulator protein that can only be activated upon binding with the autoinducer molecules. In *V. fischeri*, LuxR plays a vital role by binding to the *N*-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) through a signal-binding area in the N-terminal domain (Stevens *et al.*, 1994). The interaction in between the LuxR and 3-oxo-C6-HSL appears to be very specific (Slock *et al.*, 1990) as the C-terminal domain which contains a helix-turn-helix motif will interact with DNA at an exact site known as the "lux box" (Egland & Greenberg, 1999). Besides that, the crystal structure of TraR, a LuxR homolog in the *A. tumefaciens* also demonstrate that the AHL is completely embedded within the protein (Vannini *et al.*, 2002). The binding of the AHL to the TraR dimers can actually help to stabilize it from the degradation by the cellular proteases (Zhu & Winans, 2001).
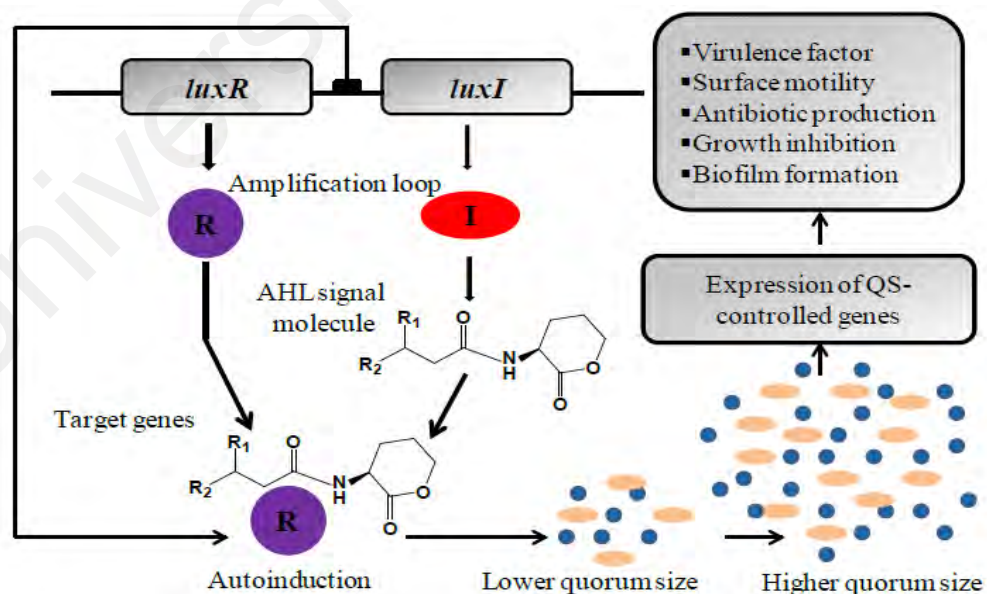
Figure 2.1: Gram-negative LuxI/R QS system. The 'R' protein is the AHL receptor while 'I' protein is AHL signal synthase (Lade *et al.*, 2014).

Table 2.1: Some examples of AHL-dependent QS systems and the phenotypes controlled (Williams, 2007).

Organism	AHLs	Phenotype
Aeromonas hydrophila	C4-HSL, C6-HSL	Biofilms, exoproteases, virulence
Aeromonas salmonicida	C4-HSL, C6-HSL	Exoproteases
Agrobacterium tumefaciens	3-oxo-C8-HSL	Plasmid conjugation
Agrobacterium vitiae	C14 : 1-HSL, 3-oxo-C16 : 1-HSL	Virulence
Acidithiobacillus ferrooxidans	3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, C12-HSL, 3-hydroxy-C12-HSL, C14-HSL, 3-oxo-C14-HSL, 3-hydroxy-C14-HSL, 3-hydroxy-C16 HSL	Not known
Burkholderia cenocepacia	C6-HSL, C8-HSL	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence
Burkholderia pseudomallei	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL	Virulence, exoproteases
Burkholderia mallei	C8-HSL, C10-HSL	Virulence
Chromobacterium violaceum	C6-HSL	Exoenzymes, cyanide, pigment
Erwinia carotovora	3-oxo-C6-HSL	Carbapenem, exoenzymes, virulence
Pantoea (Erwinia) stewartii	3-oxo-C6-HSL	Exopolysaccharide
Pseudomonas aeruginosa	C4-HSL; C6-HSL, 3-oxo-C12-HSL	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, secondary metabolites, 4-quinolone signalling, virulence
Pseudomonas aureofaciens	C6-HSL	Phenazines, protease, colony morphology, aggregation, root colonization
Pseudomonas chlororaphis	C6-HSL	Phenazine-1-carboxamide
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C12-HSL	Biofilm development
Pseudomonas fluorescens	3-oxo-C10-HSL	Mupirocin

Table 2.1, continued.

Organism	AHLs	Phenotype
Pseudomonas syringae	3-oxo-C6-HSL	Exopolysaccharide, swimming motility, virulence
Rhizobium leguminosarum bv. viciae	C14: 1-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL	Root nodulation/ symbiosis, plasmid transfer, growth inhibition, stationary phase adaptation
Rhodobacter sphaeroides	7-cis-C14-HSL	Aggregation
Serratia sp. ATCC 39006	C4-HSL, C6-HSL	Antibiotic, pigment, exoenzymes
Serratia liquefaciens MG1	C4-HSL, C6-HSL	Swarming motility, exoprotease, biofilm development, biosurfactant
Serratia marcescens SS-1	C6-HSL, 3-oxo-C6-HSL, C7-HSL, C8-HSL	Sliding motility, biosurfactant, pigment, nuclease, transposition frequency
Serratia proteamaculans B5a	3-oxo-C6-HSL	Exoenzymes
Vibrio fischeri	3-oxo-C6-HSL	Bioluminescence
Yersinia enterocolitica	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	Swimming and swarming motility
Yersinia pseudotuberculosis	C6-HSL, 3-oxo-C6-HSL, C8-HSL	Motility, aggregation

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2.1.2   Gram-positive Bacteria QS System

Unlike Gram-negative bacteria, Gram-positive bacteria produce and transport oligopeptide into their environment to regulate cell-density dependent activities. The post-translational modified oligopeptide that acts as their signalling molecules are known as autoinducing peptides (AIPs) where it contains 5–20 amino acids and sometimes with unusual side chain modifications (Lazazzera & Grossman, 1998; Williams *et al*., 2007). There are three types of AIPs classes that have been identified which includes the oligopeptide lantibiotics, 16-membered thiolactone peptides and isoprenylated tryptophan peptides (Quandri, 2002; Chan *et al*., 2004; Okada *et al*., 2005). Some examples of the well-studied QS system in Gram-positive bacterial includes ComP/ComA in *Bacillus subtilis* and AgrC/AgrA in *Staphylococcus aureus* (Solomon *et al*., 1995; Ji *et al*., 1997).

As the bacterial cell membrane is not permeable to AIPs, a dedicakted ATP-binding cassette (ABC) transporter is required to facilitate the secretion of AIPs into the extracellular environment. The detection of the AIPs is mediated by two-component sensory-transduction system where the oligopeptides are recognized by the sensor kinases and through phosphorylation, the signal is transduced from the sensor to the response regulator, thus, alters the targeted gene expression (Federle & Bassler, 2003; Reading & Sperandio, 2006).
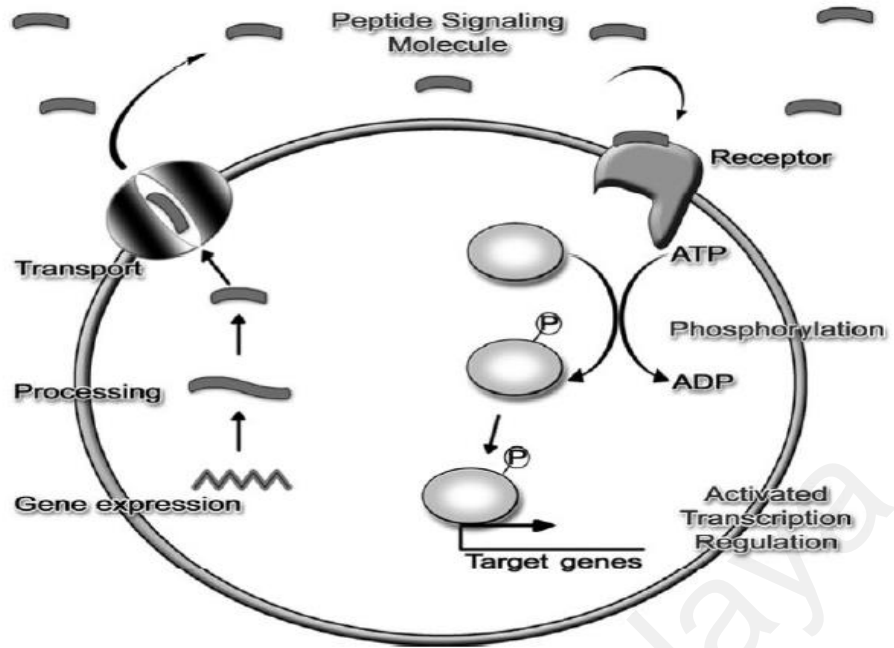
Figure 2.2: Gram-positive QS system (Turovskiy *et al*., 2007).

2.1.3   Interspecies and Interkingdom QS System

While species-specific signalling system allows other bacteria to recognize and communicate within certain species in a mixed population, a mechanism is also required for the bacteria to detect the presence of other species in order to modulate their behaviour based on the ratio of self to other in a mixed population (Federle & Bassler, 2003). Besides LuxI, there are other autoinducer synthases that plays an important role in interspecies QS system such as autoinducer-2 (AI-2) signal molecule in *V. harveyi* where it is being produced by the LuxS synthase (Surette *et al.*, 1999).

The LuxP/Q-type proteins are essential for the AI-2 type of QS system. LuxP has the ability to modulate the activity of the LuxQ sensor kinase by transducing the AI-2 signalling to the cytoplasm. LuxQ, conversely, is a hybrid two-component sensor kinase that has periplasmic sensor domain, cytoplasmic histidine kinase as well as response regulatory domains (Mok *et al.*, 2003; Henke & Bassler, 2004). When the cell densities are low, LuxQ will acts as a kinase and cross phosphorylates the histidine residues within the histidine kinase domainn and these reactions can lead to the phosphorylation of the LuxO. However, the AI-2 bound LuxP will interact with the LuxQ once the cell densities are high and converts it to a phosphatase followed by dephosphorylation of LuxQ (Neiditch *et al.*, 2005).

Interkingdom signalling system, on the other hand, involves the interaction between the autoinducer-3 (AI-3) with the hormone epinephrine/norepinephrine in an agonistic fashion (Sperandio *et al.*, 2002; Reading *et al.*, 2007). This QS system is well studied in *Escherichia coli* species especially in enterohemorrhagic *E. coli* (EHEC)

where it will respond to both the bacteria QS system and mammalian signalling system in order to adjust the transcription of the virulence genes (Sperandio *et al*., 2002).
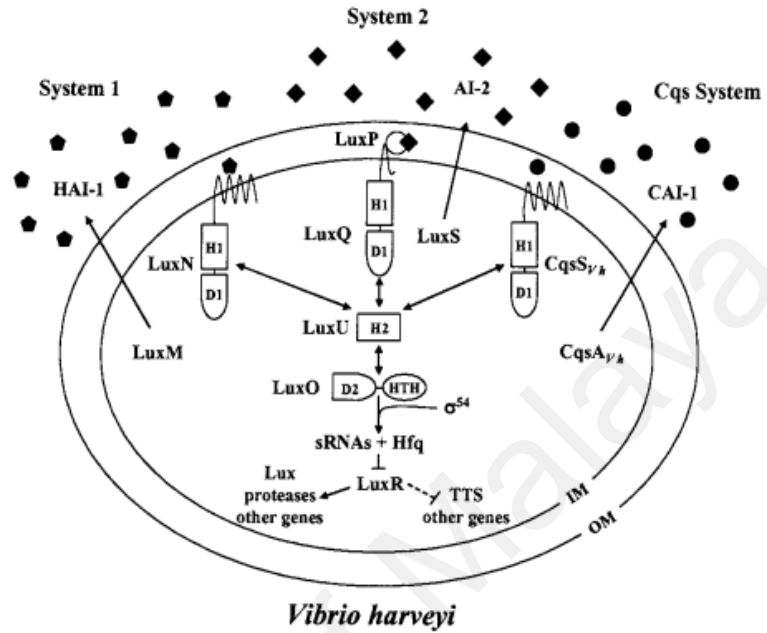
Figure 2.3: Interspecies QS system (Henke & Bassler, 2004).

2.1.4   QS Autoinducers

There are quite a number of chemical classes of microbial derived signalling molecules that have been identified with majority of them are either small (< 1000 Da) organic molecules or peptides with 5–20 amino acids (Williams *et al*., 2007). Generally, the signalling molecules can be group into two major categories where Gram-negative bacteria will utilize the fatty acid derivatives while the Gram-positive bacteria will use the amino acids and short peptides (Kleerebezem *et al*., 1997; Lazazzera & Grossman, 1998; Shapiro, 1998). The autoinducers that are produced will either diffuse freely across the cell membranes or are actively transported out of the cell (Pearson *et al*., 1999).

One of the key players of autoinducer signals in the QS system for the Gram-negative bacteria is acyl homoserine lactones (AHLs) where they have a conserved homoserine lactones (HSLs) ring with a variable acyl side chain. The AHLs can be broadly categorized as either short or long chain molecules based on the length of the acyl groups. For short chain AHLs, there are roughly 4−8 carbon atoms in the acyl moiety while the long chain AHLs have 10−18 carbon atoms. The presence of the oxo or hydroxyl substitutions at the C-3 position of the acyl chain can also give variation and specificity to the QS communication in a mixed bacterial population (Schripsema *et al*., 1996; McClean *et al*., 1997; Laue *et al*., 2000; Marketon *et al*., 2002).

Based on the previous research, there are a lot of different bacteria strains that could produce the same type of AHLs. Nevertheless, the AHLs that are made may be involved in the regulation of different phenotypes in every one of the strains. For instance, 3-oxo-C6-HSL activates the bioluminescence in the luminous marine bacterium *V. fischeri* (*P. fischeri*) but regulates exopolysaccharide production in *E. stewartii* (Watson *et al.*, 2002). This cross talk has basically allowed the scientists to come up with the QS indicator strains that are useful especially in detecting the presence of AHLs in a given sample (Llamas *et al.*, 2004).

Besides *N*-butanoyl-L-homoserine lactone (C4-HSL) and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *P. aeruginosa* also consist a third autoinducer known as *Pseudomonas* quinolone signal (PQS) which belongs to the family compounds of 2-alkyl-4-quinolones (4Qs) (Lépine & Milot, 2004). The production of the PQS is regulated by the *las* QS system but RhlR is also required for PQS activity (Pesci *et al.*, 1999). In addition, diffusible extracellular factor (DSF) produced by *Xanthomonas campestris*, which is a cabbage pathogen, is found to regulate virulence factor synthesis in response to physiological or environmental changes and is dependent on the presence of *rpfF* and *rpfB* (Barber *et al.*, 1997).

Other autoinducers that are being produced by bacteria includes autoinducer-2 (AI-2) where it was first recognized in *V. harveyi* as well as other Gram-negative bacteria such as *Salmonella, Erwinia* and *Escherichia* (Bassler *et al.*, 1993; Bassler, 2002). AI-2 is also known as a global signal molecule as it can be produce by Gram-positive bacteria even though most of them prefer linear, modified or cyclic peptides such as the autoinducing peptides (AIPs) produced by staphylococci (Williams, 2007). Streptomycetes, on the other hand, produce c-butyrolactones such as A-factor

that are structurally related to the AHLs since both compound classes belong to the butanolides (Williams, 2007).

The enzyme involved in the production of cyclic dipeptides has not been identified yet but it is believed that the nonenzymatic cyclization of linear dipeptides can generate diketopiperazines (DKPs) under extreme temperature and pH. DKPs is a novel family of signalling molecules that were discovered from cell free supernatants of *P. aeruginosa* as well as other Gram-negative bacteria that are capable of mimicking the action of AHLs. The signalling molecules also found to modulate the LuxR-dependent QS system in other Gram-negative bacteria such as the AHL biosensor strains like *C. violaceum* (CV026), *A. tumefaciens* NT1 (pDC141E33) and *E. coli* [pSB401] (Holden *et al*., 1999).

Bacteria generate cyclic dipeptides too and were recently identified in the *Pseudomonas* strains like *P. fluorescens* and *P. alkaligenes*. This particular autoinducer has the abilities to activate the AHL biosensors and has the structure of diketopiperazines (DKPs) cyclo (L-Ala-L-Val) and cyclo (L-Pro-L-Tyr) respectively. The DKPs, on the other hand, has been found in *P. mirabilis, C. freundii* and *E. agglomerans* (Holden *et al*., 1999).
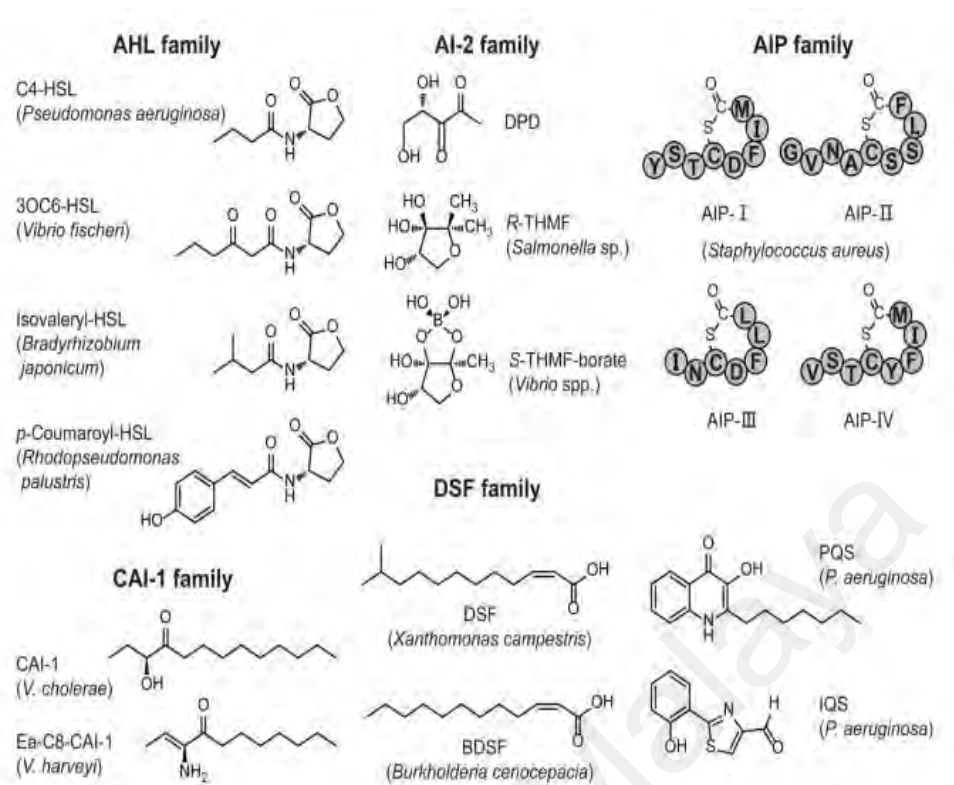
Figure 2.4: Examples of autoinducers produced by signal synthase in QS system (Henke & Bassler, 2004).

2.2   *Pseudomonas aeruginosa* QS System

Pseudomonas aeruginosa is a Gram-negative bacterium that has rod shaped, aerobic with unipolar motility, ubiquitous, non-sporing and non-capsulated. This bacterium belongs to the family Pseudomonadaceae with the characteristic of grape-like odor and green pus colonies (Rahme *et al.*, 1995; Rubin *et al.*, 2008). *P. aeruginosa* also produces diffusible pigments known as pyocyanin which is yellow-green in colour as well as fluorescein where the colonies can fluoresce green when view under the ultra violet (UV) light (Cheesbrough, 1991). *P. aeruginosa* can be found mainly in the gastrointestinal area and is a significant reservoir especially in the hospital environments such as in the humidifiers, sinks, bowls, cleaning buckets, ointments, eye drops and also weak antiseptic solutions (Cheesbrough, 1991; Sleigh & Timbury, 1994). These bacteria can be found in the environments too including soil, rhizosphere, freshwater and marine environment (Joklik *et al.*, 1992).

P. aeruginosa is an opportunistic pathogen for animals, insects, plants and also humans (Rahme *et al.*, 1995; Rubin *et al.*, 2008). It is commonly known to cause diseases like respiratory system infections, urinary tract infections, external ear infections, eye infections, septicaemia, dermatitis, soft tissue infections, gastrointestinal infections, severe burn wound as well as the bone and joints infections among the humans (Cheesbrough, 1991; Sleigh & Timbury, 1994). In addition to that, patients that are suffering from cystic fibrosis are more prone to the infections caused by *P. aeruginosa* and it will remain in the airways of the patient despite of the intensive antibiotic treatments (Pedersen, 1992; Martin *et al.*, 1993). As the infections become more severe, the patients will normally have substantial damage to the lung tissues followed by pulmonary failure (Rasmusssen & Givskov, 2006).

P. aeruginosa is considered to be a dangerous and dreaded pathogen because it is extremely difficult to control as it develops resistance towards a broad range of antibiotics (Hancock, 1998). Currently, there are a few antibiotic classes including aminoglycosides, β-lactams, quinolones, macrolides and polymyxins that are being used to combat infections caused by *P. aeruginosa* but frequent or misuse of these antibiotics results in the development of multidrug resistant *Pseudomonas* (McDaniel *et al*., 2016). The resistance occurs due to the permeability barrier of the bacteria outer membrane as well as the formation of biofilm on the cells, thus, making the bacteria to be impervious to therapeutic concentrations antibiotics. Besides that, *P. aerugianosa* also maintains its antibiotic resistance plasmids where it can subsequently transfer the genes through transduction and conjugation (Sleigh & Timbury, 1994). As a whole, there are generally three basic mechanisms that the bacteria use to resist any of the actions caused by the antimicrobial agents which is restricted uptake and efflux, drug inactivation as well as changes in the targets (Lambert, 2002).

Table 2.2: List of world's most dangerous superbugs 2017.

Priority 1: Critical
1. *Acinetobacter baumannii*, carbapenem-resistant
2. *Pseudomonas aeruginosa*, carbapenem-resistant
3. *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
Priority 2: High
1. *Enterococcus faecium*, vancomycin-resistant
2. *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
3. *Helicobacter pylori*, clarithromycin-resistant
4. *Campylobacter* spp., fluoroquinolone-resistant
5. *Salmonellae*, fluoroquinolone-resistant
6. *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: Medium
1. *Streptococcus pneumoniae*, penicillin-non-susceptible
2. *Haemophilus influenzae*, ampicillin-resistant
3. *Shigella* spp., fluoroquinolone-resistant

* information obtained from World Health Organization's website released on 27th February 2017 (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/).

In the recent years, extensive studies have been conducted related to the QS system of the *P. aeruginosa* as to understand the communication between the bacteria themselves in order to target their pathogenicity (Govan & Deretic, 1996). The bacteria are highly pathogenic due to its ability to produce and secrete various virulence factors such as the extracellular enzymes (elastase, LasA protease, alkaline protease and lipase), haemolysins (phospholipase and rhamnolipid), toxins (exoenzyme S and exotoxin A), secondary metabolites (pyocyanin and hydrogen cyanide), pilus expression and alginate production (Pollack, 1990). These toxins are thought to be the main contributors for both acute and chronic infections as the virulence can generally damage both the tissue organs as well as interfere with the human immune system's defence mechanism (Rubin *et al.*, 2008). Moreover, *P. aeruginosa* is also a formidable biofilm former and thus, making it harder to be treated with antibiotics (Davies *et al.*, 1998).
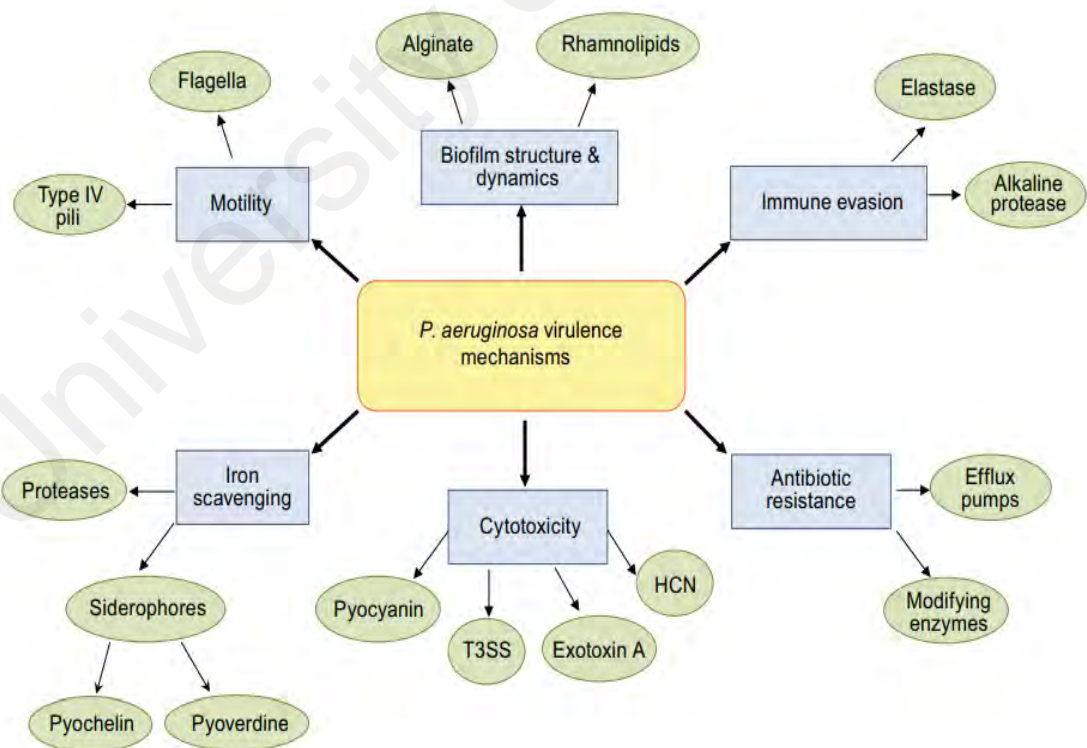
Figure 2.5: Virulence mechanisms employed during *P. aeruginosa* infections (Lee & Zhang, 2015).

P. aeruginosa has three complete, interdependent mechanisms QS systems mainly identified as *las*, *rhl* and *Pseudomonas* quinolone system (PQS). Both of *las* and *rhl* consists of genes that encodes the acylated homoserine lactone signalling molecules (*lasI* and *rhlI*) and transcriptional activator proteins (*lasR* and *rhlR*) (Winson *et al*., 1995; Pesci & Iglewski, 1999). These two QS circuits are generally arranged hierarchically because the *rhl* system is being controlled by the *las* system and transcriptome studies have proved that both the systems can regulate the expression of hundreds of genes together (Pesci *et al*., 1997; Schuster *et al*., 2003).

LasI is responsible mainly in the synthesis of *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Pesci *et al*., 1997). The *las* system was shown to regulate the expression of several virulence factors including the alkaline protease, LasA protease, LasB protease, exotoxin A and RhlR in addition of the protein secretory genes which is the *xcpP* and *xcpR* (Latifi *et al*., 1995; Winson *et al*., 1995). Research has shown that a glutathione S-transferase-LasR fusion protein can attach to the promoter of LasB in the presence of 3-oxo-C12-HSL (You *et al*., 1996). As the LasI synthesis increase, the production of 3-oxo-C12-HSL will tend to increase too followed by the increase of the LasR activator complex (Seed *et al*., 1995). In another words, the activation of the LasI/R-dependent virulence factor production by *P. aeruginosa* is closely related to 3-oxo-C12-HSL concentrations. In addition, (3-oxo-C12-HSL)-LasR also activates the expression of RhlR/I system by inducing the expression of RhlR (Pesci *et al*., 1997).

RhlI, conversely, synthesize *N*-butanoyl-L-homoserine lactone (C4-HSL) (Pearson *et al.*, 1995) and was shown to interact with the RhlR activator in order to trigger the expression of the rhlAB. The rhlAB is basically an operon that encodes a rhamnosyltransferase that are needed during the production of the rhamnolipid biosurfactants (Ochsner *et al.*, 1994) in which it can help to reduce the surface tension and thus, allows *P. aeruginosa* cells to swarm over the semi-solid surfaces (Köhler *et al.*, 2000). Current studies have also proven that the *rhl* QS system can control the expression of the genes coding for pyocyanin, rhamnolipid, lectins, hydrogen cyanide, alkaline protease, LasB protease as well as critical genes such as *rpoS, xcpP* and *xcpR* (Brint *et al.*, 1995). Like *las* system, the transcription of RhlI is also enhanced in the presence of the RhlR-C4-HSL and this will then further create an autoregulatory loop within the LasRI/RhlRI regulons (Winson *et al.*, 1995).
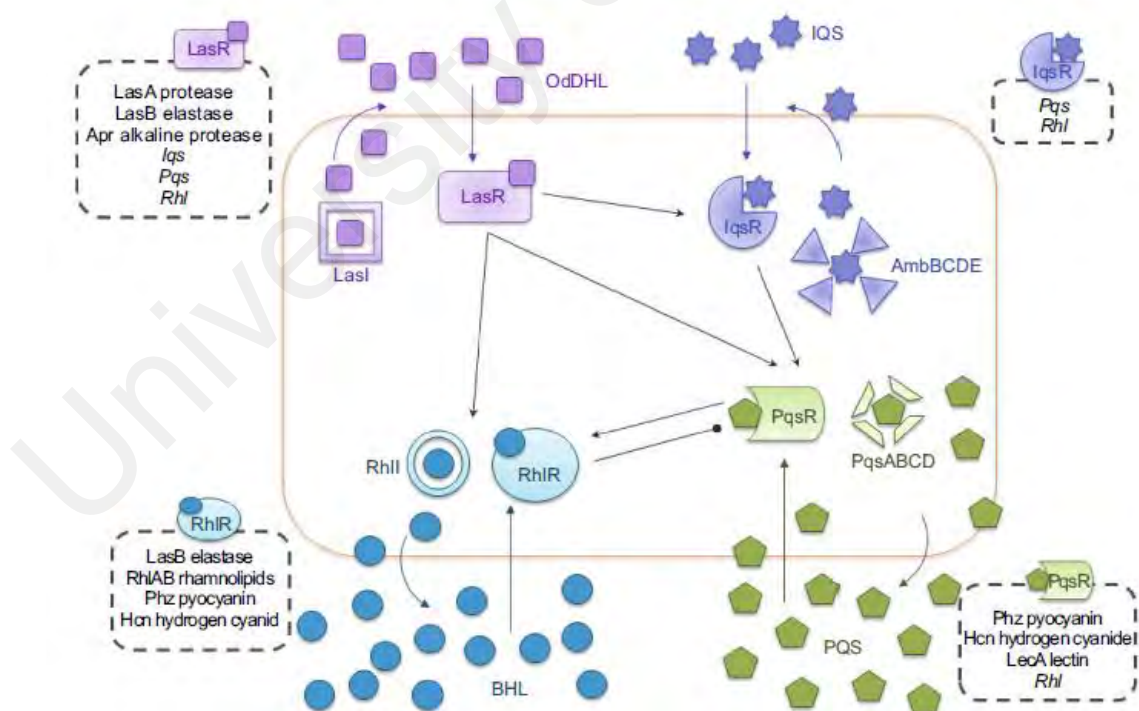
Figure 2.6: *P. aeruginosa* QS system and their respective regulons. Arrows indicate a stimulatory effect. Perpendicular lines indicate an inhibitory effect (Lee & Zhang, 2015).

P. aeruginosa also possess the quinolone signalling molecule (PQS) and this further adds a level of control in the QS network as it provides a link in between the *las* and *rhl* systems. There are at least 55 quinolones that are being produced by *P. aeruginosa* and most of them have been identified as antibiotics (Lépine & Milot, 2004). PQS and HHQ are among the quinolons which do not showed any antibiotic activity (Pesci *et al*., 1999) but were reported to behave as signalling molecules that can control the expression of various virulence factors (Diggle *et al*., 2003; Kong *et al*., 2009).

Pseudomonas quinolone signal (PQS) molecule has a very similar structure to pyo compounds (4-hydroxyquinolones) and was produced via the condensation of the anthranilate and β-keto-fatty acid followed by the conversion to PQS by monooxygenase (Bredenbruch *et al*., 2006). On the other hand, the pqsABCD gene products direct the synthesis of HHQ and was believed to be an extracellular messenger released intracellularly between *P. aeruginosa* cells. HHQ was then taken up by the neighbouring cells and was converted into PQS by the action of the predicted FAD-dependent monoxygenase PqsH (Déziel *et al*., 2004; Diggle *et al*., 2006).

The synthesis of PQS was at most optimum during the late stationary phase and this finding suggest that PQS may be involved in the signalling during the increased cell stress period (McKnight *et al*., 2000; Calfee *et al*., 2001). Previous research showed that the production of the PQS is positively regulated by the *las* system as the transcription of *pqsH* was found to be controlled by LasR but RhlR is also required for PQS activity (Pesci *et al*., 1999; Diggle *et al*., 2006; Schertzer *et al*., 2009). In addition, PQS can also activate the genes for both LasB elastase, C4-HSL synthase as well as increases the affinity of the pqsR protein for the pqsA promoter in *P. aeruginosa* and thus, places

PQS between the *las* and *rhl* QS regulatory network (McKnight *et al*., 2000; Diggle *et al*., 2003; Wade *et al*., 2005; Xiao *et al*., 2006)
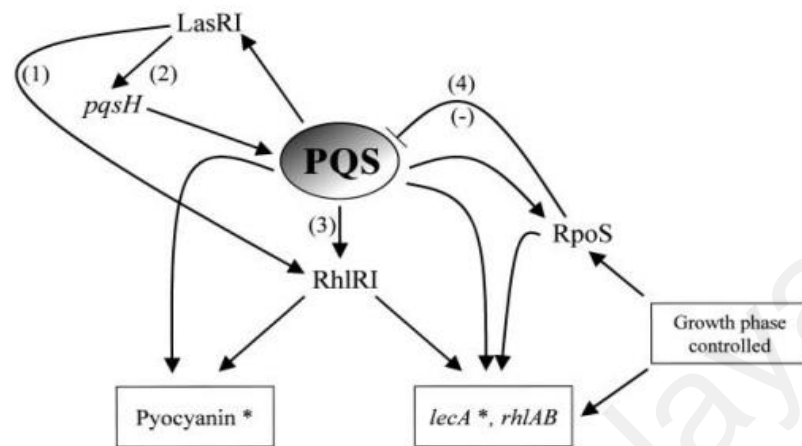
Figure 2.7: A detail mechanism for *P. aeruginosa* PQS system (Diggle *et al*., 2006).

The discovery of the fourth QS signal, named as integrated QS signalling system (IQS) revealed the complexity of the QS system in *P. aeruginosa* and it plays a vital role in modulation of bacteria pathogenesis. IQS (2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde) allows the bacteria to regulate the QS system along with the environmental stresses and was synthesised from a non-ribosomal peptide synthase gene cluster ambBCDE (Lee *et al*., 2013). Study showed that the production of PQS and C4-HSL signals as well as virulence factors such as pyocyanin, rhamnolipids and elastase was affected when there is disruption of IQS signal. Furthermore, IQS has the ability to partially control the las system functions when the bacteria are under phosphate depletion stress conditions (Lee *et al*., 2013; Lee & Zhang, 2015). The pivotal findings allowed the researchers to gain better understanding on the *P. aeruginosa* QS system especially on studies that harbour mutated *lasI* or *lasR* genes (Ciofu *et al*., 2010).
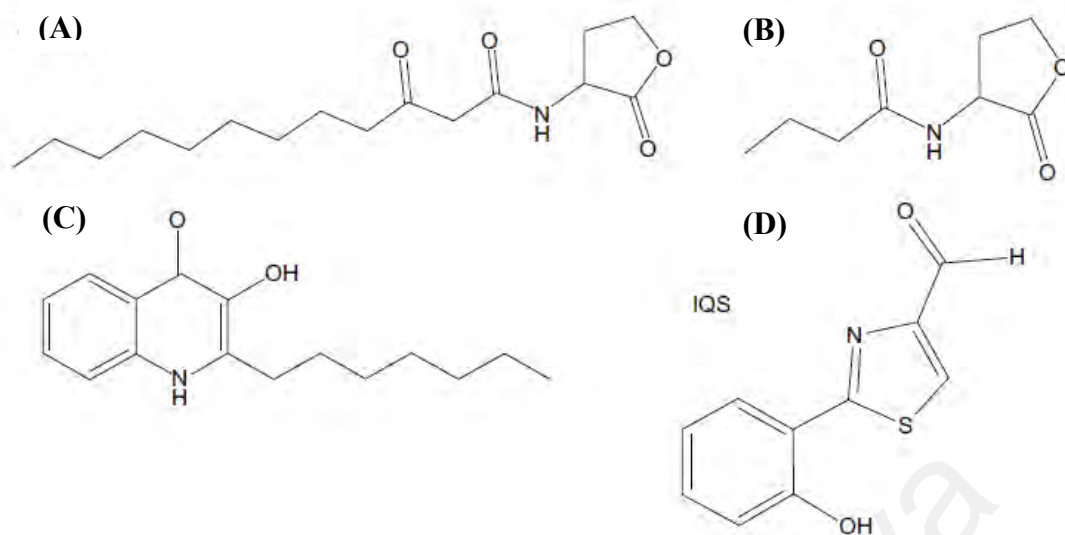
Figure 2.8: *P. aeruginosa* PQS signals (A) *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (B) *N*-butanoyl-L-homoserine lactone (C4-HSL) (C) 2-heptyl-3-hydroxy-4-quinolone (PQS) (D) 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) (Lee & Zhang, 2015).
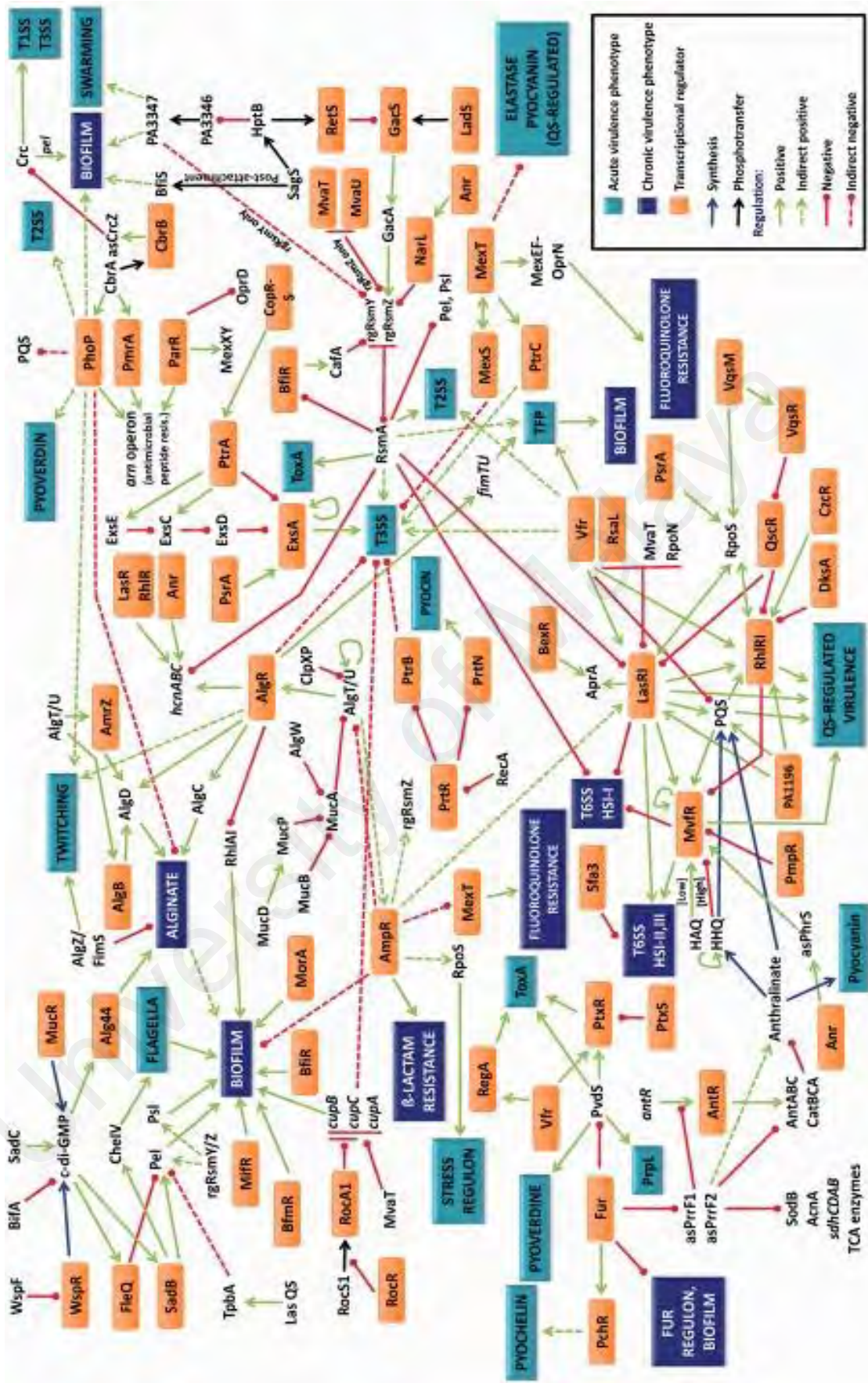
Figure 2.9: *P. aeruginosa* PAO1 virulence regulatory network (Balasubramanian *et al.*, 2013).

2.3   Quorum Quenching (QQ)

The communication among the living organisms appears to be prevalent in the nature but the ability to interfere with these communications is also known to be widespread as it can change the expression of specific characters (Williams, 2007). The continuous emergence of the multiple drug resistant bacteria strains has caused the prokaryotes and eukaryotes to come up with strategies that can interfere with the bacteria QS system (Dong *et al.,* 2002). The disruption of the bacteria QS system is generally known as quorum quenching (QQ) and it has been suggested that the AHL antagonists can basically inhibit the expression of the bacteria pathogenic traits without affecting the growth. This serves as an advantage as it can help to minimize the possibility of the bacteria from becoming resistance mutants (Hentzer & Givskov, 2003).

Researchers have recently discovered that there are a few QQ strategies that have been used by the organism mainly as a defence mechanism against the competitors (Dong *et al*., 2007). One of the ways to combat the bacteria communication is by blocking the signal generation and accumulation but to date, it is the least investigated strategy to target the QS system (Rasmussen & Givskov, 2006). There are two main prospective targets that are identified in the synthesis of the AHL-type signals, which is *S*-adenoysl methionine (SAM) and also enoyl-ACP reductase (ENR) (Zhang, 2003). Triclosan, a commonly used biocide for its antibiotic properties has been found to impede the enoyl-ACP reductase (Hoang & Schweizer, 1999). Closantel, conversely, is a broad spectrum salicylanilide anthelmintic that has the capabilities to inhibit the histidine kinase sensor of both the system (Stephenson *et al*., 2000) by suppressing the AHL gene (Zhang, 2003).

The inhibition of reception of the signalling molecule by the antagonist is another method that can be used to the control the bacterial QS (Antunes & Ferreira, 2009). The antagonist molecule plays a vital role in the QQ mechanism as it can compete or interferes with the bacteria signalling molecules from binding to the AHL receptor protein. Once the receptor fails to be activated, the bacteria will lose its abilities to express any virulence factors (Kalia & Purohit, 2010). Diketopiperazines (DKPs) are cyclic dipeptides that has the same structure as the signalling dipeptides in the mammalian tissues (Draganov *et al.*, 2005) and is normally produced by bacteria like *P. aeruginosa, Proteus mirabilis, Citrobacter freundii* as well as *Pantoea agglomerans* (Holden *et al.*, 1999) in addition to yeast, fungi and lichens (Draganov *et al.*, 2000). DKPs will then acts as AHL antagonists in the LuxR based QS system while as agonist in the rest (Holden *et al.*, 1999).

Besides that, AHLs such as the *N*-decanoyl-L-homoserine lactone (C10-HSL) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) acyl side chains are found to inhibit the *N*-butanoyl-L-homoserine lactone (C4-HSL) dependant expression of exproteases in the *Aeromonas hydrophila* (Swift *et al.*, 1999) as well as the suppression of filament in the pathogenic *Candida albicans* (Hogan *et al.*, 2004). 3-oxo-C12-HSL from *P. aeruginosa*, conversely, showed to inhibit the production of exotoxins and cell wall fibronectin as well as inhibit the *agr* expression of *Staphylococcus aureus* (Qazi *et al.*, 2006). For the *S. aureus* group, these bacteria used the thiolactone based autoinducing peptides to regulate the *agr* virulence and also to inhibit the virulence of other *S. aureus* group (Ji *et al.*, 1997; Schauder & Bassler, 2001). Fungi such as *Penicillium radicicola* and *Penicillium coprobium* produced panicillic acid and patulin respectively where it can target the LasR and RhlR in the *P. aeruginosa* system (Koch *et al.*, 2005; Rasmussen *et al.*, 2005).

The antagonists can also come from the natural compounds such as the furanones and enones that are extracted for the marine macro alga *Delisea pulchra* (Manefield *et al.*, 2001; Ren *et al.*, 2004) where it can attach to the AHL receptor and thereby, affect the binding of the bacteria signalling molecules (Manefield *et al.*, 2002). Meanwhile, alga *Laminaria digitata* produced oxidized halogens that can react specifically with the oxo group of AHLs and destroy their signalling ability by penetrating the biofilm (Borchardt *et al.*, 2001). Research has also showed that garlic and bark of *Combretum albiflorum* extracts consist of different QS inhibitors (QSI) that can quench the virulence factors of *P. aeruginosa* (von Bodman *et al.*, 2008; Vandeputte *et al.*, 2010). In other words, plants generally produced compounds that can mimic the AHL signals where it can either stimulate or inhibit the QS responses in the bacteria (Cook, 1999; Teplitski *et al.*, 2000).

Studies has also shown that there are three different mechanisms that can degrade the QS signals molecules which is through chemical degradation, metabolism of the AHLs and enzymatic destruction. For chemical degradation, the pH is important as it can affect the structure of the signalling molecules that is being produced by the bacteria. When the pH is increase to above 7, the lactone ring will tend to open (Yates *et al.*, 2002) and thus, inactivates the activity of the AHL signal as well as prevent the expression of the QS controlled genes (Byers *et al.*, 2002). When the pH turns back into acidic condition, the lactone ring will re-cyclizes and restored back the activity (Yates *et al.*, 2002).

Enzymatic degradation, on the other hand, has been observed in a handful of prokaryotes as well as a few eukaryotes. In this kind of degradation, the QQ enzymes will hydrolyze the signalling molecules either the lactone ring (Dong *et al*., 2000) or the amide bond (Lin *et al*., 2003). These two reactions are mediated largely by enzymes like AHL-lactonase, AHL-acylase, oxidoreductases as well as lactonase like enzymes known as paraoxonases (Kalia & Purohit, 2011). It is highly possible that a single bacterium can have both AHL-lactonase plus AHL-acylase and this can help to facilitate the metabolism of AHL. On top of that, enzymes that degrade the AHL can also play a part in regulating the QS as it has been found in those bacteria that produce AHLs such as *P. aeruginosa* and *Agrobacterium tumefaciens* (Carlier, 2003; Huang, 2003). For the non-AHL producing bacteria, the degrading enzymes that are being produced is used specially for defence purposes where it can go against the antibiotic-producing bacteria (Gonzalez & Keshavan, 2006).

Another method that can be used to stop the bacteria cell-to-cell communication is by metabolizing the AHL signalling molecules. Bacteria such as *P. aeruginosa* and *Variovorax paradoxus* have been reported to be able to proliferate with the AHL as sole source of carbon, nitrogen as well as energy. The bacteria tend to secrete an amino acylase in which it can cleave the peptide bond of the signalling molecules. The side chain is basically used as the carbon source while the ring part is used as the energy donor. The nitrogen source, conversely, comes from the amide bond that is made available as ammonium via the action of the lactonase (Leadbetter & Greenberg, 2000; Huang *et al*., 2003).
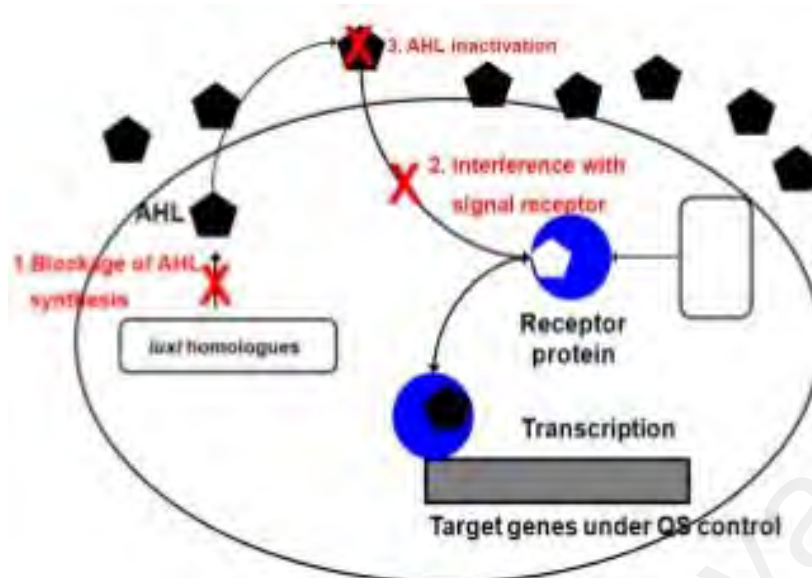
Figure 2.10: Inhibition of QS system (Bhardwaj *et al*., 2013).
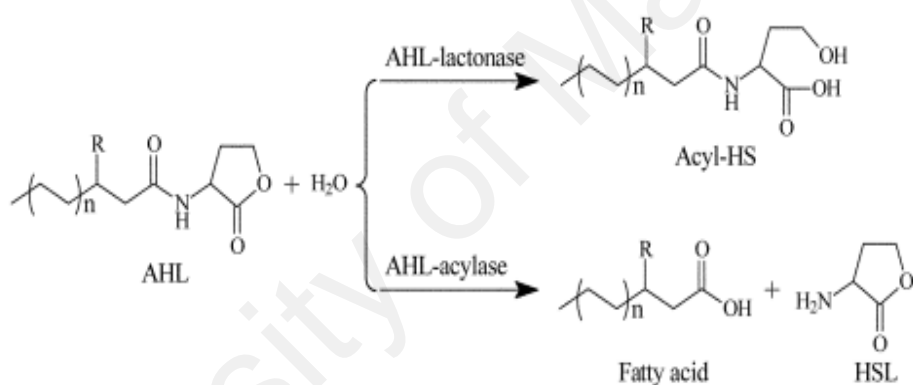
Figure 2.11: The corresponding degradation mechanisms of AHL-lactonase and AHL-acylase (Dong & Zhang, 2005).

2.4   Natural Products as Anti-QS Antagonists

Natural products medicines can come from different source of materials including the terrestrial plants, terrestrial microorganisms, marine organisms as well as the terrestrial vertebrates and invertebrates (Newman *et al.*, 2000). As natural products have a rich treasure of therapeutic effects, it has the potential of becoming the active ingredients to the new modern drugs (Vickers & Zollman, 1999). The natural compounds have shown to have a great diversity of chemical structures that can help to target the problems that provoked the diseases caused by the pathogens (Ganesan, 2008). With such a thriving record, the identification of new metabolites from the living organisms might be expected to be the core of the pharmaceutical discovery efforts (Jesse *et al.*, 2009).

The value of the natural products as medicine can basically be assessed by using three main criteria, which is the number of diseases that have been treated or prevented by the substances, the amount of times that the substances have been used to treat the disease in addition to the rate of the introduction of the new chemical with wide structural diversity that also serves as templates for semi-synthetic and total synthetic modification (Chin *et al.*, 2006). Even though there are some pharmacological uses such as antiviral, anticancer, antihelminthic, anti-inflammatory, antimalarials, antimicrobial and immunosuppressants, lipid control agents, vasodilatory effects that have been scientifically verified, there are still many of the compounds in the nature that remains to be explored and research (Vickers *et al.*, 1999; Adonizio *et al.*, 2006) especially in the work of QS.

Plants have been exploited as medicines for thousands of years and these medicines initially took the form of crude extracts such as the teas, powders, tinctures, poultices as well as other herbal formulations (Balick & Cox, 1997). Just more than 200 years ago, a pharmacist by the name of Friedrich Sertürner has successfully isolated the first pharmacologically active pure compound known as morphine from the opium that are produced by the cut seed pods of the *Papaver somniferum* (Hamilton & Baskett, 2000). After the Second World War, the research continues on once the penicillin has been discovered and extensive studies have been conducted massively on screening different plants in order to create more new drugs for treating and preventing any infectious diseases caused by the microbes (Koehn & Carter, 2005; Harvey, 2008). Among the focus of the studies in the use of plants as medicine is to isolate any active compounds that are produced followed by the purification and characterization of the pure compounds (Kinghorn, 2001; Samuelsson, 2004; Jesse *et al.*, 2009).

Plants have an almost limitless ability to produce aromatic substance of which most of them are phenols or their oxygen-substituted derivatives (Lim *et al.*, 2006). Among the secondary metabolites that are being synthesized includes catechins, quinolones, flavonones, polyphenolics, alkaloids, lignans, carvacrol, alkaloid, coumarins, anthraquinone, thionin, resin, saponins, tannins, polyacetylene, monosaccharide, sulphated terpenoids and terpenoids (Harborne, 1999; Bourgaud *et al.*, 2001). In many cases, the compounds secreted are responsible for the plant odors or flavours such as terpenoid capsaicin from the chilli peppers. Besides that, compounds like quinones and tannins are found to play an important role in the plant pigmentation (Cowan, 1999). Nevertheless, the compounds also serve as the plant defence mechanisms against predation by the insects, herbivores and especially the microorganism where it can target the pathogens and work through a non-species

specific mechanism like disrupting the bacteria cell membranes (Cowan, 1999; Dewick, 2002).

The discovery and explanation of the QS systems has been paralleled by the growing interest in the ability to manipulate the signal reception and transduction, thus, making the systems eligible as the drug target (Rasmussen *et al*., 2005). There are numerous studies that have been globally established and the controlled QS regulon in *P. aeruginosa* PAO1 is one of it where about one third of these bacteria genes encode the virulence factors (Schuster *et al*., 2003). Most of the bacteria nowadays have become resistant to majority of the antibiotics available in the market. Therefore, it is important to come up with a solution and one of it is by inhibiting the QS system as it can offer an alternative to the antibiotic mediated bactericidal or bacteristatatic approach. The bacteria communication or signals that have been attenuated by the QS inhibitors can generally lessen the risk of producing antibiotic resistant genes as well as to prevent the development of the bacterial virulence and thus, can help to decrease the establishments of the diseases (Schauder & Bassler, 2001; Raffa *et al*., 2005; Rasmussen & Givskov, 2006b). In addition, the QS inhibitors can also disturb the integrity of the biofilms and therefore, makes the bacteria more susceptible to antibiotics and minimize the possibility of becoming resistant (Dong *et al*., 2002; Hentzer & Givskov, 2003).

The applications of the plants are typically straight forward where the compound of the plants usually targets the Gram-negative bacteria QS system via three different ways. One of the most apparent strategies is to screen for any compounds that can stop the signalling molecules from being synthesized by the LuxI encoded AHL synthase (Suga & Smith, 2003). When there is no AHL being produced, bacteria will have

difficulties in sensing the communication even though the quorum has been reached and hence, unable to activate the QS virulence controlled genes (Gonzalez & Keshavan, 2006).

Besides that, the signalling molecules can also be targeted if the production of the signals cannot be prevented. The degradation of the signalling molecules can be done through metabolic, chemical or enzymatic (Rasmussen & Givskov, 2006b). Increased pH and temperature levels are found to increase the rate of the ring of AHL molecules to open and undergoes lactonolysis (Byers *et al*., 2002; Yates *et al*., 2002). Higher organisms seem to take this advantage as their defence mechanism when they are being attacked by bacteria that produce AHLs. Previous research has shown that plants tend to increase their pH at the site that are infected by *E. carotovora* which uses QS to control their expression of virulence factors (Byers *et al*., 2002). Other research has also confirmed that plant root-associated fungi such as *Phialocephala fortinii* and *Meliniomyces variabili* as well as Ascomycete isolate can hinder the bacteria virulence by degrading their signalling molecules and communication system (Dong *et al*., 2001; Uroz & Heinonsalo, 2008).

Another one of the most thorough research done for the inhibition of QS was based on the use of the secondary metabolites from plants to target the LuxR signal receptor and is a classical pharmacological strategy to receptor antagonism (Suga & Smith, 2003; Rasmussen & Givskov, 2006b). Some of the compounds that are produced by the natural products have the capabilities to mimic the AHL signals and competitively bind to the LuxR receptor (Teplitski *et al*., 2000). Once the compounds of the plants bind to the LuxR protein, the QS system will then be interfered as the LuxR can no longer act as transcriptional receptor (Suga & Smith, 2003). Research conducted

by Rasmussen's team have identified a few compounds like para-benzoquinone, 2,4,5-tri-bromo-imidazole, indole, 3-nitro-benzene-sulfonamide and 4-nitro-pyridine-N-oxide (4-NPO) that can block both LuxR and LasR-based QS (Rasmussen *et al.*, 2005b).

One of the most extensively studied QS mimic compounds are the halogenated furanones in which it is produced by the red marine alga known as *D. pulchra* (Givskov *et al.*, 1996). In the year of 1995, Peter Steinberg who is a marine ecologist has approached Staffan Kjelleberg to help him overlook the patent application for one of his compounds that were effective in preventing the thick build-ups of bacteria, algae and invertebrates on the marine surface or better known as biofouling (Bauer *et al.*, 2005). Kjelleberg was hesitated at first but then was later amazed by the furanones compound structure as it has the similar structure as the AHL signalling molecules that are used by many Gram-negative bacteria such as *S. liquefaciens* and *P. aeruginosa* (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997).

The furanones were shown to strongly and specifically inhibit the QS-regulated behaviours in most of the bacteria species where it will bind competitively to the AHL receptor proteins (Manefield *et al.*, 1999). Due to that, the furanones extracted from the *D. pulchra* are said to be a genuine AHL signal-mimic compound that are structurally analogs to the AHL and can affect the AHL-regulated behaviours (Kjelleberg *et al.*, 1997). In addition, furanones also promote their rate of proteolytic degradation in contrast to the stabilizing effects if the AHL signal binding (Zhu & Winans, 2001; Manefield *et al.*, 2002). In the recent research, the furanones AHL mimics have been shown to affect the biofilm structure of the *P. aeruginosa in vitro* as well as inducing abnormal, uncoordinated swarming motility on *P. mirabilis* (Gram *et al.*, 1996; Hentzer *et al.*, 2002).

In addition to that, furanones can blocked the initiation of the normal virulence functions by attenuating the total exoprotease activity in the *S. liquefaciens, V. harveyi, E. carotovora* and *P. aeruginosa* significantly without affecting the growth (Gram *et al*., 1996; Hentzer *et al*., 2003). Furanones also play a very important role in decreasing the light emission among the *Vibrio* species such as *V. fischeri* and *V. harveyi*, hinder the pigment production in *C. violaceum* and stop the swarming motility in *S. liquefaciens* (Givskov *et al*., 1996; Rasmussen *et al*., 2000; Martinelli *et al*., 2004).

Besides *D. pulchra*, there are also other higher plants like various fruits and vegetables that are found to possess anti QS properties (Teplitski *et al*., 2000). Among the examples include garlic, carrot, chamomile, water lily as well as an array of peppers that has been proved to have anti QS activity against the *luxI*-gfp reporter strain. On top of that, studies have shown that the garlic can inhibit the formation of the biofilm that are produced by the *P. aeruginosa* (Rasmussen *et al*., 2005). The pea seedlings and root exudates are also found to inhibit the pigment production, exochitinase activity and protease activity in *C. violaceum* (Teplitski *et al*., 2000). Some of the plants like the buttonwood, graceful sandmat, bottlebrush, black olive, Florida clover ash and live oak have been tested positive for QQ activities in *C. violaceum* and *A. tumefaciens* biosensors strains (Adonizio *et al*., 2006).

Medicago truncatula, rice, crown fetch, tomato and soybean was among the higher plants that can produce substances that can mimic the activities of the AHL as well as having specific effects on the QS-regulated behaviour in the bacteria (Teplitski *et al*., 2000; Daniels *et al*., 2002). The fruits and herbs was also found to have anti-QS activity in a bioassay using *C. violaceum* CV026 as biosensor strain and also against the swarming motility of *E. coli* and *P. aeruginosa*. Among the fruits and herbs that exhibit

a moderate inhibition of QS-controlled system are the raspberry, blueberry, blackberry, cranberry, grape, thyme, basil, kale, oregano as well as the turmeric (Vattem *et al.*, 2007). Other than the signal mimics compounds like the furanones and synthetic derivatives, there are other alternative compounds such as ellagic acid, tannic acid and epigallocatechin gallate that can inhibit communication in the *E. coli* and *P. aeruginosa* biosensor strains (Huber *et al.*, 2004).

As a whole, numerous anti-QS compounds have been identified and studied extensively throughout these few years in the field of QS (Rasmussen & Givskov, 2006b). However, in order to prevent bacteria from being resilient toward QS inhibitors, a more innovative and novel strategy woud have to be employ so as to extend the range of the anti-QS activity against the multidrug resistant bacteria (Kalia *et al.,* 2014).

Table 2.3: Antagonist of QS against selected bacteria and pathogens (Koh *et al*., 2013).

Plant source	Inhibition against	Reference
Melicope lunu-ankenda (leaves) *Syzygium aromaticum* (bud)	*E. coli* [pSB401] *E. coli* [pSB1075] *C. violaceum* CV026 *P. aeruginosa* PA01 *P. aeruginosa* lecA::lux	Krishnan *et al*., 2012; Tan *et al*., 2012
Garlic (bulbs)	*P. aeruginosa*	Bjarnsholt *et al*., 2005; Rasmussen *et al*., 2005b
Vanilla planifolia (beans)	*C. violaceum* CV026	Choo *et al*., 2006
Tremella fuciformis (whole)	*C. violaceum* CV026	Zhu & Sun, 2008
Panax notoginseng (flowers and roots) *Areca catechu* (seeds) *Prunus armeniaca* (kernel of seed) *Prunella vulgaris* (whole) *Nelumbo nucifera* (leaves) *Punica granatum* (bark) *Imperata cylindrical* (stem) *Panax ginseng* (roots)	*C. violaceum* CV026 *P. aeruginosa* PA01	Song *et al*., 2010; Koh & Tham, 2011
Moringa oleifera (leaves and fruits)	*C. violaceum* ATCC 12472	Singh *et al*., 2009a
Capparis spinosa (fruits)	*C. violaceum* CV026 *P. aeruginosa* *E. coli* *P. mirabilis* *S. marcescens*	Issac Abraham *et al*., 2011
Laurus nobilis (fruits, flowers, leaves, bark)	*C. violaceum* ATCC 12427	Al-Hussaini & Mahasneh, 2009
Acacia nilotica (green pod)	*C. violaceum* ATCC 12472	Singh *et al*., 2009b
Quercus virginiana (leaves) *Chamaesyce hypericifolia* (aerial) *Tetrazygia bicolor* (leaves) *Conocarpus erectus* (leaves) *Bucida burceras* (leaves) *Callistemon viminalis* (leaves, inflorescence)	*C. violaceum* ATCC 12472 *C. violaceum* CV026 *A. tumefaciens* NTL4	Adonizio *et al*., 2006
Ananas comosus *Musa paradiciaca* *Manilkara zapota* *Ocimum sanctum*	*C. violaceum* ATCC 12472 *C. violaceum* CV026 *P. aeruginosa* PA01	Musthafa *et al*., 2010
Scutellaria baicalensis	*C. violaceum* CV026	Song *et al*., 2012

Table 2.3, continued.

Plant source	Inhibition against	Reference
Scorzonera sandrasica	*C. violaceum* ATCC 12472 *C. violaceum* CV026	Bosgelmez-Tinaz *et al*., 2007
Orange	*Y. enterocolitica*	Truchado *et al*., 2012
Lonicera alpigena *Castanea sativa* *Juglans regia* *Ballota nigra* *R. officinalis* *Leopoldia comosa* *Malva sylvestris* *Cyclamen hederifolium* *Rosa canina* *R. ulmifolius*	*S. aureus*	Quave *et al*., 2008
Tea tree Rosemary	*C. violaceum* CV026	Alvarez *et al*., 2012
Lippia alba *Ocotea* sp. *Elettaria cardamomum* *Swinglea glutinosa* *Myntotachys mollis* *Zingiber officinale*	*P. putida* [pRK-C12] *E. coli* [pJBA132]	Jaramillo-Colorado *et al*., 2012
Piper bredemeyeri (leaves) *P. brachypodom* (leaves) *P. bogotence* (whole)	*C. violaceum* CV026	Olivero *et al*., 2011
Phellinus igniarius	*C. violaceum* CV026	Zhu *et al*., 2012
Exudates from pea (*Pisum sativum*)	*S. liquefaciens* MG44 *S. faciens*MG44	Teplitski *et al*., 2000
Ganoderma lucidum	*C. violaceum* CV026	Zhu *et al*., 2011
Sulforaphane Erucin	*P. aeruginosa*	Ganin *et al*., 2013

2.4.1 Plants Tested for Anti-QS Activities

Angelica dahurica (root of the Holy Ghost) is a type of medicinal plants that grows wild in Korea, China, Japan and Russia (Chevallier, 2001). The herb is primarily used to treat headache, migraine, swelling, contusions, toothache, leukorrhea, supraorbital neuralgia and haemorrhage (Yang *et al*., 2003). The herb is also claimed to be effective against acne, erythema, sinusitis, cold as well as flu (Wagner, 1990). In addition, previous research proved that extracts from *A. dahurica* can inhibit violacein production in *C. violaceum* CV12472 and swarming motility in *P. aeruginosa* as well as anti-staphylococcal activity (Lechner *et al*., 2004; Damte *et al*., 2013).

Rhizoma cibotii (chain fern rhizome) is commonly found in China and is used as Chinese herbal medicine to tonify the liver and kidneys, strengthen the bones and tendons, expel wind and dampness as well as relax the meridian (China Pharmacopoeia Committee, 2010; Wang *et al.,* 2013) In addition, the herb can also help to treat lumbar and leg pain, numbness, hemiplegia, leucorrhea spermatorrhea, women bleeding, osteoma, osteosarcoma, brain tumors along with multiple myeloma. It is also typically used to mix with other Chinese herbs to pickle drinking wine for disease treatment such as waist sour and rheumatism (Xu *et al*., 2000; Zhao *et al*., 2007). Pharmacological investigations showed that the herb possess anti-inflammatory, antioxidant, antiviral and anticancer (Jia & Zhang, 1996; Liu *et al*., 2002; Zhang *et al*., 2008; Mai *et al*., 2012).

Prunella vulgaris (self-heal) is a perennial herb that is usually found throughout Europe and Asia especially in China such as Anhui, Jiangsu, Henan and Zhejiang provinces (Chen *et al.*, 2010). The herb is traditionally used as herbal medicine to relieve sore throat, fever, tuberculosis of the lymphatic glands, headache, inflammation of the eyes, dizziness and wound healing (Wee & Keng, 1990; Pinkas *et al.*, 1994; Marková *et al.*, 1997; Cheung & Zhang, 2008). In modern application, the extract of this plant showed lipoperoxidation suppression, antiestrogenic, antioxidative, antimicrobial, antiviral and anti-QS activities (Laranjinha *et al.*, 1994; Liu *et al.*, 2002; Psotova *et al.*, 2003; Osakabe *et al.*, 2004; Koh & Tham, 2011).

Schizonepeta tenuifolia (Japanese catnip) is a medicinal herb that can be found in China, Japan and Korea. The herb can be used as hemostatic and antibiotic as well as to relieve body aches, common cold, fever, sore throat, psoriasis, allergic dermatitis, eczema and ostitis media (Ding *et al.*, 1993; Fung & Lau, 2002). Previous studies have also shown that the herbs exhibit anti-inflammatory, antipyretic, antioxidant and immunomodulatory (Yoon *et al.*, 2007; Kim *et al.*, 2008; Crozier *et al.*, 2009; Kang *et al.*, 2010).

Forsythia suspense (weeping forsythia) was first described scientifically in 1784 as *Ligustrum suspensum* and can be found in China, Korea, Japan and Europe (Rouf *et al.*, 2001; Bown, 2002). The herb is mainly used on patients with seasonal febrile disease, exogenous wind-heat syndrome together with fever, thirst and mild chillness, sthenia-heat syndrome with high fever, excessive thirst, full and large pulse, pericardium syndrome, skin infections, scrofula, clearing away the heat and toxic materials, drive out the wind and the heat as well as reduce inflammation (Leung, 1990; Dharmananda, 2002; Park *et al.*, 2005). The fruit of the plant also possesses other

effects such as antibacterial, antiviral, cholerectic, antipyretic, antidote, antiphlogistic, antitussive, diuretic, febrifuge and laxative effects (Rouf *et al.*, 2001; Ping *et al.*, 2004). The water extract of *F. suspense* was found to inhibit production of violacein in *C. violaceum* CV12472 and also QS-regulated virulence factors production in *P. aeruginosa* (Zhang & Chu, 2017).

Houttuynia cordata (fish leaf) is a type of plant that comes from the family of Saururaceae and is often found in places like the China, Korea, Japan, Himalayas as well as in the Southeast Asia (Leung, 1990). Traditionally, this herb is used to help to relieve patients that are suffering from dysuria, get rid of the toxin, cough due to lung-heat, lung abscess with purulent expectoration, acute urinary tract infections, dermatitis, phlegm cough, pneumonia, refractory hemoptysis, acute dysentery, malignant pleural effusion, carbuncles, fevers, mastitis, urticaria, anal prolapse, conjunctivitis, anaphylaxis as well as sores too (Sriwanthanal *et al.*, 2007; Lau *et al.*, 2008). Besides that, the herb also has therapeutic activity against the herpes simplex, chronic sinusitis, allergies, cancer and reducing the effects of radioactivity in the body especially in the Japan and Vietnam (Bown, 2002; Lee *et al.*, 2008). Modern application of *H. cordata* includes antibacterial, antiphlogistic, antiviral, antifungal, antioxidative, anti-inflammatory, anticancer, depurative, diuretic, emmenagogue, febrifuge, laxative as well as ophthalmic effects (Sriwanthanal *et al.*, 2007). Additional study showed that the herb extract can inhibit biofilm formation and motility in *P. aeruginosa*, *Staphylococcusepi dermidis* and *C. albicans*. (Shao *et al.*, 2012; Shao *et al.*, 2013a; Shao *et al.*, 2013b).

Garcinia mangostana (mangosteen) is a tropical tree that is widely grown throughout Malaysia, India, Myanmar, Philippines, Sri Lanka and Thailand (Pedraza-Chaverrí *et al*., 2008). The pericarp, fruit, bark and leaves of the tree is commonly being used as traditional medicine that helps to reduce inflammation, fever, abdominal pain, diarrhea, dysentery, wound infection, suppuration, chronic ulcer, suppurations, eczema, hyperkeratosis, psoriasis, cystitis, gonorrhoea and gleet (Farnsworth & Bunyapraphatsara, 1992; Gopalakrishnan *et al*., 1997; Sato *et al*., 2004; Sakagami *et al*., 2005; Suksamram *et al*., 2006). Results from previous research proved that the secondary metabolites produced by *G. mangostana* have various pharmacological activities such as antioxidant, antitumor, anti-inflammatory, antiallergy, antibacterial, antifungal, antiviral, antiparasitic, antiproliferative, astringent as well as cardiovascular protective effects (Gopalakrishnan *et al*., 1997; Jiang *et al*., 2004; Chomnawang *et al*., 2007; Chen *et al*., 2008; Pedraza-Chaverrí *et al*., 2008; Abachi *et al*., 2016).

Mangifera foetida (horse mango) is distributed throughout Malaysia, Thailand, Myanmar, Indochina, Indonesia and Borneo Island (Kostermans & Bompard, 1993; Khoo *et al.*, 2010). In Indonesia, women with postpartum symptoms will normally eat the leaves together with rice as medication (Grosvenor *et al.*, 1995a). Based on previous research, the crude extracts from the fruits showed antioxidant activity while the extracts from the leaves displayed anticancer and antibacterial activities (Grosvenor *et al.*, 1995b; Murakami *et al*., 1995; Ikram *et al*., 2009). In addition, the twigs of *M. foetida* was found to cause antibacterial activity in MDR *S. aureus* and *E. coli* (Panthong *et al*., 2014). The seeds are also used to treat patients with scabies, eczema and trichophytosis (Lim, 2012).

Syzygium samarangense (java apple) is widely cultivated throughout Malaysia, Thailand, Indonesia, Taiwan, India and Bangladesh (Khandaker *et al.*, 2012; Shahreen *et al.*, 2012). The leaves, roots, bark, flowers and fruits of the plant possess various medicinal and pharmacological activities like anti-diabetic, antibacterial, antiviral, spasmolytic, immunomodulatory, antihyperglycaemic, analgesic, protease inhibitory, anti-inflammatory, antipyretic, antiscorbutic, diuretic, high blood pressure, carminative, fever as well as wound healing effect (Kao, 1996; Khandaker *et al.*, 2012; Lim, 2012; Shahreen *et al.*, 2012). In addition, the flowers have astringent properties while the leaves are being used to heal cracked tongues, cold, itches and waist pain (Rahmatullah *et al.*, 2009; Mollika *et al.*, 2014).

Averrhoa carambola (star fruit) is believed to have been originated from Ceylon and Moluccas but can now be found in Malaysia, Thailand, Indonesia, India, Taiwan, Philippines, China, Australia, Brazil, Florida and Israel (Ghani, 2003). The tree is commonly used to treat headache, vomiting, coughing, hangovers, eczemas, cardiovascular disease, antioxidative, anticancer, poisonous bites as well as kidney and bladder disease (Chau *et al.*, 2004; Tadros & Sleem, 2004; Carolino *et al.*, 2005; Shui & Leong, 2006). The leaves extract of *A. carambola* can also help to relieve aphthous stomatitis, angina, oliguria, pyodermas, diabetes, antipruritic, antipyretic, anthelmintic, anti-ulcer, postpartum edema and gastroenteritis (Avinash *et al.*, 2012; Dasgupta *et al.*, 2013).

Pandanus amaryllifolius (pandan leaves) is a plant that is widely cultivated in tropical and subtropical regions such as Thailand, Malaysia, Indonesia, India, Africa and Australia (Sun, 1992; Nor *et al.*, 2008). The leaves are commonly used in cooking as spice, food colouring and fragrant flavouring as well as potpourri and air fresheners

due to the distinct aroma of nutty to fresh hay flavour (Sun, 1992). The leaves have also been used as traditional medicine in helping to reduce fever, relieve indigestion and flatulence while the oil extracted from the leaves have antispasmodic effects and is effective against headaches, sore throat, rheumatism and epilepsy (Cheeptham & Towers, 2002; Nor *et al.,* 2008).

Cymbopogon citratus (lemongrass) is a perennial grass that is widely found in tropical countries like Southeast Asia and is normally used in flavoring, fragrances, cosmetics as well as aromatherapy. Traditionally, the plant is used as antiseptic, anticonvulsant, analgesic, rheumatic, sore throat hypotensive, fever as well as treatment for nervous and gastrointestinal disorders (Filipoy, 1994; Shah *et al.*, 2011). Besides that, there are also various scientific studies that proved the plant produce pharmacological activities such as antibacterial, antifungal, anti-inflammatory, anti-amoebic, antifilarial, antioxidant, antimycobacterial, antimalarial and hypoglycemic (Blasi *et al.*, 1990; Lemos *et al*., 1990; Suresh *et al.,* 1990; Melo *et al*., 2001; Abe *et al*., 2003; Tchoumbougnang *et al*., 2005; Adeneye & Agbaje, 2007).

Momordica charantia (bitter gourd) is a type of medicinal plant that is commonly used in Ayurveda for treating carious diseases. The plant can be found cultivated in countries like China, Brazil. Colombia, Cuba, Ghana, India, Mexico, Malaysia, New Zealand, Panama and Peru (Virdi *et al*., 2003; Grover & Yadav, 2004). The fruits are traditionally used to treat patients with diabetes, malaria, anemia, jaundice, cholerea, anthelmintic, antiemetic, carminative, purgative and peptic ulcer (Ross, 1999; Gurbuz *et al.*, 2000; Virdi *et al*., 2003; Dengiz & Gursan, 2005). Besides that, previous research has also shown that the fruits possess various pharmacological activities like hypoglycaemic, antifungal, inhibition of p-glycoproteins, antihyperlipidemic,

antioxidant, antibacterial, antiviral, antitumor, antileukemic, anthelmintic, antimutagenic, antimycobacterial, anti-inflammatory, hypotensive and immunostimulant (Ng *et al*., 1992; Raman & Lau, 1996; Basch *et al*., 2003; Chen & Li, 2005; Sathishsekar & Subramanian, 2005; Schmourlo *et al*., 2005; Zheng *et al*., 2005).

Petroselinum crispum (parsley) is a type of herb that is commonly used as a condiment, garnish or flavoring ingredient and can be found in Europe and Western Asia (*Zhang et al*., 2006). Essential oil extracted from the leaves and seed of parsley is used commercially as flavoring agent or fragrance in perfumes, soaps and creams while the essential oil attained from the fruit has strong effects on the central nervous system (Fejes *et al*., 1998; Petropoulos *et al*., 2004). The herb is also widely known for their actions on digestion, stomach, kidney, blood, liver, treatment of allergy, cancer chemopreventive agent, antioxidant, antibacterial, chronic inflammatory disorders as well as suppressing the cellular and humoral immune response (Zheng *et al*., 1992; Gazzani, 1994; Fejes *et al*., 1998; Wong & Kitts, 2006; Yousofi *et al*., 2012).

Mentha spicata (spearmint) can be considered as one of the most widely used spices throughout the world especially in the cosmetic, pharmaceutical, confectionary, beverage and food industries (Díaz-Maroto *et al*., 2003; Kanatt *et al*., 2007). The leaves are commonly used to relieve hiccup, flatulence, allergy, pain relieving, giddiness, inflammation, bronchitis as well as to control vomiting during pregnancy (Zheng *et al*., 2003; Kumar & Chattopadhyay, 2007). In terms of pharmacological activities, the extract was found to be antimicrobial, antispasmodic, antioxidant, antiviral, antihistaminic, anti-inflammatory, haemostatic and antiplatelet (Yamamura *et al*., 1998; Samarth & Kumar, 2003; Zheng *et al*., 2003; Ozgen *et al*., 2006; Tognolini *et al*., 2006).
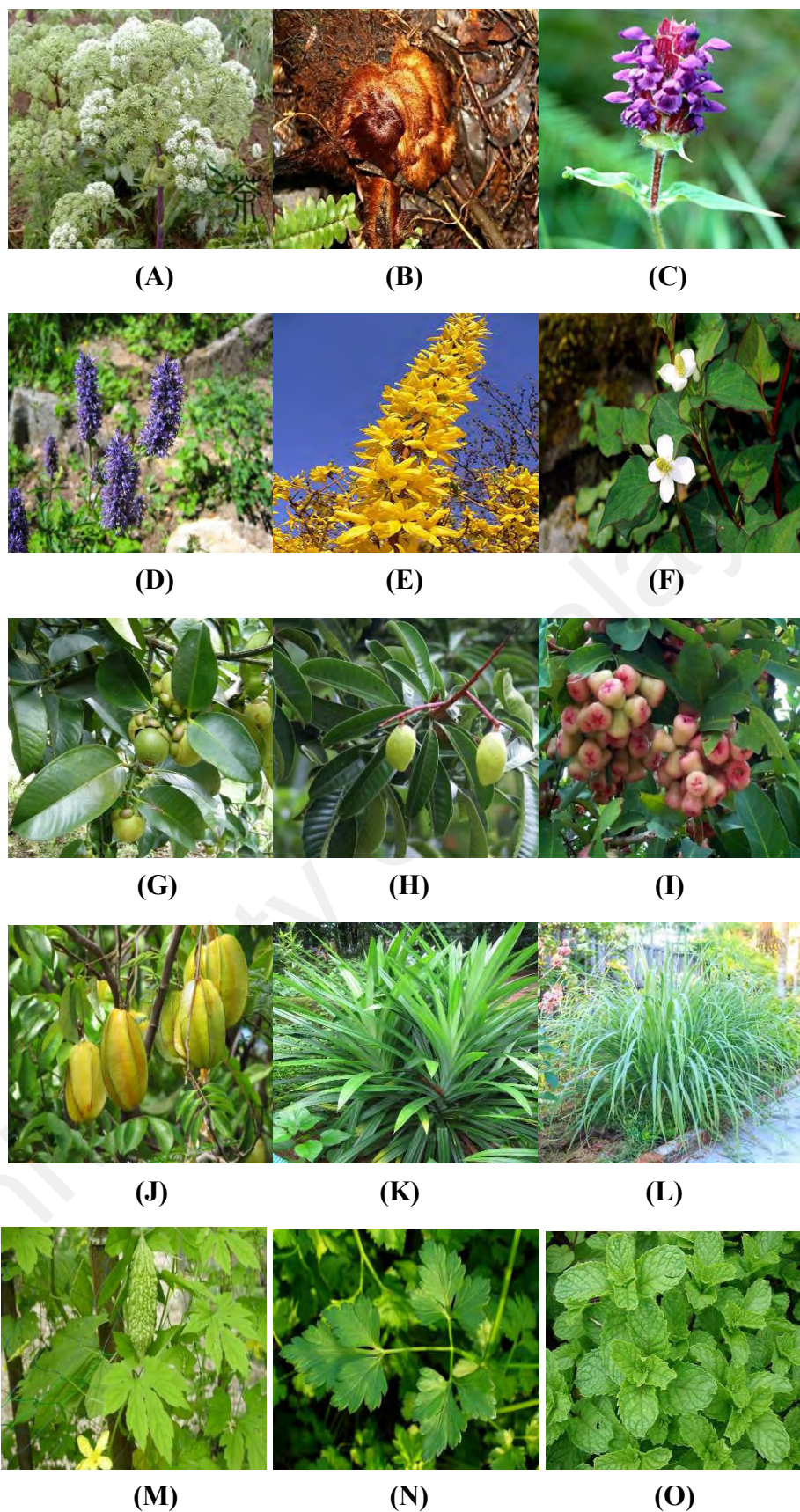
Figure 2.12: Plants tested for anti-QS activities (A) *Angelica dahurica* (B) *Rhizoma cibotii* (C) *Prunella vulgaris* (D) *Schizonepeta tenuifolia* (E) *Forsythia suspense* (F) *Houttuynia cordota* (G) *Garcinia mangostana* (H) *Mangifera foetida* (I) *Syzygium samarangense* (J) *Averrhoa carambola* (K) *Pandanus amaryllifolius* (L) *Cymbopogon citratus* (M) *Momordica charantia* (N) *Petroselinum crispum* (O) *Mentha spicata*

2.5   Biosensors for the Detection of AHLs and Screening of QS Antagonist

In order to study the effects of the plants extracts in quenching *P. aeruginosa* PAO1 QS system, several biosensors or signal molecule reporter strains have been used. The biosensors used carry a functional LuxR protein that is cloned with a target promoter and they do not produce any AHLs. The transcription of the reporter genes that is controlled by QS, such as bioluminescence and violacein production, will only be activated once they are being supplied with synthetic AHLs (Steindler & Venturi, 2007). The use of the biosensors can also help to determine if the plants extracts is targeting *P. aeruginosa* PAO1 autoinducer synthase (LasI/RhlI) or signal receptor (LasR/RhlR) (Chang *et al*., 2014).

Chromobacterium violaceum is a Gram-negative bacterium that is commonly found in soil and water. The bacteria produce violet colonies on solid media due to its inherent ability in biosynthesis of antibiotic known as violacein. The production of the violacein is controlled by QS system regulated by *N*-hexanoyl-L-homoserine lactone (C6-HSL). The CV026 biosensor was constructed by subjecting *C. violaceum* to mini-Tn*5* transposon mutagenesis in order to obtain a double Tn*5* insertion, violacein-negative, white mutant CV026. This biosensor lost its ability to produce AHLs and will only induce the producion violacein when it is exposed to exogenous short chain AHLs (C4-HSL–C8-HSL) and their oxo-derivatives (McClean *et al*., 1997). Plant extracts with QQ activities will degrade the production of violacein when they are being cultured together with CV026 supplemented with synthetic AHLs (Chong *et al*., 2011).

Like CV026, the lux-based biosensor strains, *E. coli* [pSB401] and *E. coli* [pSB1075] will produce bioluminescence when exogenously supplied with AHLs. The plasmids for both the biosensors confer a bioluminescent phenotype in the presence of AHLs activating the LuxR protein. *E. coli* [pSB401] contains fusion of *luxRI'::luxCDABE* on a pACYC184 plasmid backbone while the *E. coli* [pSB1075] carries a fusion of *laxRI'::luxCDABE* on a pUC18 plasmid backbone (Winson *et al*., 1998). In order to determine the transcriptional regulation of LecA, *lecA::luxCDABE* gene fusions were constructed in *P. aeruginosa* PAO1 (Winzer *et al*., 2000).

Two L-arabinose-dependent expression plasmids, pBAD-*lasI* and pBAD-*rhlI* harbouring *P. aeruginosa* PAO1 *lasI* and *rhlI* were constructed and transformed into AHL-negative *E. coli* MG1655 for the purpose of screening AHLsynthase inhibitors. Both of the expression of *lasI* and *rhlI* genes were driven by the L-arabinose inducible promoter in the pBAD expression plasmid (Chang *et al*., 2014).
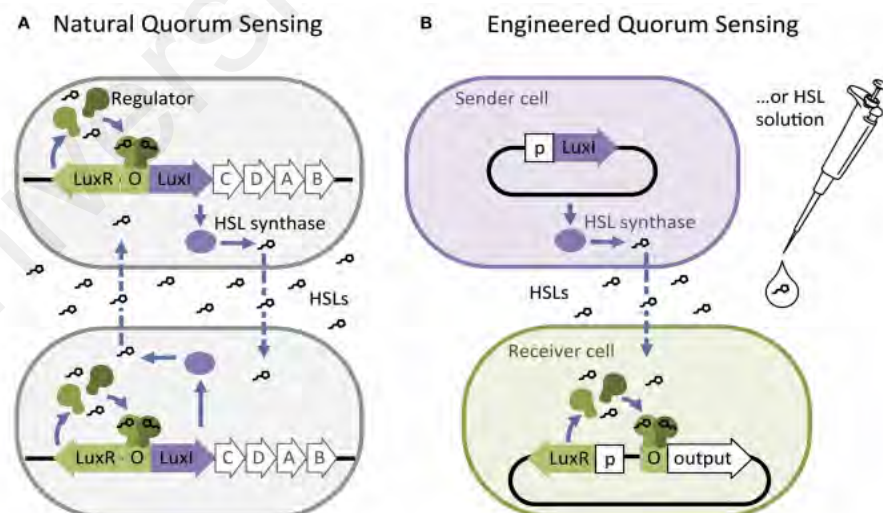
Figure 2.13: The structure of natural and artificial homoserine lactone networks **(A)** Natural HSL QS networks such as the luciferase expression system in *V. fischeri* regulate the expression of gene clusters (e.g., LuxR, I, C, D, A, and B) **(B)** QS networks have been decoupled and used to build engineered, synthetic systems to control the expression of any gene of interest (output). O = "Operator" binding site for the regulator protein, p = constitutive promoter (Davis *et al*., 2015).

2.6   Transcriptomic: RNA-sequencing

Transcriptome represents a complete set of transcripts from protein coding (mRNA) to noncoding RNA (tRNA, rRNA, lncRNA, pri-miRNA) of a specific type of cell or entire organism (Ishii, 2014; Thompson *et al.*, 2016). The analysis of the transcriptome data can help to identify the genes or the pathways that are differentially expressed in different conditions and this can offer a whole perspective on the molecular activity in the cells (Liang, 2013; Ishii, 2014). In addition to that, the massive information produced can helps the scientist to gain better understanding in the genomic variations and disease mechanisms as well as resistance in which it is important in the development of better diagnostics or therapies. The methods that were used to analyse RNA was once limited to either Northern blots or quantitative PCR (qPCR) but with microarrays and next generation sequencing (NGS) like RNA-sequencing, the studies of high throughput transcriptomics has then become possible (Liang, 2013).

Both microarray and RNA-sequencing has the same workflow that includes (a) experimental design (b) data acquisition and (c) data analysis and interpretation. However, RNA-sequencing seems to show strong prospective to replace microarray for whole-genome transcriptome profiling as it can overcome some limitations such as sensitivity, discovery and range of expression (Trapnell *et al.*, 2009; Mutz *et al.*, 2013). RNA-sequencing produced lower background signal as compared to microarray and can eliminate cross-hybridization or non-ideal hybridization (Wang *et al.*, 2009; Zhao *et al.*, 2014). Furthermore, RNA-sequencing allows researchers to detect known transcripts as well as exploring the new ones while microarrays are confined to the reference information available during the production (Wang *et al.*, 2009; Zhao *et al.*, 2014). RNA-sequencing also has the ability to quantify a huge dynamic range of expression

levels even with organisms that are lacking in reference genome. RNA-sequencing can also be used to study complex transcriptomes as well as to reveal sequence variations like single nucleotide polymorphisms (SNPs) (Cloonan *et al*., 2008).

There are a few available sequencing platforms to conduct RNA-sequencing and this includes Illumina (Marioni *et al*., 2008), Roche/454 FLX (Chun *et al*., 2010), Applied Biosystems SOLiD™ (Cloonan *et al*., 2008), Pacific Biosciences SMRT (English *et al*., 2012) and Helicos HeliscopeTM (Wang *et al*., 2009). A comparison study between Illumina platform and other existing technologies has shown that the data obtained from Illumina were found to be highly reproducible with low technical variation. Illumina sequencing also allow the identification of differentially expressed genes as well as other analysis such as alternative splice variants and novel transcripts (Marioni *et al*., 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

Table 3.1: List of bacterial strains/plasmids used in this study.

Strains or plasmids	Relevant genotype/Description	Reference/Source
P. aeruginosa PAO1	Wild type	Chan *et al.*, 2011
S. aureus	Wild type	University Malaya Medical Centre
C. violaceum CV026	Double mini-Tn*5* mutant derived from ATCC 31532 Kan[R], Hg[R], *cviI*::Tn*5* *xylE*, plus spontaneous Str[R] AHL biosensor	McClean *et al.*, 1997
E. coli [pSB401]	luxRluxI (*P. fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 299999]) pACYC184-derived, Tet[R]	Winson *et al.*, 1998
E. coli [pSB1075]	lasRlasI (*P. aeruginosa* PAO1)::*luxCDABE* (*Photorhabdus luminescens* [ATCC 299999]) fusion in Tet[R]	Winson *et al.*, 1998
P. aeruginosa PAO1 *lecA::lux*	*lecA::luxCDABE* genomic reporter fusion in PAO1	Winzer *et al.*, 2000
E. coli (MG1655::pBAD24-*lasI*)	L-arabinose-dependent expression plasmids that were constructed and transformed into *E.coli* MG1655, harbouring *P. aeruginosa* PAO1 las*I* genes	Chang *et al.*, 2014
E. coli (MG1655::pBAD24-*rhlI*)	L-arabinose-dependent expression plasmids that were constructed and transformed into *E.coli* MG1655, harbouring *P. aeruginosa* PAO1 rhl*I* genes	Chang *et al.*, 2014

Legends:
* Kan[R], Hg[R], Str[R], Tet[R] – resistant to kanamycin, mercury, streptomycin and tetracycline respectively
* Mini-Tn*5* – mini transposon insertion
* ATCC – American Type Culture Collection

3.1.2   Growth Media

All media were sterilized by autoclaving at 121℃, 15 psi for 20 minutes, unless stated otherwise. Heat-sensitive solutions such as antibiotics and glucose were sterilized by filtration through sterile syringe filters with 0.22 µm pore size (Sartorius Minisart, Germany).

3.1.2.1 Lysogeny Broth (LB) Medium

LB medium (Scharlab, Spain) was used in this study. Both agar and broth was prepared based on the protocol given by the manufacturer.

3.1.2.2 Swarming agar

Swarming plates were prepared based on the method modified as described previously (Chen *et al.*, 2007) where it consists of 0.6% (w/v) Bacto™ agar (BD, USA), 0.6% (w/v) Bacto™ Peptone (BD, USA), 0.2% (w/v) yeast extract (BD, USA) and 0.5% (w/v) glucose (Merck, USA) in 1 L of distilled water. The plates were then leave to air-dry in the laminar flow for 15 minutes prior to using it.

3.1.2.3 AHL Extraction Medium

For the extraction of AHL, bacterial cells were grown in LB buffered with 50 mM MOPS (3-[*N*-morpholino] propanesulfonic acid) (Merck, USA) adjusted to pH 6.8 in order to avoid lactonolysis (Yates *et al.*, 2002).

3.1.3　Chemical Reagents

All the chemical reagents used were of analytical grade or of the highest grade obtained from Amresco®, USA; BD, Difco™ Laboratories, USA; Calbiochem, Germany; Cayman Chemicals, USA; Fisher Scientific, USA; Merck KGaA, Germany; MP Biomedicals, USA and Sigma-Aldrich, USA. High performance liquid chromatography (HPLC) grade solvents for acetonitrile, chloroform, cyclohexane, ethanol, ethyl acetate, dichloromethane, dimethyl sulfoxide (DMSO), hexane, hydrochloric acid, methanol and methanol-D4 were obtained from Merck KGaA, Germany.

3.1.4　Commercial Kits

Table 3.2: List of commercial kits used in this study.

Instruments	Manufacturer/Origin
Agilent Bioanalyzer-High Sensitivity DNA Chip	Agilent Technologies, USA
Agilent Bioanalyzer-RNA 6000 Nano Kit	Agilent Technologies, USA
HiSeq Rapid V2 SBS (200 cycles)	Illumina, USA
KAPA Library Quantification Kit	KAPA Biosystems, USA
MasterPure RNA Purification Kit with DNase treatment	Epicentre, USA
MiSeq Reagent Kit V2 (50 cycles)	Illumina, USA
Qubit® dsDNA High Sensitivity (HS) Assay Kit	Life Technologies, USA
RiboZero™ rRNA Removal Kit (Bacteria)	Epicentre, USA
RiboGuard™ RNase Inhibitor	Epicentre, USA
TruSeq Stranded mRNA Library Preparation Kit	Illumina, USA

3.1.5 Instruments

Table 3.3: List of instruments used in this study.

Instruments	Manufacturer/Origin
Agilent 2100 Bioanalyzer	Agilent Technologies, USA
Autoclave	Hirayama, Japan
Cary 60-UV Vis Spectrophotometer	Agilent Technologies, USA
Centrifuge	Eppendorf, Germany
Class II, Type A2 Biological Safety Cabinet	Thermo Scientific, USA
HiSeq 2500 Sequencing System	Illumina, USA
Incubators	Memmert, Germany
Industrial grade blender	Waring, USA
Illumina ECO™ Real-Time PCR System	Illumina, USA
Laminar flow	Esco, USA
Rapid Resolution Liquid Chromatography (RRLC)	Agilent Technologies, USA
Preparative High Performance Liquid Chromatography (HPLC) Pump System	Gilson, UK
Liquid Chromatography - Nuclear Magnetic Resonance Mass Spectrometry System (LC NMR/MS) [Bruker Avance III +Maxis Impact]	Bruker, Switzerland
Microfluidic Bioflux Shear Flow System	Bioflux, USA
Milli-Q® Integral Water Purification System for Ultrapure water	Merck, Germany
Nanodrop 2000 Spectrophotometer	Thermo Scientific, USA
Pipettes	Eppendorf, Germany
Qubit® 2.0 Fluorometer	Life Technologies, USA
Rotary evaporator	EYELA, Japan
Shaking incubator (Certomat® BS-1)	Sartorius Stedim biotech, France
Sonicator	Branson, USA
Tecan infinite M200 microplate reader	Tecan, Switzerland
Thermomixers	Eppendorf, Germany
Vortex BenchMixer™	Benchmark Scientific, USA
Weighing machines	Sartorius AG, Germany

3.1.6   Plants and Purified Compounds

3.1.6.1   Selection of Plant Materials

A total of 15 plant samples were collected from various places and the identity of the plants were identified at Rimba Ilmu, University of Malaya. The plant samples were chosen based on the numerous finding regarding to their medicinal properties.

Table 3.4: Plants tested for anti-QS activities.

No.	Plant	Scientific Name	Part of Plant
1	Root of the holy ghost	*Angelica dahurica*	Roots
2	Chain fern rhizome	*Rhizoma cibotii*	Rhizomes
3	Self-heal	*Prunella vulgaris*	Stem, leaves, flowers and fruits
4	Japanese catnip	*Schizonepeta tenuifolia*	Stem, leaves, flowers and fruits
5	Weeping forsythia	*Forsythia suspense*	Fruit
6	Fish leaf	*Houttuynia cordota*	Stem, leaves and flowers
7	Mangosteen	*Garcinia mangostana*	Leaves
8	Horse mango	*Mangifera foetida*	Leaves
9	Java apple	*Syzygium samarangense*	Leaves
10	Star fruit	*Averrhoa carambola*	Leaves
11	Pandan leaves	*Pandanus amaryllifolius*	Leaves
12	Lemongrass	*Cymbopogon citratus*	Leaves
13	Bitter gourd	*Momordica charantia*	Fruit
14	Parsley	*Petroselinum crispum*	Leaves
15	Spearmint	*Mentha spicata*	Leaves

3.1.6.2   Purified Compounds

Table 3.5: Purified compounds tested for anti-QS activities.

Compounds	Sources	Part of plant
Catechin	Purified from *Garcinia mangostana* (Table 3.4)	Leaves
Malabaricone C	Obtained from Department of Pharmacology, Faculty of Medicine, University of Malaya (collaboration work); purified from *Mystrica cinnamomea*	Bark

3.1.7   Stock Solutions

3.1.7.1 Synthetic N-acyl-homoserine Lactones (AHLs)

The synthetic AHLs used in this study was obtained from Sigma-Aldrich (USA) and Cayman Chemicals (USA). The synthetic AHLs was prepared by dissolving the powder with acetonitrile and stored in -20°C before dissolving into desired concentrations for further usage later.

3.1.7.2 Antibiotics

Ampicillin was purchased from Amresco® (USA) while tetracycline was from Calbiochem (Germany). For ampicillin, a stock solution of 100 μg/mL was prepared by dissolving the powder in distilled water. Tetracycline, on the other hand, was dissolved in ethanol with the concentration of 20 μg/ml. Both the antibiotics were filtered sterilized and stored at -20°C for further use.

3.1.7.3 Crude Extracts and Purified Compounds

The crude extracts and purified compounds used in this study were dissolved with absolute dimethyl sulfoxide (DMSO) (Merck KGaA, Germany) at 10 mg/mL concentration, unless stated otherwise, followed by sterilization through a syringe filter with pore size of 0.22 μm (Sartorius Minisart, Germany). The stock solutions can then be stored in -20°C and were diluted with sterile ultrapure water to the desire concentration prior to use.

3.1.7.4 Synthetic Compounds

Synthetic catechin obtained from Sigma-Aldrich (USA) was dissolved with DMSO (Merck KGaA, Germany) at 10 mg/mL concentration and was used for 2 purposes:

a) to confirm the structure of the catechin purified from *G. mangostana* leaves

b) as a positive control for malabaricone C publication (Chong *et al*., 2011)

Synthetic trans-cinnamaldehyde from Sigma-Aldrich (USA) was used as control for *E. coli* MG1655 carrying pBAD-*lasI* and pBAD-*rhlI* bioassays while synthetic gallic acid from Sigma-Aldrich (USA) was used as control in swarming assays for catechin and malabaricone C.

3.1.8 Nuclear Magnetic Resonance (NMR)

Methnol-D4 (Merck KGaA, Germany) was used to dissolve the bioactive compound purified from *G. mangostana* leaves in order to confirm the structure in Liquid Chromatography-Nuclear Magnetic Resonance Mass Spectrometry System (LC-NMR/MS) [Bruker Avance III+Maxis Impact] (Bruker, Switzerland).

3.2   Methods

3.2.1   Growth Conditions

All the bacteria strains were grown on Lysogeny (LB) medium (Scharlab, Spain) at 37°C except for *C. violaceum* CV026 which was incubated at 28°C. Broth cultures were grown with shaking at 220 rpm with the liquid filling up to no more than one-fifth of the flask total volume in order to obtain optimal aeration. For some QS bioassays, the media will be supplemented with antibiotics (Amresco®, USA; Calbiochem, Germany), glucose (Merck, USA) and L-arabinose (MP Biomedicals, France).

3.2.2   Growth Curve

The growth of the bacterial was measured using previously reported method (Hayouni *et al.*, 2008) with slight modification in order to make sure that the crude extracts samples and purified compounds do not cause any antibacterial activity that may lead to false anti-QS results. Overnight bacteria cultures were diluted to OD_{600nm} of 0.1 before adding into 96-well microtitre plate consisted of 230 µL of diluted bacteria cultures and 20 µL of samples. The bacteria were then incubated at their optimum temperature and the optical density OD_{600nm} were determined every 30 minutes for 24 hours by Tecan Infinite M200 microplate reader (Switzerland).

3.2.3   Extraction of Plant Samples

3.2.3.1 Crude Extracts and *Garcinia mangostana*

The plant samples that were used in this research were typically obtained from the shops, market or orchard. The samples were first cleaned with distilled water followed by soaking with 95% (v/v) ethanol (Merck KGaA, Germany) and dried for 3-5 days with oven at 45ºC before ground into fine powder form with industrial grade blender (Waring, USA). Approximately 50 g of the crude extracts dried powders were then sequentially soaked and extracted with 100% (v/v) hexane, chloroform and methanol (Merck KGaA, Germany). For purification of *G. mangostana*, a total weight of 750 g dried powder were soaked and extracted with the solvents mentioned above. The extracts were then filtered by using Whatmann No. 1 filter paper (Macherey-Nagel, Germany) and were subsequently removed under low vacuum pressure by using the rotary evaporator (EYELA, Japan) adjusted to 45ºC. A crude condense concentrated extracts were subsequently resuspended with 100% (v/v) DMSO (Merck KGaA, Germany) to a final concentration of 10 mg/mL as stock solution, unless stated otherwise, and can then be diluted with sterile ultrapure water to desire concentration before used to test against the anti-QS activities. The concentration used to test the QS bioassays was 1 mg/mL for both crude extracts and bioactive compounds fractionated from *G. mangostana*.

3.2.3.2 *Mystrica cinnamomea*

Malabaricone C was obtained from the Department of Pharmacology, Faculty of Medicine, University of Malaya. Briefly, 50 g of dried powdered bark of *M. cinnamomea* was extracted with methanol, filtered and dried *in vacuuo* to yield the crude extracts. Extracts were resuspended in 100% (v/v) DMSO (Merck KGaA, Germany) to a concentration of 50 mg/mL and then diluted with sterile ultrapure water to form plates with 1, 2 and 3 mg/mL concentration for the screening of anti-QS activities.

3.2.4   Purification of Plant Samples

3.2.4.1 *G. mangostana*

Column chromatography with silica gel was attached to a preparative high performance liquid chromatography (HPLC) pump system (Gilson, UK) in order to separate and purify the bioactive compounds found in *G. mangostana*. A final weight of 384 g of crude extract that was extracted with chloroform was obtained. The extract was then dissolved in methanol followed by sonication before adding silica gel. The silica gel that was infused with crude extracts dissolved in methanol was then subjected to evaporation before adding into the column. The column filled with the extract was eluted with cyclohexane–ethyl acetate (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 10;90; 0:100) followed by ethyl acetate–methanol (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 10:90; 0:100). The bioactive molecules obtained was then subjected to ^{1}H NMR identification with Liquid Chromatography-Nuclear Magnetic Resonance Mass

Spectrometry System (LC-NMR/MS) [Bruker Avance III+Maxis Impact] (Bruker, Switzerland).

3.2.4.2 *M. cinnamomea*

The mass spectra profile was obtained using a Shimadzu UFLC-IT-TOFMS mass spectrometer. Eluent flow was split 1:4 post UV detector using a passive splitter between the mass spectrometer and a Gilson FC204 fraction collector for collection of fractions into 96-well microtitre plates. Two methods were used: a) Column Hypersil BDS C18, 4.6×150 mm (particle size 5 μm) at 40°C, solvents H_2O (0.1% (v/v) formic acid)/acetonitrile (0.1% (v/v) formic acid), flow rate 1.0 mL/min, gradient: 0-14 min 10-100% (v/v) MeCN, 14-18 minutes, 100% (v/v) acetonitrile, 18 fractions collected at 1 minute intervals and b) Column Waters Xbridge C18, 2.2×50 mm, (particle size 2.5 μm) at 40°C, solvents H_2O (0.1% (v/v) formic acid)/acetonitrile (0.1% (v/v) formic acid), flow rate 0.5 mL/min, gradient: 0-7 minutes 10-100% (v/v) acetonitrile, 7-9 minutes 100% (v/v) acetonitrile, 18 fractions collected at 0.5 minutes intervals. Fractions were dried using a centrifugal evaporator and re-prepared with sterile ultrapure water for the relevant anti-QS assays.

3.2.5 Screening of Crude Extracts and Purified Compounds for Anti-QS Activities

3.2.5.1 Quantitative Analysis of Violacein Production in *C. violaceum* CV026

The quantitative analysis of violacein production was done based on the reported method (Chong *et al*., 2011) with slight modification. Overnight culture of *C. violaceum* CV026 was first adjusted to OD_{600nm} of 1.2 before supplemented with 0.125 µg/mL of synthethic C6-HSL. Next, 100 µL of CV026 diluted culture was transferred into the 96-wells microtitre plate followed by the addition of 10 µL of samples. The microplate was incubated for 16 hours at $28^{o}C$ with agitation of 220 rpm. The plate was then dried at $60^{o}C$ until all the medium had evaporated before adding 100 µL of DMSO to each of the well in order to dissolve the dried violacein. The plate was subsequently incubated for another additional 2 hours at $28^{o}C$ with shaking. The absorbance for each of the well was measured at OD_{590nm} with Tecan Infinite M200 microplate reader (Switzerland). All the experiments were done in triplicate, in three independent experiments.

3.2.5.2 Bioluminescence Assay

The bioluminescence assay was determined by using the method reported previously (Winzer *et al*., 2000) with modification. *E. coli* [pSB401] and *E. coli* [pSB1075] was grown overnight in LB medium at $37^{o}C$ with addition of 20 µg/mL tetracycline added into the LB medium. In the 96-well microtitre plate, 20 µL of samples were added to 230 µL of the bacteria culture that was diluted to OD_{600nm} of 0.1. For *E. coli* [pSB401], 0.001 µg/mL of synthetic 3-oxo-C6-HSL was added while 0.0125 µg/mL of 3-oxo-C10-HSL was added into *E. coli* [pSB1075]. The luminescence and

turbidity of the cultures were read every 30 minutes for 24 hours with Tecan Infinite M200 microplate reader (Switzerland) at OD_{600nm}. A graph was plot based on luminescence given in relative light units (RLU) per unit of turbidity (OD_{600nm}). All the experiments were done in triplicate, in three independent experiments.

3.2.5.3 *P. aeruginosa* PAO1 *lecA::lux* Expression Assay

Briefly, 20 µL of samples were added into the 96-well microtitre plate filled with 230 µL of *P. aeruginosa* PAO1 *lecA::lux* culture that was diluted to OD_{600nm} of 0.1 (Tan *et al.*, 2012). The plate was then incubated at 37°C and the luminescence were taken every 30 minutes for 24 hours using Tecan Infinite M200 microplate reader (Switzerland) at OD_{600nm}. A graph was plot based on luminescence given in relative light units (RLU) per unit of turbidity (OD_{600nm}). All the experiments were done in triplicate, in three independent experiments.

3.2.5.4 Pyocyanin Assay

Pyocyanin was extracted from overnight *P. aeruginosa* PAO1 culture supernatant as previously described (Chong *et al.*, 2011). Briefly, 500 µL of samples were supplemented into 4.5 mL of overnight culture that was diluted to OD_{600nm} of 0.1 and incubated at 37°C for 24 hours. The treated cell culture was then extracted with 3 mL of chloroform (Merck KGaA, Germany) and vortexed for 5 minutes followed by centrifuged at 9000 rpm for 10 minutes. Next, the chloroform layer was transferred to a fresh tube and 1 mL of 0.2 M hydrochloric acid (Merck KGaA, Germany) was added by mixing thoroughly. After centrifugation at 9000 rpm for 10 minutes, the top layer of the mixture was removed and the absorbance was read at 520 nm by using Tecan Infinite

M200 microplate reader (Switzerland). All the samples for this assay were done in triplicate, in three independent experiments.

3.2.5.5 Elastolytic Assay

The elastolytic activity was generally the cleavage of the ECR in which it will releases soluble red pigment and this can be ascertained by using the elastin-Congo red (ECR) (Merck, Germany) as stated previously (Al-Ansari, 2009). A total volume of 500 µL samples were cultured together with 4.5 mL of overnight *P. aeruginosa* PAO1 that was diluted to OD_{600} of 0.1 and incubated at 37°C for 13 hours. The co-cultured was then centrifuged for 10 minutes at 9000 rpm and the supernatant was subsequently filtered using the filter membrane. Next, 1 mL of the filtered supernatant was added to 1 mL of the ECR buffer (100 mM Tris-HCl and 1 mM $CaCl_2$ with pH 7.2) that contains 20 mg of ECR and was then incubated at 37°C for 3 hours with shaking. After the incubation, 0.2 mL of 0.12 M Na_2EDTA was immediately added in order to stop the reaction. The insoluble ECR was later removed by centrifugation for 10 minutes at 9000 rpm and the absorption of the supernatant was measured with Tecan Infinite M200 microplate reader (Switzerland) at the absorbance of 495 nm. All the experiments were done in triplicate, in three independent experiments.

3.2.5.6 Staphylolytic Assay

LasA protease activity was quantified by determining the ability of *P. aeruginosa* PAO1 culture supernatants to lyse the boiled *S. aureus* cells based on the previous research (Diggle *et al*., 2002) with slight modification. A 30 mL volume of the overnight culture of *S. aureus* was boiled for 10 minutes followed by centrifuged at

9000 rpm for 10 minutes. The pellet was then resuspended with 0.02 M of Tris-HCl with pH 8.5 to an OD_{600} of approximately at 0.8. Next, 100 µL of diluted overnight culture of *P. aeruginosa* PAO1 was cultured together with 100 µL of samples. The mixture was then centrifuged for 10 minutes at 9000 rpm and the supernatant was consequently added to 900 µL of the *S. aureus* suspension. The controls for this assay include DMSO and *P. aeruginosa* PAO1.The reading was determined at OD_{600} for every 5 minutes interval for 1 hour duration with the use of Tecan Infinite M200 microplate reader (Switzerland). All the experiments were done in triplicate, in three independent experiments.

3.2.5.7 Swarming Motility Assay

The swarming plates were based on 0.6% (w/v) LBA supplemented with final concentration of 0.5% (w/v) glucose and the method was modified as described previously (Chen *et al*., 2007). For crude extracts, 1 mL of the samples were mixed together with 30 mL of agar before pouring into the petri dish. For fractionated and purified compounds (catechin and malabaricone C), the volume was adjusted to 150 µL of samples with 5 mL of agar poured into 6-well plates. The plates were then leave to air-dry for 15 minutes and 1 µL of the overnight culture of *P. aeruginosa* PAO1 with OD_{600nm} of 0.1 was point inoculated at the center of the agar surface. The plates were incubated statically at 37°C for 16 hours. All the experiments were done in triplicate, in three independent experiments.

3.2.5.8 Biofilm Assay

Both of the purified compounds, catechin and malabaricone C, were screened for the inhibition of the biofilm using static microtiter plate assay based on the method as described previously (Vandeputte *et al.*, 2010). Overnight culture of *P. aeruginosa* PAO1 was first diluted to OD_{600} of 0.1 before adding 50 µL of the diluted culture into 930 µL of LB medium supplemented with 0.5% (w/v) of filter-sterilised glucose (Merck, USA) and 20 µL of malabaricone C and catechin. The *P. aeruginosa* PAO1 cells were cultured in the sterile 50 mL plastic tubes and were incubated statically for 18 hours at 37°C. The planktonic bacteria were removed by washing five times with sterile distilled water. The tubes were then air dried for 15 minutes and was stained with 1 mL of 1% (v/v) crystal violet (Merck KGaA, Germany) for 45 minutes. The crystal violet was discarded and the stained biofilms were washed with sterile distilled water for five times. The quantitative analysis of biofilm production was done by adding 2 mL of 95% (v/v) ethanol (Merck KGaA, Germany) and 100 µL of the resulting solution was transferred to a new 96-well microtitre plate and the absorbance of the solution was read at 590 nm with Tecan Infinite M200 microplate reader (Switzerland). All the experiments were performed in triplicate, in three independent experiments.

3.2.6 Statistical Analysis

All the assays were performed in triplicate and it represents the average of three independent experiments. The significance of the data was presented as mean $\pm$ standard deviation (SD) and analyzed using ANOVA test ($p < 0.05$) using GraphPad Prism Software v7.

3.2.7 Determine the Mode of Action of Catechin and Malabaricone C against *P. aeruginosa* PAO1 QS System

3.2.7.1 Bacterial Strains

The screening of catechin and malabaricone C as QS signal synthase antagonists or transcriptional regulator protein antagonists were carried out based on previous research with slight modifications (Chang *et al.*, 2014) by using *E. coli* MG1655::pBAD24-*lasI* and *E. coli* MG1655::pBAD24-*rhlI*. Both of the *E. coli* consist of arabinose-dependent expression plasmids with pBAD-*lasI* and pBAD-*rhlI* harboring *P. aeruginosa* PA01 *lasI* and *rhlI*, respectively.

3.2.7.2 AHLs Extraction

E. coli MG1655::pBAD24-*lasI* and *E. coli* MG1655::pBAD24-*rhlI* were grown overnight at 37°C for 24 hours in 10 mL LB broth buffered with 50 mM MOPS (Merck, USA) adjusted to pH 6.8 in order to avoid spontaneous degradation of AHLs (Yates *et al.*, 2002). Overnight cultures were then diluted at 1:50 ratio into 10 mL of fresh LB broth supplemented with 50 mM MOPS, 1% (w/v) L-arabinose (MP Biomedicals, France) and 1 mg/mL of catechin or malabaricone C. The mixture was incubated at 37°C for 5 hours. At desired time point, the AHLs were extracted using ethyl acetate (Merck KGaA, Germany) and were subsequently evaporated to complete dryness and stored in -20°C for further analysis.

3.2.7.3 Detection of AHLs with *E. coli* [pSB401] and *E. coli* [pSB1075]

The extracted AHLs from the culture supernatant were dissolved in sterile ultrapure water and tested using the biosensors *E. coli* [pSB401] and *E. coli* [pSB1075] where it can detect short and long chain AHLs, correspondingly. In the 96-well microtitre plate, 5 µL of samples were added to 245 µL of the bacteria culture that was diluted to OD_{600nm} of 0.1. The luminescence and turbidity of the cultures were read every 30 minutes for 24 hours at 37ºC with Tecan Infinite M200 microplate reader (Switzerland) at OD_{600nm}. A graph was plot based on luminescence given in relative light units (RLU) per unit of turbidity (OD_{600nm}). All the experiments were done in triplicate, in three independent experiments.

3.2.8 Whole Transcriptome Shotgun Sequencing or RNA-Sequencing Analysis of Catechin and Malabaricone C

3.2.8.1 Bacteria and Culture Conditions

All the glassware was treated with RNaseZAP™ (Sigma-Aldrich, Germany) and rinsed with RNase-free water in order to remove any surface-contaminant. The glassware and consumables such as tips and tubes were then autoclaved at 121°C for 15 psi for 30 minutes.

P. aeruginosa PAO1 was first streaked on LB agar and incubated for 18 hours at 37°C to make sure the colonies obtained were pure. Next, a single colony was picked from the LB agar and inoculated into fresh, sterile LB broth with 18 hours incubation at 37°C, 220 rpm in a shaking incubator. A total volume of 500 µL of the overnight culture was transferred into fresh flask that contains 50 mL LB broth pre-warmed to 37°C. In this experiment, 3 flasks will be supplemented with 1 mg/mL of catechin and malabaricone C, respectively, 3 flasks added with DMSO as solvent control and another 3 flasks without any compounds or solvents as it will act as untreated *P. aeruginosa* PAO1. The culture was then incubated with shaking at 37°C to mid-exponential phase and was extracted when the OD_{600} reached 0.5. Lastly, 1 mL of the extracted culture was transferred into a new, sterile 2 mL microcentrifuge tubes in which the cells were immediately subjected to RNA extraction. Each of the treatments were conducted separately in triplicate, in three independent experiments.

Catechin and malabaricone C were resuspended in 100% (v/v) DMSO to a concentration of 20 mg/mL. Both of the samples and DMSO were diluted a few rounds with sterile ultrapure water (20 mg/mL → 15 mg/mL → 10 mg/mL → 8 mg/mL → 6 mg/mL → 4 mg/mL → 2 mg/mL → 1 mg/mL) before adding into the LB media in order to avoid solvent toxicity.

3.2.8.2 RNA Extraction and Purification

MasterPure RNA Purification Kit with DNase treatment (Epicentre, USA) was used to extract and purify the total RNA. Based on the manufacturer's protocol, there were 3 steps involved starting with the lysis of the cell samples, precipitation of the total nucleic acid and finally the removal of the contaminating DNA from the total nucleic acid preparation. The extracted RNA was eluted with 40 µL of sterile, RNAse-free water in addition of 1 µL of RiboGuard^{TM} RNase Inhibitor (Epicentre, USA).

3.2.8.3 Determination of RNA Quality, RNA Concentration and RNA Integrity Number (RIN)

In order to obtain optimal cDNA library preparations and sequencing quality, all the extracted RNA must be subjected to strict quality check. The qualities of the extracted RNA samples were quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) where samples with values of A_{260}/A_{280} and A_{260}/A_{230} more than 2.0 were selected for further work. If the value given by NanoDrop is lower than 2.0, it indicates that the samples extracted is either contaminated or contain proteins (Teare *et al.*, 1997).

Agilent Bioanalyzer-RNA 6000 Nano Kit (Agilent Technologies, USA) was used to check the integrity and level of degradation of RNA samples as the RNA Integrity Number (RIN) will determine if the samples can be used for downstream work. RNA samples with RIN value more than 7.5 were selected for rRNA depletion by using the RiboZero™ rRNA Removal Kit (Bacteria) (Epicentre, USA) as the kit can remove ribosomal RNA (rRNA) form the intact and partially degraded samples. There are 4 steps in the protocol given by the manufacturer which include preparation of magnetic beads, treating the samples with rRNA Removal Solution, removal of rRNA and purification of rRNA-depleted samples before proceeding with the library preparation. In addition, RNA samples were also assessed for the loss of intact rRNA with Agilent Bioanalyzer-RNA 6000 Pico Kit (Agilent Technologies, USA) according to the manufacturing user guide.

3.2.8.4 cDNA Library Preparation and RNA-Sequencing

For library preparation, 5 µL of rRNA-depleted RNA was used as input while TruSeq Stranded mRNA Library Preparation Kit (Illumina, USA) was used to perform library construction based on the given protocol. The library size and quality were then determined with Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA), Agilent Bioanalyzer-High Sensitivity DNA Chip (Agilent Technologies, USA) as well as KAPA Library Quantification Kit (KAPA Biosystems, USA) coupled with Illumina ECO™ Real-Time PCR System (Illumina, USA). All libraries were normalized to 4 nM according to the quality check result and pooled 5 µL each before further proceed with MiSeq Reagent Kit V2 (50 cycles) (Illumina, USA) to check for the libraries distribution and cluster efficiency. Finally, once the libraries were re-pooled based on the MiSeq LibQC performance to get even distribution, all the samples were

then subjected to HiSeq Rapid V2 SBS (200 cycles) (Illumina, USA) using the HiSeq 2500 Sequencing System (Illumina, USA).

3.2.8.5 Sequence Analysis

For data analysis, *P. aeruginosa* PAO1 obtained from National Centre for Biotechnology Information (NCBI) was used as the reference genome (GenBank accession number: AE004091). CLC Genomics Workbench version 7.5 (CLC Bio, Denmark) was used to trimmed the raw transcriptomics data obtained from the sequencing run (fastq) with default parameters and also to analyze the BAM files. ANOVA was used to determine the significant differentially expressed genes with filter parameters of $\geq$ 2-fold change, $\leq$ 2-fold change and *p* value < 0.05. Pseudomonas Genome Database (Winsor *et al*., 2015) was used to confirmed the sequences and functions of the genes obtained upon analysis.

CHAPTER 4: RESULTS

4.1   Crude Extracts

There were 15 plant samples (Table 3.4) used in this study. The samples were sterilized and were sequentially extracted with hexane, chloroform and methanol. A total of 45 crude extracts were screened for anti-QS activities which include CV026 violacein production, bioluminescence assay (*E. coli* [pSB401] and *E. coli* [pSB1075]), expression of *P. aeruginosa* PAO1 *lecA::lux* as well as *P. aeruginosa* PAO1 QS-related virulence such as pyocyanin assay, elastase assay, staphylolytic assay and swarming motility assay.

4.1.1   Bacteria Growth Curve

The growth of the tested bacteria (as shown in Appendixes A−E) cultured with 1 mg/mL of crude extracts were determined for a period of 24 hours in order to eliminate any antibacterial effects. All of the experiments were done in triplicate with absorbance read at OD_{600} and 10% (v/v) DMSO as negative control.

4.1.2 Screening of crude extracts for anti-QS Activities

4.1.2.1 Quantitative Analysis of Violacein in *C. violaceum* CV026

Crude extracts were screened for anti-QS by using *C. violaceum* CV026 as the preliminary bioassay. The production of the purple pigment in *C. violaceum* CV026 will only be produce when there was presence of synthetic short chain AHLs. *C. violaceum* CV026 was grown overnight with 1 mg/mL crude extracts supplemented with C6-HSL in 96-well microtitre plate at $28^{\circ}C$ with shaking. The plate was dried at $60^{\circ}C$ and 100% (v/v) DMSO was added to the dried violacein. The values of violacein production were expressed at OD 590 nm. DMSO was used as negative control. All of the experiments were done in triplicate. Based on the results, *Syzygium samarangense* in hexane extracts showed the strongest inhibition followed by *Pandanus amaryllifolius* extracts.
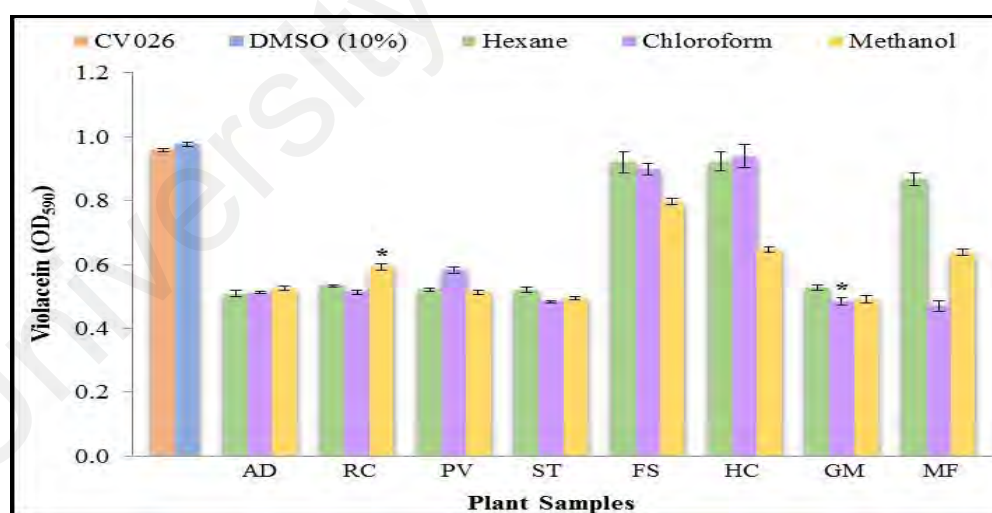
Figure 4.1a: The effects of crude extracts towards the inhibition of violacein production in *C. violaceum* CV026. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

AD	– *Angelica dahurica*	FS	– *Forsythia suspense*
RC	– *Rhizoma cibotii*	HC	– *Houttuynia cordota*
PV	– *Prunella vulgaris*	GM	– *Garcinia mangostana*
ST	– *Schizonepeta tenuifolia*	MF	– *Mangifera foetida*
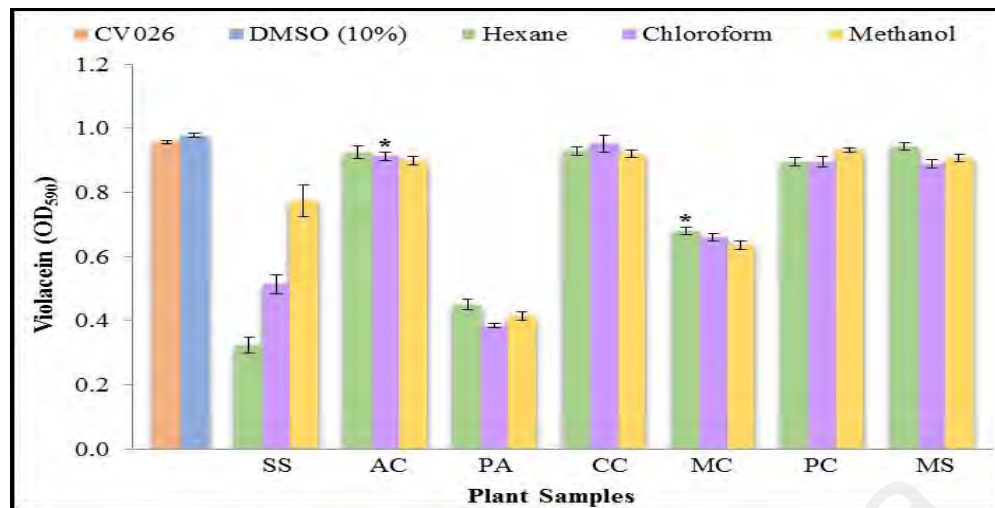
Figure 4.1b: The effects of crude extracts towards the inhibition of violacein production in *C. violaceum* CV026. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

SS	– *Syzygium samarangense*	MC	– *Momordica charantia*
AC	– *Averrhoa carambola*	PC	– *Petroselinum crispum*
PA	– *Pandanus amaryllifolius*	MS	– *Mentha spicata*
CC	– *Cymbopogon citratus*		

4.1.2.2 Inhibition of *lux*-based Biosensors in *P. aeruginosa* PAO1

The luminescence expression for both *Escherichia coli* DH5α biosensors were measured every 30 minutes for 24 hours by co-culturing with 1 mg/mL crude extracts. Synthetic 3-oxo-C6-HSL was added into *E. coli* [pSB401] while *E. coli* [pSB1075] required synthetic 3-oxo-C10-HSL in order to induce bioluminescence. The luminescence was determined by relative light unit (RLU) divided by OD_{600}. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. Table 4.1 summarize the results of the screening. The results showed that all the extracts from *A. dahurica* and *R. cibotii* can inhibit both lux-based biosensors while *G. mangostana* extracted with chloroform displayed strongest inhibition towards *E. coli* [pSB401] and *E. coli* [pSB1075].

Table 4.1: Plants tested for luminescence expression in *E. coli* DH5α biosensors.

No	Plants	*E. coli* [pSB401]			*E. coli* [pSB1075]		
		H	**C**	**M**	**H**	**C**	**M**
1	*Angelica dahurica*	**	**	*	**	**	**
2	*Rhizoma cibotii*	**	**	**	**	**	**
3	*Prunella vulgaris*	X	X	X	X	*	*
4	*Schizonepeta tenuifolia*	X	X	X	X	X	X
5	*Forsythia suspense*	X	X	*	X	X	X
6	*Houttuynia cordota*	X	*	*	X	X	X
7	*Garcinia mangostana*	**	***	X	*	***	*
8	*Mangifera foetida*	X	X	X	X	X	X
9	*Syzygium samarangense*	X	X	**	X	X	**
10	*Averrhoa carambola*	*	*	X	X	**	X
11	*Pandanus amaryllifolius*	X	X	X	X	X	*
12	*Cymbopogon citratus*	X	X	X	*	X	*
13	*Momordica charantia*	X	X	X	*	*	*
14	*Petroselinum crispum*	X	**	*	X	X	X
15	*Mentha spicata*	X	X	*	X	X	X

Note 1: The symbol "*" indicates weak inhibition; "**" indicates intermediate inhibition; "***" strong inhibition; "X" indicate negative results.
Note 2: 'H' indicates hexane; 'C' indicates chloroform; 'M' indicates methanol.

4.1.2.3 Inhibition of *lecA::lux* Expression in *P. aeruginosa* PAO1

The expression of *P. aeruginosa* PAO1 *lecA::lux* biosensors were measured every 30 minutes for 24 hours by co-culturing the biosensor with 1 mg/mL of crude extracts. The luminescence was determined by relative light unit (RLU) divided by OD_{600}. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. Table 4.2 summarize the results of the screening with *G. mangostana* extracted in hexane and chloroform showed the strongest inhibition as compared to the rest of the samples.

Table 4.2: Plants tested for expression in *P. aeruginosa* PAO1 *lecA::lux* biosensor.

No	Plants	*P. aeruginosa* PAO1 *lecA::lux*		
		Hexane	**Chloroform**	**Methanol**
1	*Angelica dahurica*	*	*	*
2	*Rhizoma cibotii*	X	*	X
3	*Prunella vulgaris*	X	X	X
4	*Schizonepeta tenuifolia*	X	X	X
5	*Forsythia suspense*	X	X	X
6	*Houttuynia cordota*	X	X	*
7	*Garcinia mangostana*	**	**	X
8	*Mangifera foetida*	X	*	X
9	*Syzygium samarangense*	*	X	X
10	*Averrhoa carambola*	X	X	X
11	*Pandanus amaryllifolius*	X	X	X
12	*Cymbopogon citratus*	X	X	X
13	*Momordica charantia*	X	*	X
14	*Petroselinum crispum*	X	*	X
15	*Mentha spicata*	X	X	X

Note: The symbol "*" indicates weak inhibition; "**" indicates intermediate inhibition; "***" strong inhibition; "X" indicate negative results.

4.1.2.4 Inhibition of Pyocyanin Production

P. aeruginosa PAO1 was grown overnight with 1 mg/mL crude extracts incubated at 37ºC with 10% (v/v) DMSO used as negative control. All of the experiments were done in triplicate, in three independent experiments. Some of the extracts such as *F. suspense*, *H. cordota*, *S. samarangense*, *A. carambola*, *P. crispum* and *M. spicata* displayed weak or no inhibition at all. Extracts of *G. mangostana*, on the other hand, exhibited the strongest inhibition.
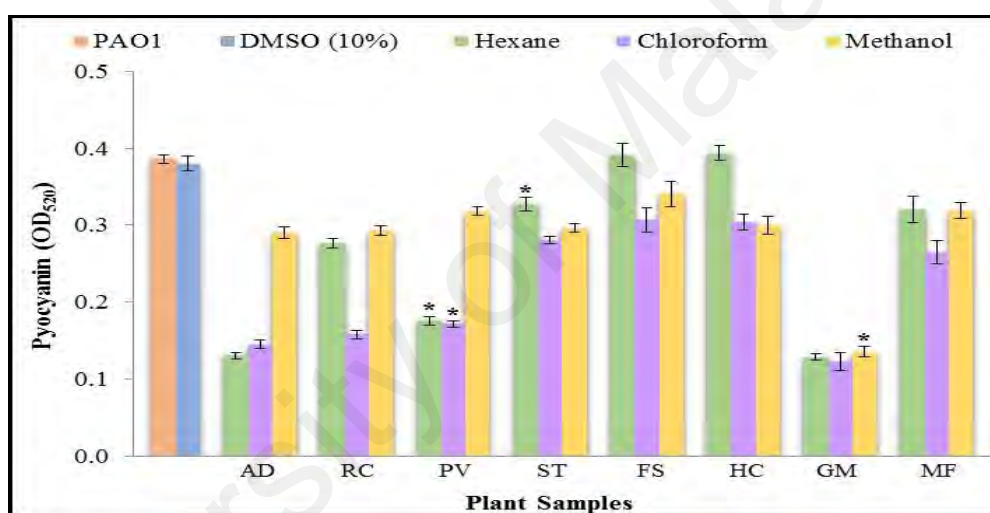
Figure 4.2a: The effects of crude extracts against the production of pyocyanin in *P. aeruginosa* PAO1. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

AD	– *Angelica dahurica*	FS	– *Forsythia suspense*
RC	– *Rhizoma cibotii*	HC	– *Houttuynia cordota*
PV	– *Prunella vulgaris*	GM	– *Garcinia mangostana*
ST	– *Schizonepeta tenuifolia*	MF	– *Mangifera foetida*
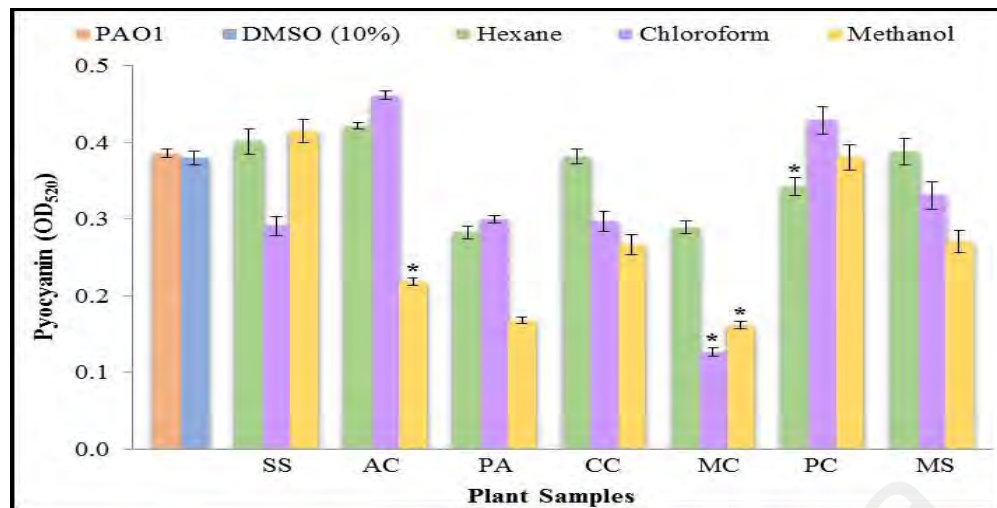
Figure 4.2b: The effects of crude extracts against the production of pyocyanin in *P. aeruginosa* PAO1. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

SS	– *Syzygium samarangense*	MC	– *Momordica charantia*
AC	– *Averrhoa carambola*	PC	– *Petroselinum crispum*
PA	– *Pandanus amaryllifolius*	MS	– *Mentha spicata*
CC	– *Cymbopogon citratus*		

4.1.2.5 Inhibition of Elastase Production

The effects of crude extracts against the production of elastase in *P. aeruginosa* PAO1 was measured with Tecan Infinite M200 at the OD of 495 nm. DMSO with 10% (v/v) concentration act as negative control. All of the experiments were done in triplicate, in three independent experiments. *A. dahurica* and *S. samarangense* extracts were shown to strongly inhibit the production of elastase while *P. vulgaris* and *P. amaryllifolius* were the weakest.
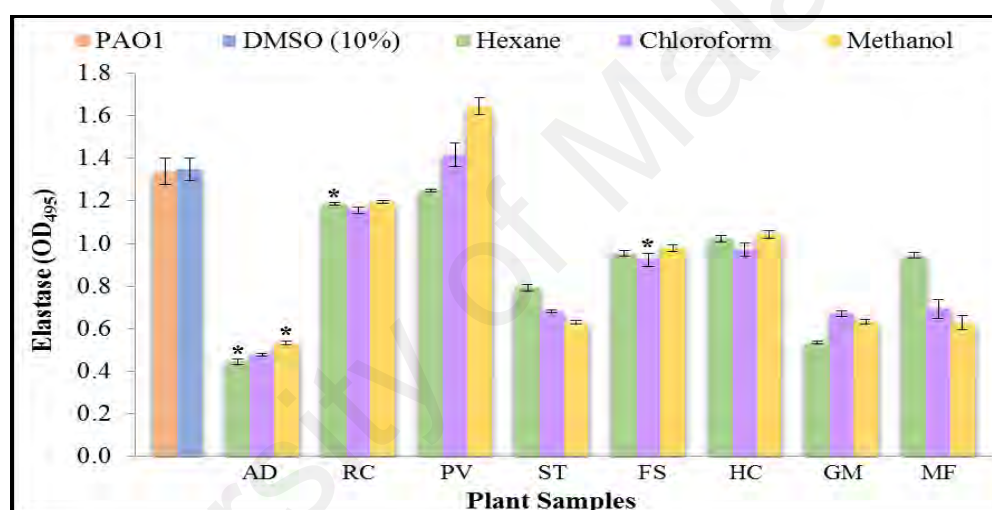
Figure 4.3a: The effects of crude extracts against elastase production in *P. aeruginosa* PAO1. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

AD	– *Angelica dahurica*	FS	– *Forsythia suspense*
RC	– *Rhizoma cibotii*	HC	– *Houttuynia cordota*
PV	– *Prunella vulgaris*	GM	– *Garcinia mangostana*
ST	– *Schizonepeta tenuifolia*	MF	– *Mangifera foetida*
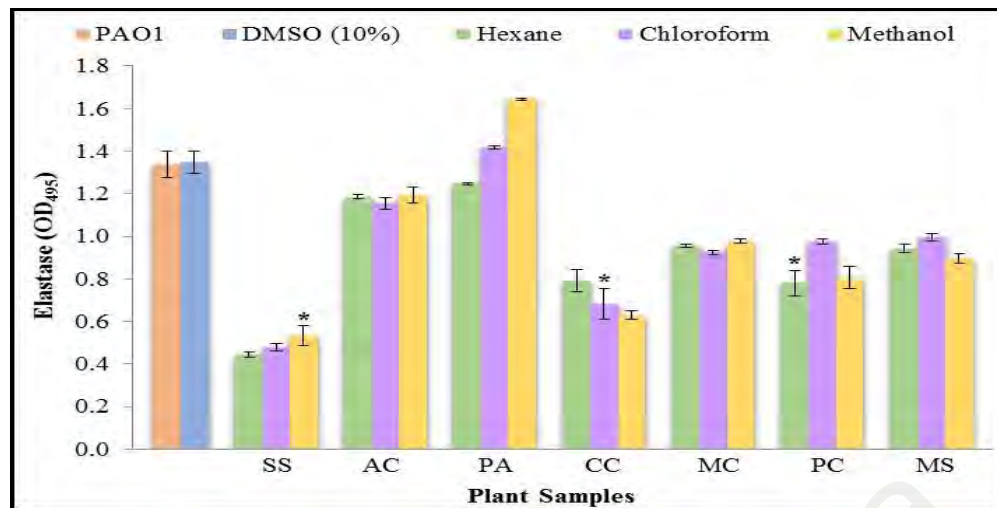
Figure 4.3b: The effects of crude extracts against elastase production in *P. aeruginosa* PAO1. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

Legends:

SS   – *Syzygium samarangense*   MC   – *Momordica charantia*
AC   – *Averrhoa carambola*   PC   – *Petroselinum crispum*
PA   – *Pandanus amaryllifolius*   MS   – *Mentha spicata*
CC   – *Cymbopogon citratus*

4.1.2.6 Inhibition of *P. aeruginosa* PAO1 Staphylolytic Activity

Analysis of the LasA protease activity caused by the lysis of *S. aureus* cells was measured every 5 minutes interval for 1 hour at the absorbance of 600 nm. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. All of the extracts presented negative results except for *G. mangostana* extracted with chloroform.
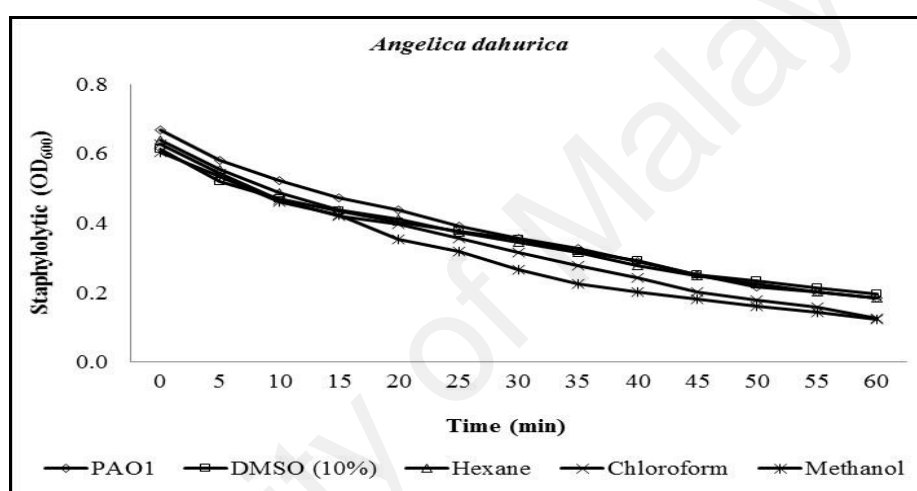
(A)
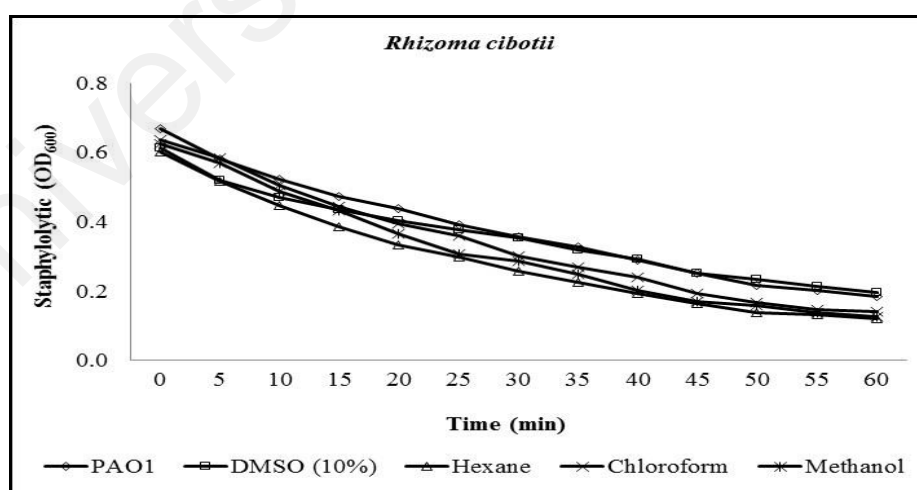
(B)

Figure 4.4: Inhibition of the LasA protease activity by crude extracts at 1 mg/mL concentration of (A) *Angelica dahurica* (B) *Rhizoma cibotii* (C) *Prunella vulgaris* (D) *Schizonepeta tenuifolia* (E) *Forsythia suspense* (F) *Houttuynia cordota* (G) *Garcinia mangostana* (H) *Mangifera foetida* (I) *Syzygium samarangense* (J) *Averrhoa carambola* (K) *Pandanus amaryllifolius* (L) *Cymbopogon citratus* (M) *Momordica charantia* (N) *Petroselinum crispum* (O) *Mentha spicata*
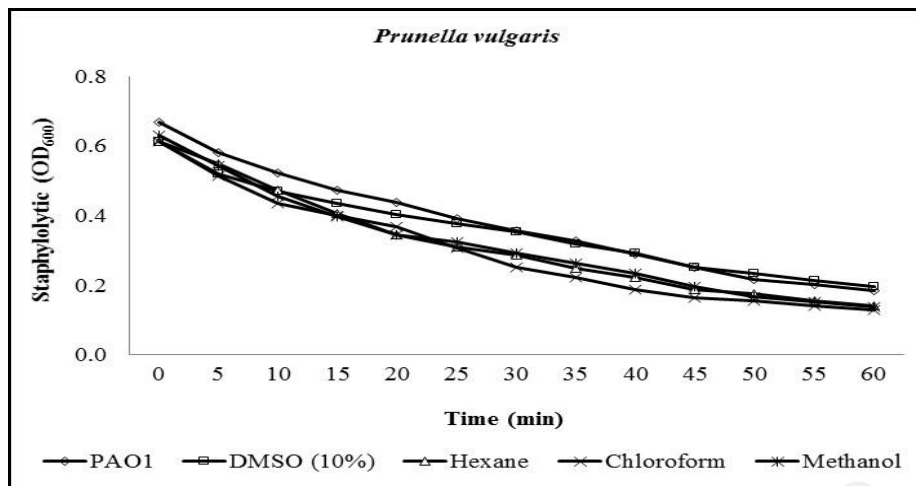
(C)
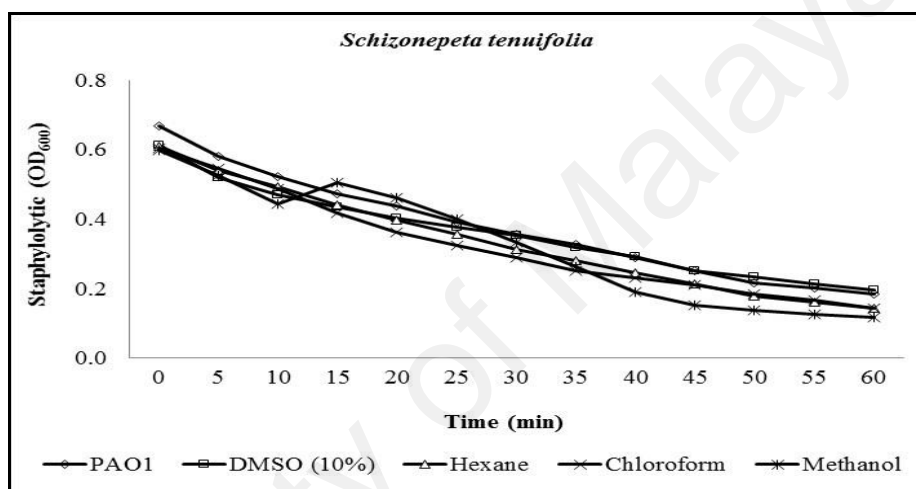
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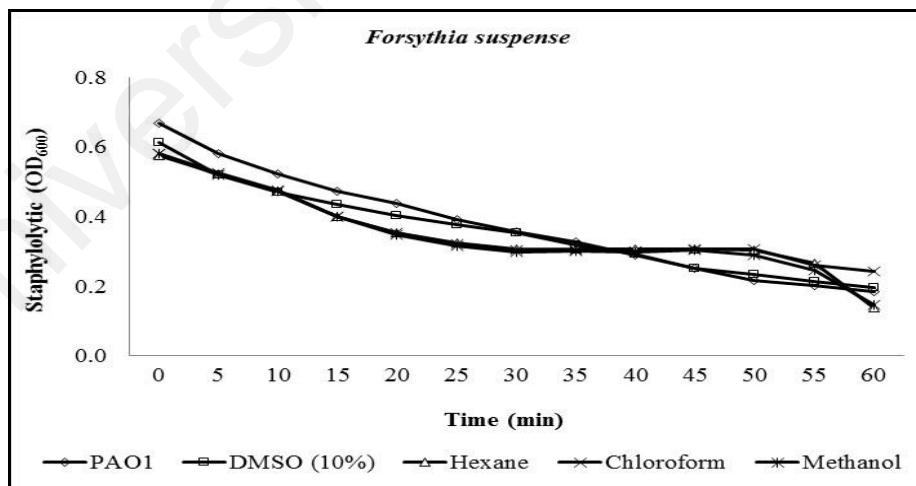
(E)

Figure 4.4, continued.
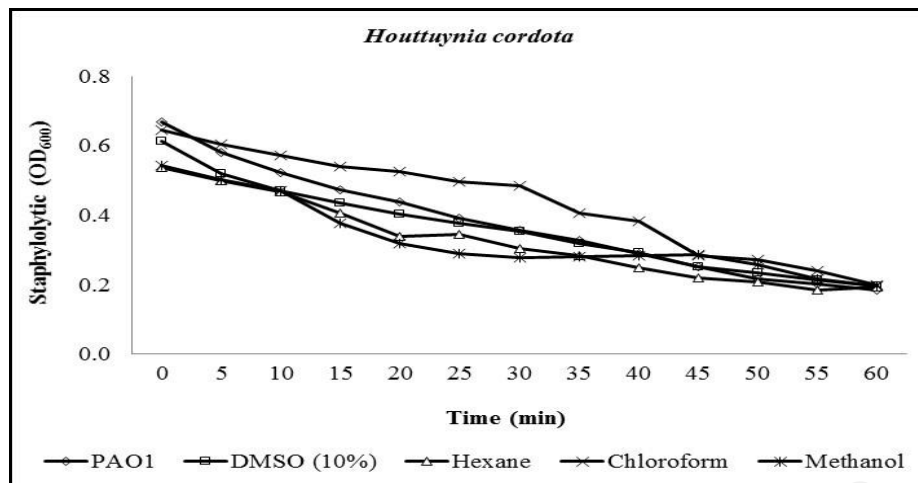
(F)
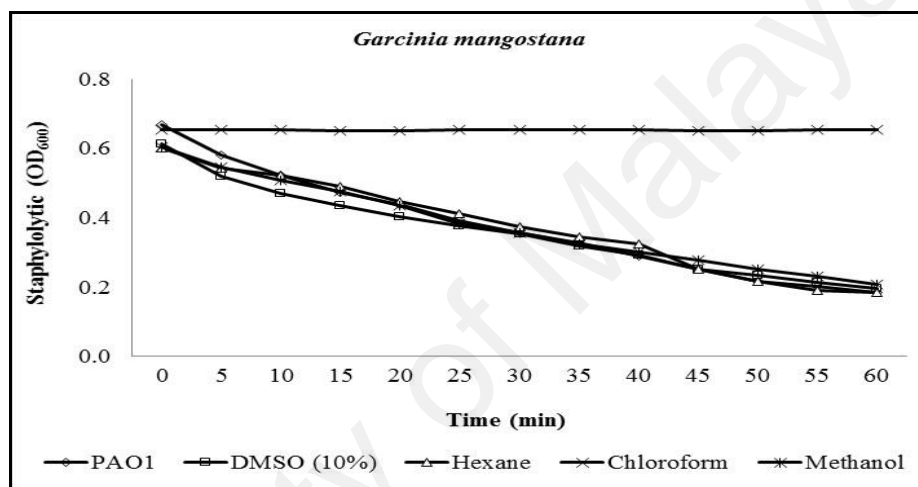
(G)
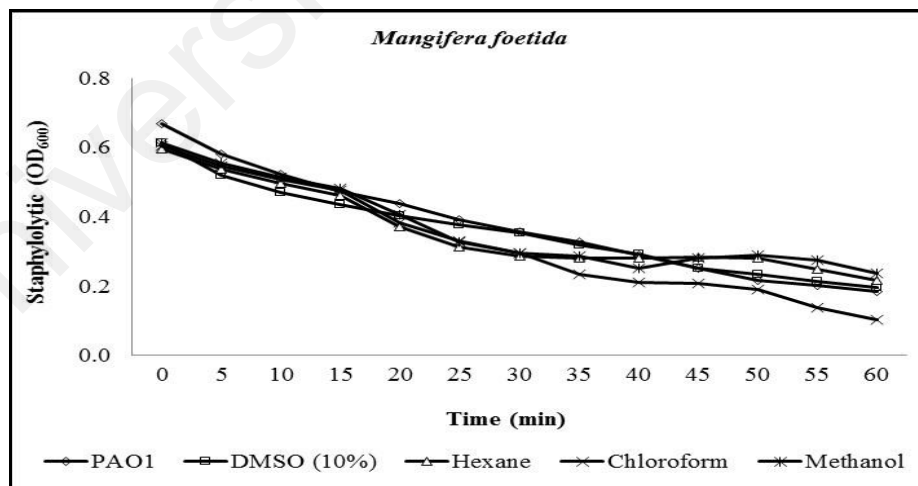
(H)

Figure 4.4, continued.
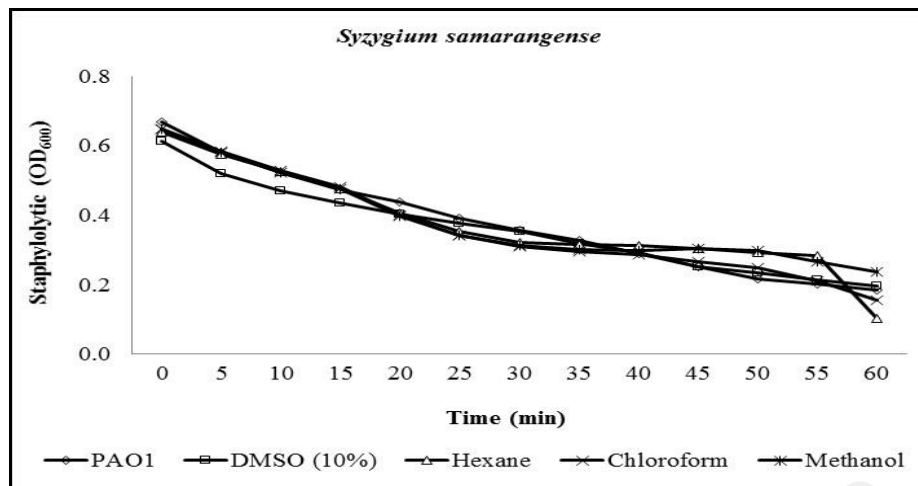
(I)
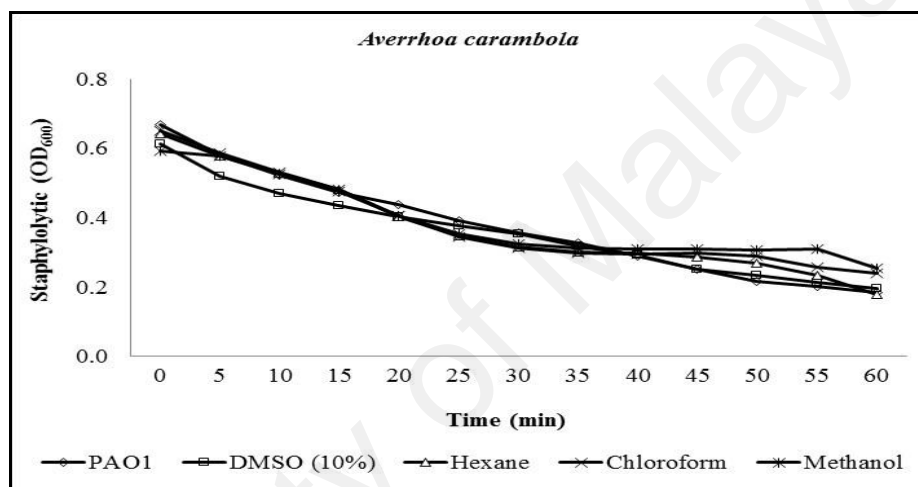
(J)
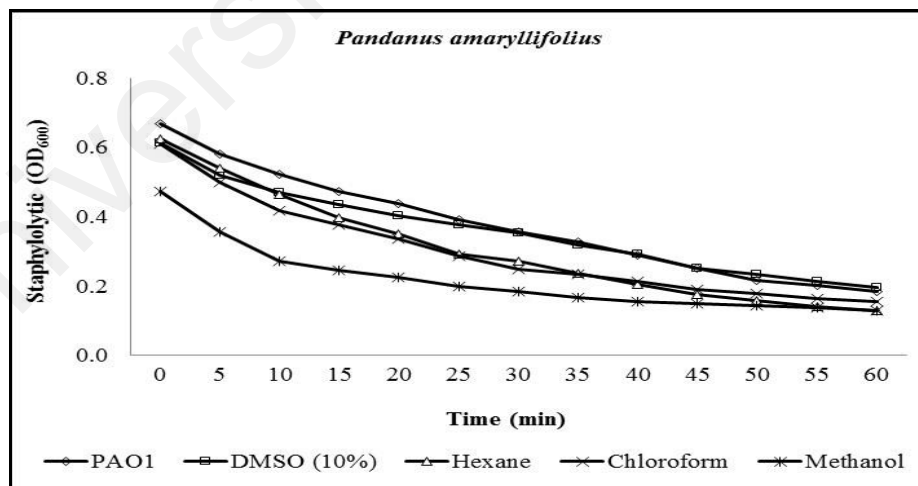
(K)

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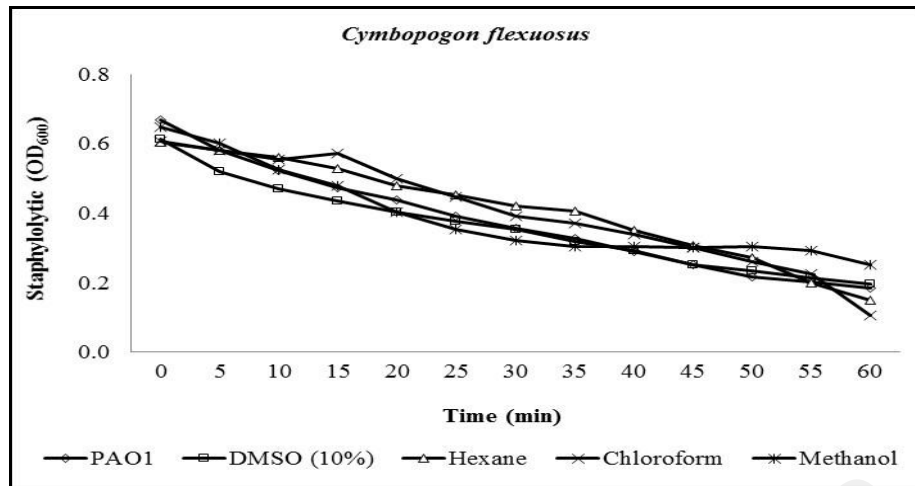
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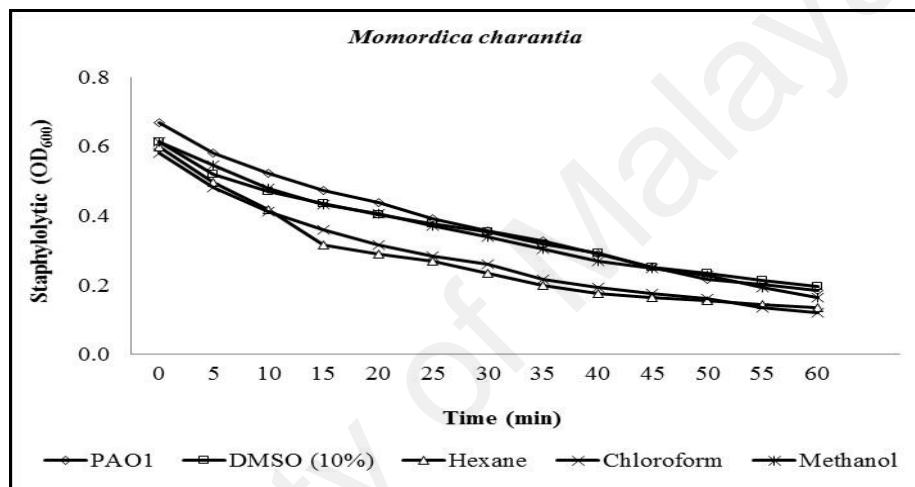
(M)
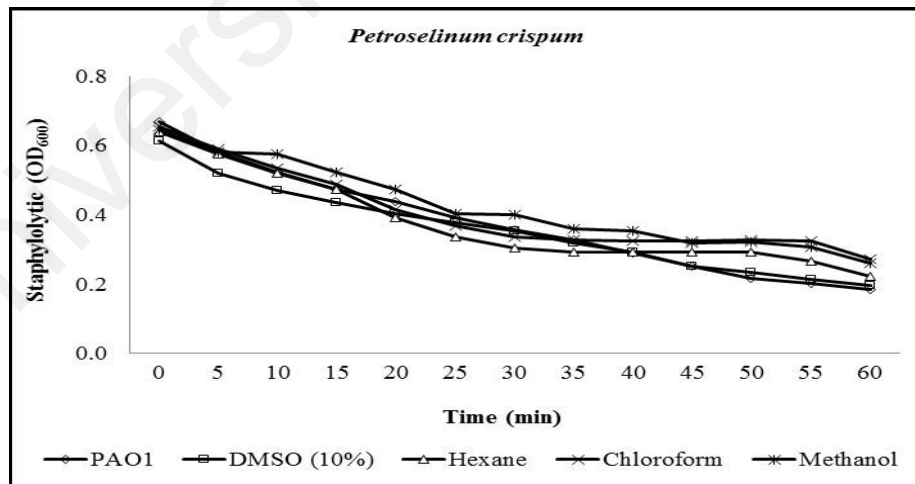
(N)

Figure 4.4, continued.
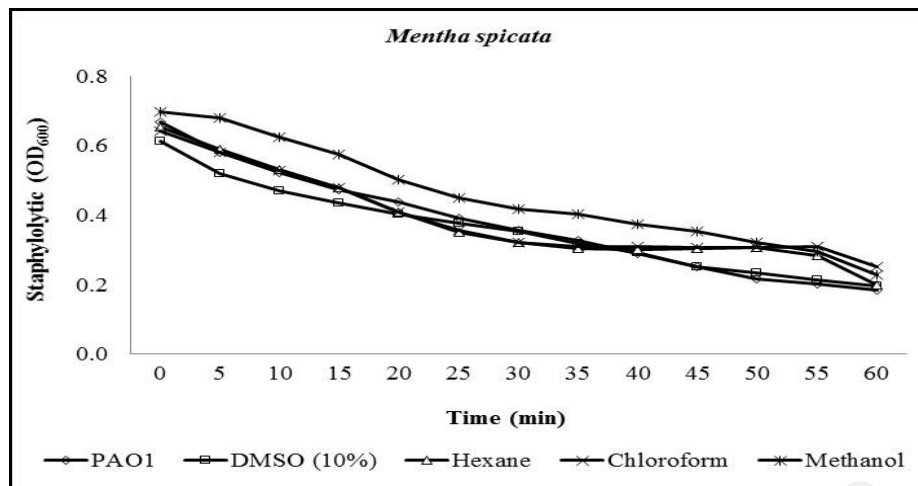
(O)

Figure 4.4, continued.

4.1.2.7 Inhibition of Swarming Motility

Swarming plays an important role in *P. aeruginosa* PAO1 and was proved to be associated with its virulence factor. The ability of the crude extracts to inhibit swarming motility was put into test by using semi-solid agar supplemented with glucose. The swarming plates were then incubated statically for 16 hours at 37°C All of the experiments were done in triplicate, in three independent experiments. Chloroform extracts of *G. mangostana* displayed the strongest inhibition toward *P. aeruginosa* PAO1 swarming motility followed by the chloroform extracts of *S. tenuifolia*.
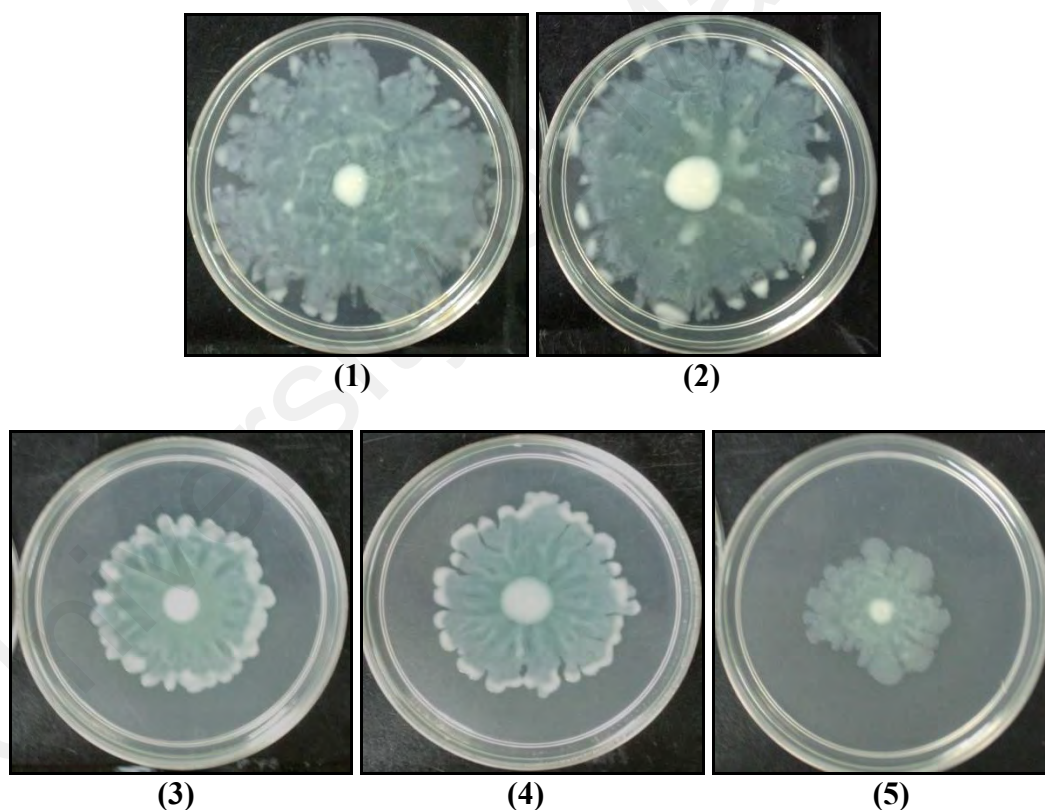
Figure 4.5: Swarming agar inoculated with (1) *P. aeruginosa* PAO1, supplemented with (2) 10% (v/v) DMSO and 1 mg/mL of crude extracts in hexane, chloroform and methanol sequentially (3−5) *Angelica dahurica* (6−8) *Rhizoma cibotii* (9−11) *Prunella vulgaris* (12−14) *Schizonepeta tenuifolia* (15−17) *Forsythia suspense* (18−20) *Houttuynia cordota* (21−23) *Garcinia mangostana* (24−26) *Mangifera foetida* (27−29) *Syzygium samarangense* (30−32) *Averrhoa carambola* (33−35) *Pandanus amaryllifolius* (36−38) *Cymbopogon citratus* (39−41) *Momordica charantia* (42−44) *Petroselinum crispum* (45−47) *Mentha spicata*
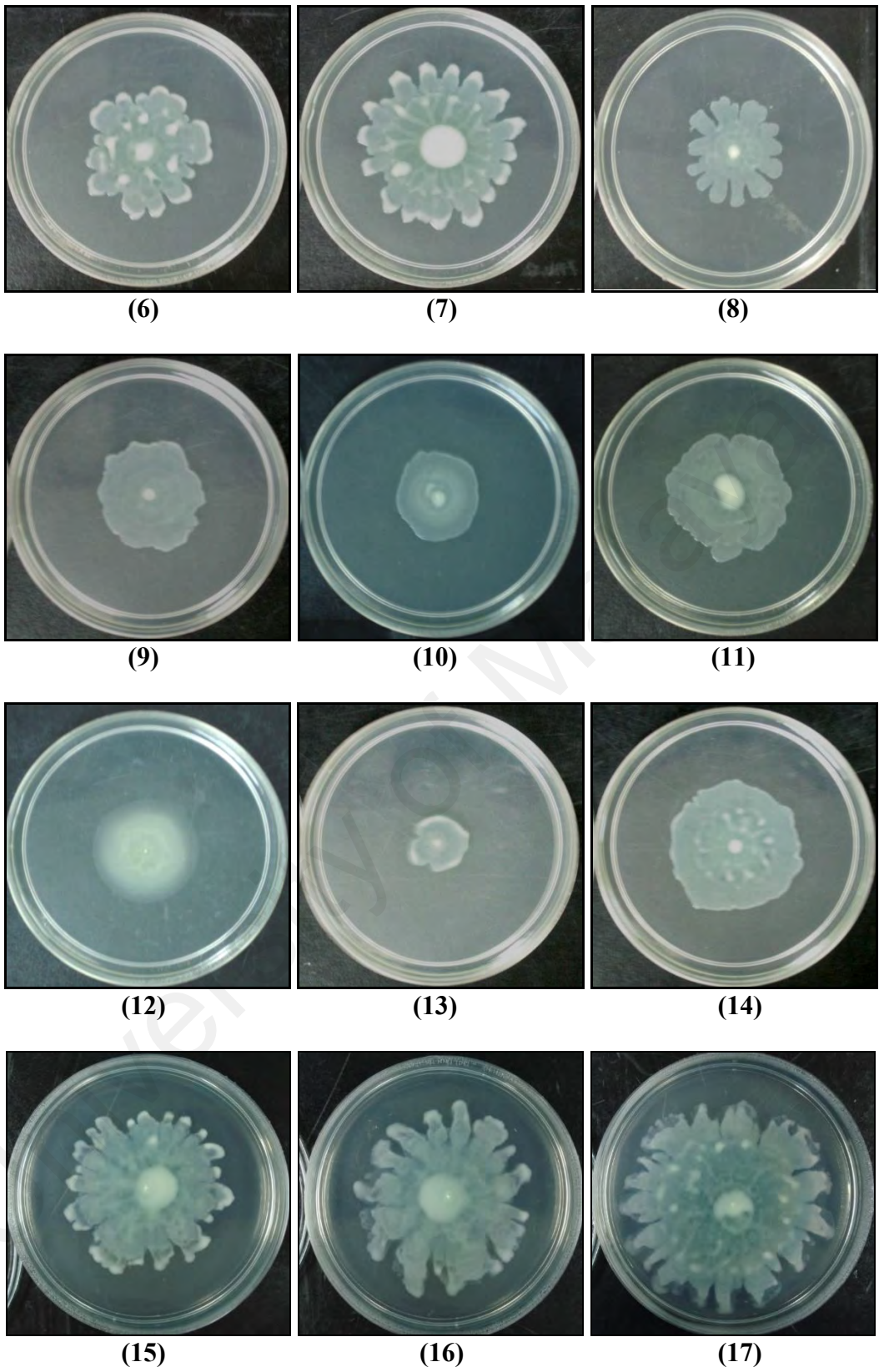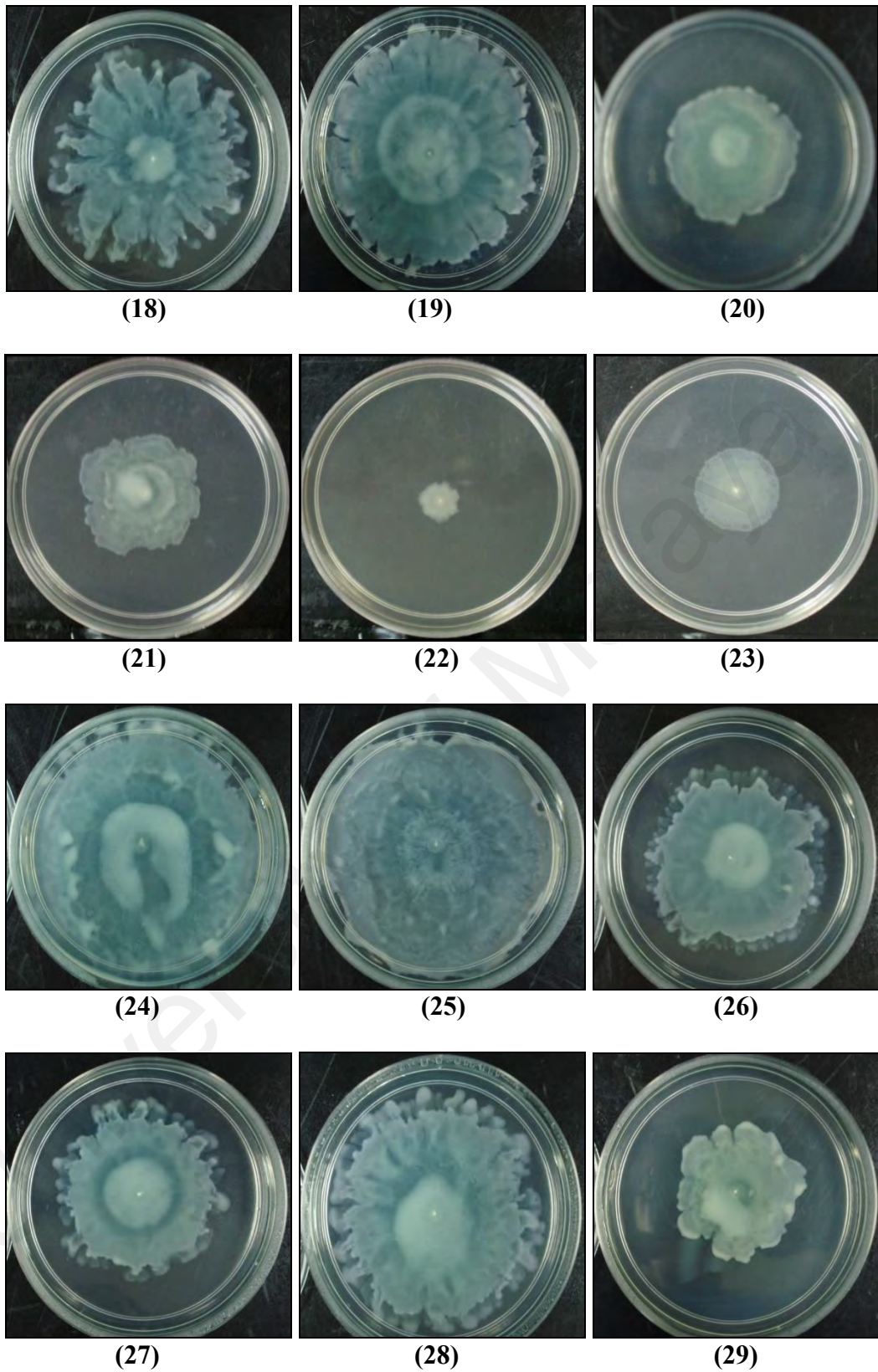
(6)   **(7)**   **(8)**

(9)   **(10)**   **(11)**

(12)   **(13)**   **(14)**

(15)   **(16)**   **(17)**

Figure 4.5, continued.

(18) (19) (20)

(21) (22) (23)

(24) (25) (26)

(27) (28) (29)

Figure 4.5, continued.

(30)　　　　　　(31)　　　　　　(32)

(33)　　　　　　(34)　　　　　　(35)

(36)　　　　　　(37)　　　　　　(38)

(39)　　　　　　(40)　　　　　　(41)

Figure 4.5, continued.

(42)   (43)   (44)

(45)   (46)   (47)
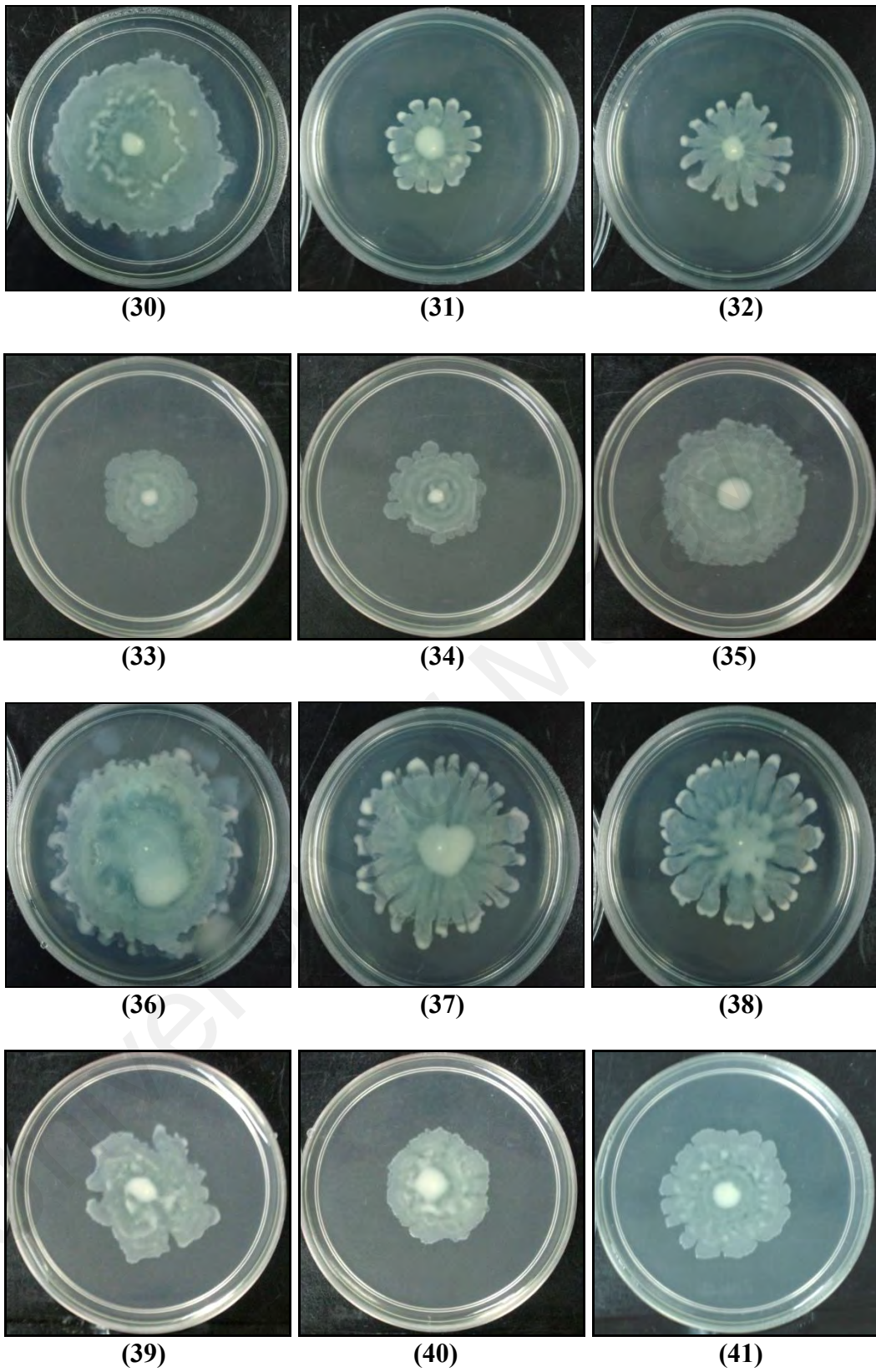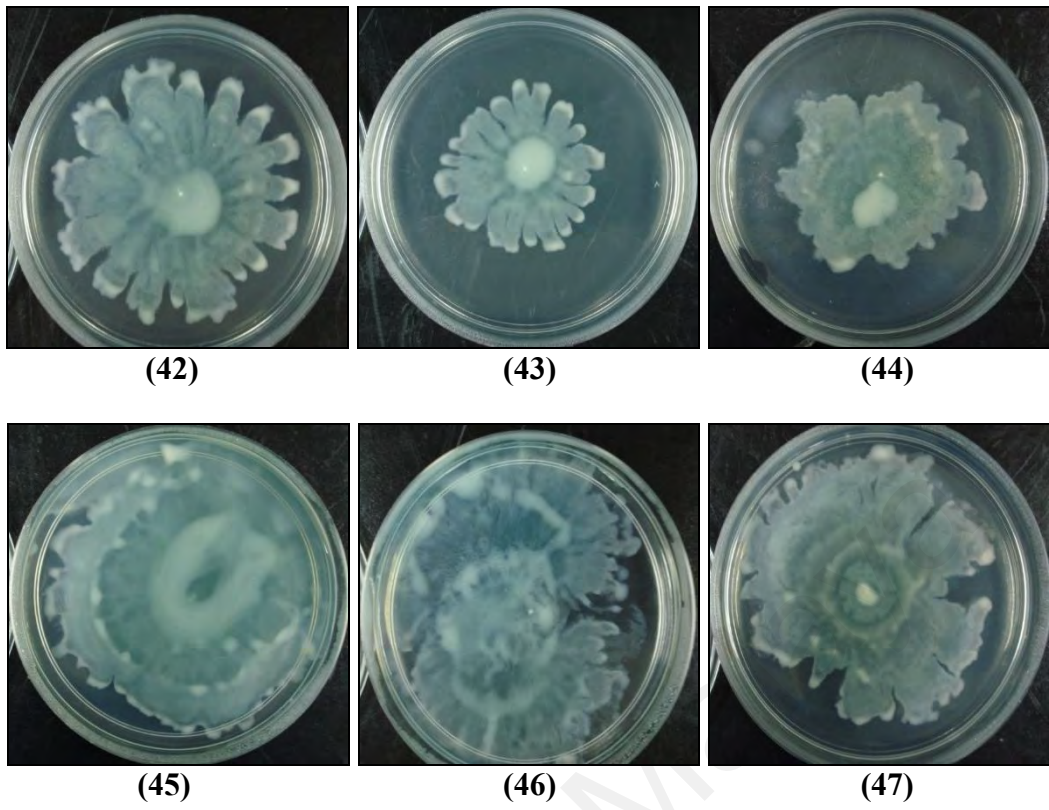
Figure 4.5, continued.

4.2   Catechin (Purified from *G. mangostana*)

Based on the results obtained from Section 4.1, *G. mangostana* extracted with chloroform was chosen for further downstream work as this was the only plant sample that showed positive results for staphylolytic activity on top of other anti-QS bioassays. The extraction for *G. mangostana* was done according to the methods in Section 3.2.3.1 with 750 g of dried powder whereas the purification of the chloroform extract was done based on Section 3.2.4.1.

A total of 54 fractions (labelled as GA1–54) were obtained during the first purification (Table 4.3) while 40 fractions (labelled as GB1–40) were acquired during the second purification (Table 4.4). In the first purification, GA20–21 at 1 mg/mL concentration was the only fractions that shown to produce positive results for staphylolytic activity. These two fractions were then combined as they contained the same compounds based on TLC results (Appendix F) and was then proceed to the second purification. In the second purification, GB24–26 was the only one that gives positive results for staphylolytic assay. Based on the NMR results, the active compound was found to be catechin (Figure 4.7) and the data were shown in Appendix G. Synthetic catechin from Sigma was used as control to confirm the structure of the catechin that was purified from *G. mangostana*.
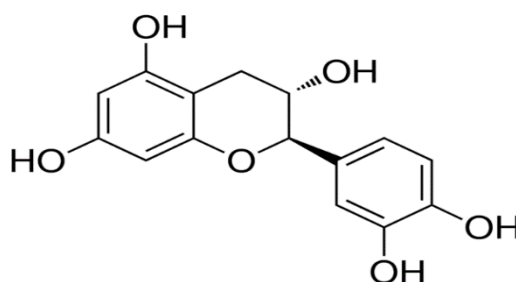
Figure 4.6: The structure of catechin.

Table 4.3: First purification of *G. mangostana* extracted from chloroform screened for anti-QS activities.

Fractions (GA)	CV026 quantification assay	Bioluminescence assay			Pyocyanin assay	Elastase assay	Staphylolytic assay	Swarming motility assay
		pSB401 biosensor	pSB1075 biosensor	PAO1 *lecA::lux*				
1	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X
7	√	X	X	X	X	X	X	√
8	√	X	X	X	X	X	X	√
9	√	X	X	X	X	X	X	√
10	X	X	X	X	√	X	X	√
11	X	X	X	X	X	X	X	X
12	X	√	X	X	X	√	X	X
13	X	√	X	X	X	√	X	√
14	X	X	X	X	X	X	X	√
15	√	X	X	X	X	X	X	X
16	√	X	X	X	X	√	X	X
17	X	X	X	X	X	X	X	√
18	X	X	X	X	X	X	X	X
19	√	√	√	X	X	X	X	√
20	√	√	√	√	√	√	√	√

Note: The symbol "√" indicates positive results for the anti-QS tested while "X" indicates negative results.

Table 4.3, continued.

Fractions (GA)	CV026 quantification assay	Bioluminescence assay			Pyocyanin assay	Elastase assay	Staphylolytic assay	Swarming motility assay
		pSB401 biosensor	pSB1075 biosensor	PAO1 *lecA::lux*				
21	√	√	√	√	√	√	√	√
22	√	X	X	X	X	X	X	X
23	X	X	X	X	√	X	X	X
24	X	X	X	X	√	X	X	X
25	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X
29	X	X	X	X	X	√	X	X
30	X	X	X	X	X	√	X	X
31	X	X	X	X	X	X	X	X
32	X	X	X	X	√	X	X	√
33	X	X	X	X	√	X	X	√
34	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	√
36	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X
38	X	X	X	X	X	√	X	X
39	X	X	X	X	X	√	X	X

Note: The symbol "√" indicates positive results for the anti-QS tested while "X" indicates negative results.

Table 4.3, continued.

Fractions (GA)	CV026 quantification assay	Bioluminescence assay			Pyocyanin assay	Elastase assay	Staphylolytic assay	Swarming motility assay
		pSB401 biosensor	pSB1075 biosensor	PAO1 *lecA::lux*				
40	X	X	X	X	X	X	X	X
41	√	X	X	√	X	X	X	X
42	√	X	X	√	X	X	X	X
43	√	X	X	√	X	X	X	X
44	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X
48	√	X	X	X	X	X	X	X
49	√	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X
52	X	X	X	X	√	X	X	√
53	X	X	X	X	√	X	X	√
54	X	X	X	X	X	√	X	X

Note: The symbol "√" indicates positive results for the anti-QS tested while "X" indicates negative results.

Table 4.4: Second purification of *G. mangostana* (GA20–21) extracted from chloroform screened for anti-QS activities.

Fractions (GB)	CV026 quantification assay	Bioluminescence assay			Pyocyanin assay	Elastase assay	Staphylolytic assay	Swarming motility assay
		pSB401 biosensor	pSB1075 biosensor	PAO1 *lecA::lux*				
1	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X
3	√	X	X	X	X	X	X	X
4	√	X	X	X	X	X	X	X
5	√	X	X	X	X	X	X	X
6	X	√	√	X	√	X	X	X
7	X	√	√	X	√	X	X	X
8	X	X	X	X	√	X	X	X
9	√	X	X	X	√	√	X	X
10	√	X	X	X	√	√	X	X
11	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	√
14	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X
16	√	X	X	√	X	X	X	X
17	√	X	X	√	X	X	X	X
18	X	X	X	√	X	X	X	X
19	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X

Note: The symbol "√" indicates positive results for the anti-QS tested while "X" indicates negative results.

Table 4.4, continued.

Fractions (GB)	CV026 quantification assay	Bioluminescence assay			Pyocyanin assay	Elastase assay	Staphylolytic assay	Swarming motility assay
		pSB401 biosensor	pSB1075 biosensor	PAO1 *lecA::lux*				
21	X	X	X	X	X	X	X	X
22	X	X	X	X	X	√	X	X
23	X	X	√	X	X	√	X	X
24	√	√	√	√	√	√	√	√
25	√	√	√	√	√	√	√	√
26	√	√	√	√	√	√	√	√
27	X	X	X	X	X	X	X	X
28	X	X	X	X	X	√	X	X
29	X	X	X	X	X	X	X	X
30	√	X	X	X	X	√	X	X
31	√	X	X	X	X	√	X	X
32	√	X	X	X	X	√	X	X
33	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X
36	√	X	X	X	X	X	X	X
37	√	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X
40	X	X	X	√	√	X	X	X

Note: The symbol "√" indicates positive results for the anti-QS tested while "X" indicates negative results.

4.2.1 Bacteria Growth Curve

The growth of the bacteria was monitored in order to make sure that 1 mg/mL of catechin did not affect the growth of the bacteria tested. All of the experiments were done in triplicate, in three independent experiments, with absorbance read at OD_{600} and 10% (v/v) DMSO as negative control.
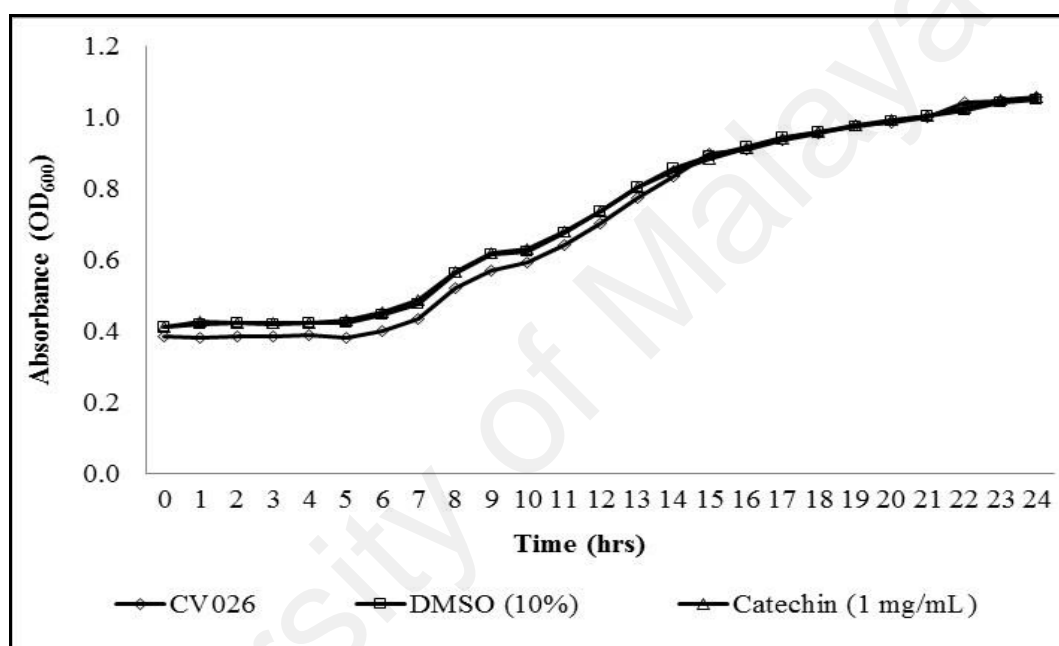
Figure 4.7: The growth of *C. violaceum* CV026 with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
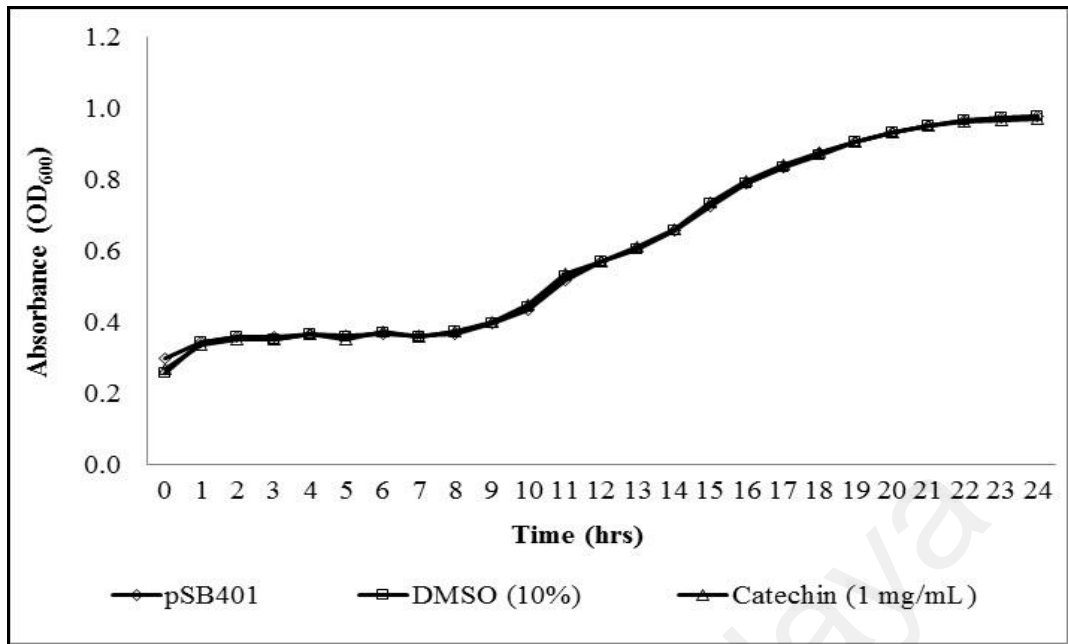
Figure 4.8: The growth of *E. coli* [pSB401] with with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
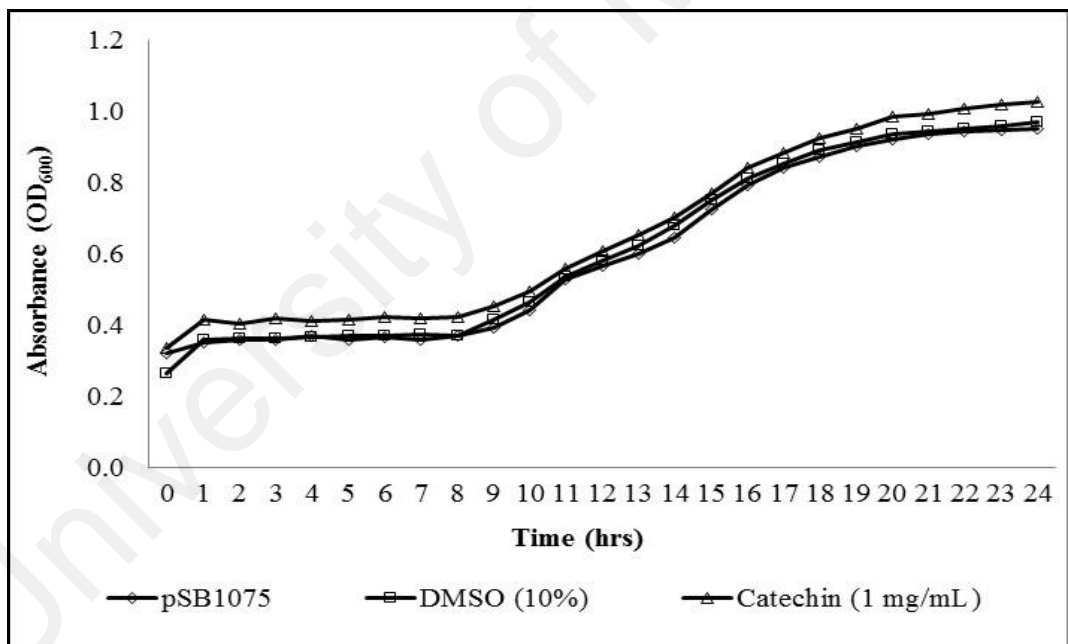
Figure 4.9: The growth of *E. coli* [pSB1075] with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
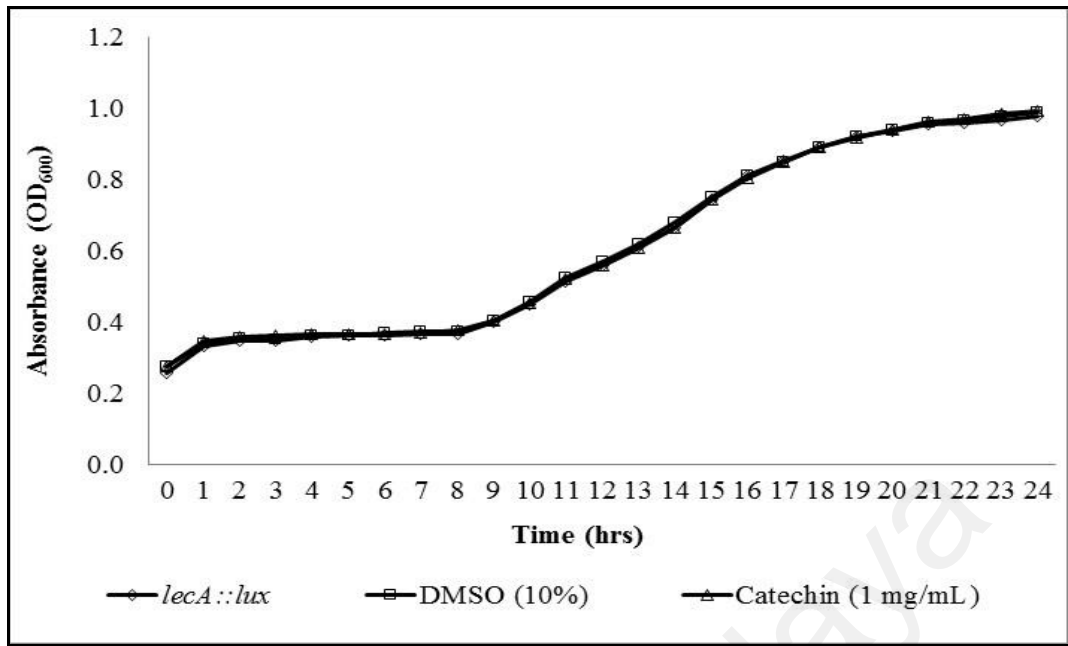
Figure 4.10: The growth of *P. aeruginosa* PAO1 *lecA::lux* with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
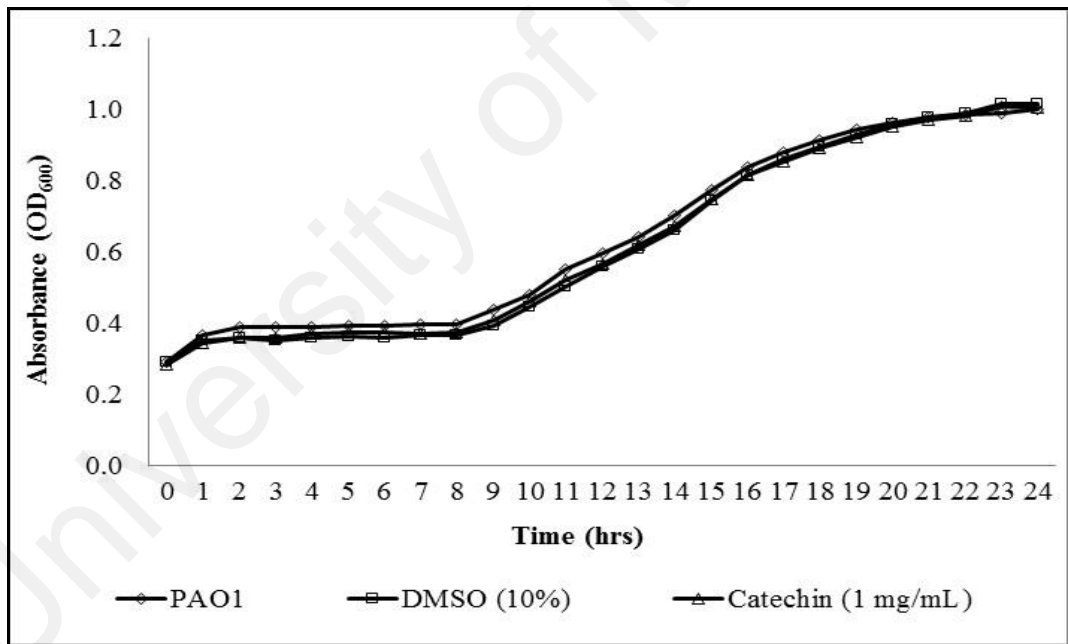
Figure 4.11: The growth of *P. aeruginosa* PAO1 with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
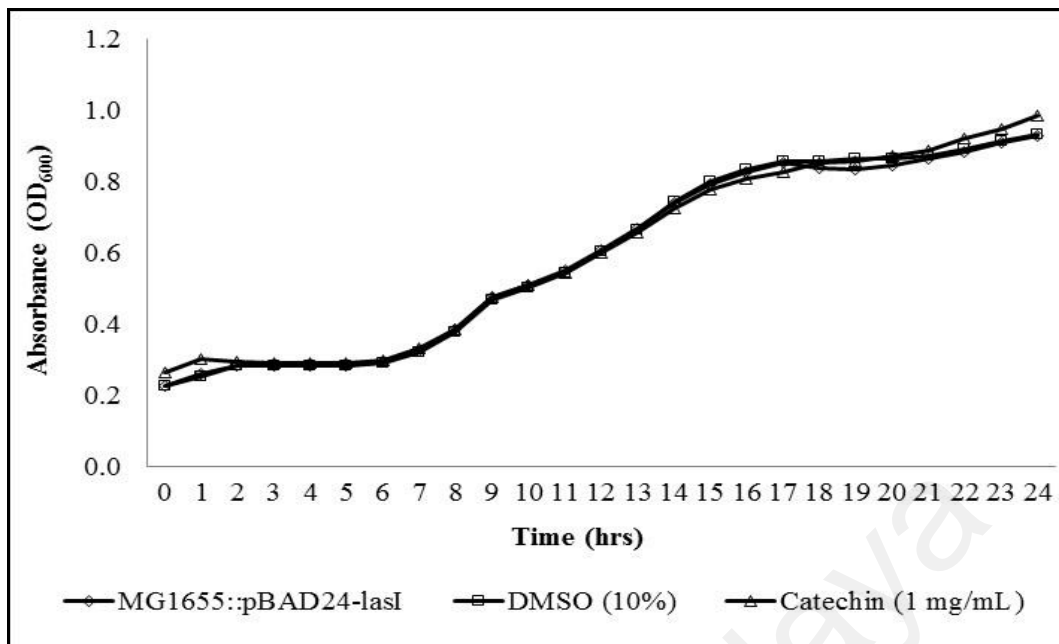
Figure 4.12: The growth of *E. coli* MG1655::pBAD24-*lasI* with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.
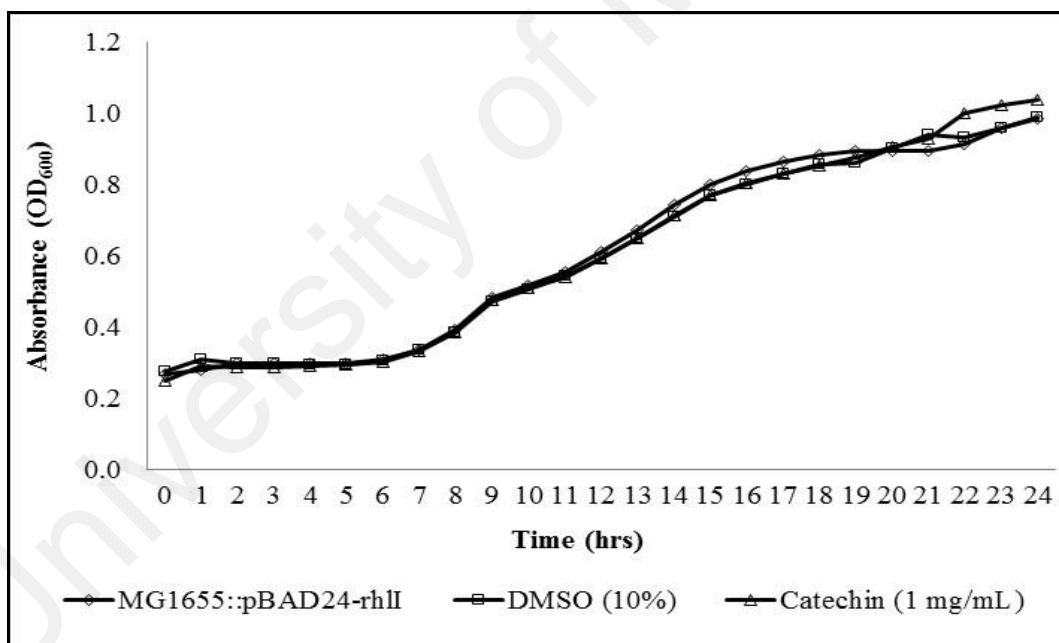
Figure 4.13: The growth of *E. coli* MG1655::pBAD24-*rhlI* with 1 mg/mL catechin and 10% (v/v) DMSO as control. Statistical analysis was conducted using ANOVA test.

4.2.2   Screening of Catechin for Anti-QS Activities

The results shown were based on the anti-QS activities tested with 1 mg/mL of catechin purified from *G. mangostana* chloroform extact.

4.2.2.1 Quantitative Analysis of Violacein in *C. violaceum* CV026

Catechin was preliminary screened for anti-QS using *C. violaceum* CV026 and based on the results, the compound was shown to inhibit the production of violacein. Briefly, *C. violaceum* CV026 was grown overnight with 1 mg/mL catechin supplemented with C6-HSL in 96-well microtitre plate at 28ºC with shaking. The plate was dried at 60ºC and 100% (v/v) DMSO was added to the dried violacein. The values of violacein production were expressed at OD 590 nm. DMSO was used as negative control. All of the experiments were done in triplicate, in three independent experiments.
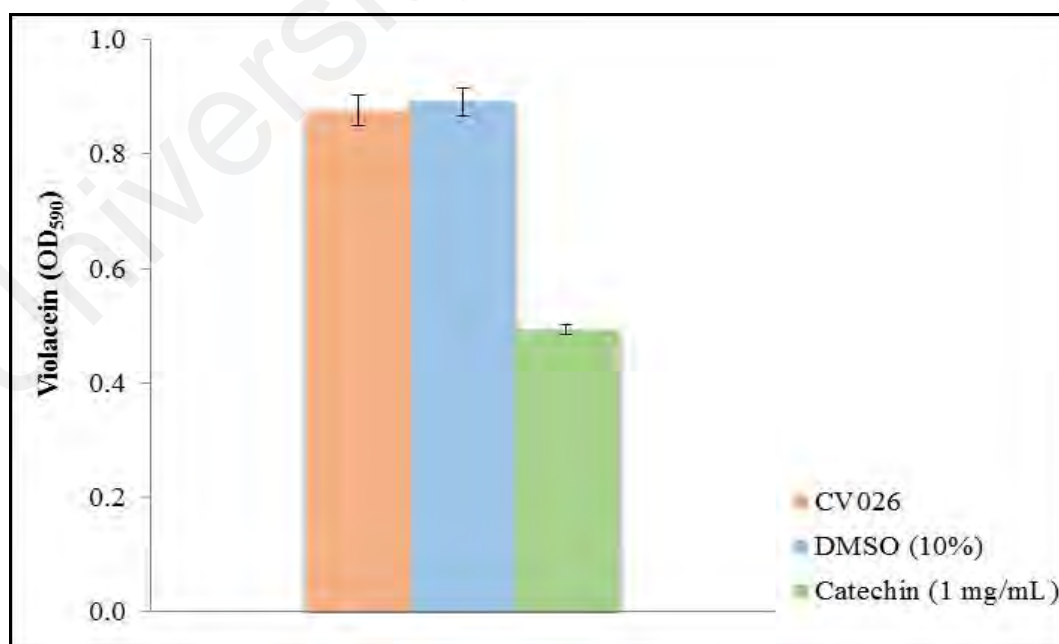
Figure 4.14: Inhibition of violacein production in *C. violaceum* CV026 by 1 mg/mL of catechin. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.2.2.2 Inhibition of *lux*-based Biosensors in *P. aeruginosa* PAO1

The luminescence expression for *E. coli* DH5α biosensors used in this study contained the *lux* reporter plasmid pSB401 and pSB 1075. Both the biosensors were co-cultured together with 1 mg/mL catechin, in addition with synthetic 3-oxo-C6-HSL and 3-oxo-C10-HSL, respectively. The reading was taken every 30 minutes for 24 hours and the luminescence was determined by relative light unit (RLU) divided by OD_{600}. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results.

(A)
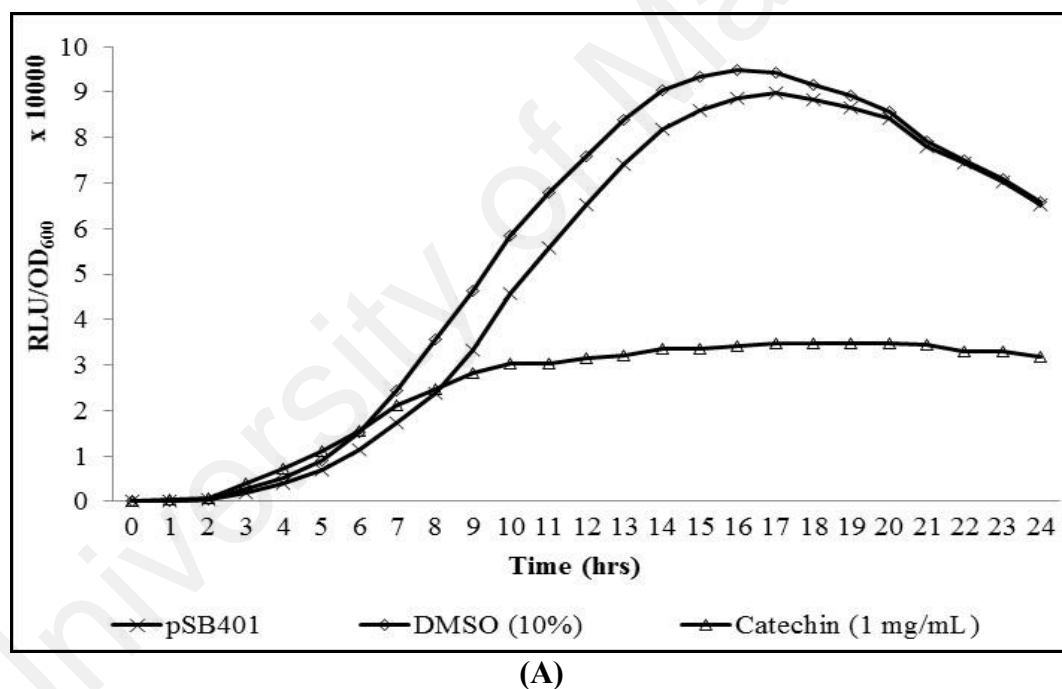
Figure 4.15: Inhibition of *lux*-based biosensors by 1 mg/mL of catechin in (A) *E. coli* [pSB401] (B) *E. coli* [pSB1075]. Statistical analysis was conducted using ANOVA test.
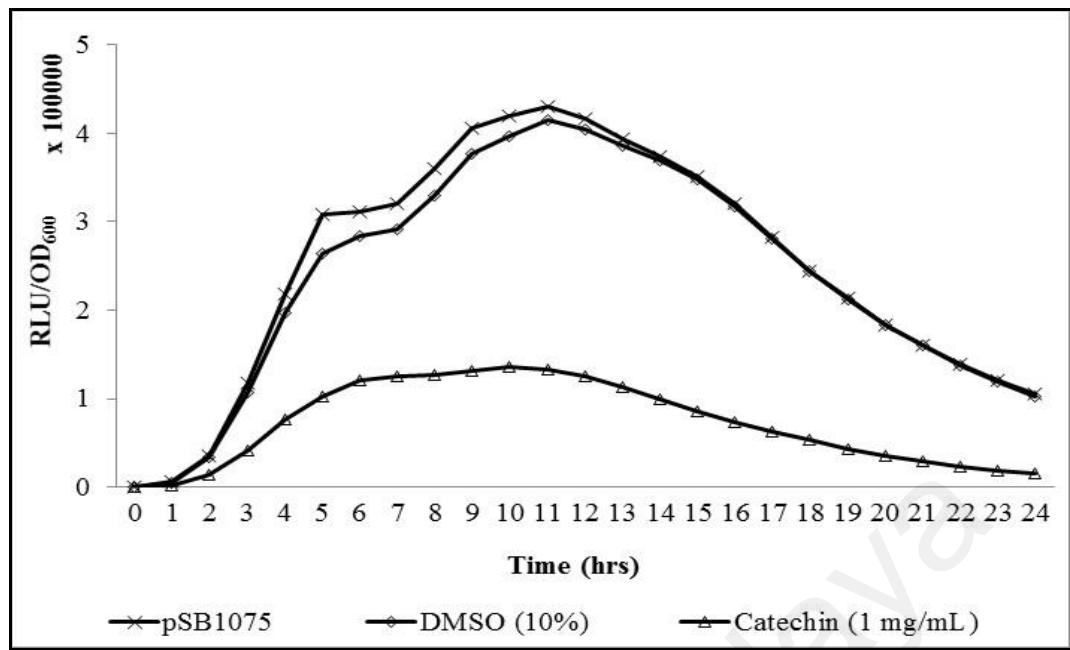
(B)

Figure 4.15, continued.

4.2.2.3 Inhibition *lecA::lux* Expression in *P. aeruginosa* PAO1

The expression of *P. aeruginosa* PAO1 *lecA::lux* co-cultured with 1 mg/mL catechin was measured using the Tecan Infinite M200 with 10% (v/v) DMSO as negative control. The reading was taken every 30 minutes for 24 hours and the luminescence was given in relative light unit (RLU) divided by OD_{600}. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results.
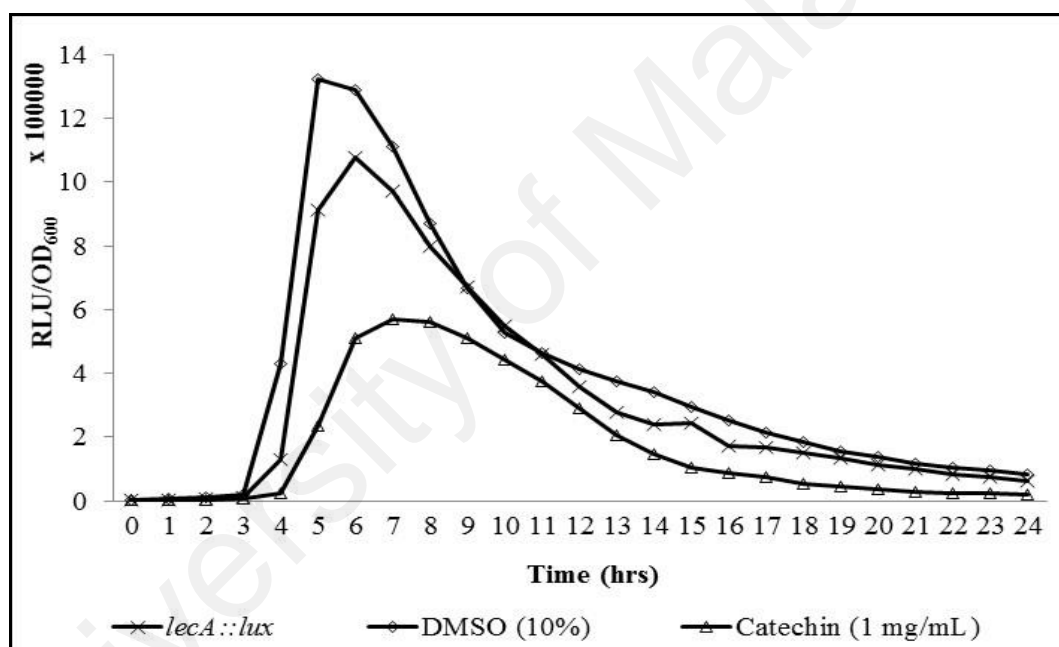
Figure 4.16: Inhibition of *P. aeruginosa* PAO1 *lecA::lux* expression by 1 mg/mL of catechin. Statistical analysis was conducted using ANOVA test.

4.2.2.4 Inhibition of Pyocyanin Production

P. aeruginosa PAO1 was grown overnight with 1 mg/mL catechin at 37°C with 10% (v/v) DMSO used as negative control. All of the experiments were done in triplicate, in three independent experiments. Based on the results, there was a significant reduction in the production of pyocyanin in *P. aeruginosa* PAO1.
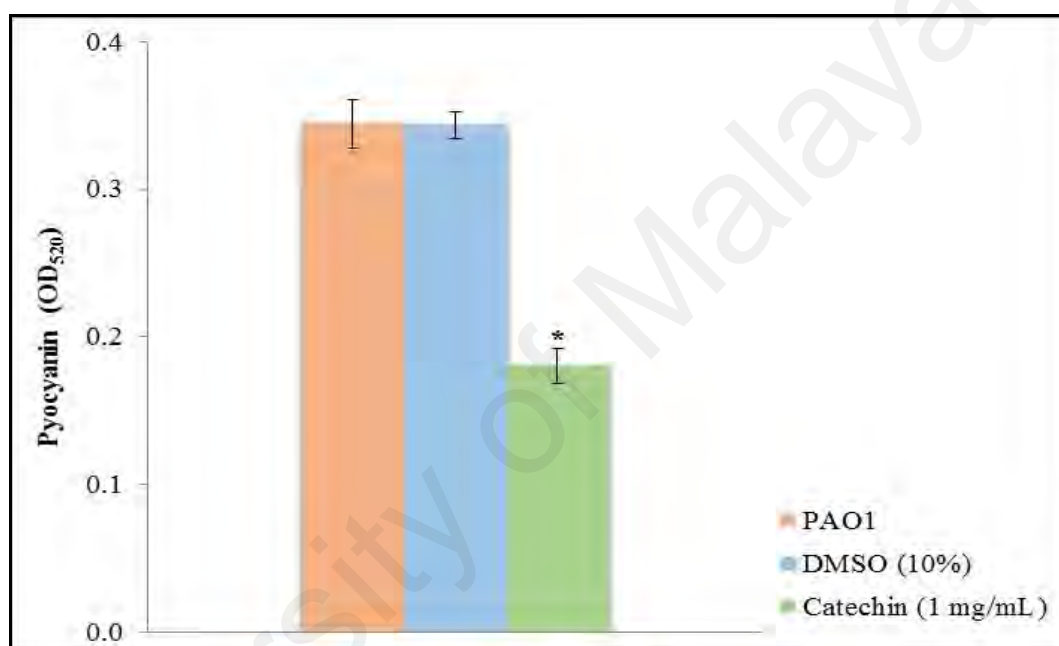
Figure 4.17: Inhibition of pyocyanin production in *P. aeruginosa* PAO1 by 1 mg/mL of catechin. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.2.2.5   Inhibition of Elastase Production

The effect of catechin against the production of elastase in *P. aeruginosa* PAO1 was measured with Tecan Infinite M200 at the OD of 495 nm. DMSO with 10% (v/v) concentration acts as negative control. All of the experiments were done in triplicate, in three independent experiments.
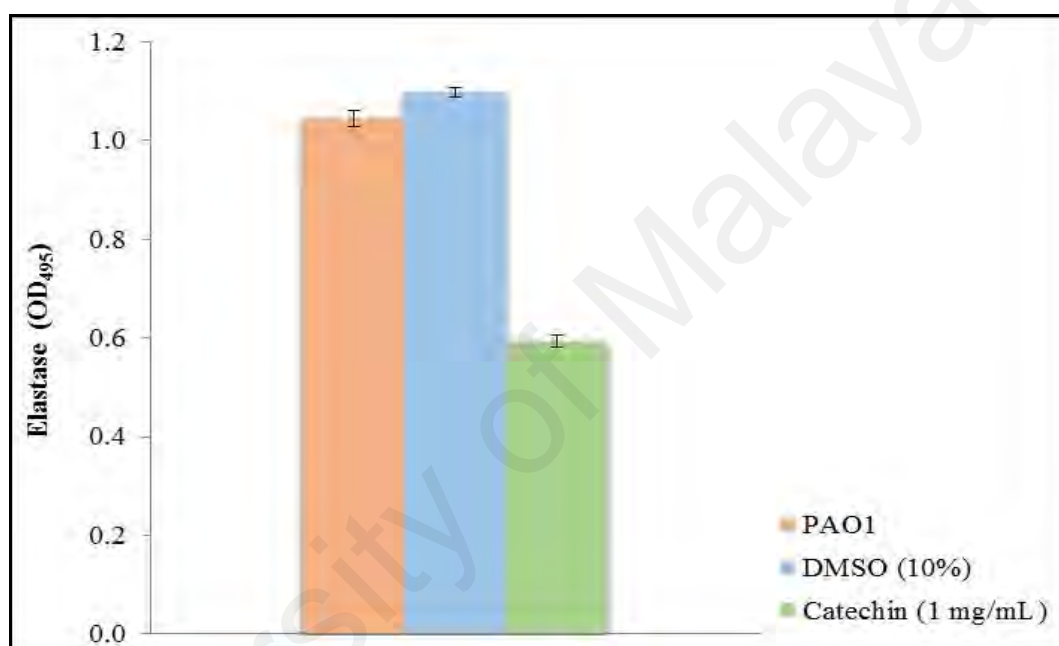
Figure 4.18: Inhibition of elastase in *P. aeruginosa* PAO1 by 1 mg/mL of catechin. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.2.2.6 Inhibition of *P. aeruginosa* PAO1 Staphylolytic Activity

The staphylolytic activity in *P. aeruginosa* PAO1 was measured every 5 minutes interval for 1 hour at the absorbance of 600 nm. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. The result obtained proved that catechin purified from *G. mangostana* chloroform extracts can reduced the staphylolytic activity.
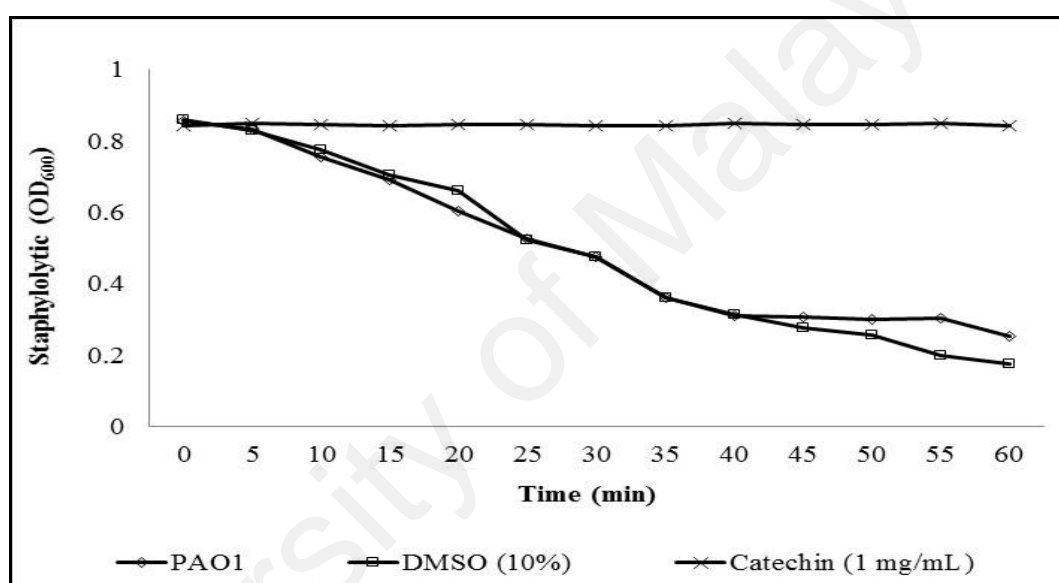
Figure 4.19: Inhibition of the LasA protease activity by 1 mg/mL of catechin. Statistical analysis was conducted using ANOVA test.

4.2.2.7 Inhibition of Swarming Motility

The effects of catechin against the *P. aeruginosa* PAO1 swarming motility was assessed by using the swarming plates that were mixed with samples. DMSO was used as negative control while synthetic gallic acid acts as positive control. All of the experiments were done in triplicate, in three independent experiments.
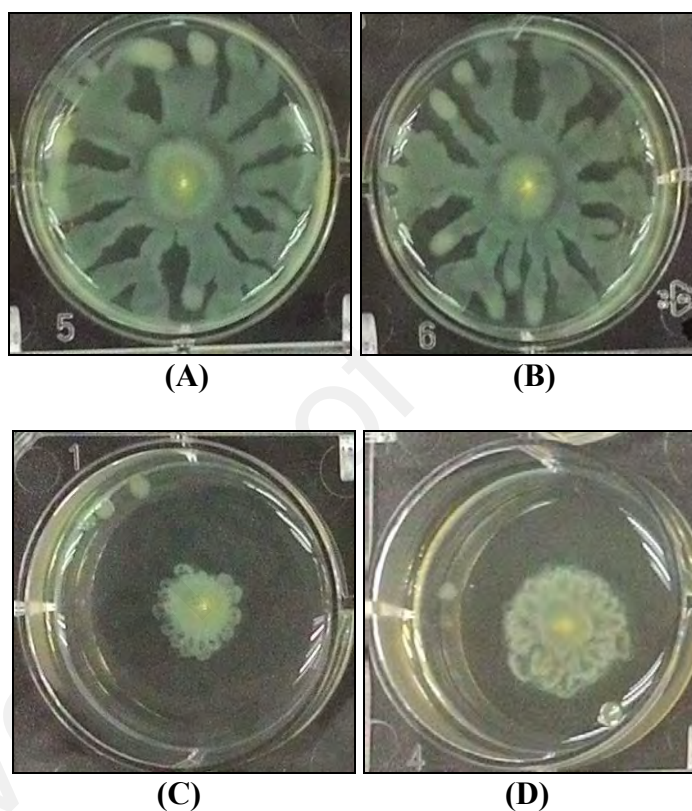
Figure 4.20: Swarming agar inoculated with (A) *P. aeruginosa* PAO1 and supplemented with (B) 10% (v/v) DMSO (C) 1 mg/mL catechin (D) 1 mg/mL synthetic gallic acid

4.2.2.8   Screening of Biofilm Inhibition

      The static microtiter plate assay was used to screen the effects of catechin inhibiting the production of biofilm produced by *P. aeruginosa* PAO1. The values of biofilm production were expressed at OD 590 nm. DMSO was used as negative control. All of the experiments were done in triplicate, in three independent experiments.
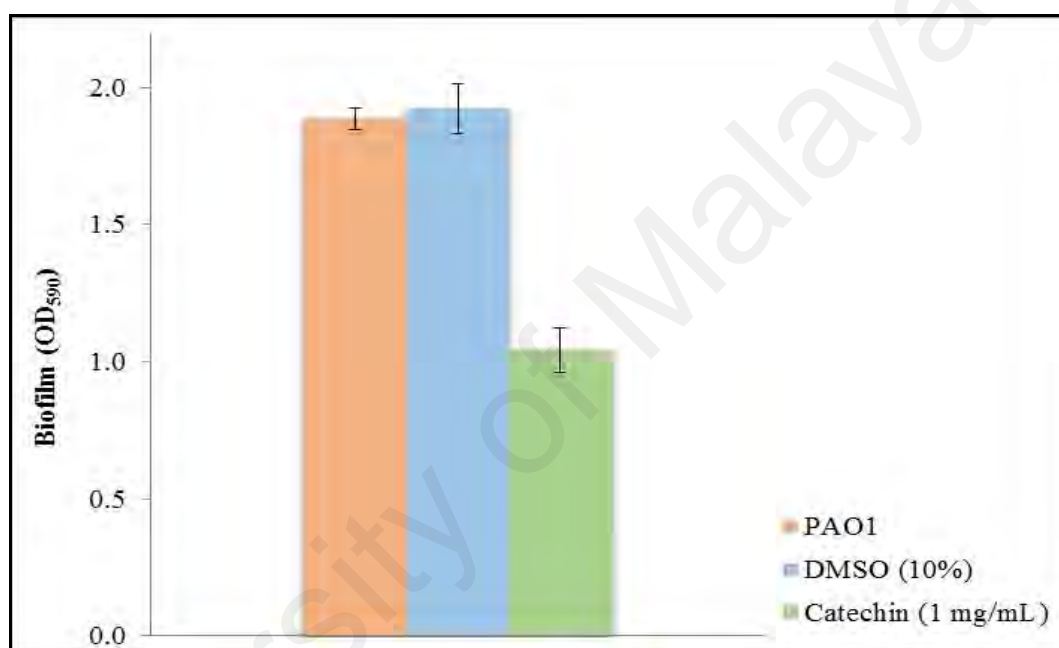
Figure 4.21: Inhibiton of biofilm produce by *P. aeruginosa* PAO1 by 1 mg/mL of catechin. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.2.3 Discover the Mode of Action of Catechin against *P. aeruginosa* PAO1 QS System

The screening of catechin as *P. aeruginosa* PAO1 QS signal synthase antagonists or transcriptional regulator protein antagonists were carried out using *E. coli* which contains arabinose-dependent expression plasmids with pBAD-*lasI* and pBAD-*rhlI* harboring *P. aeruginosa* PAO1 *lasI* and *rhlI*, respectively. The presence of arabinose will induce the production of C4-HSL and 3-oxo-C12-HSL in *E. coli* MG1655::pBAD24-*rhlI* and *E. coli* MG1655::pBAD24-*lasI*, respectively. AHLs that were extracted from the pBAD biosensors treated with 1 mg/mL catechin were then analysed using *E. coli* [pSB401] and *E. coli* [pSB1075] as these biosensors can detect both short and long chain AHLs, correspondingly. Trans-cinnamaldehyde was used as positive control and the experiments were done in triplicate.
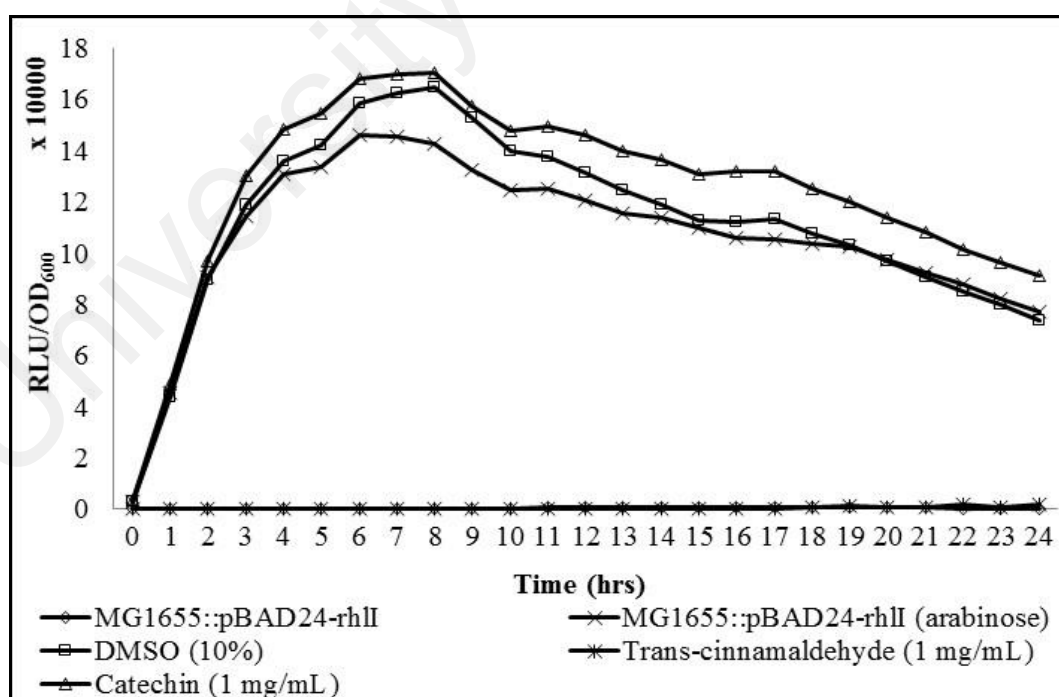
Figure 4.22: Analysis of *E. coli* MG1655::pBAD24- *rhlI* extracted AHLs treated with 1 mg/mL catechin using *E. coli* [pSB401]. Statistical analysis was conducted using ANOVA test.
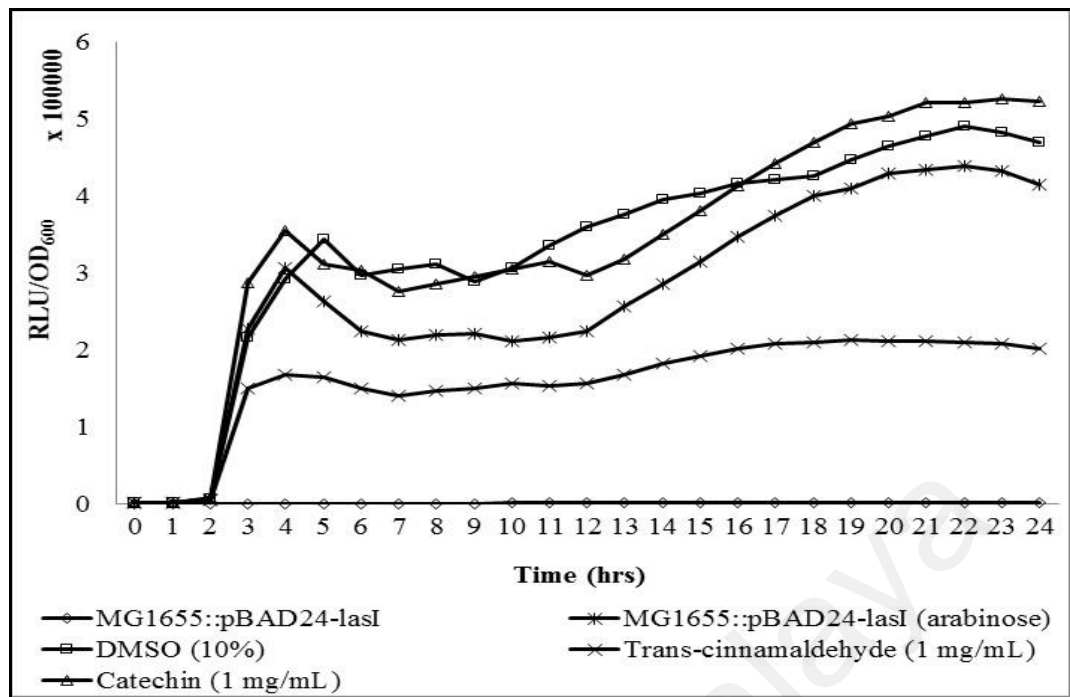
Figure 4.23: **A**nalysis of *E. coli* MG1655::pBAD24-*lasI* extracted AHLs treated with 1 mg/mL catechin using *E. coli* [pSB1075]. Statistical analysis was conducted using ANOVA test.

4.3   Malabaricone C (Purified from *M. cinnamomea*)

Malabaricone C from *Mystrica cinnamomea* was extracted and purified by the Department of Pharmacology, Faculty of Medicine, University of Malaya and the NMR data for malabaricone C were shown in Appendix H.
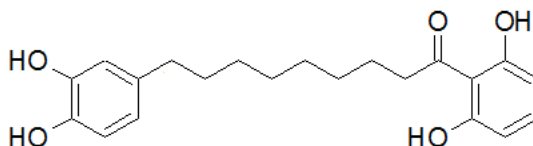
Figure 4.24: The structure of malabaricone C.

4.3.1   Bacteria Growth Curve

Before proceed with any of the anti-QS bioassays, bacteria growth curve was first plotted in order to make sure that concentration tested for malabaricone C, synthetic catechin, synthetic gallic acid and synthetic trans-cinnamaldehyde (positive control) and DMSO (negative control) does not caused any antibacterial effects on the bacteria tested. All of the experiments were done in triplicate, in three independent experiments, with absorbance read at OD_{600}.
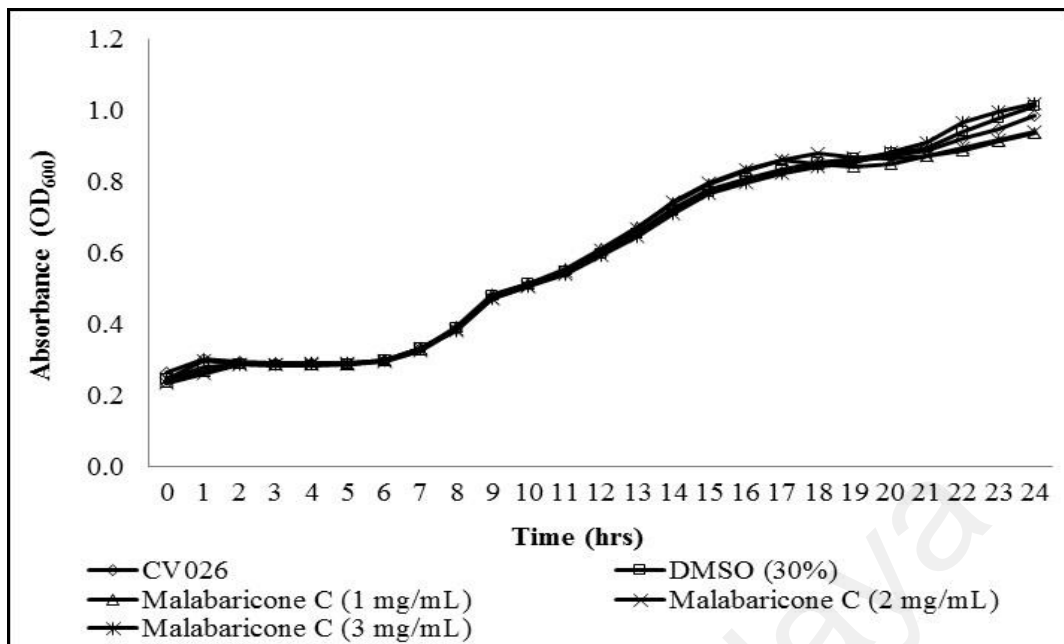
(A)
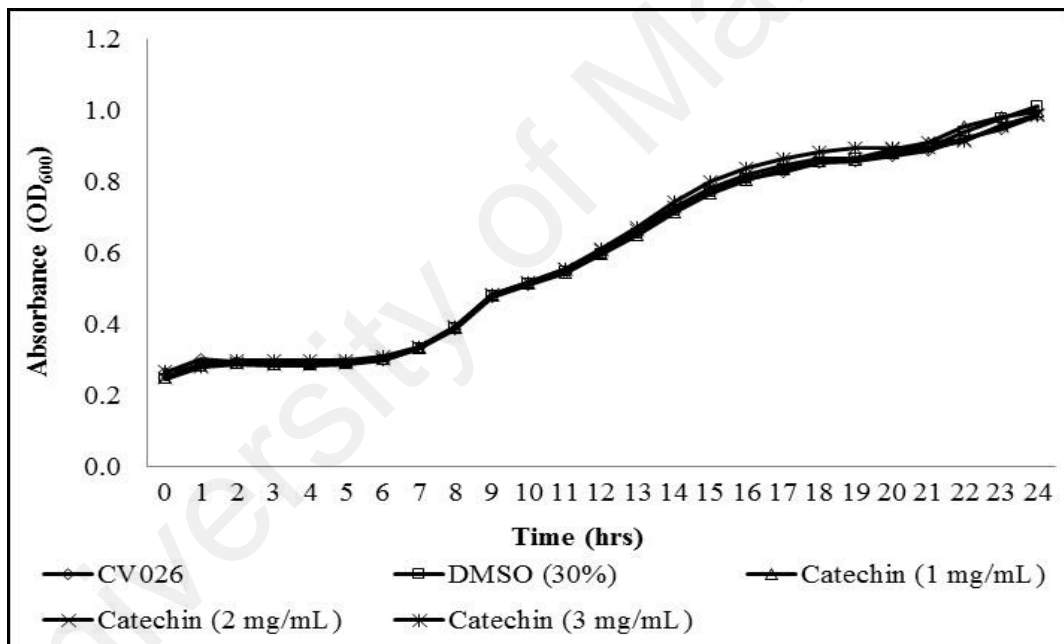
(B)

Figure 4.25: The growth of *C. violaceum* CV026 with 30% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1, 2 and 3 mg/mL (B) synthetic catechin at 1, 2 and 3 mg/mL. Statistical analysis was conducted using ANOVA test.
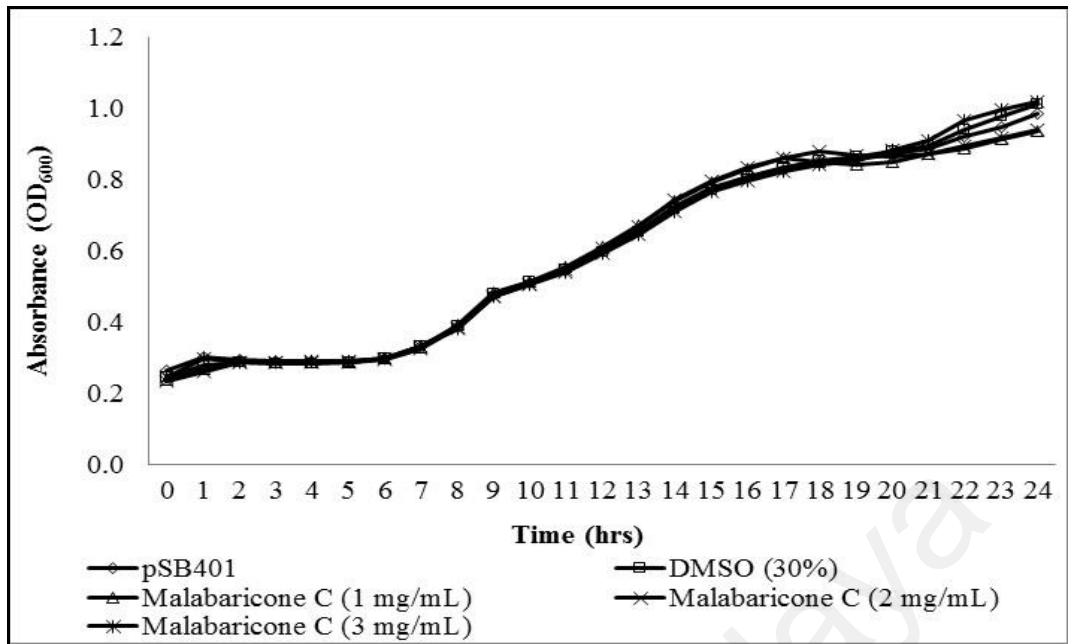
(A)
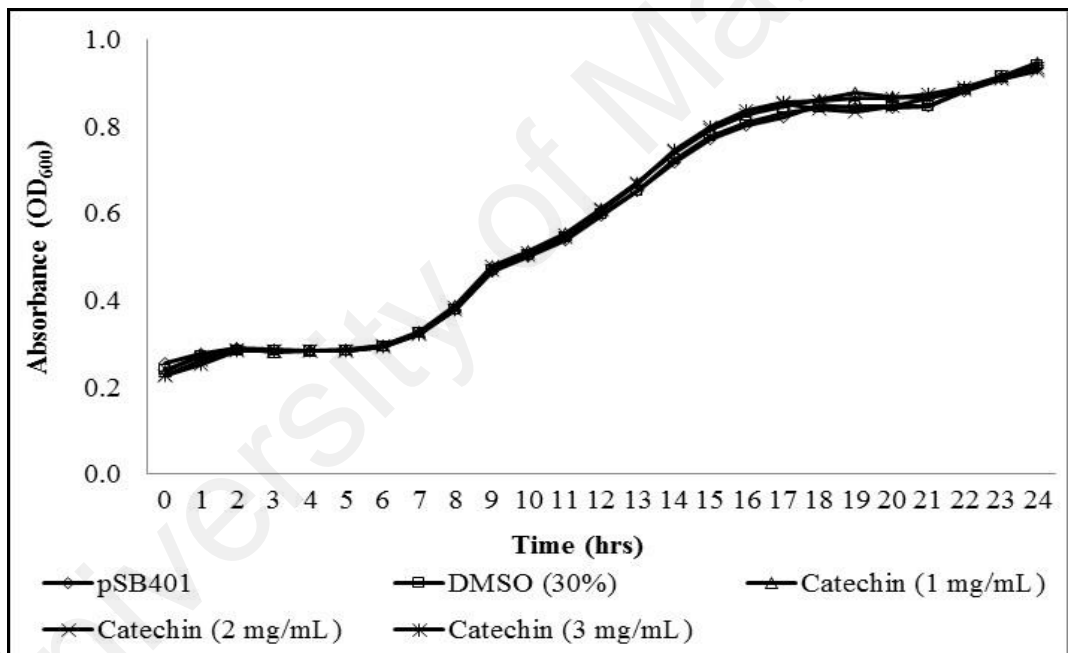
(B)

Figure 4.26: The growth of *E. coli* [pSB401] with 30% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1, 2 and 3 mg/mL (B) synthetic catechin at 1, 2 and 3 mg/mL. Statistical analysis was conducted using ANOVA test.
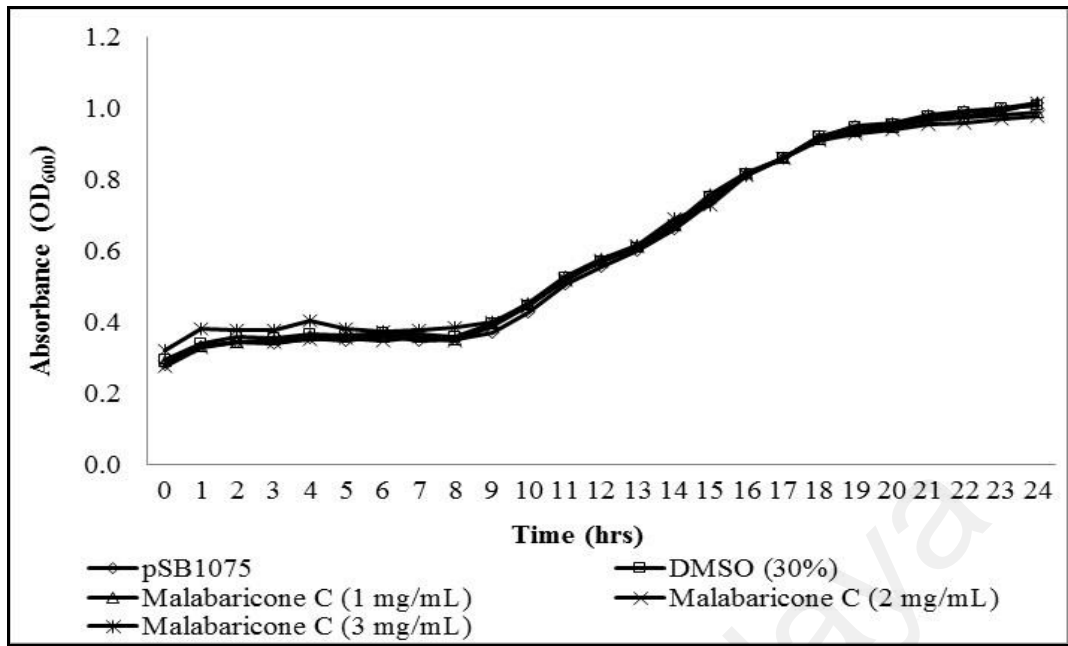
(A)
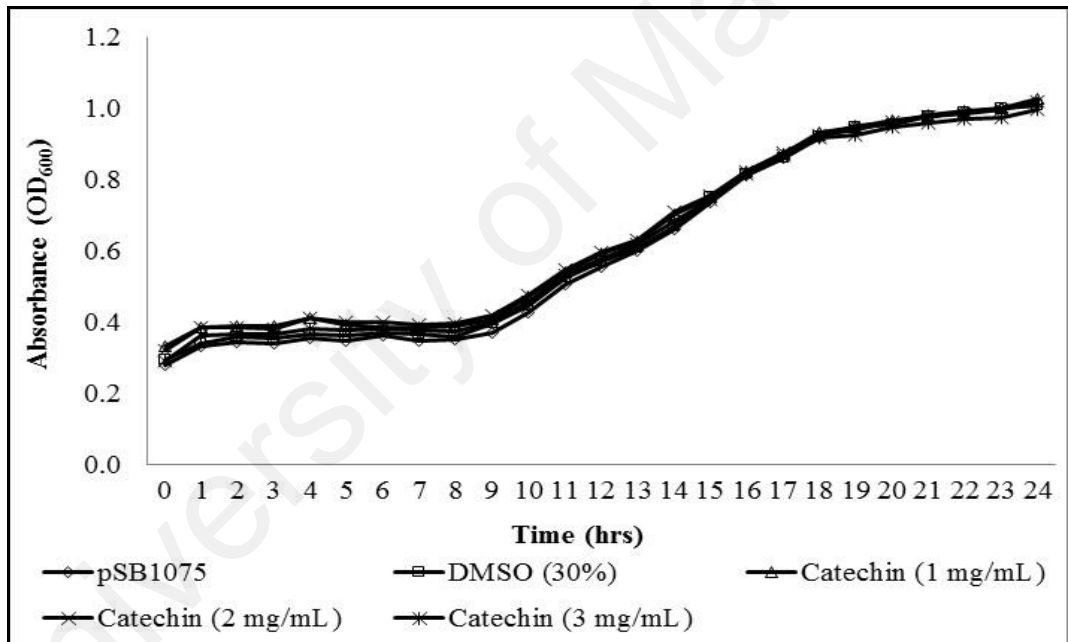
(B)

Figure 4.27: The growth of *E. coli* [pSB1075] with 30% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1, 2 and 3 mg/mL (B) synthetic catechin at 1, 2 and 3 mg/mL. Statistical analysis was conducted using ANOVA test.
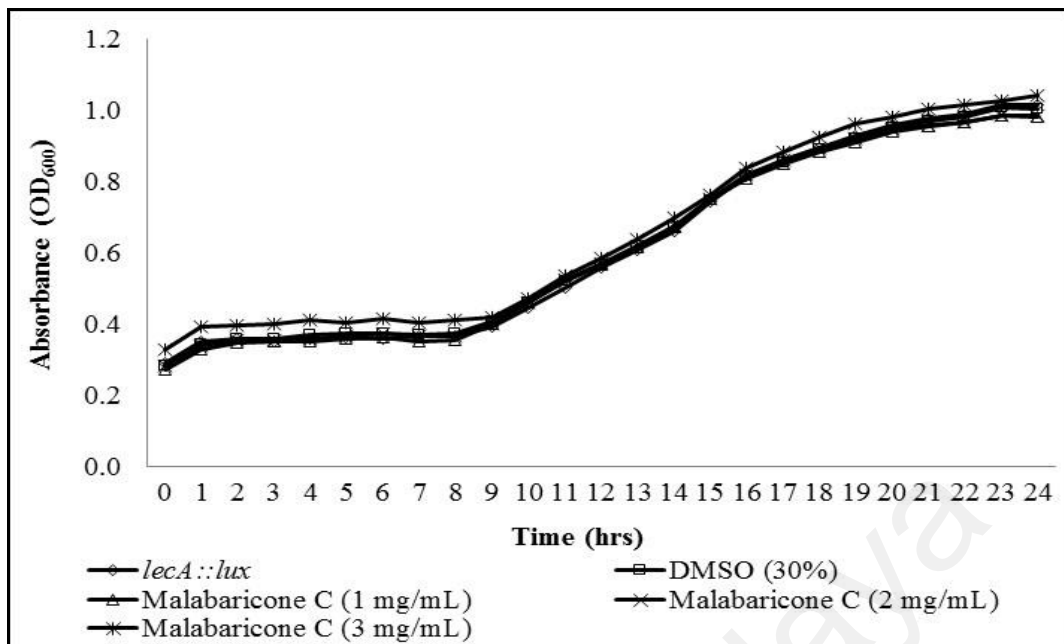
(A)
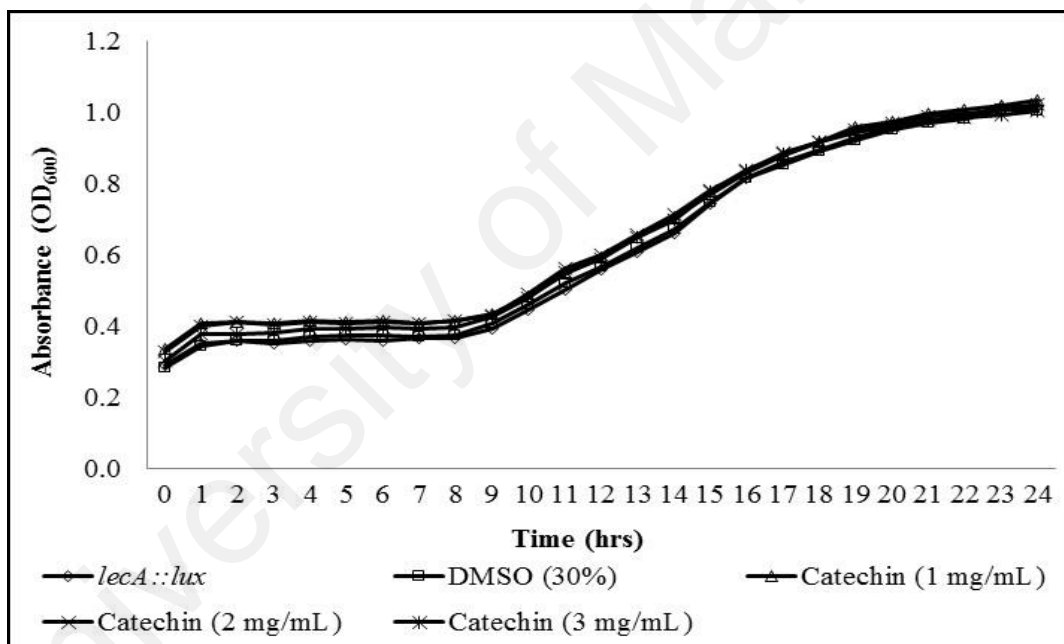
(B)

Figure 4.28: The growth of *P. aeruginosa* PAO1 *lecA::lux* with 30% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1, 2 and 3 mg/mL (B) synthetic catechin at 1, 2 and 3 mg/mL. Statistical analysis was conducted using ANOVA test.
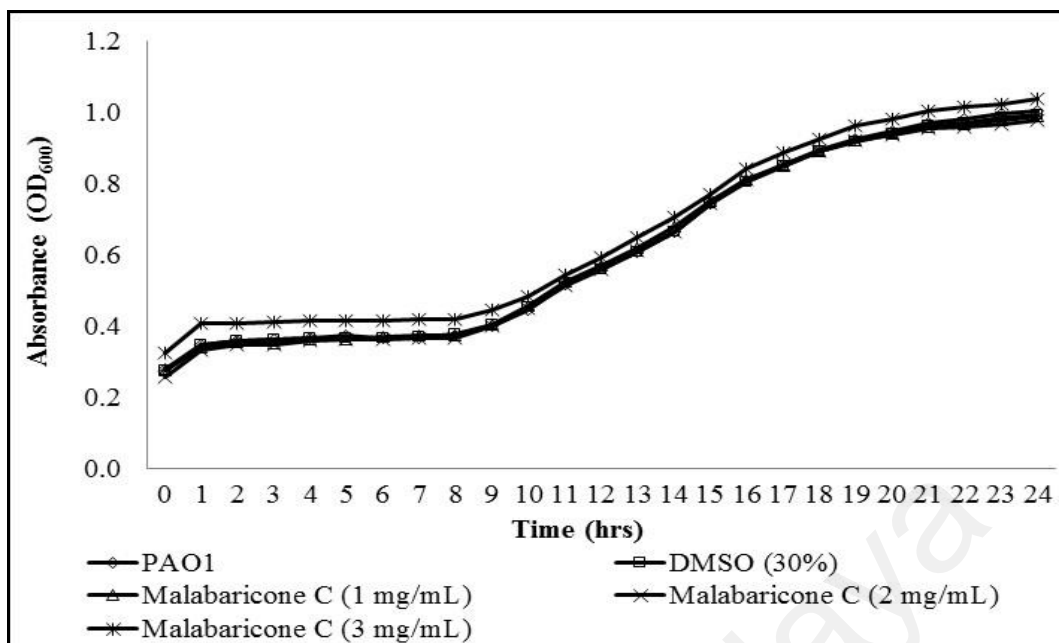
(A)
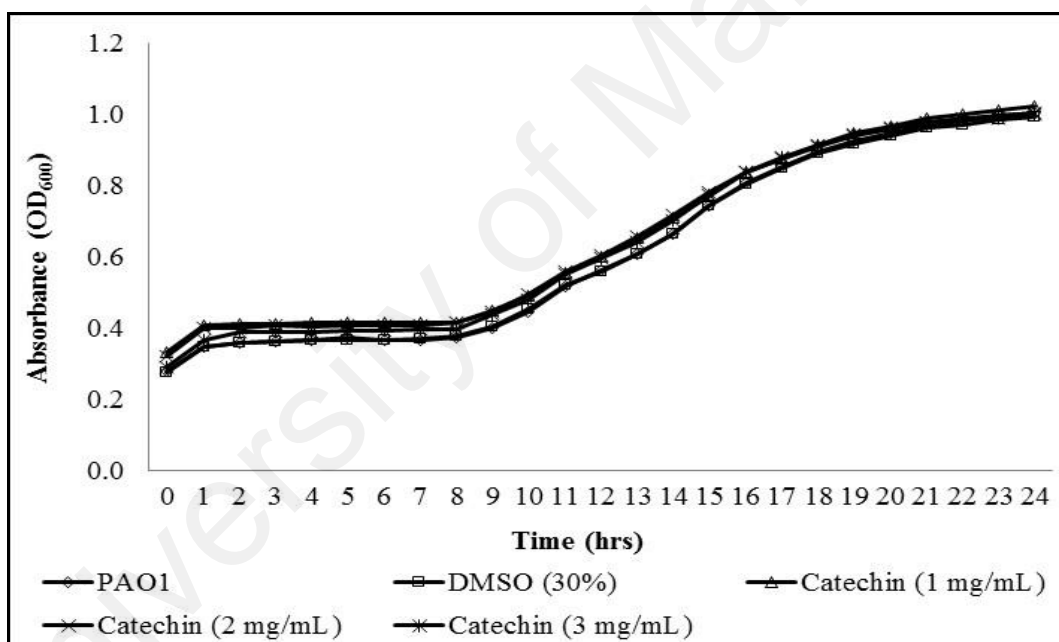
(B)

Figure 4.29: The growth of *P. aeruginosa* PAO1 with 30% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1, 2 and 3 mg/mL (B) synthetic catechin at 1, 2 and 3 mg/mL (C) synthetic gallic acid at 1, 2 and 3 mg/mL. Statistical analysis was conducted using ANOVA test.
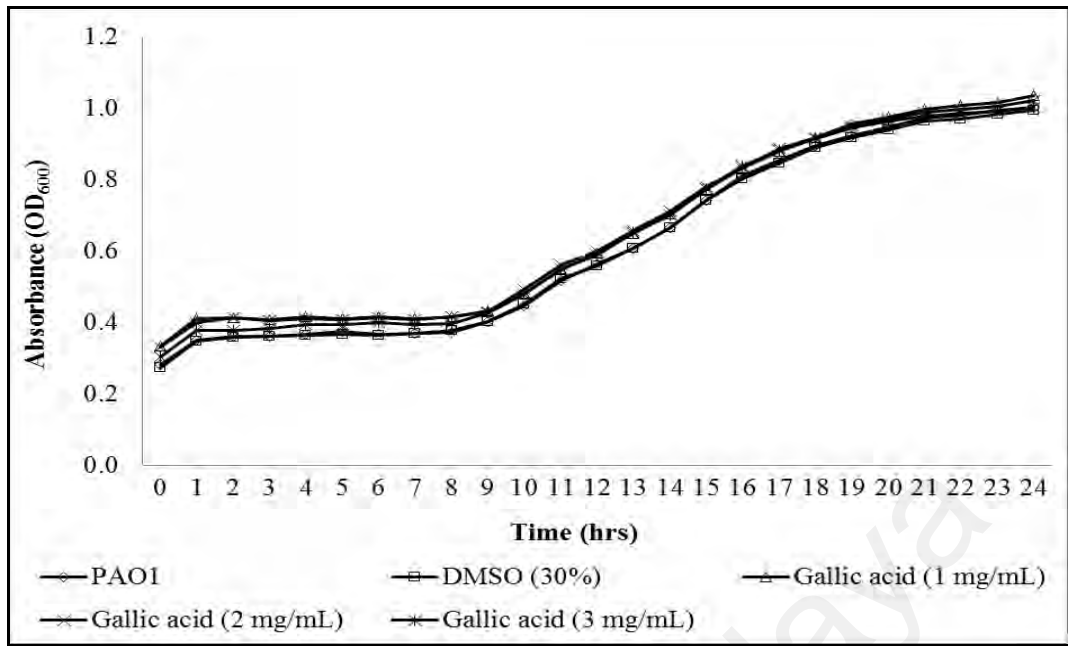
(C)

Figure 4.29, continued.
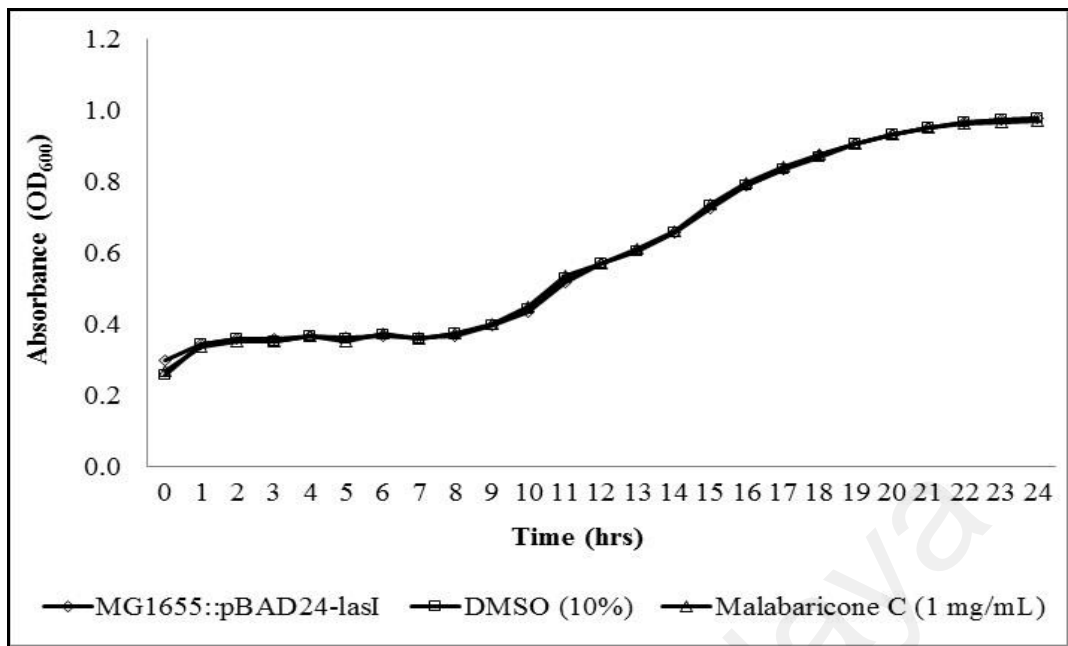
(A)
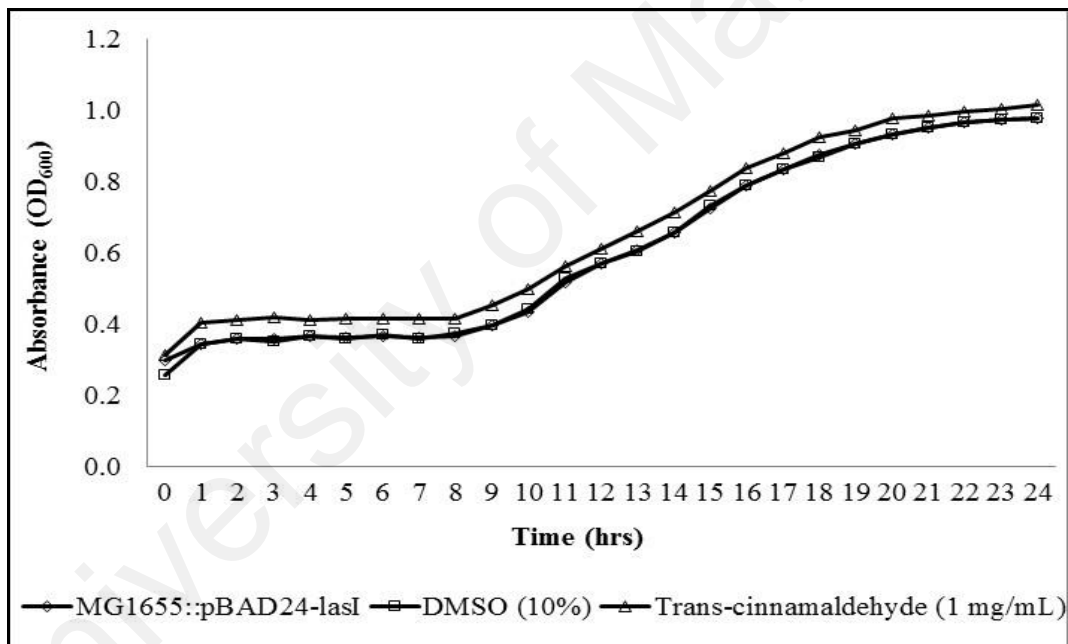
(B)

Figure 4.30: The growth of *E. coli* MG1655::pBAD24-*lasI* with 10% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1 mg/mL (B) synthetic trans-cinnamaldehyde at 1 mg/mL.
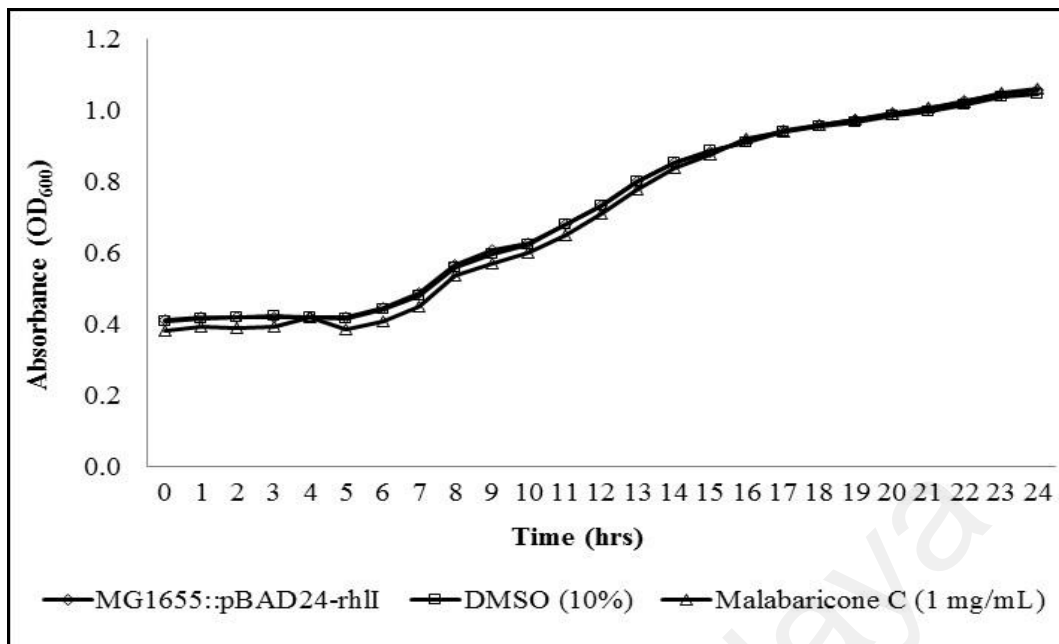
(A)
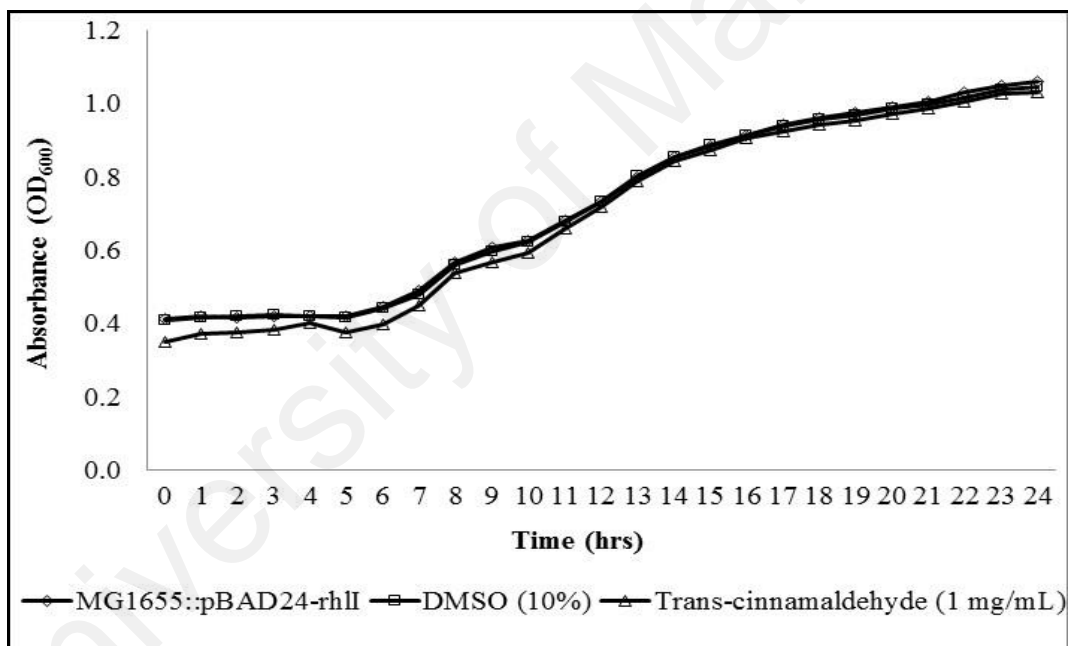
(B)

Figure 4.31: The growth of *E. coli* MG1655::pBAD24-*rhlI* with 10% (v/v) DMSO as control and in the presence of (A) malabaricone C at 1 mg/mL (B) synthetic trans-cinnamaldehyde at 1 mg/mL.

4.3.2 Screening of Malabaricone C for Anti-QS Activities

Malabaricone C was screened at 1, 2 and 3 mg/mL comcentration for anti-QS activities. Synthetic catechin served as positive control, unless stated otherwise.

4.3.2.1 Quantitative Analysis of Violacein in *C. violaceum* CV026

Malabaricone C was screened for anti-QS by using *C. violaceum* CV026 as the preliminary bioassay. *C. violaceum* CV026 was grown overnight with 1, 2 and 3 mg/mL of malabaricone C and synthetic catechin supplemented with C6-HSL in 96-well microtitre plate. The values of violacein production were expressed at OD 590 nm. DMSO was used as negative control while synthetic catechin was used as positive control. All of the experiments were done in triplicate, . The results showed that the production of violacein decreased as the concentration increased.
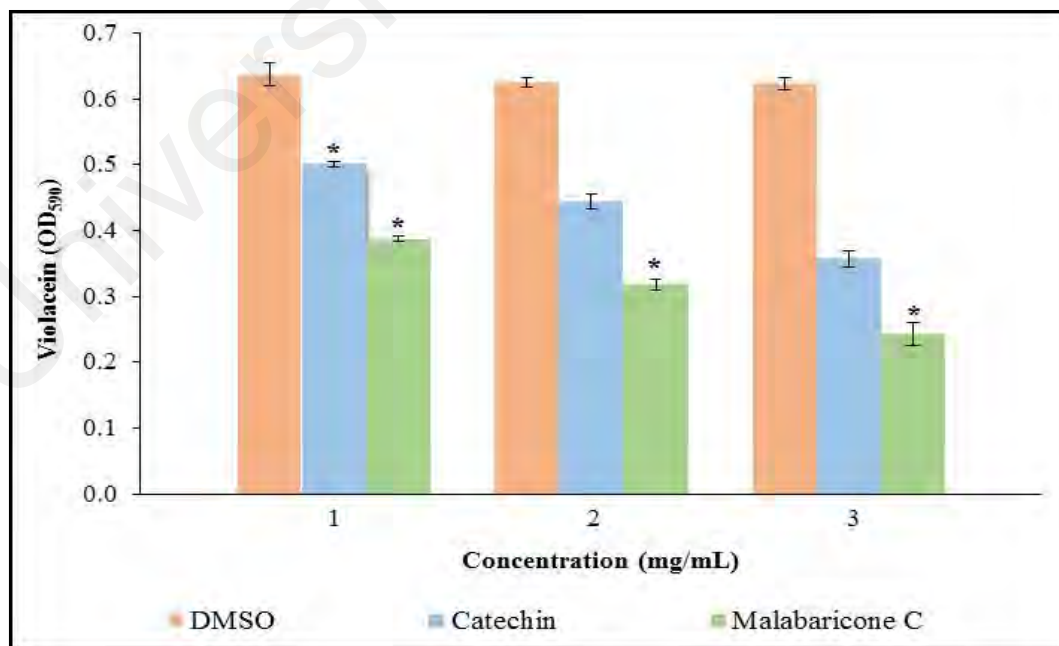
Figure 4.32: Inhibition of violacein production in *C. violaceum* CV026 by malabaricone C. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.3.2.2   Inhibition of the *lux*-based Biosensors

The luminescence expression for *E. coli* DH5α biosensors that contain the *lux* reporter plasmid pSB401 and pSB1075 was measured every 30 minutes for 24 hours using the Tecan Infinite M200. The luminescence was determined by relative light unit (RLU) divided by OD_{600}. Both pSB401 and pSB1075 was supplemented with synthetic 3-oxo-C6-HSL and 3-oxo-C10-HSL, respectively. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. The results displayed negative results for both lux-based biosensors.

(A)
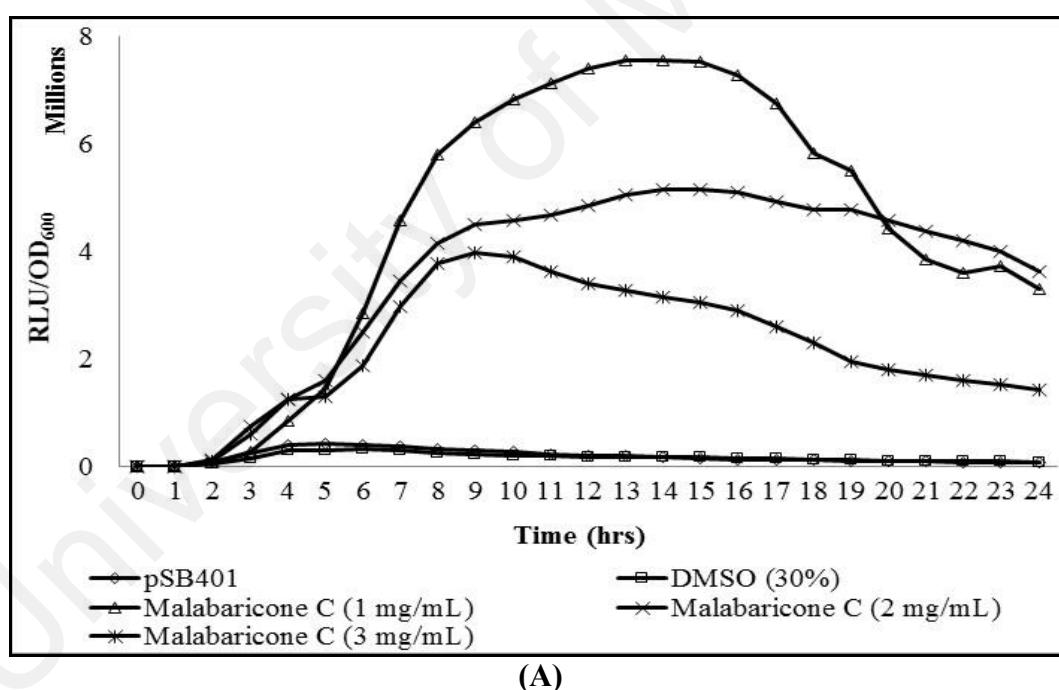
Figure 4.33: Inhibition of *lux*-based biosensors by malabaricone C in (A) *E. coli* [pSB401] (B) *E. coli* [pSB1075]. Statistical analysis was conducted using ANOVA test.
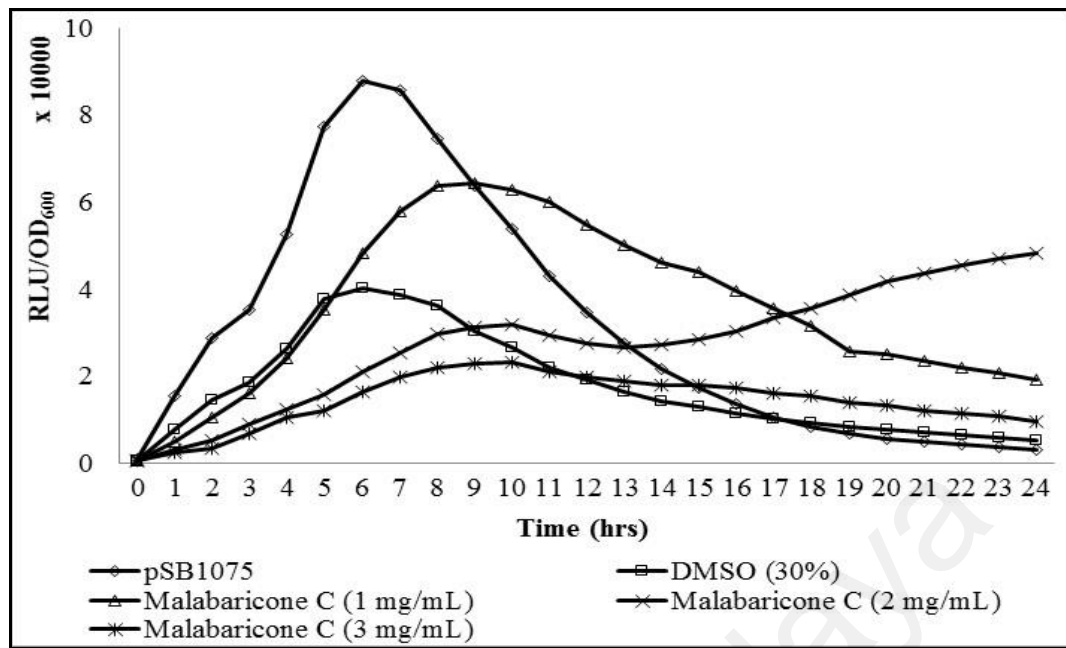
(B)

Figure 4.33, continued.

4.3.2.3 Inhibition *lecA::lux* Expression in *P. aeruginosa* PAO1

The expression of the *lecA::lux* in the *P. aeruginosa* PAO1 was determined by culturing *P. aeruginosa* PAO1 *lecA::lux* with 1, 2 and 3 mg/mL concentration of malabaricone C. The reading was taken by Tecan Infinite M200 every 30 minutes for 24 hours and the luminescence is given in relative light unit (RLU) divided by OD_{600}. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. The reading showed negative results.
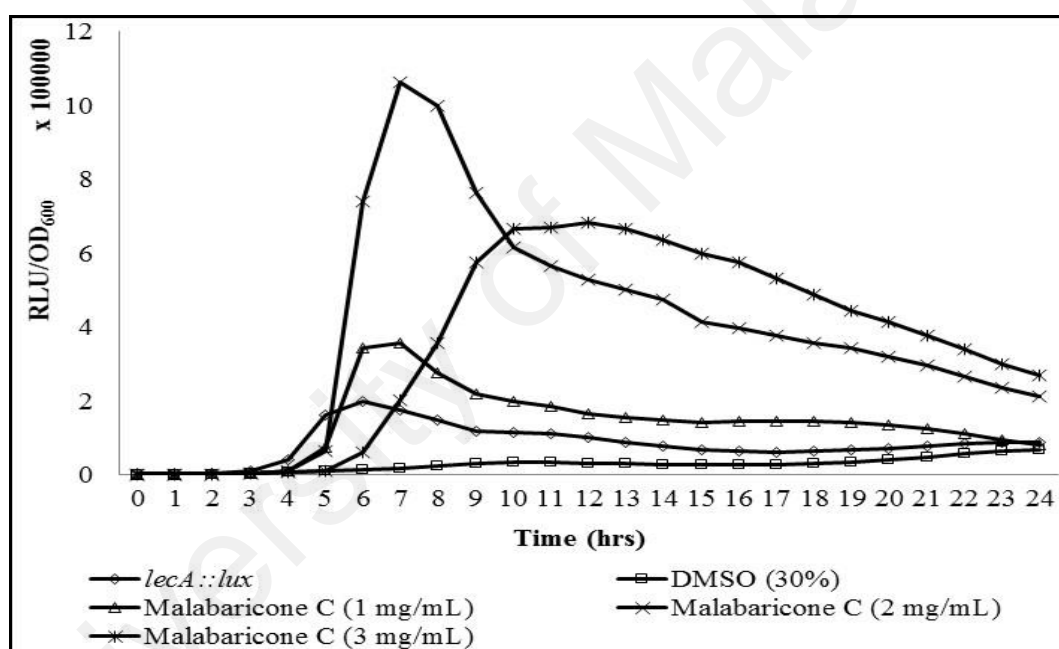
Figure 4.34: Inhibition of expression in *P. aeruginosa* PAO1 *lecA::lux* by malabaricone C. Statistical analysis was conducted using ANOVA test.

4.3.2.4 Inhibition of Pyocyanin Production

P. aeruginosa PAO1 was grown overnight with 1, 2 and 3 mg/mL of malabaricone C and synthetic catechin at 37°C with shaking. DMSO was used as negative control while synthetic catechin was used as positive control. The values of pyocyanin levels were expressed at OD of 520 nm. All of the experiments were done in triplicate, in three independent experiments. Malabaricone C was shown to decrease the production in violacein especially at 3 mg/mL concentration.
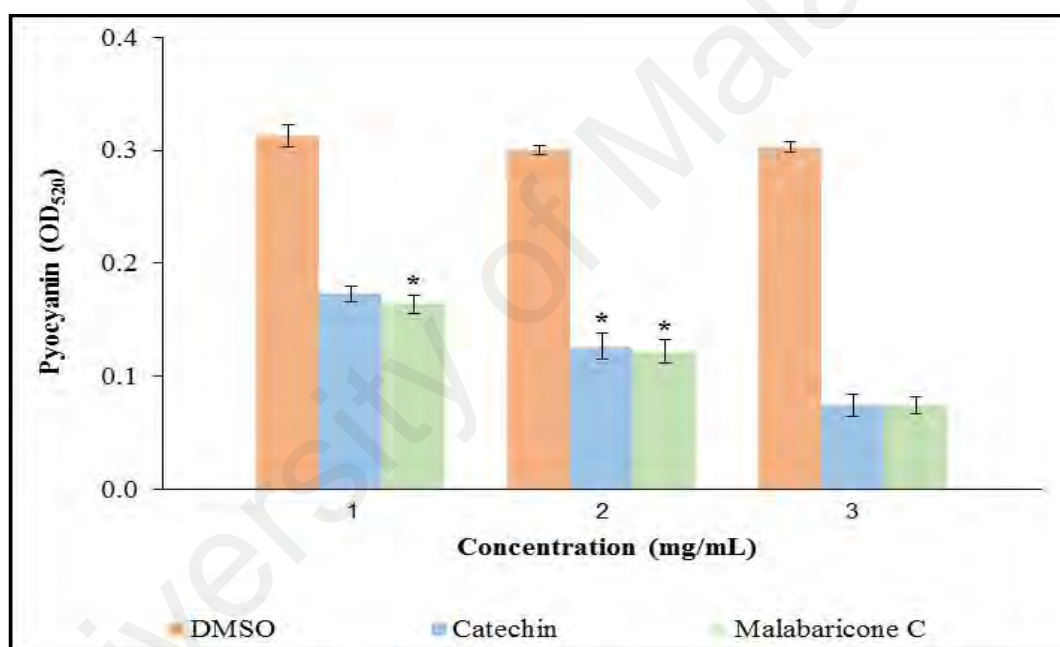
Figure 4.35: Inhibition of pyocyanin production in *P. aeruginosa* PAO1 by malabaricone C. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.3.2.5 Inhibition of Elastase Production

The elastolytic activities were determined by culturing *P. aeruginosa* PAO1 with malabaricone C at 1, 2 and 3 mg/mL. DMSO act as negative control while synthetic catechin act as positive control. The absorption of the supernatant was then measured at the OD of 495 nm. All of the experiments were done in triplicate, in three independent experiments. Based on the results obtained, malabaricone C did not inhibit the elastase production in *P. aeruginosa* PAO1.
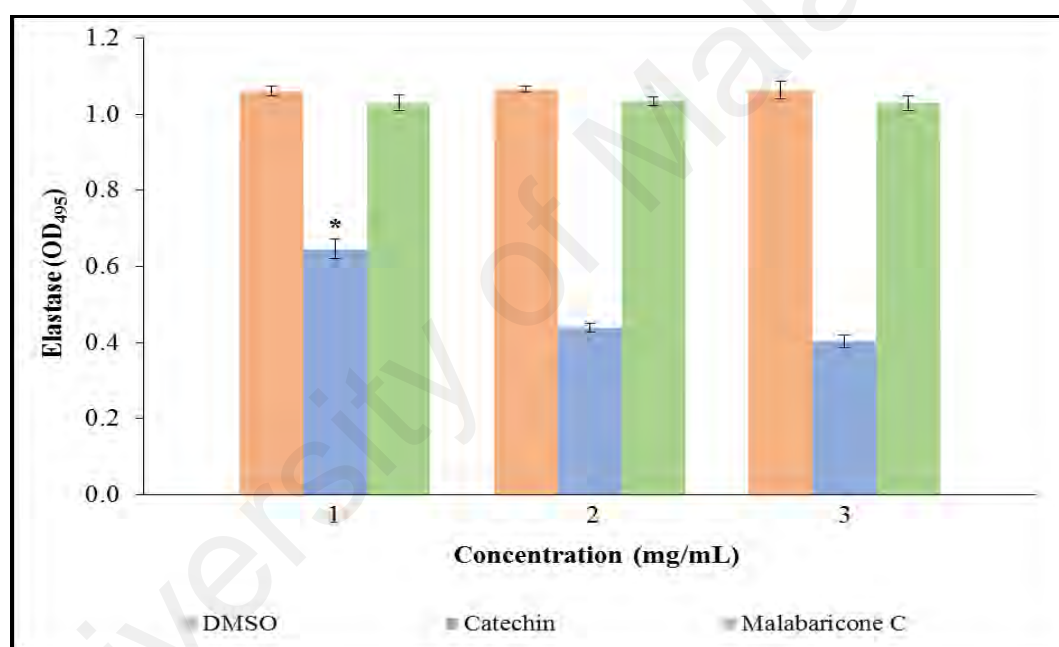
Figure 4.36: Inhibition of elastase production in *P. aeruginosa* PAO1 by malabaricone C. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.3.2.6 Inhibition of *P. aeruginosa* PAO1 Staphylolytic Activity

The LasA protease activity was conducted by measuring the changes in the absorbance caused by the lysis of *S. aureus* cells after every 5 minutes interval for 1 hour. Malabaricone C with the concentration of 1, 2 and 3 mg/mL was subsequently co-cultured with the *P. aeruginosa* PAO1 for 13 hours at 37°C. The changes of the activities were measured at the absorbance of 600 nm. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. Malabaricone C did not exhibit positive results in the inhibition of staphylolytic activity,
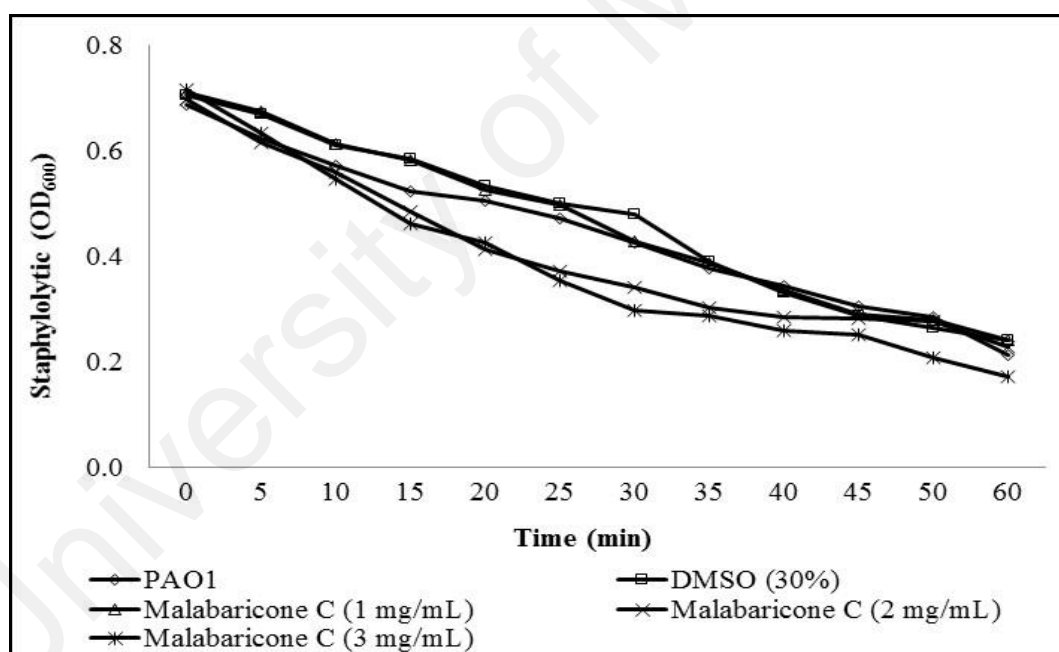
Figure 4.37: Inhibition of staphylolytic activity by malabaricone C. Statistical analysis was conducted using ANOVA test.

4.3.2.7 Inhibition of Swarming

The effects of malabaricone C against the *P. aeruginosa* PAO1 swarming motility was assessed by using the swarming plates supplemented with glucose. DMSO was used as negative control while gallic acid acts as positive control. All of the experiments were done in triplicate, in three independent experiments.


(A)   **(B)**

(C)   **(D)**   **(E)**

(F)   **(G)**   **(H)**
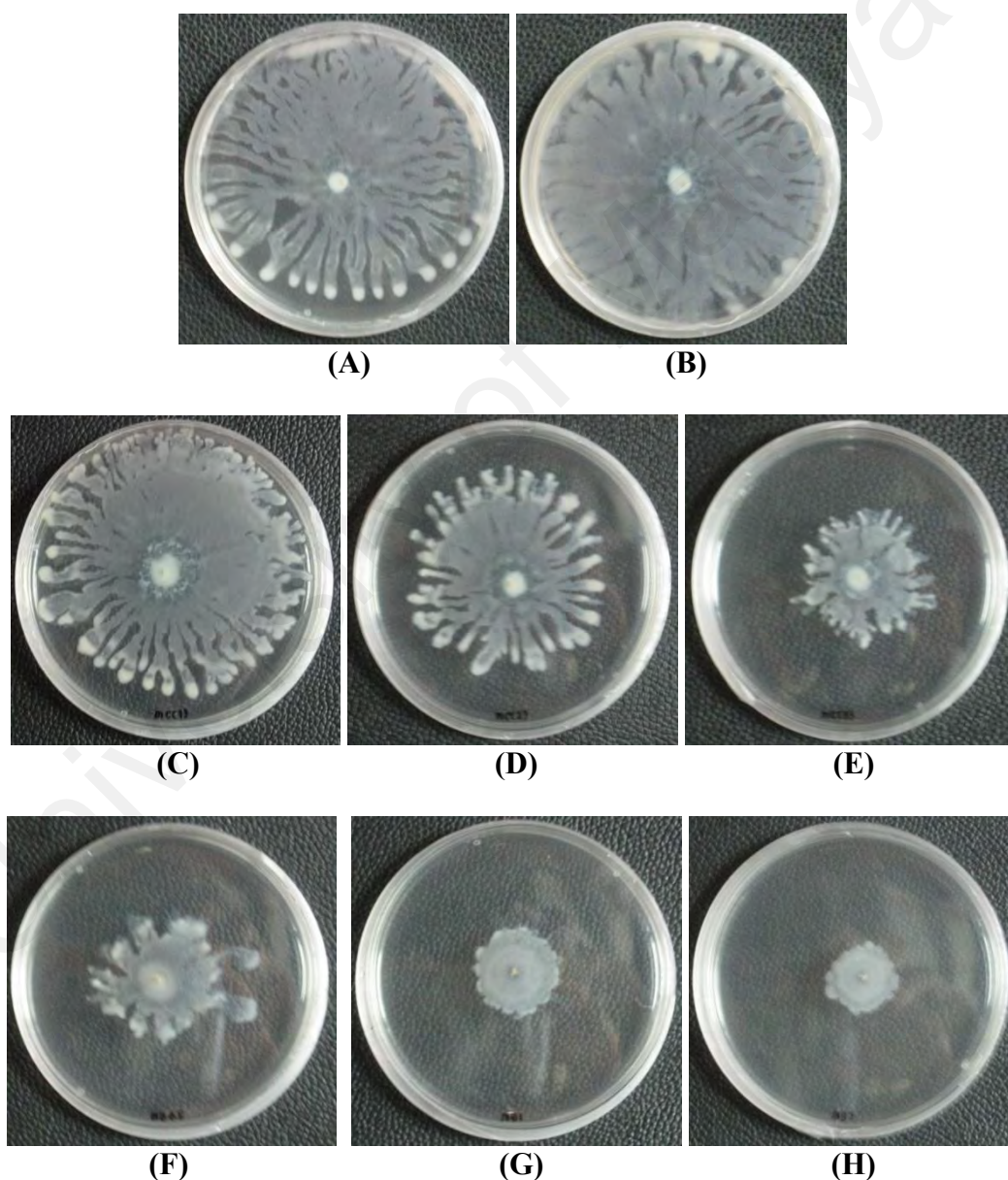
Figure 4.38: Swarming agar inoculated with (A) *P. aeruginosa* PAO1 and supplemented with (B) 30% (v/v) DMSO (C) 1 mg/mL malabaricone C (D) 2 mg/mL malabaricone C (E) 3 mg/mL malabaricone C (F) 1 mg/mL synthetic gallic acid (G) 2 mg/mL synthetic gallic acid (H) 3 mg/mL synthetic gallic acid

4.3.2.8   Screening of Biofilm Inhibition

Biofilm plays an important role in *P. aeruginosa* PAO1 and static microtiter plate assay was used to screen the ability of malabaricone C in inhibiting the production of biofilm. The values of biofilm production were expressed at OD of 590 nm. DMSO was used as negative control while synthetic catechin was used as positive control. All of the experiments were done in triplicate, in three independent experiments.
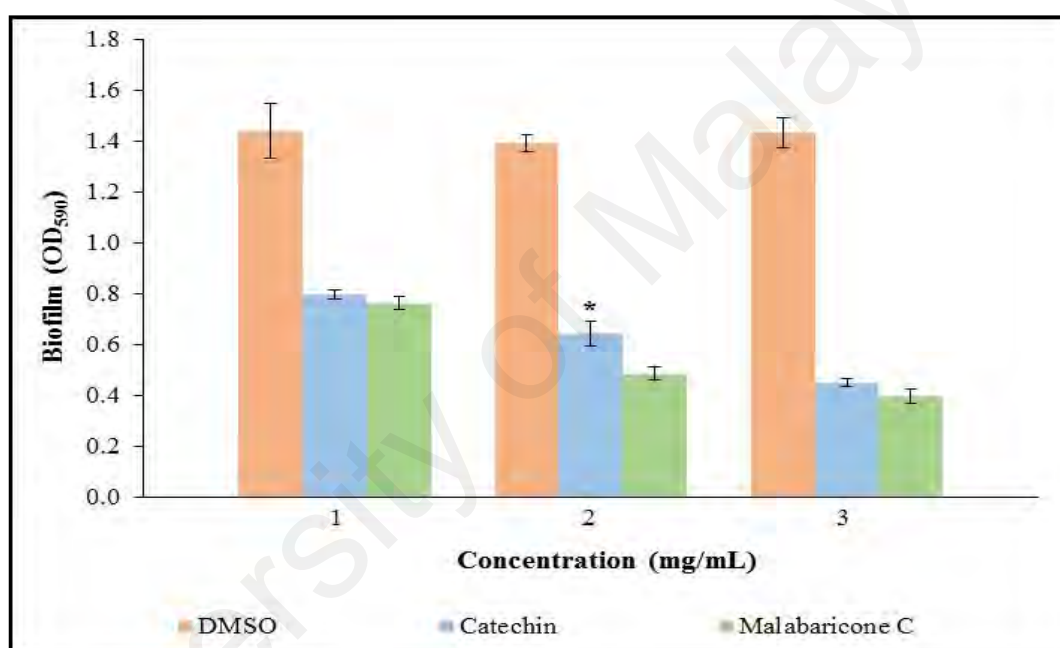
Figure 4.39: Inhibition of biofilm in *P. aeruginosa* PAO1 by malabaricone C. Error bars indicate standard deviations (n = 3) while * indicates $p < 0.05$. Statistical analysis was conducted using ANOVA test.

4.3.3   Discover the Mode of Action of Malabaricone C against *P. aeruginosa* PAO1 QS System

E. coli which contains arabinose-dependent expression plasmids with pBAD-*lasI* and pBAD-*rhlI* were used to screen if malabaricone C targets the QS signal synthase or transcriptional regulator protein antagonists. With the arabinose, both *E. coli* MG1655::pBAD24-*rhlI* and *E. coli* MG1655::pBAD24-*lasI* will produce C4-HSL and 3-oxo-C12-HSL, respectively. AHLs that were extracted from the pBAD biosensors treated with 1 mg/mL malabaricone C were subsequently analysed using *E. coli* [pSB401] and *E. coli* [pSB1075] as these biosensors can detect short and long chain AHLs, correspondingly. Trans-cinnamaldehyde was used as positive control and the experiments were done in triplicate.
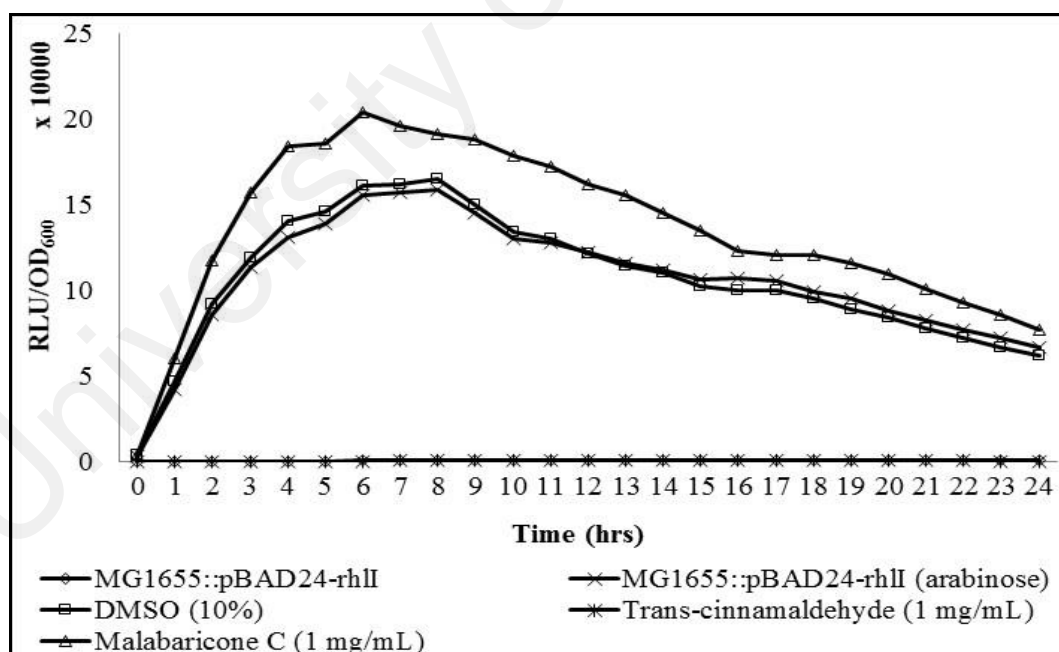
Figure 4.40: Analysis of *E. coli* MG1655::pBAD24- *rhlI* extracted AHLs treated with 1 mg/mL malabaricone C using *E. coli* [pSB401]. Statistical analysis was conducted using ANOVA test.
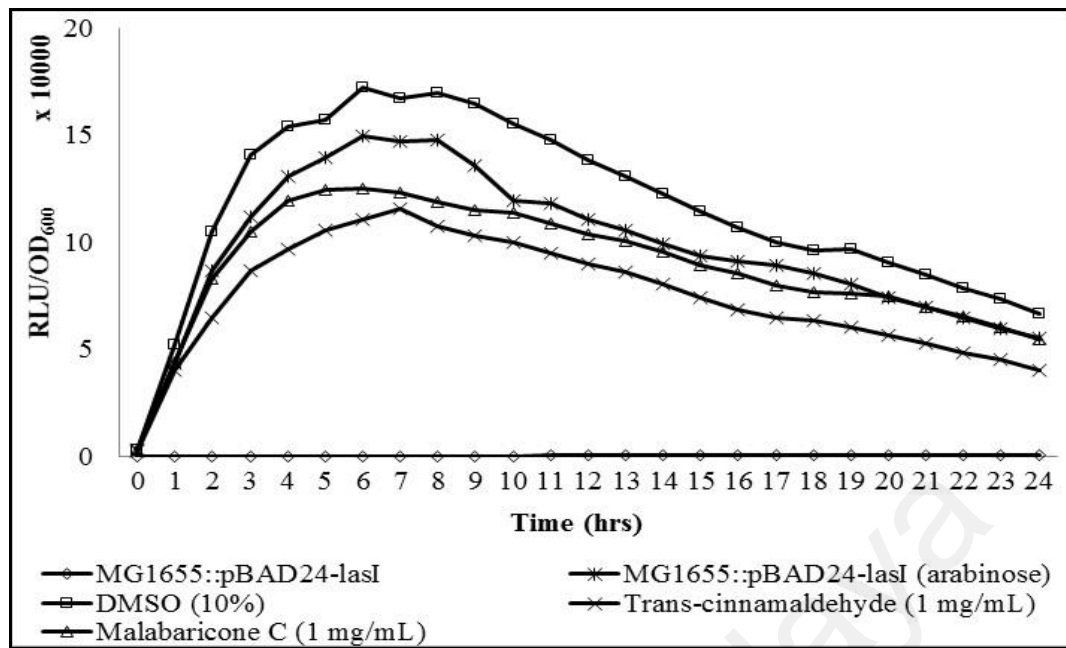
Figure 4.41: Analysis of *E. coli* MG1655::pBAD24-*lasI* extracted AHLs treated with 1 mg/mL malabaricone C using *E. coli* [pSB1075]. Statistical analysis was conducted using ANOVA test.

4.4   Transcriptome Analysis of *P. aeruginosa* PAO1 Treated with Catechin and Malabaricone C

4.4.1   RNA Quality Check and Library Preparation

The quality and total volume of the extracted RNA for both catechin (Appendixes I−J) and malabaricone C (Appendixes K−L) were checked prior to sequencing.

4.4.2   Transcriptome Data Analysis

The differential expression analysis of catechin and malabaricone C against *P. aeruginosa* PAO1 were performed with RNA-seq technique using HiSeq 2500 Sequencing System (Illumina, USA). The raw data for each pool of samples were trimmed separately and assembled with CLC Genomics Workbench version 7.5 (CLC Bio, Denmark). The reads were then mapped using the reference sequences obtained from GenBank (accession number: AE004091).

The proportion test method was used to detect the differentially expressed genes with false discovery rate (FDR) and p value < 0.05. The expression values were estimated as reads per kilobase of exon model per million mapped reads (RPKM). Statistical analysis using Empirical analysis of DGE (EDGE) tool was then carried out after obtaining the quantile normalization and transformation of the RPKM values.

The box plot for each samples was plotted where it indicates the $\log_2$ transformed values of the differentially expressed genes in *P. aeruginosa* PAO1. It is normal to have all the box in the same size as the figures were generated based on the normalised data while data that were not normalized will present irregular heights of the box plot. The volcano plot gives a general view of the differentially expressed genes with $p < 0.05$. The dots on the left represents down-regulated genes while the dots on the right represents up-regulated genes. The genes ID and the functions of the genes affected were obtained from Pseudomonas Genome Database.

4.4.2.1 Catechin
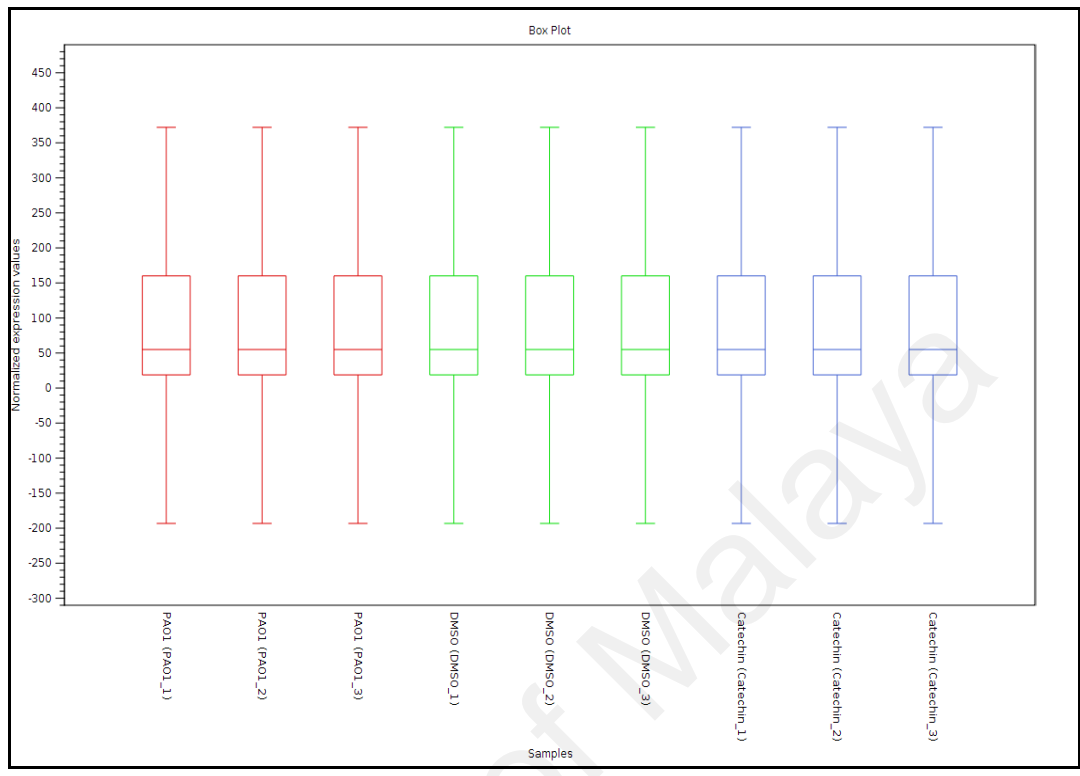
Figure 4.42: Box plot of normalized values for catechin.
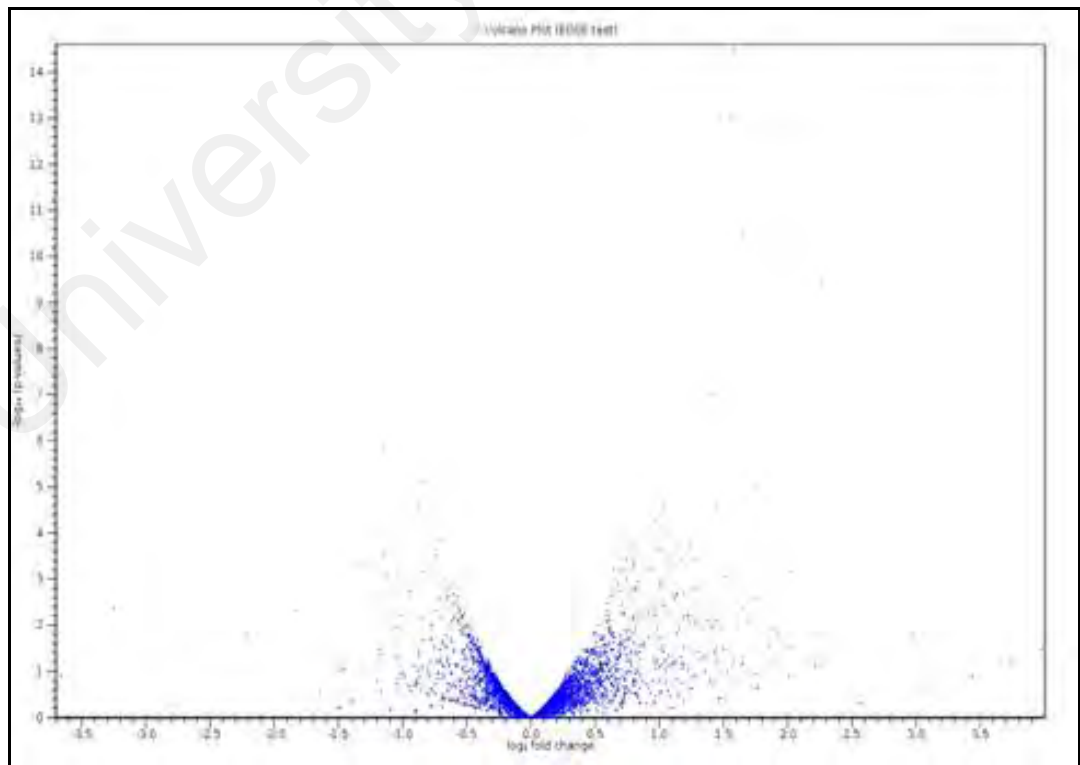
Figure 4.43: Volcano plot of normalized values for catechin.

Table 4.5: Significantly down-regulated genes of *P. aeruginosa* PAO1 treated with 1 mg/mL catechin.

Gene ID	p-value	Fold change	Gene function
PA5482	0.00238364	-9.64054	Hypothetical protein
eraR	0.0129258	-7.0445	Response regulator EraR. Ethanol oxidation pathway
pchC	0.0025391	-5.68064	Pyochelin biosynthetic protein PchC
pchG	0.0000386407	-4.92952	Pyochelin biosynthetic protein PchG
PA1349	0.0387751	-4.58197	Conserved hypothetical protein
pchR	0.00180231	-3.92383	Transcriptional regulator PchR
ampO	0.00233737	-3.72321	Antibiotic resistance and susceptibility
PA5481	0.00460401	-3.50422	Hypothetical protein
PA4222	0.0000602514	-3.33776	Probable ATP-binding component of ABC transporter
nasS	0.008756	-3.10251	Nitrate transport ATP-binding protein
PA4223	0.0000485658	-3.09349	Probable ATP-binding component of ABC transporter
PA4911	0.00650151	-2.95056	Probable permease of ABC branched-chain amino acid transporter
ampP	0.00000910672	-2.87945	Antibiotic resistance and susceptibility
pchD	0.000603104	-2.80866	Pyochelin biosynthesis protein PchD
pchF	0.0000025049	-2.78886	Pyochelin synthetase
pchE	0.0000000204648	-2.78049	Dihydroaeruginoic acid synthetase
PA4738	0.0121458	-2.48036	Conserved hypothetical protein
lipH	0.0327223	-2.39759	Lipase modulator protein
fptA	0.000118422	-2.31028	Fe(III)-pyochelin outer membrane receptor precursor
PA4826	0.0240966	-2.23411	Hypothetical protein
PA1364	0.0083424	-2.22189	Probable transmembrane sensor
PA4097	0.00339916	-2.19072	Probable alcohol dehydrogenase (Zn-dependent)
PA2166	0.00274566	-2.1749	Hypothetical protein
PA1280	0.0128704	-2.17805	Hypothetical protein
PA4140	0.00903837	-2.1417	Hypothetical protein
yieF	0.0270685	-2.12799	NAD(P)H quinone oxidoreductase
PA0632	0.00244913	-2.07802	Hypothetical protein
PA1606	0.03708	-2.0708	Hypothetical protein
ibpA	0.00699956	-2.06948	Heat-shock protein IbpA
PA1044	0.0162241	-2.3038	Hypothetical protein
PA1168	0.032826	-2.26164	Hypothetical protein
napB	0.0227153	-2.48711	Cytochrome c-type protein NapB precursor
pscQ	0.049003	-2.42244	Translocation protein in type III secretion
mntP	0.0226195	-2.08074	Conserved hypothetical protein
pchA	0.000141668	-2.00706	Salicylate biosynthesis isochorismate synthase

Table 4.6: Significantly up-regulated genes of *P. aeruginosa* PAO1 treated with 1 mg/mL catechin.

Gene ID	p-value	Fold change	Gene function
PA1924	0.00937993	5.82194	Hypothetical protein
PA1883	0.0321246	4.97777	Probable NADH-ubiquinone/plastoquinone oxidoreductase
PA0252	0.00729721	3.04361	Hypothetical protein
PA4155	0.0367964	2.70018	Hypothetical protein
PA3506	0.00689311	2.62367	Probable decarboxylase
PA0474	0.0475768	2.61398	Hypothetical protein
PA4703	0.0299064	2.43747	Hypothetical protein
cdhC	0.00258771	2.41434	Carnitine dehydrogenase-related gene C
PA2550	0.00108262	2.36544	Probable acyl-coA dehydrogenase
PA3432	0.0260953	2.36489	Hypothetical protein
PA0384	0.00310588	2.34808	Hypothetical protein
shaE	0.00663461	2.33277	Membrane proteins
PA0817	0.00837155	2.30743	Probable ring-cleaving dioxygenase
PA2913	0.0155691	2.26998	Hypothetical protein
PA2947	0.0437007	2.22759	Hypothetical protein
PA2600	0.0249448	2.22348	Hypothetical protein
PA5404	0.0257857	2.22093	Hypothetical protein
PA1029	0.00593058	2.18992	Hypothetical protein
PA3338	0.0254185	2.17263	Hypothetical protein
moaD	0.00648457	2.17202	Molybdopterin converting factor, small subunit
PA0048	0.0198794	2.16826	Probable transcriptional regulator
moaB1	0.00609477	2.05497	Molybdopterin biosynthetic protein B1
PA2280	0.000929979	2.01279	Oxidoreductase
xylZ	0.048415	2.00543	Toluate 1,2-dioxygenase electron transfer component

4.4.2.2 Malabaricone C
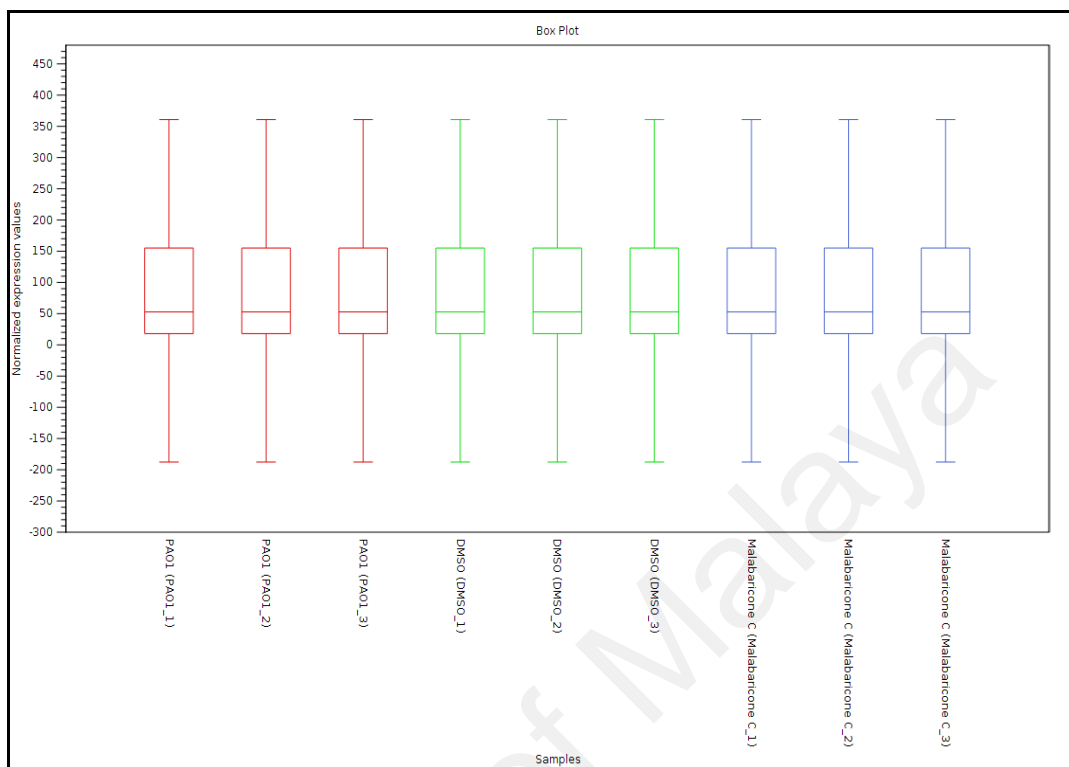
Figure 4.44: Box plot of normalized values for malabaricone C.
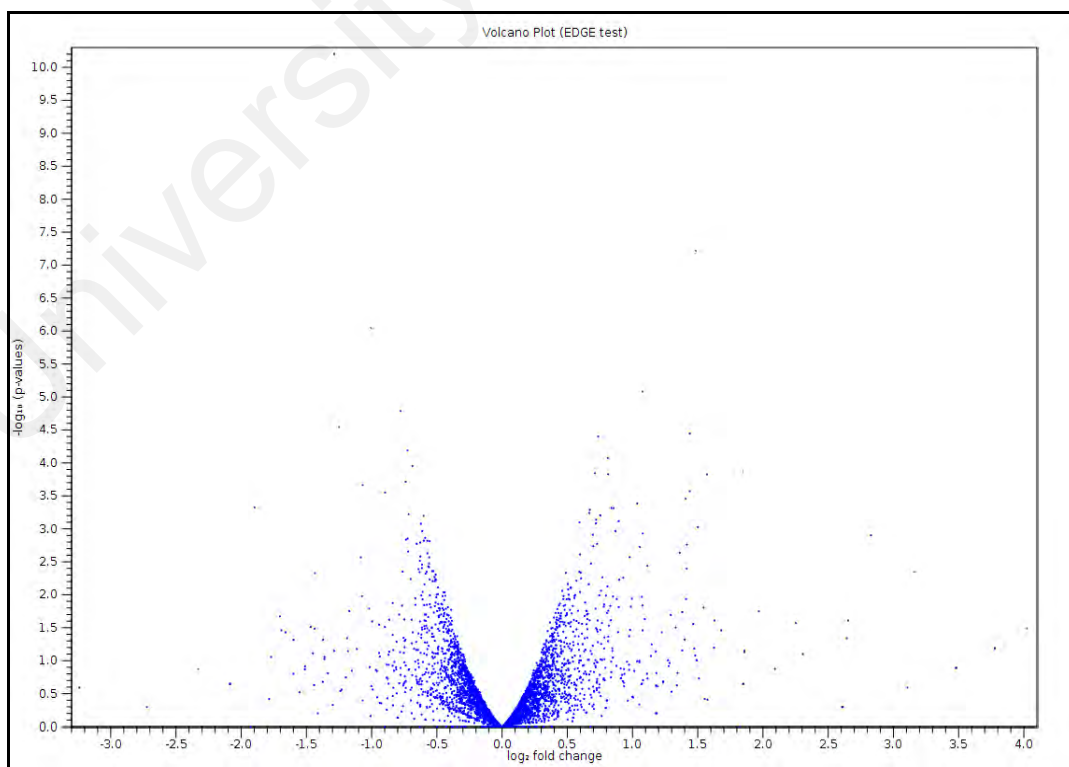
Figure 4.45: Volcano plot of normalized values for malabaricone C.

Table 4.7: Significantly down-regulated genes of *P. aeruginosa* PAO1 treated with 1 mg/mL malabaricone C.

Gene ID	p-value	Fold change	Gene function
lhpM	0.0174206	-8.78833	Permease of ABC transporter, LhpM
PA0111	0.00691644	-8.31171	Hypothetical protein
PA3422	0.0154037	-7.27997	Probable ATP-binding component of ABC transporter
PA2156	0.0237749	-7.20894	Conserved hypothetical protein
PA5482	0.0179112	-7.05733	Hypothetical protein
PA2087	0.0323303	-6.17907	Hypothetical protein
PA4077	0.0124059	-5.27019	Probable transcriptional regulator
PA1349	0.0327367	-4.73112	Conserved hypothetical protein
PA0489	0.0344653	-3.16482	Probable phosphoribosyl transferase
PA2045	0.0396702	-2.95368	Conserved hypothetical protein
nasS	0.014739	-2.93989	Nitrate transport ATP-binding protein
PA0818	0.0152019	-2.89286	Hypothetical protein
PA1364	0.0274298	-2.87379	Probable transmembrane sensor
PA0570	0.000550493	-2.85868	Hypothetical protein
PA0627	0.0119955	-2.8564	Conserved hypothetical protein. R-type pyocin, related to P2 phage; tail formation
PA4738	0.00848462	-2.74757	Conserved hypothetical protein
PA3507	0.0051916	-2.72443	Probable short-chain dehydrogenase
PA5211	0.0222638	-2.72132	Conserved hypothetical protein. Membrane proteins
PA1892	0.0145622	-2.67981	Hypothetical protein
PA3720	0.0203993	-2.5779	Hypothetical protein
PA1697	0.0188071	-2.56678	ATP synthase in type III secretion system
atuE	0.044463	-2.49394	Putative isohexenylglutaconyl-coA hydratase
PA3496	0.0360025	-2.37566	Hypothetical protein
PA3445	0.00783169	-2.28604	Conserved hypothetical protein
PA1237	0.0179579	-2.13004	Probable multidrug resistance efflux pump
mtlD	0.0296501	-2.09071	Mannitol dehydrogenase
PA1044	0.0369927	-2.08662	Hypothetical protein. Membrane proteins
yieF	0.0342117	-2.06842	NAD(P)H quinone oxidoreductase
PA1301	0.010589	-2.04476	Probable transmembrane sensor

Table 4.8: Significantly up-regulated genes of *P. aeruginosa* PAO1 treated with 1 mg/mL malabaricone C.

Gene ID	p-value	Fold change	Gene function
PA2422	0.000470977	12.4688	Hypothetical protein
PA2936	0.0276632	3.85422	Hypothetical protein
PA3381	0.0496921	3.50713	Probable transcriptional regulator
PA4570	0.0253336	2.86018	Hypothetical protein
PA5397	0.041842	2.84666	Hypothetical protein
hptA	0.000159794	2.80107	Histidine phosphotransfer protein HptA
PA0980	0.0404589	2.79332	Hypothetical protein
sdsA1	0.00021316	2.73724	SDS hydrolase SdsA1
PA3843	0.0209964	2.65284	Hypothetical protein
PA2669	0.00334425	2.42901	Hypothetical protein
PA0741	0.0252758	2.3467	Conserved hypothetical protein
PA2922	0.00108958	2.32951	Probable hydrolase
PA0529	0.00406629	2.31899	Conserved hypothetical protein
PA0498	0.000287906	2.24253	Hypothetical protein
PA3598	0.00203754	2.20723	Conserved hypothetical protein
PA4724.1	0.0191764	2.17346	Conserved hypothetical protein
PA2346	0.0149258	2.12679	Conserved hypothetical protein
PA1134	0.00583319	2.12164	Hypothetical protein
PA2182	0.00918062	2.10787	Hypothetical protein
PA1921	0.036616	2.07069	Hypothetical protein
PA0466	0.0451473	2.06542	Hypothetical protein

CHAPTER 5: DISCUSSION

5.1   Crude Extracts

5.1.1   Processing of Plant Samples

Secondary metabolites produced by plants have been studied for over the last 50 years where it plays a vital role in the adaptation of the plants to their environment as well as an important source in the pharmaceuticals area (Bourgaud *et al.*, 2001). Secondary metabolites are biosynthetically derived from the primary metabolites. Unlike the primary metabolites, the secondary metabolites are not made through the metabolic pathways and are not required for the growth and development of the plants. Among the examples of secondary metabolites produced by the plants includes alkaloids, phenolics, steroids, essential oils, lignins, phenols, flavones, flavonoids, terpenoids, resins and tannins (Cowan, 1999). In this study, 15 plants samples (Table 3.4) were screened for anti-QS activities in *P. aeruginosa* PAO1.

The first step of the work is to process and prepare the plant samples accordingly in order to preserve the secondary metabolites in the plants before extraction. Plants such as *G. mangostana*, *M. foetida*, *S. samarangense*, *A. carambola*, *P. amaryllifolius*, *C. citratus*, *M. charantia*, *P. crispum* and *M. spicata* are considered fresh samples while the rest are dried samples. The samples were initially cleaned and dried with oven at 45ºC before ground into fine powder form with industrial grade blender. The fine powder form is better than the ground form as it gave more homogenized and smaller sized particles, therefore, having a better surface contact with the extraction solvents (Azwanida, 2015). The process used to prepare the samples are similar to the

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maceration technique where it involved soaking the powdered form samples of the with solvents and allowed to stand at room temperature for a period of time with frequent agitation. This will then soften the samples and thus, break the plant's cell wall and release the soluble phytochemicals. The mixture was then filtered to remove the solid plant material followed by evaporation with rotary evaporator, leaving only the crude extracts (Handa *et al*., 2008). The collected crude extracts were then further dried in the fume hood to ensure complete evaporation of the extraction solvents or the water vapour formed during evaporation. This step was conducted in order to decrease the effects of any residual solvents in the bioassay that might give false positive results or to prevent any samples contamination.

Serial exhaustive extraction (SEE) was used to extract the biomolecules from the powdered form of the plant samples. This method involves successive extraction using various solvents with different polarity and hence, improved the isolation of the compounds from the complex crude extracts (Das *et al*., 2010). Three extraction series were used sequentially in this study with a range of polarities varying from non-polar (hexane) followed by intermediate polarity (chloroform) and finally polar solvent (methanol). Hexane was used to extracts volatile oils as well as to remove the plant's chlorophyll while chloroform was used to extracts terpenoid and flavonoids (Cowan, 1999; Cos *et al*., 2006). Methanol, on the other hand, was used to extract numerous secondary metabolites such as anthocyanins, terpenoids, saponins, tannins, xanthoxyllines, totarol, quassinoids, lactones, flavones, phenones and polyphenols (Cowan, 1999).

Before preparing the crude extracts stock solutions, the final weight of the extracts was first measured. Dimethyl sulfoxide (DMSO) was used to prepare the stock solution due to its ability to completely dissolve a wide range of compounds found in the crude extracts. Methanol and ethanol was not recommended as the solvents evaporate easily as compared to DMSO. In addition to that, there are advantages of using 100% DMSO in preparing the stock solutions: (a) reduce the need of sterilization due to microbial contamination and (b) assuring good solubility of crude extracts during dilution process (Cos *et al.*, 2006). As DMSO is potentially toxic to many microorganisms and cells, it is significant to test the extracts diluted in DMSO with minimum inhibitory concentration (MIC) assay in order to avoid interference in the biological test systems and produce false positive results. The stock solution for all the crude extracts will then be kept in -20°C.

5.1.2   Anti-QS Activities of Crude Extracts

Natural products are an important source of chemical diversity for the potential identification and development of new anti-pathogenic drugs (Vandeputte *et al.*, 2010). There are three different targets that the natural products can aim, namely, the signal generator, signal molecule and the signal receptor. An obvious strategy is to screen for any compounds in the natural products that can either prevent the signal molecules from being synthesized and thus, decrease the expression of the *luxI*-encoded AHL synthase or compounds that can mimic the autoinducers produced by the bacteria and compete with the AHLs to bind to the signal receptor (Givskov *et al.*, 1996; Rasmussen & Givskov, 2006).

In the recent years, extensive studies have been conducted related to the *P. aeruginosa* PAO1 QS system so as to understand the communication between the bacteria themselves in order to target their pathogenicity (Govan & Deretic, 1996). *P. aeruginosa* PAO1 is a well-known opportunistic human pathogen that can secrete various QS-related virulence determinants and secondary metabolites that includes elastase, alkaline protease, LasA protease, exotoxin A, phospholipase C, exoenzyme S, hydrogen cyanide as well as pyocyanin (Hirakate *et al.*, 1995; Preston *et al.*, 1995). The toxins produced are thought to be the main contributors for both acute and chronic infections among the human (Rubin *et al.*, 2008). Moreover, *P. aeruginosa* PAO1 is also a formidable biofilm former and thus, making it harder to be treated with antibiotics (Davies *et al.*, 1998). Even though the expression of the virulence factors is normally dependent on the environmental stimulus like iron, nitrogen availability, temperature or osmolarity, a high cell density is also required in order to induce the virulence (Pearson *et al.*, 1997).

The plants that are selected in this study have long been used as remedies for illness and the results obtained appear to provide some additional information regarding their usage. The ability of the extracts to quench the QS-regulated virulence in *P. aeruginosa* PAO1 such as swarming, elastase, pyocyanin, staphylolytic as well as bioluminescence was tested. For preliminary screening, 1 mg/mL concentration was used to test the QS activities before selecting the best extract for purification purpose. The present results have shown that the extracts from the samples listed in Table 3.4 has the ability to reduce the production of QS-regulated virulence factors in *P. aeruginosa* PAO1. Throughout the entire work, the growth of the bacteria was measured in order to confirm that it was not affected by the extracts or DMSO that was used to dissolve the extracts. Several biosensors as stated in Table 3.1 have been used to screen for the anti-QS activities. The samples that were tested showed broad spectrum effects as it can modulate the QS system at multiple levels mediated by different AHL molecules regardless of short or long chain (Pesci *et al*., 1999; Adonizio *et al*., 2006).

A preliminary screening was conducted on the crude extracts using *C. violaceum* CV026. Wild-type *C. violaceum* produce violet colonies on solid media due to its inherent ability to synthesis antibiotic known as violacein. The production of the violacein is controlled by the QS system regulated by the major C6-HSL. The CV026 biosensor was constructed by subjecting *C. violaceum* to mini-Tn*5* transposon mutagenesis in order to obtain a double Tn*5* insertion, violacein-negative, white mutant CV026, making it incapable of producing any AHLs. The synthesis of violacein will be restored once the biosensor is supplemented with synthetic C6-HSL exogenously (McClean *et al*., 1997). In the quantitative analysis of CV026 assay, some of the crude extracts were found to inhibit the production of violacein as compared to the controls that were being used. Among the extracts that showed significant results include

A. dahurica, *R. cibotii, P. vulgaris, S. tenuifolia, G. mangostana* and *P. amaryllifolius* while hexane extracts from *S. samarangense* exhibited the strongest inhibition. This indicates that the biomolecules found in the plants may have the ability to act as antagonist and target the receptor, CviR. In addition, the molecules may have also induced the degradation of the AHLs or encouraged the AHLs to be transported out of the bacteria cells (Kalia, 2013).

There were two *lux*-based biosensors used in this study which was *E. coli* [pSB401] and *E. coli* [pSB1075]. Synthetic 3-oxo-C6-HSL was added into *E. coli* [pSB401] while *E. coli* [pSB1075] required synthetic 3-oxo-C10-HSL in order to induce the bioluminescence (Winson *et al*., 1998). Based on the results listed in Table 4.1, all the three solvent extracts from *A. dahurica* and *R. cibotii* showed intermediate positive results for both the *lux*-based biosensors. Only chloroform extracts from *G. mangostana* showed the strongest activity against *E. coli* [pSB401] and *E. coli* [pSB1075]. This showed that the plant samples were capable of intervening with the short and long chain AHLs, suggesting a broad range of anti-QS activity. The extracts were also unlikely to inhibit the synthesis of AHLs as the biosensors consist of a defective *luxI* synthase gene.

As a pathogenic bacterium, *P. aeruginosa* PAO1 has the capability to attach to the host tissues mediated by the host cell surface glycoconjugates and caused infections (Karlsson, 2001). The adhesion is basically facilitated by a glycanic recognition pattern that involves some adhesins including lectins. *P. aeruginosa* produces two types of sugar-binding lectins known as LecA (PA-IL) and LecB (PA-IIL) which specifically bind to galactose and fucose, respectively (Johansson *et al*., 2008). The productions of these lectins are associated with virulence factors especially in the development of

biofilm and are regulated by the QS system (Diggle *et al*., 2006). LecA was found to cause respiratory epithelial injury due to the cytotoxic effect on the respiratory epithelial cells (Bajolet-Laudinat *et al*., 1994). Besides that, LecA can also induce a permeability defect in the intestinal epithelium and thus, increased the absorption of an important extracellular virulence factor, exotoxin A (Laughlin *et al*., 2000). Previous research has proved that the expression of *lecA* gene is tightly regulated by the RhlI/R-C4-HSL QS system and the mutation of *rpoS* gene caused the eradication of lectin synthesis in *P. aeuginosa* PAO1 (Winzer *et al*., 2000). In this study, the expression of the *lecA* was determined by using biosensor with *luxCDABE* gene region from *Photorhabdus luminescence* that was cloned into a functional *lec A* gene region of *P. aeruginosa* PAO1 (Winzer *et al*., 2000). Only hexane and chloroform extracts of *G. mangostana* was found to show intermediate inhibition towards the *P. aeruginosa* PAO1 *lecA::lux* biosensor while the rest displayed weak or no inhibition at all. The results obtained might suggest that the compounds found in *G. mangostana* can either cause effects on the RhlI/R-C4-HSL QS system or on the expression of the *rpoS* sigma factor.

P. aeruginosa PAO1 has been shown to cause various infections in a number of body systems in the humans including the respiratory tract, urinary tract and central nervous system especially in the immunocompromised host (Chuang *et al*., 1999; Lyczak *et al*., 2000; Mittal *et al*., 2009). Various studies showed that *P. aeruginosa* PAO1 produces various virulence factors known as phenazines and pyocyanin is one of them (Lau *et al*., 2004). Pyocyanin is a blue-redox toxic phenazine pigment that plays a major role in *P. aeruginosa* PAO1 virulence factor as well as electron transfer facilitator (Hernandez & Newman, 2001; Lau *et al*., 2004). The pigment is commonly found in large quantities in the sputum of the patients with cystic fibrosis (Lau *et al*., 2004). The production of the pyocyanin is regulated by the Rhl system and it can control a limited

set of genes known as PYO stimulon during the stationary phase including the genes involved in efflux and redox processes (Dietrich *et al*., 2006; Jimenez *et al*., 2012). Studies have proved that both PQS and PqsE directly control the biosynthesis of phenazine and pyocyanin itself was also shown to be responsible for the up-regulation of this gene. In addition, the disruption of the IQS system will also affect the pyocyanin production (Lee *et al*., 2013). These findings indicate the complexity of the signalling networks that were present in *P. aeruginosa* PAO1 (Deziel *et al*., 2005; Dietrich *et al*., 2006). Among the 15 plants tested for pyocyanin assay, extracts from *G. mangostana* portrayed the most significant results as compared to the rest of the samples. This proved that the compounds from *G. mangostana* were capable in interfering with the production of pyocyanin in *P. aeruginosa* PAO1.

P. aeruginosa PAO1 also has the ability to secrete various toxic compounds and degradative enzymes. One of secreted virulence is elastase (LasB), a protease with high activity and broad substrate specificity (Grimwood *et al*., 1993). Elastase plays a vital part in the pathogenicity during host infection as the enzymes are capable to degrade or inactivate the vital biologic tissues and immune systems including the immunoglobulin, serum complement factors, fibrin and elastin (Heck *et al*., 1990; Hong & Ghebrehiwet, 1992). The production of elastase is controlled by both the Las and Rhl QS system (Pearson *et al*., 1997; Berre *et al*., 2008). One of the study showed that both *rhlI* and *rhlR* mutants showed defects in the production of elastase when compared with the wild type strain (Brint & Ohman, 1995). In addition, another group of researchers reported that the LasB deletion mutant showed significant decreased in the attachment of the bacterial, formation of the microcolony as well as the extracellular matrix linkage in biofilm that are associated with decreased biosynthesis of rhamnolipids. Further transcriptomic analysis also proved that the LasB mutant can down-regulate the

synthesis of rhamnolipid. The results obtained indicate that LasB could promote biofilm formation partly through the rhamnolipid-mediated process (Yu *et al*., 2014). My study showed that some of the crude extracts tested gave positive results by decreasing the production of elastase especially extracts from *A. dahurica* and *S. samarangense*. This could mean that the compounds found in the extracts have the possibilities to affect either the Las or Rhl or both systems in *P. aeruginosa* PAO1.

LasA, on the other hand, is an extracellular proteolytic enzyme that is generally produced by *P. aeruginosa* PAO1 during the infection period. The enzyme was shown to significantly enhance the activity of LasB in the degradation of elastin (Kessler *et al*., 1997). Furthermore, LasA can enhance virulence by promoting the shedding of syndecan-1, the predominant cell-surface heparin sulfate proteoglycan of epithelial cells (Park *et al*., 2000). LasA is also known as the staphylolytic protease as the enzyme can lyse the cells of *S. aureus* by cleaving the peptidoglycan pentaglycine interpeptides over a broad range of pH (Kessler *et al*., 1993; Park & Galloway, 1995). The staphylolytic activity gave advantage to *P. aeruginosa* PAO1 to overcome *S. aureus* during the colonization of the cystic fibrosis lung infection (Grande *et al*., 2007). Like elastase, LasA biosynthesis was also controlled by the Las and Rhl QS systems (Jimenez *et al*., 2012). Previous research showed that the production of LasA was inhibited in both *rhlI* and *rhlR* mutants (Brint & Ohman, 1995). Of all the crude extracts tested for staphylolytic activity, only chloroform extracts of *G. mangostana* showed inhibition in the production of LasA. We can then hypothesize that the *G. mangostana* molecules extracted in chloroform solvent has the ability to overcome the infection caused by *P. aeruginosa* PAO1 due to the presence of the potent staphylolytic enzyme.

Majority of the bacteria including *P. aeruginosa* PAO1 uses motility as part of their colonization and with the help of the flagella or pili, the bacteria can spread across the surfaces within a thin liquid film (Kearns, 2010). There are three types of motility used by bacteria: swimming, twitching and swarming (Rashid & Kornberg, 2000). For this study, I focused on the effects of the crude extracts against the *P. aeruginosa* PAO1 swarming activity. The results showed that chloroform extracts of *G. mangostana* displayed the strongest inhibition toward *P. aeruginosa* PAO1 swarming motility followed by *S. tenuifolia*. Swarming depends greatly on the bacterial cell density, nutrient growth medium as well as surface condition moistness (Wang *et al*., 2004). Various researches reported that *P. aeruginosa* PAO1 normally swarm in semisolid media in between 0.4%−0.7% concentration and is dependent on both flagella and type IV pili (Kohler *et al*., 2000). Swarming in *P. aeruginosa* PAO1 was also shown to be regulated by the QS system where the *lasI/R* mutant decreased and delayed the swarming progression while the *rhlI/R* mutant totally eradicated the swarming motility (Kohler *et al*., 2000). Furthermore, the swarming activity is also highly influenced by the rhamnolipid production (Deziel *et al*., 2003; Caiazza *et al*., 2005). Rhamnolipids are basically biosurfactants that can modulate the swarming motility pattern by improving the bacterial surface translocation with their wetting properties. This will then allow the swarming cells to overcome the strong surface tension of the water that surround the swarming cells (Noordman & Janssen, 2002; Deziel *et al*., 2003; Caiazza *et al*., 2005). Previous work showed that *P. aeruginosa* PAO1 with mutant *rhlB* (monorhamnolipds production) and *rhlC* (dirhamnolipids production) genes were found to alter the swarming patterns by producing abnormal shaped tendrils (Caiazza *et al*., 2005). Like swarming, the production of rhamnolipids is also control by the Rhl system and studied showed that *P. aeruginosa* which harbours *rhlI* mutant was not able to synthesize rhamnolipids and hence, reduced the rhamnosyltransferase activity (Brint & Ohman,

1995; Ochsner & Reiser, 1995). Other studies have also addressed the importance of swarming in the formation of biofilm and antibiotic resistance of *P. aeruginosa* PAO1 (Caiazza *et al*., 2007; Merritte *et al*., 2007; Overhage *et al*., 2008). Therefore, the compounds found in the chloroform extracts of *G. mangostana* and *S. tenuifolia* may have impeded either the *P. aeruginosa* PAO1 QS system or the synthesis of the rhamnolipids or both. The inhibition of the swarming by the extracts could be helpful in decreasing the formation of biofilm as well as antibiotic resistance issues.

In summary, the plants that were selected in this study have shown to reduce the QS-regulated virulence in *P. aeruginosa* PAO1 without having any bactericidal or bacteriostatic effects. From the assays that have been conducted using the biosensors, we can assume that the extracts may have interfered with the receptor protein instead of the AHL synthase as the biosensors used do not have *lux*-based synthase. However, further work will be done in order to confirm if the bioactive molecules in the plants are targeting the signal synthase or receptor. The ability of the plants to inhibit the virulence might contribute to the research database and hopefully can lead to the discovery of new drugs without increasing the risk of antibiotic resistance.

5.2   Catechin

5.2.1   Purification of Catechin from *G. mangostana* Chloroform Extracts

The results obtained in Section 4.1.2 showed that most of the plant samples tested for anti-QS activities showed encouraging results. However, only chloroform extracts of *G. mangostana* was chosen for downstream work as this is the only extracts that displayed positive results for all the bioassays especially the staphylolytic activity. Serial exhaustive extraction (SEE) technique was used to extract the active biomolecules in *G. mangostana* followed by fractionation and purification of the chloroform extract using column chromatography with silica gel.

In the first fractionation, a total of 54 fractions (labelled as GA1–54) were acquired and tested for anti-QS activities. Based on Table 4.3, fractions labelled with GA20 and GA21 were found to show positive results for all the bioassays and were then screened with TLC in order to make sure that both of the molecules are the same before combined for second purification. The second purification yields 40 fractions (labelled as GB1–40) and Table 4.4 presented that GB24, GB25 and GB26 portrayed significant positive results and based on the TLC results, all the three fractions were found to be the same molecule. In addition to that, the TLC results also displayed that the molecule are pure and was subsequently subjected to ^{1}H NMR analysis with the intentions to identify the molecule. The bioactive molecule responsible for the inhibition of QS activities was found to be catechin. Synthetic catechin from Sigma was used as control to confirm the structure.

5.2.2 Anti-QS Activities of Catechin

Plants have been known to produce secondary metabolites that plays a vital role in their adaptation and survival to the surrounding environment. In the pharmaceuticals area, the secondary metabolites become an important source of active compounds for designing drugs that can help to overcome the antibiotic resistant problems (Bourgaud *et al.*, 2001). The classification of the secondary metabolites generally depends on the basis of the chemical structure, composition, solubility as well as the biosynthetic pathway. Flavonoids are one of the secondary metabolites produced by the plants that are polyphenolic with a phenyl benzopyrone structure (Middleton *et al.*, 2000). They are basically water soluble and is part of the plant's natural pigments that leads to the large variety of colours in the flowers (Temidayo, 2013). Depending on the saturation level, the C-ring substitution pattern as well as the opening of the central pyran ring, flavonoids can be categorized into flavones, flavanols, isoflavones, flavonols, flavavones, flavanonols and chalcones (Middleton *et al.*, 2000). A few beneficial effects of flavonoids include antioxidant, anti-inflammatory, anti-allergic, antiviral, and anti-carcinogenic properties (Middleton, 1998).

Catechin that was purified from the leaves of the *G. mangostana* belongs to the flavanols or flavan-3-ol group class. The compound is commonly found in foods consumed by humans such as fruits, vegetables, wine, black chocolate and green tea (Arts *et al.*, 2000; Pignatelli *et al.*, 2000). Catechin has been proven to have various pharmacological activities such as antiviral (Xu *et al.*, 2017), antifungal activity against *C. albicans* (Hirasawa & Takada, 2004), antimicrobial activity (Ahn *et al.*, 1990; Mendel, 2007) as well as antitoxicity activity (Mendel, 2007).

In addition to that, catechin also possess high antioxidant activities where the compound can attenuate the development of atherosclerotic lesion by decreasing the susceptibility of the low density lipoprotein (LDL) to oxidation and aggregation (Hayek *et al*., 1997). Catechin was also found to reduce the peroxynitrite-induced nitration of tyrosine due to the alteration on the limit surface charge of LDL (Pannala *et al*., 1997). Furthermore, catechin provides cytoprotective activity and protects the cells with their high iron chelating effects (Morel *et al*., 1993). Catechin also acts synergistically with quercetin in the inhibition of platelet adhesion to collagen as well as collagen-induced platelet aggregation (Pignatelli *et al*., 2000). Orally administered catechin was also found to decrease the plasma glucose levels by obstructing the intestinal α-amylase or sucrose, hence, prevent the digestion of starch or sucrose in the body (Matsumoto *et al*., 1993).

In this study, catechin was found to decrease the production of violacein in *C. violaceum* CV026 and this indicates that the compound was targeting the CviR as the biosensor used is a mutant that have lost the ability to produce AHLs. In addition to that, catechin was also shown to inhibit some of the virulence factors in *P. aeruginosa* PAO1 including bioluminescence, LecA, pyocyanin, elastase, staphylolytic as well as swarming. The virulence factors tested in this study was regulated by the QS system as discussed on Section 5.1.2. Previous work done by Vandeputte's team on the catechin extracted from *Combretum albiflorum* bark extract showed positive results too in the inhibition of violacein in *C. violaceum* CV026 as well as pyocyanin, elastase and biofilm in *P. aeruginosa* PAO1 (Vandeputte *et al*., 2010). This shows that the results acquired by the Vandeputte's team are the same with the data obtained in my study. To the best of my knowledge, this is the first documentation that reports the effects

G. magostana leaves against the QS-regulated virulence in *P. aeruginosa* PAO1 without affecting the growth of the bacteria or biosensors.

Catechin was also found to reduce the biofilm production in *P. aeruginosa* PAO1. Biofilm or "city of microbes" plays a very important role in organizing the bacteria communities by holding the cells together to the surface through the matrix of extracellular polymeric substances (EPS) (Watnick & Kolter, 2000; Donlan, 2002). EPS is mainly make up of biomolecules, exopolysaccharides, extracellular DNA (eDNA) and polypeptide in which it will then forms a hydrated polar mixture that helps to hold the structure of the biofilm together (Sutherland, 2001; Flemming & Wingender, 2010). The formation of biofilm generally involves few different stages where it consists of attachment, proliferation as well as differentiation (Davies *et al.*, 1998). *P. aeruginosa* PAO1 synthesis three types of polysaccharides known as alginate, Pel and Psl which can help to determine the stability of the biofilm structure (Ryder *et al.*, 2007).

The biofilm formation and maturation in *P. aeruginosa* PAO1 is mainly regulated by the Las system. Previous research showed that the bacteria cells with the *lasI* mutant seems to look flat, undifferentiated and was dispersed rapidly from the surface once exposed to the sodium dodecyl sulfate (Davies *et al.*, 1998). Another research proved that the LasR can regulate the expression of Psl by binding to the promoter region of *psl* operon (Sakuragi & Kolter, 2007). Further work was done where they found that the Rhl system can interfere with the formation of biofilm by enhancing the synthesis of Pel polysaccharide (Sakuragi & Kolter, 2007). Other group showed that the PQS system was also involved where the biofilm produced by the *pqsA* mutant seems to contain less amount of eDNA when compared to the wild type (Allesen-Holm *et al.*, 2006; Yang *et al.*, 2007). In addition, there is also an indirect link in between the

formation of the biofilm with swarming and twitching motilities, rhamnolipids as well as the lectins production (Rasamiravaka *et al.*, 2014).

Natural products play a dominant role for treating and preventing any infectious diseases among the humans (Koehn & Carter, 2005). As plants have been living in an environment with a very high bacteria cell density such as soil, they have long been shown to have protective mechanisms against the bacterial infections. The compound of the plants usually targets the bacteria QS system via three different ways either by targeting the signalling molecules from being synthesized by the *luxI* encoded AHL synthase, degrade the signalling molecules and/or target the *luxR* signal receptor (Suga & Smith, 2003). Majority of the compounds that are produced by the natural products have the capabilities to mimic the AHL signals and competitively bind to the *luxR* receptor (Teplitski *et al*., 2000).

Biosensors *E. coli* MG1655::pBAD24-*lasI* and *E. coli* MG1655::pBAD24-*rhlI* was used to determine if catechin was targeting the AHL synthase or receptor of *P. aeruginosa* PAO1. Both of the biosensors contain arabinose-dependent expression plasmids that harbour *P. aeruginosa* PA01 *lasI* and *rhlI*, respectively. The production of C4-HSL and 3-oxo-C12-HSL will be produced by *E. coli* MG1655::pBAD24-*rhlI* and *E. coli* MG1655::pBAD24-*lasI*, respectively, when being supplied with arabinose. The AHLs was then extracted from the pBAD biosensors that are treated with catechin and subjected to be tested with biosensors *E. coli* [pSB401] and *E. coli* [pSB1075] where it can detect both short and long chain AHLs, correspondingly. The results obtained showed that catechin could not inhibit the *lasI* and *rhlI* AHL synthase of *P. aeruginosa* PAO1. Therefore, we can assume that catechin inhibits *P. aeruginosa* PAO1 QS system

by targeting the transcriptional regulator/receptor protein without causing any bactericidal or bacteriostatic effects.

As a whole, the results obtained in this study showed that catechin has a broad range of anti-QS activities as it can target both short and long chains of AHLs in addition to the virulence regulated by the *P. aeruginosa* PAO1 QS system. RNA-sequencing was subsequently performed in order to do a more in-depth study about the effects of catechin on *P. aeruginosa* PAO1 in the gene level.

5.3   Malabaricone C

In this study, the malabaricone C obtained from the Department of Pharmacology, Faculty of Medicine, University of Malaya was isolated from the bark of the *Myristica cinnamomea* King (Myristicaceae) with methanol solvent. The tree is commonly known as cinnamon nutmeg and can be found in Malaysia, Singapore, Borneo and Philippines. The outer part of the bark is dark brown, rugose with fine grid cracks while the inner part is pale brown. The shape of the leaves is oblong to oblanceolate with bright green colour on the top and pale silvery brown underneath. The fruit on the plant is yellow and has the structure of globose to broadly globular-oblong. The seed, on the other hand, is red in colour and is commonly used as spice by the locals (Seidemann, 2005).

Malabaricone C or 1-(2,6-Dihydroxyphenyl)-9-(3,4-dihydroxyphenyl) nonan-1-one is an acylphenols with the molecular formula of $C_{21}H_{26}O_5$. The compound contains two phenolic hydroxyl groups as well as a resorcinol moiety in which it can induce irreversible, hydrogen peroxide-dependent loss of activities of the heme-containing peroxides like myeloperoxidase (MPO) (Forbes *et al*., 2004). Various research has been conducted that proved the use of malabaricone C in different pharmacological activities. (Forbes *et al*., 2004; Chelladurai *et al*., 2007).

Malabaricone C was found to possess antimicrobial activity against a selection of bacteria which includes *S. aureus* and *C. albicans* (Orabi *et al*., 1991). In addition to that, the compound was shown to prevent the removal of sialic acid from the surface glycan of the host cell and thus, inhibit the pneumococcal sialidases (NanA, NanB and NanC) in *Streptococcus pneumoniae* (Park *et al*., 2017). Malabaricone C also inhibit the

growth of mycelial in pathogenic fungi. The same research showed strong antifungal activity against the rice blast, tomato late blight, wheat leaf rust and red pepper anthracnose by the compound as well (Choi *et al*., 2008a).

Choi's team showed that malabaricone C had 100% mortality rate against *Bursaphelenchus xylophilus* (Choi *et al*., 2008b). Furthermore, malabaricone C was proved to impede the PDGF-induced proliferation and migration of RASMCs via the Nrf2 activation and HO-1 induction through the PI3K/AKT pathway. These results indicated the potential of the compound as HO-1 inducer for preventing or treating patients with vascular diseases (Lee *et al*., 2012). A research done by Patro's team mentioned the effects of malabaricone C on the Cu (II)-dependent nuclease activity. They found that the compound can facilitate the DNA nicking due to the ability of malabaricone C bind with the Cu(II) and DNA through a site that specifically generate the Cu(I)-peroxo complex. Malabaricone C also displayed cytotoxicity activity against the human breast cancer cell line, MCF-7, where it was found to kill the cells through the apoptotic pathway that involves oxidative damage towards the cellular DNA (Patro *et al*., 2010).

Malabaricone C exhibited maximum 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity as it can prevent Fe(II)- and 2,2'-azobis(2-amidinopropane) dihydrochloride-induced lipid peroxidation (LPO) of rat liver mitochondria. Besides, the compound was shown to inhibit the γ-ray-induced damage of pBR322 plasmid DNA (Patro *et al*., 2005). By promoting angiogenesis, malabaricone C was also found to have healing activity against the indomethacin-induced stomach ulceration in mice (Banerjee *et al*., 2008). Due to its high antioxidant activity, the compound was suggested the possibilities of being used as a novel natural antioxidant in food (Hou *et al*., 2012).

Another research team demonstrated that the production of nitric oxide (NO), prostaglandin E (PGE), interleukin-6 (IL-6), and interferon-γ (INF-γ) was inhibited by malabaricone C too. This action generally leads to the suppression of lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) expression and the promoter activities of COX-2 and iNOS. The pre-treatment of the compound also stopped the LPS-induced nuclear factor-kappa B (NF-κB) activation by inhibiting the phosphorylation of IκB kinase (IKK), phosphorylation and degradation of IκBα as well as the nuclear translocation of NF-κB. Additional study showed that the reduction of reactive oxygen species (ROS) accumulation by malabaricone C can inhibit LPS-induced Akt phosphorylation without disturbing the phosphorylation of the mitogen-activated protein kinases (MAPKs) (Kang *et al.*, 2012).

The data collected in this study showed that malabaricone C displayed anti-QS activity against *P. aeruginosa* PAO1 in addition to reducing the bacterial phenotype expression. Out of all the bioassays tested, the compound showed positive results in the inhibition of violacein, pyocyanin, biofilm as well as swarming motility. To the best of my knowledge, this is the first report regarding to the effects of malabaricone C against the *P. aeruginosa* PAO1 QS system.

Based on the preliminary screening using *C. violaceum* CV026, malabaricone C was found to significantly decrease the production of violacein in a concentration dependent manner. The synthesis of violacein in *C. violaceum* is controlled by the QS system, regulated by short chain AHLs especially C6-HSL. The results indicated that the compound generally interfered with the signal receptor, CviR, as the biosensor used contained defective *luxI* synthase gene and production of violacein will only occurred if AHLs are being supplied (McClean *et al.*, 1997).

As the concentration of malabaricone C increased, the virulence factors produced by *P. aeruginosa* PAO1 such as pyocyanin and biofilm decreased as well. The results indicated that malabaricone C was able to affect the QS system as both pyocyanin and biofilm was controlled by the Rhl and Las system, respectively. In addition to that, the compound also affects the motility of the bacteria by reducing the ability of *P. aeruginosa* PAO1 to swarm. All of this virulence was regulated by the QS system and therefore, plays important roles in the development of acute and chronic infections as well as multi-drug resistance in *P. aeruginosa* PAO1 (Davies *et al.*, 1998; Rumbaugh *et al.*, 2000; Dietrich *et al.*, 2006).

The screening of malabaricone C as the QS transcriptional regulator protein antagonist was determined by using *E. coli* MG1655::pBAD24-*lasI* and *E. coli* MG1655::pBAD24-*rhlI*. Both of the biosensors were supplemented with arabinose in order to induce the synthesis of 3OC12-HSL and C4-HSL, respectively. Based on the results attained, the possibility of malabaricone C for inhibiting the autoinducer synthase, LasI and RhlI, can be ruled out and thus, confirmed that the compound inhibit the QS system through the signal receptor.

In comparison, the effects of malabaricone C against the *P. aeruginosa* PAO1 QS is less significant than catechin but the data obtained is still important as it can contribute in the drug discovery process. Further transcriptome work was subsequently done to determine the *P. aeruginosa* PAO1 genes that were affected by malabaricone C.

5.4   Transcriptome analysis of *P. aeruginosa* PAO1

5.4.1   Catechin

Based on the filter parameters of $\geq$ 2-fold change, $\leq$ 2-fold change and *p* value < 0.05, *P. aeruginosa* PAO1 treated with catechin resulted in 35 down-regulated genes (Table 4.5) and 24 up-regulated genes (Table 4.6).

Iron is an essential trace element that is required by most living organisms including bacteria for growth, development and infection purpose. Iron acts as a catalyst in some of the most fundamental enzymatic processes such as oxygen metabolism, electron transfer as well as and DNA and RNA synthesis (Braud *et al*., 2009). However, under aerobic conditions, iron is not freely available to bacteria as it forms ferric hydroxides that have poor solubility in the environment. In addition to that, irons in the human hosts are usually bound to proteins like transferrin and lactoferrin in extracellular fluid or to ferritin, hemoglobin and heme-containing enzymes in cells (Otto *et al*., 1992; Wooldridge *et al*., 1993; Youard *et al*., 2007). In order to acquire the iron, the bacteria have come up with some strategies that include an iron uptake system mediated by high-affinity iron chelators called siderophores and also a system for heme uptake via specific receptors (Marinez *et al.*, 1990; Wooldridge *et al.*, 1993). *Pseudomonas aeruginosa* produces two major siderophores that are involved in the synthesis of QS-regulated virulence factors known as pyoverdine and pyochelin (Ochsner *et al*., 2002; Lee *et al*., 2009). When the pyoverdine signalling pathway was interrupted, pyochelin biosynthesis will take over and continue to obtained more iron (Zaborin *et al*., 2009).

Our findings showed that catechin down-regulated genes involved in pyochelin: *pchC* (fold change: -5.68064), *pchG* (fold change: -4.92952), *pchR* (fold change: -3.92383), *pchD* (fold change: -2.80866), *pchF* (fold change: -2.78886), *pchE* (fold change: -2.78049), *pchA* (fold change: -2.00706) and *fptA* (fold change: -2.31028). Pyochelin is a condensation product between salicylate and two cysteinyl residues. The synthesis of pyochelin requires proteins that are encoded by two divergent operons pchDCBA and pchEFGHI (Serino *et al*., 1995; Reimmann *et al*., 2001). PchA enzymes will convert chorismate to isochorismate while PchB enzyme will catalyse isochorismate to salicylate (Gaille *et al*., 2002; Gaille *et al*., 2003). Salicylate is then coupled to the cysteine moieties by a thiotemplate mechanism which involves the salicylate adenylating enzyme PchD, the peptide synthetases PchE and PchF as well as the reductase PchG (Quadri *et al*., 1999; Patel & Walsh, 2001; Reimmann *et al*., 2001). During the formation of the thiazoline ring and methylation of the nitrogen in the thiazolidine ring, two domains, PchE (E domain) and PchF (MT domain), will be responsible for the epimerization of the L-cysteinyl to the D-cysteinyl residue (Patel & Walsh, 2001; Patek *et al*., 2003). In addition, the expression of the pyochelin biosynthetic genes also depends on the AraC-type regulator PchR as it is required for the production of the FptA ferric pyochelin receptor (Heinrichs & Poole, 1993; Michel *et al*., 2005). A thioesterase encoded by the PchC was found to be necessary for maximum production of pyochelin as well as its precursor, antibiotic dihydroaeruginoate (Dha). Furthermore, PchC also plays a role in removing wrongly charged molecules from the *pch* domain during the synthesis of pyochelin (Reimmann *et al*., 2004). When the pyochelin is secreted into the extracellular environment, it will start to chelates iron and transport it back to the bacterial cytoplasm through the specific outer membrane receptor FptA (Michel *et al*., 2007).

The rise of the antibiotic resistance among the pathogenic bacteria has now become a major threat in the modern medicine (Bonhoeffer *et al*., 1997). The resistance can happen either through the acquisition of foreign antibiotic-resistance genes or through the mutation in indigenous genes encoding the target or the transport systems of the drugs. The concentration of antibiotics that actually works against the bacteria also depends on the permeability of bacterial envelopes, production of drug-inactivating enzymes as well as the presence of various efflux pump systems in pumping out the different types of antibiotics from the bacteria cells (Nikaido, 1994). The β-lactam class such as penicillins, cephalosporins, monobactams and carbapenems are among the antibiotics that are commonly used to treat *P. aeruginosa* PAO1 infections (Poole, 2004). The β-lactam antibiotics will basically block the peptidoglycan biosynthesis by binding to the cell wall transpeptidases (Masuda *et al*., 1999). However, the prolonged use of the drugs resulted in *P. aeruginosa* developing resistance to β-lactam antibiotics and is partly mediated by a group of genes that belongs to amp system (Balasubramanian *et al*., 2012). The suppression of *ampO* gene (fold change: -3.72321) and *ampP* gene (fold change: -2.87945) by catechin indicates that the compound can decrease the antibiotic resistance in *P. aeruginosa* PAO1, making it more susceptible to treatment.

Quinones play an important role in the electron transport chain for most prokaryotes and eukaryotes either in the form of ubiquinones or menaquinones. The quinones that are involved in the electron transport chain are normally hydrophobic and lipid soluble while the water soluble quinones are part of the cytotoxic anti-bacterial compounds (Ryan *et al*., 2014). In addition, the water soluble quinones are also being used as defence mechanism as they can covalently modify DNA and proteins as well as their ability to undergo redox cycling (Bolton *et al*., 2000; Cerenius *et al.*, 2008). As

bacteria also produce quinones as antibiotics, it is therefore significant for the bacteria to be able to detoxify these cytotoxic compounds as they are harmful for their growth. The mechanism of quinone detoxification is done through the two electron reduction reactions carried out by a group of flavoenzymes known as NAD(P)H quinone oxidoreductases (Ryan *et al*., 2014). The enzyme is commonly known as the detoxification enzyme since it possesses the ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones (Siegel *et al*., 2004). Based on the data obtained, there is a down-regulation of *yieF* gene (fold change: -2.12799) in *P. aeruginosa* PAO1 treated with catechin and thus, decrease the capability of the bacteria to detox the quinone from their system.

Bacteria normally have a close relationship with their host organisms in ways that will either produce benefits, neutral or harmful effects. In order for the interaction to happen, the bacteria will secrete proteins into the extracellular environment where it will then interact with the host cell targets. Type III secretion system (T3SS) is responsible for the transport of the proteins across the inner bacterial membrane, peptidoglycan layer and the outer bacterial membrane before crossing into the host cell barriers (Ghosh, 2004). The system comprised of cytosolic ATPase, inner and outer membrane rings as well as an extracellular needle. Majority of the human pathogens employ T3SS to secrete virulence factors and caused infections (Hueck, 1998; Ghosh, 2004). The results showed that the *pscO* gene (fold change: -2.42244) that function as translocation protein in type III secretion was down-regulated. In contrast to secretion, the translocation process does not occur through the flagellar apparatus and is strictly dependent on the T3SS (Lee & Galan, 2004). T3SS will generally accumulate the supermolecular structures on the bacteria surface and become a dedicated machinery for

the translocation of pathogenicity proteins into the cytosol of the host cells (Ginocchio *et al*., 1994; Roine *et al*., 1997).

eraR gene (fold change: -7.0445) that is involved in ethanol oxidation pathway was also down-regulated in *P. aeruginosa* PAO1 treated with catechin. It functions as the EraR response regulator and is part of the luxR family (Mern *et al*., 2010). Previous research showed that the gene was also indirectly involved in the biofilm-specific antibiotic resistance (Beaudoin *et al*., 2012).

P. aeruginosa PAO1 secretes proteins such as toxins, proteases, phospholipases and lipases into the extracellular medium (Potvin *et al*., 2003). Among the lipolytic enzymes secreted by the bacteria includes LipA and LipC. LipA is encoded within the *lipA/lipH* operon along with the cognate foldase LipH. Both LipA and LipC required the action of the lipase-specific LipH in order to acquire proper folding and enzyme activity (Martinez *et al*., 1999; Rosenau & Jaeger, 2000; Rosenau *et al*., 2010). In addition to that, LipH is important for the expression of active extracellular lipase in *P. aeruginosa* PAO1 (Pandey *et al*., 2017). Research shown that LipC can affects the motility, biofilm formation as well as rhamnolipid production in *P. aeruginosa* (Rosenau *et al*., 2010). Table 4.5 showed that *lipH* gene (fold change: -2.39759) was down-regulated in *P. aeruginosa* PAO1 treated with catechin.

Catechin also down-regulated the nitrate transport ATP-binding protein, *nasS* gene (fold change: -3.10251). The Nas enzymes are responsible in converting the inorganic nitrate to nitrite and consecutively to ammonium by the siroheme-dependent NADH-nitrite reductase (Lin & Stewart, 1997; Moreno-Vivian *et al*., 1999; Richardson *et al*., 2001). Recent study found that QS can regulates the activities of denitrification

enzymes in anaerobically grown *P. aeruginosa* cells in which the level of enzyme activities are higher in *rhlR* mutant as compared to its parent strains (Yoon *et al*., 2002).

In order to adapt to the various conditions, bacteria must regulate their metabolism by sensing the environmental signals. Under anaerobic conditions, *P. aeruginosa* PAO1 will use N-oxides as terminal electron acceptors and denitrification is normally induced under low-oxygen conditions (Ka *et al*., 1997; Arai *et al*., 1999). There are four different types of nitrate reductases that catalyse the reduction of nitrate to nitrite: eukaryotic assimilatory nitrate reductases, cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and periplasmic dissimilatory (Nap) nitrate reductases (Moreno-Vivian *et al*., 1999). *P. aeruginosa* normally form biofilm under oxygen limitation (Govan & Deretic, 1996) and by down-regulating the *napB* gene (fold change: -2.48711), it can reduce the possibilities of bacteria undergoing denitrification and hopefully the biofilm formation as well. In addition, recent work suggests that QS regulates the activities of denitrification enzymes (Yoon *et al*., 2002).

cdhC gene (fold change: 2.41434) that acts a carnitine dehydrogenase-related gene C was up-regulated in *P. aeruginosa* PAO1 treated with catechin. Most of the bacteria use carnitine in either aerobic or anaerobic environments to regulate a variety of cellular functions including as an electron acceptor, as a compatible solute to survive harsh surrounding or as a sole carbon, nitrogen and energy source. Additionally, carnitine also acts as osmoprotectant where it can help to enhance thermotolerance, cryotolerance and barotolerance. Bacteria can acquire carnitine either by transporting into the cell or acquired from metabolic precursors in which it can serve directly as a compatible solute for stress protection or be metabolized through one of a few distinct pathways as a nutrient source (Meadows & Wargo, 2015). *P. aeruginosa* PAO1

metabolize carnitine to the osmolyte glycine betaine and utilise it as osmoprotectant and osmolyte (Wargo & Hogan, 2009; Bastard *et al*., 2014).

Catechin also up-regulated *moaD* gene (fold change: 2.17202) and *moaB1* gene (fold change: 2.05497) that are involved in the biosynthesis of molybdopterin. Molybdenum (Mo) is generally the catalytic component of some vital enzymes involved in nitrogen, sulphur and carbon metabolism in prokaryotes and eukaryotes. With the exception of nitrogenase, the molybdenum will coordinate to the unique pterin compound molybdopterin (MPT) and formed the molybdenum cofactor (Moco) (Zhang *et al*., 2011; Iobbi-Nivola & Leimkühler, 2013). MoaD was required to carry the sulfur used for generation of Moco in form of a C-terminal thiocarboxylate group and this mechanism occurs in a MoaD/MoeB complex (Iobbi-Nivola & Leimkühler, 2013).

5.4.2   Malabaricone C

A total of 29 down-regulated genes (Table 4.7) and 21 up-regulated genes (Table 4.8) were affected when malabaricone C co-cultured with *P. aeruginosa* PAO1.

lhpM (fold change: -8.78833) that function as permease of ABC transporters was top on the list of the genes that was being down-regulated. ABC (ATP binding cassette) transporters are consider as one of the largest classes of transporters and consist of permease proteins, ATPase proteins as well as substrate binding proteins (Garmory & Titball, 2004; Higgins & Linton, 2004). In order to power the translocation of a diverse assortment of substrates such as ions and macromolecules across the membranes, the transporters will utilise the energy obtained from the binding and hydrolysis of ATP (Rees *et al*., 2009). In addition to that, most of the ABC transporters also depends on a high affinity solute binding protein which is found located in the periplasmic space in between the inner and outer membrane of Gram-negative bacteria when it comes to the uptake of nutrients or molecules (Davidson *et al*., 2008). ABC transporters have two main functions, which is as (a) importers where they will bring in nutrients and other molecules into the cells and (b) exporters that help to pump toxins, lipids as well as drugs such as antibiotics across the membrane (Rees *et al*., 2009). Due to that, ABC transporters play a vital role in bacteria's fitness and survival as the transporters are connected with the uptake of various nutrients and have been associated with the virulence caused by the pathogenic bacteria as well as the development of multidrug resistance bacteria (Garmory & Titball, 2004; Rees *et al*., 2009). The resistance occurred when the ABC transporter increased the expulsion of the drugs from the bacteria cell (Leonard *et al*., 2003). The down-regulation of this gene can therefore increase the effects of the antibiotics against the bacteria, making the treatment more

effective. In addition, options like inhibiting ABC transporters with ABC inhibitory drugs or using antibiotics that bypass the resistance mechanism can also be applied to reduce the multidrug resistance situation (Choi, 2005).

Malabaricone C was also found to suppress PA1237 gene (fold change: -2.13004) that acts as probable multidrug resistance efflux pump. The efflux pumps are part of the key that caused multidrug resistance due to their high efficiency in releasing any harmful substrates such as antibiotics with broad structure specificities (Sun *et al.*, 2014). Bacterial efflux pumps basically fall into five classes: (a) major facilitator superfamily (MFS) (b) ATP (adenosine triphosphate)-binding cassette (ABC) superfamily (c) small multidrug resistance (SMR) family (d) multidrug and toxic compound extrusion (MATE) family and also (e) resistance-nodulation-division (RND) family that can only be found in Gram-negative bacteria (Piddock, 2006; Poole, 2007). *P. aeruginosa* PAO1 is considered to be one of the most versatile microbial organisms and is basically resistant to many antimicrobial agents (Dotsch *et al.*, 2009; Kobayashi *et al.*, 2009). The low permeability of the bacteria outer membrane by a complex set of efflux pump systems as well as secretion of alginate during the formation of the biofilm are among the factors which allow *P. aeruginosa* PAO1 to become highly virulent and resistant to multiple antibiotic agents. In another words, the efflux pump plays a very important role in preventing the antibiotics, dyes and detergents from entering into the bacterial cells by actively exporting the substance out of the cells (Piddock, 2006). Besides that, *P. aeruginosa* PAO1 also produce other exo-products such as lipopolysaccharides, elastase as well as other toxins that can cause harmful pathogenesis resulting in the destruction of the host's tissues (Porras-Gómez *et al.*, 2012). The involvement of the multidrug resistance efflux pump in QS system has also been reported in *P. aeruginosa* PAO1. One of the studies showed that the efflux pump

MexGHI-OpmD exported PQS autoinducer precursor as well as anthranilate, a toxic metabolite, out of the bacteria cells. The team found that the deletion of *mexG* or *opmD* genes caused the bacteria failed to produce 3-oxo-C12-HSL and PQS as a result of high level of anthranilate accumulated inside the cells (Rahmati *et al*., 2002).

The transcriptome analysis also showed that malabaricone C affects the *P. aeruginosa* PAO1 ATP synthase in type III secretion system, PA1697 (fold change: -2.56678). For metabolism purpose, bacteria including *P. aeruginosa* PAO1 produced adenosine triphosphate (ATP) either by substrate-level phosphorylation of fermentable carbon sources or by oxidative phosphorylation using the respiratory chain and ATP synthase. Recent research proved that a new clinical diarylquinoline drug (TMC207) can be used to block the ATP synthase enzyme in *Mycobacterium tuberculosis* (Andries *et al*., 2005; Koul *et al*., 2007). The acute infections caused by *P. aeruginosa* PAO1 normally depends on the expression of certain virulence factors such as flagella, pili, exotoxin as well as type III secretion system (T3SS) (Sadikot *et al*., 2005). Chronic infections like pulmonary infections in cystic fibrosis, on the other hand, are regularly accompanied by biofilm communities and thus, becomes the main obstacle to eradicate *P. aeruginosa* PAO1 (Parsek & Singh, 2003; Brencic *et al*., 2009; Morita *et al*., 2014). The T3SS found in *P. aeruginosa* PAO1 contains a number of proteins that can form macromolecular complex extend across the inner bacterial membrane, periplasmic space, peptidoglycan layer, outer bacterial membrane, extracellular space as well as the host cell membrane (Moraes *et al*., 2008). T3SS is basically a specialized needle-like structure that delivers effector toxins directly from the bacteria into the host cytosol in a highly regulated manner. The activation of the T3SS system will then interferes with the signal transduction and thus, causes cell death or alterations in host immune responses (Galle *et al*., 2012).

Malabaricone C also down-regulated the expression of genes involved in *P. aeruginosa* PAO1 R-type porin related to P2 phage, PA0627 gene (fold change: -2.8564). Majority of the bacteria produce bactericidal compounds known as bacteriocins that are usually only effective towards the same or closely related species. The bacteriocins produced by *P. aeruginosa* PAO1 are known as pyocins and located on the chromosome. There are three different types of pyocins produced which is R-type, F-type and S-type (Michel-Briand & Baysse, 2002; Galvez *et al.*, 2008). The R-type pyocins structure looks like the inflexible and contractile tails of bacteriophages. Five groups of R-type pyocins have been identified based on their bactericidal spectra and is classified under R1, R2, R3, R4 and R5 (Michel-Briand & Baysse, 2002). Each of the pyocin spectrum is someway connected with one another: R4 comprises of R3 spectrum, R2 comprises of R3 and R4 spectra while R5 has the broadest spectrum that includes R1 which is not related to R2, R3 and R4 (Scholl *et al.*, 2009). *P. aeruginosa* produced R2 pyocin which is related to P2 phage and is important for competitive growth advantage by binding their tail fibers to the targeted bacterial surface receptors and then killed the target cell membrane by forming pores (Nakayama *et al.*, 2000).

Like catechin, malabaricone C was also found to down-regulate the *yieF* gene (fold change: -2.06842) that acts as NAD(P)H quinone oxidoreductase required for quinones detoxification as well as the nitrate transport ATP-binding protein, *nasS* gene (fold change: -2.93989). The will affect the bacteria ability to detoxify the quinones or nitrites produced by themselves and thus, can caused harmful effects as it is toxic to the bacteria cells (Ryan *et al.*, 2014).

Most of the bacteria uses the two-component systems (2CS) to help them response and adapt to the changing environmental conditions or challenges. The 2CS consists of a sensor kinase that can help to detects specific environmental stimuli as well as a response regulator which will be phosphorylated by the sensor kinase and thus, regulate the expression of necessary response phenotypes (Hoch, 2000; Bijlsma & Groisman, 2003). Histidine-containing phosphotransfer (Hpt) is part of 2CS and has the function to carry out multistep phosphotransferring reaction to a cognate response regulator (Hsu *et al.*, 2008).

Besides being a ubiquitous environmental bacterium and an opportunistic human pathogen, *P. aeruginosa* PAO1 can also degrade and metabolize commercial detergent, sodium dodecyl sulfate (SDS). Previous research has proved that the SdsA1 secreted by *P. aeruginosa* act as SDS hydrolase which allows the bacterium to use primary sulfates such as SDS as their sole carbon or sulfur source. This enzyme generally allows the bacteria to survive and thrive under bacteriocidal conditions (Hagelueken *et al.*, 2006).

Malabaricone C was found to up-regulate both the *hptA* gene (fold change: 2.80107) and *sdsA1* gene (fold change: 2.73724). This can be due to the reason that *P. aeruginosa* PAO1 was trying to adapt to a new environment especially when the compound was found to affect the detoxification of quinones and accumulation of pyocins in the bacteria cells.

5.5   Future Work

More in depth studies on the effects of catechin and malabaricone C are currently being carried out using *P. aeruginosa* PAO1 PEE 6032 GFP biosensor and confocal microscope to measure the thickness of the biofilm formed. Further work related to the mechanism of catechin and malabaricone C as QS inhibitors would be carried out using molecular docking analysis to determine the protein ligand interactions as well as the binding ability of the compounds towards the genes obtained in the RNA-sequencing studies. In addition, the compounds could also be tested on other QS-regulated Gram-negative and Gram-positive bacteria.

CHAPTER 6: CONCLUSION

The excessive and discriminate uses of antibiotics have led to the emergence of bacteria with resistance towards majority of the available drugs, making it a global threat to public health. Researchers have been exploring different alternatives to overcome the issues and one of them is by targeting bacterial QS system, a mechanism commonly used by a variety of bacteria in coordinating their communal behaviour (Dong *et al.,* 2002; Henke & Bassler, 2004). Natural products have a rich treasure of therapeutic effects with great diversity of secondary metabolites (Vickers *et al.*, 1999; Ganesan, 2008). Numerous studies conducted on natural compounds against QS activities have shown promising results and could potentially render the virulence caused by the bacteria (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997; Teplitski *et al.*, 2000; Rasmussen *et al.*, 2005; Chong *et al.*, 2011; Koh *et al.*, 2013).

In summary, the plants and compounds that were selected in this study have been shown to reduce the QS-regulated virulence in *P. aeruginosa* PAO1 without having any bactericidal or bacteriostatic effects. Both catechin and malabaricone C also proved to target the signal receptor of the *P. aeruginosa* PAO1 QS system rather than their signal inducer. The ability of the plants to inhibit the QS-related virulence might contribute to the research database and thus, allows other researchers to conduct a more focused and better research in the quest of understanding these potent inhibitors of QS. More importantly, the discovery of the QS inhibitors can become a therapeutic weapon that lead to the discovery of new drugs to target opportunistic pathogenic bacteria without the risk of antibiotic resistance. Besides that, the QS inhibitors could be used together with the antibiotics to produce better synergistic effects against the dangerous pathogens especially the multidrug resistant (MDR) bacteria.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. List of Publications:

Chong, Y. M., Yin, W. F., Ho, C. Y., Mustafa, M. R., Hadi, A. H. A., Awang, K., … Chan, K. G. (2011). Malabaricone C from *Mystrica cinnamomea* exhibits anti-quorum sensing activity. *Journal of Natural Products*, *74*(10), 2261–2264.

Koh, C. L., Sam, C. K., Yin, W. F., Tan, L. Y., Krishnan, T., **Chong, Y. M.**, & Chan, K. C. (2013). Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors*, *13*(5), 6217–6228.

Chang, C. Y., Krishnan, T., Wang, H., Chen, Y., Yin, W. F., **Chong, Y. M.**, … Chan, K. G. (2014). Non-antibiotic quorum sensing inhibitors acting against *N*-acyl homoserine lactone synthase as druggable target. *Scientific Reports*, *4*, 7245.

Chong, Y. M., How, K. Y., Yin, W. F., & Chan, K. C. (2018). The effects of Chinese herbal medicines on the quorum sensing-regulated virulence in *Pseudomonas aeruginosa* PAO1. *Molecules*, *23*(4), 972–985.

B. List of Presentations:

Chong, Y. M., Yin, W. F., & Chan, K. C. (2012). The discovery of mangosteen (*Garcinia mangostana* L.) leaves extracts exhibiting anti-quorum sensing properties. Poster Presentation at International Congress on Natural Products Research 2012, New York, USA.

Chong, Y. M., Yin, W. F., & Chan, K. C. (2013). The discovery of *Angelica dahurica* roots extracts exhibiting anti-quorum sensing properties. Poster Presentation at 54th Annual Meeting of the American Society of Pharmacognosy 2013, St. Louis, USA.

Chong, Y. M., Yin, W. F., & Chan, K. C. (2014). The effects of bitter gourd (*Momordica charantia*) extracts on *Pseudomonas aeruginosa* PAO1 quorum sensing system. Poster Presentation at 2014 American Society of Pharmacognosy (ASP) Annual Meeting & 14th Annual International Conference on the Science of Botanicals (ICSB), Mississippi, USA.