CAFFEINE AS A NOVEL QUORUM SENSING INHIBITOR

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ABSTRACT

Quorum sensing (QS) is a system used by bacteria to control the gene expression in response to cell density. QS involves in the regulation of variety bacterial physiological functions such as biofilm formation, pyocyanin production and swarming which has been recorded to contribute to bacterial pathogenesis. Therefore, QS would be an interesting target to attenuate pathogens for treating bacterial pathogenicity and infections. In this work, the anti-QS activity of caffeine; a natural occurring alkaloid found at varying quantities in seeds, leaves and fruits of some plants were tested against *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PA01. Quantification assay on the violacein produced by *C. violaceum* CV026 showed that caffeine inhibited violacein synthesis in concentration dependent manner. In agreement to that, qualitative studies of *C. violaceum* CV026 showed the extent of violacein reduction is approximately 68% for caffeine applied at the concentration of 0.3mg/mL. Subsequently, this study also showed that caffeine inhibited swarming motility of *P. aeruginosa* PA01 as observed by the reduction of *P. aeruginosa* PA01 migration distance from the spot-inoculated centre on the swarming agar. In addition, caffeine also inhibited *P. aeruginosa* PA01 pyocyanin production. Qualitative study conducted on *P. aeruginosa* PA01 has confirmed that *P. aeruginosa* PA01 pyocyanin production was reduced up to 77% when treated with caffeine at 0.3mg/mL. To the best of our knowledge, this is the first documentation providing evidence on the presence of caffeine anti-quorum sensing activity. This study will allow caffeine to be explored as an alternative for anti-infective drug in future.

ABSTRAK

Pengesahan Quorum (QS) merupakan suatu sistem yang digunakan oleh bakteria untuk mengawal gen sebagai tindak balas kepada kepadatan sel. QS terlibat dalam pencaturan pelbagai fungsi fisiologi bakteria seperti pembentukan biofilem, pengeluaran pyocyanin dan motiliti berkerumun yang telah direkodkan sebagai penyumbang kepada patogenesis bakteria. Oleh itu, QS telah menjadi sasaran menarik untuk melemahkan patogen bagi tujuan merawat jangkitan bakteria. Dalam karya ini kafein; yang merupakan sejenis alkaloid semulajadi yang mudah didapati di dalam biji, daun dan buah-buahan sesetengah tumbuhan pada pelbagai kuantiti, telah diuji aktiviti anti-QSnya terhadap *Chromobacterium violaceum* CV026 dan *Pseudomonas aeruginosa* PA01. Kajian kuantitatif pada violacein yang dihasilkan oleh *C. violaceum* CV026 menunjukkan bahawa kafein menghalang sintesis violacein dengan cara yang bergantung kepada kepekatan. Selain itu, kajian kualitatif *C. violaceum* CV026 pula menunjukkan pengurangan dalam penguluaran violacein dimana pengurangan berlaku sebanyak 68% apabila diuji dengan kafein pada kepekatan 0.3mg/mL. Seterusnya, kajian ini juga telah mencatatkan bahawa kafein menghalang motiliti berkerumun *P. aeruginosa* PA01. Ini dapat dilihat dengan pengurangan jarak penghijrahan *P. aeruginosa* PA01 dari pusat agar berkerumun di mana *P. aeruginosa* PA01 telah disuntik. Di samping itu, kafein juga menghalang pengualaran pyocyanin *P. aeruginosa* PA01. Kajian kualitatif yang dilakukan pada *P. aeruginosa* PA01 telah mengesahkan bahawa pengeluaran pyocyanin *P. aeruginosa* PA01 telah berkurangkan sehingga 77% apabila dirawat dengan kafein pada kepekatan 0.3mg/mL. Sepanjang pengetahuan kami, ini adalah dokumentasi yang pertama membuktikan kehadiran aktiviti anti-QS pada kafein. Kajian ini memberi ruang kepada yang lain untuk menterokai kafein sebagai alternatif kepada ubat anti-jangkitan pada masa akan datang.

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LIST OF ABBREVIATIONS

%	Percentage
˚C	Degree Celsius
3- hydroxy- C14-HSL	*N*-3-hydroxy-tetradecanoyl-L-Homoserine lactone
3-Oxo-C6-HSL	*N*-3-oxohexanoyl-L-homoserine lactone
3-Oxo-C10-HSL	*N*-3-oxodecanoyl-L-homoserine lactone
3-Oxo-C12-HSL	*N*-3-oxododecanoyl-L-homoserine lactone
ACN	Acetonitrile
AHL	*N*-acyl-homoserine lactone
AI2	Autoinducer 2
ANOVA	Analysis of variance
BC	Before Christ
C4-HSL	*N*-butanoyl-L-homoserine lactone
C6-HSL	*N*-hexanoyl-L-homoserine lactone
C8-HSL	*N*-octanoylhomoserine lactone
C10-HSL	*N*-decanoyl-L-Homoserine lactone
C12-HSL	*N*-dodecanoyl-L-Homoserine lactone
$C_8H_{10}N_4O_2$	1,3,7-trimethylxanthine
CF	Cystic Fibrosis
cm	Centimetre
DMSO	Dimethyl Sulfoxide
DPD	4,5-dihydroxy-2,3-pentanedione
et al.	*et alia* (and others)
h	Hour
HAA	3-(3-hydroxyalkanoyloxy) alkanoic acid
HCL	Hydrochloric acid

HHQ	2-heptyl-4-(1H)-quinolone
L	Liter
LB	Luria-Bertani
MDRO	Multidrug resistant organisms
mg	Milligram
min	Minute
mL	Milliliter
mM	Milimolar
MOPS	3-(N-Morphalino) propanesulfonic acid
nm	Nanometre
OD	Optical Density
PBS	Phosphate Buffer Saline
PCN	Pyocyanin
PQS	*Pseudomonas* quinolone signal
psi	Pounds per square inch
QS	Quorum Sensing
Rpm	Revolutions per minute
sec	Second
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
µg	Microgram
µL	Microliter
µM	Micromolar

CHAPTER 1.0

INTRODUCTION

Quorum sensing (QS) is first introduced by Fuqua *et al.* (Fuqua, Clairborne, Winans, 1994) to describe bacterial cell-to-cell interaction through small diffusible signaling molecules known as autoinducers. Autoinducers allow bacteria to synchronize a global gene expression in response to population density as well as changes or stresses that occurs in the environment. Since the discovery of *Vibrio harveyi and Vibrio fisheri,* that employ QS to regulate luminescence during symbiotic partnership with marine host, numerous other groups of bacteria have been discovered for its cooperative behavioral patterns (Eberhard, 1972). In addition, decades of research have revealed that QS played a significant role in regulating bacterial virulence and pathogenicity. Among the well studied pathogens includes gram positive *Staphylococcus aureus* and gram negative *Pseudomonas aeruginosa* (Smith & Iglewski, 2003; Yarwood *et al.*, 2004).

The capability of bacteria to interact and act as a group has provided them several advantages for survival and conform resistance to antibiotics. Via QS, bacteria are capable to form biofilm, produce cytotoxic proteins and toxic by-products, as well as swarming motility (Smith & Iglewski, 2003). These attributes have been the reasons for the difficulty in the eradication of these pathogens especially in the cases of hospital-acquired infections.

According to the World Health Organization (WHO), the numbers of virulent and drug-resistant bacteria are increasing at an alarming rate. The numbers of antibiotics available to combat multidrug resistant organisms (MDRO) are dwindling fast as compared to the finding of novel antibiotics classes. However, due to excellent discovery of QS during bacterial pathogenesis, the direction of combating this crisis has shifted towards anti-QS or anti-pathogenic drugs to combat this issue. Therefore, much

of the research conducted has been focused on the search for QS inhibiting compounds. QS-inhibiting compounds are classified in terms of their origin; bacterial-originated compound or non bacterial-originated compound. Some of the non bacterial-originated anti-QS compounds that have been documentated are halogenated furanones (from red alga *Delisea pulchra*) (Manefield *et al.*, 1999) and also catechin (from *Combretum albiflorum* bark extract) (Vandeputte *et al.*, 2010). However, to date, no study has been conducted to investigate the potential of caffeine as anti-QS compound against the nosocomial pathogen, *P. aeruginosa* (Norizan *et al*., 2013). In this study, caffeine is preliminary screened for its effectiveness in inhibiting QS by using a QS biosensor; *Chromobacterium violaceum* CV026 followed by its ability to inhibit QS-control virulence determinants of *P. aeruginosa* PA01. *P. aeruginosa* PA01 was chosen as the model QS-bacteria because of its well-studied intricate QS system that regulates its virulence determinants.

The objectives of this study were:

1) To quantitatively measure the inhibition of QS by caffeine using *Chromobacterium violaceum* CV026 as QS biosensor.
2) To investigate the inhibition of caffeine against QS-controlled virulence determinants of *Pseudomonas aeruginosa* PA01 (i.e.: swarming and pyocyanin production).

CHAPTER 2.0

LITERATURE REVIEW

2.1 Quorum Sensing

Quorum sensing (QS) is a bacterial communication system that relies on cell density which allows bacteria to communicate through the diffusible signal molecules known as "autoinducers". Autoinducers are produced and released into the environment as the bacterial population increases. This continues until a threshold concentration of autoinducers in the environment is reached (Williams, 2007). Acting like an "on-and-off" switch, a feedback regulatory loop is activated when these autoinducers achieve adequate concentrations (when bacterial concentrations are at high densities) (Rice *et al*., 2008). This later initiate the expression of genes when the autoinducers directly binding together with a transcriptional regulator or by indirectly via the activation of a signaling cascade (Fuqua *et al.*, 2001). As a result, coordinated behavioral change in bacteria is initiated, followed by the expression of QS dependent target genes which expresses several bacterial phenotypes in the population of cells. Biofilm formation (Shih and Huang, 2002; Cvitkovitch *et al.*, 2003; Parsek and Greenberg, 2005), bioluminescence (Engebrecht *et al.*, 1981), pyocyanin production (Lau *et al.*, 2004) and swarming (Tremblay *et al.*, 2007; Shrout *et al.*, 2006) are some of the phenotypes regulated by QS that are known to contribute to bacterial pathogenesis for both Gram-positive and Gram-negative bacteria.

The first discovery of QS was found in a marine bioluminescent bacterium, *Vibrio fischeri* which exhibit symbiotic partnership with several marine animals (Engebrecht *et al.*, 1981). During this partnership, the marine animal host utilizes the light produced by *V. fischeri* as tool to attract prey, keep away from predators, or for mating (Li and Tian, 2012). Eventually, it was discovered that bioluminescence

produced by *V.fischeri* only occurred at high cell number, and is regulated by QS. This discovery has led to the subsequent research in the discovery of more novel QS bacteria.

Bacterial QS signal molecules are chemically diverse and generally can be characterized in three groups which are: (1) LuxI/LuxR QS in Gram-negative bacteria that utilizes *N*-acyl-homoserine lactones (AHL) for signaling molecules; (2) *luxS*-encoded autoinducer 2 (AI-2) system that exist in both Gram-positive and Gram-negative bacteria; (3) oligopeptide-two-component-type QS in Gram-positive bacteria, which combine the use of molecules signal and small peptides (Dunny and Leonard, 1997; Fletcher *et al.*, 2007). However, the most commonly studied is the first two types of QS systems.

2.1.1 LuxI/LuxR-type QS

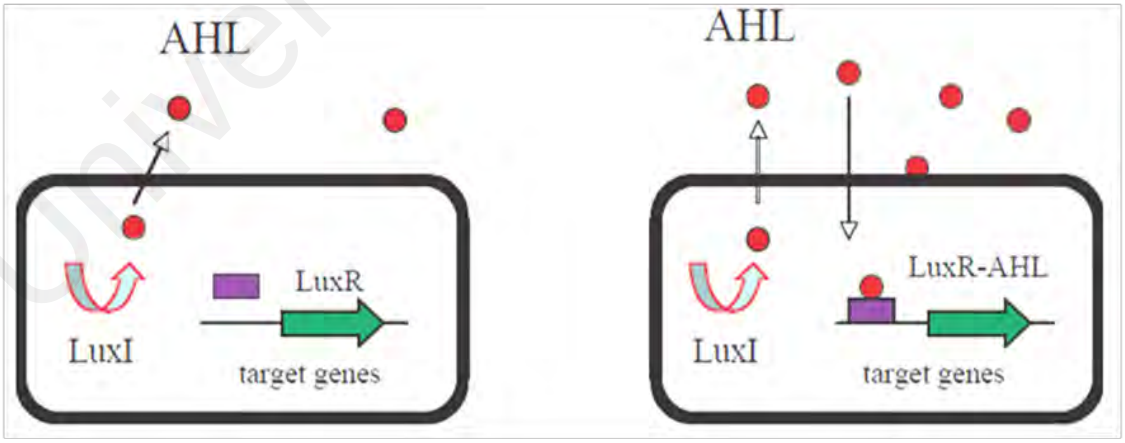
The Gram-negative LuxI/LuxR QS system utilizes AHL as signal molecules. Each of the species has developed their own distinctive AHL or combination of AHL that only can be detected by the same members of the species resulting to species specific interactions (Waters and Bassler, 2005; von Bodman *et al.*, 2008; Fuqua and Greenberg, 2002). These AHL QS signals are highly conserved as they have the same homoserine lactone moiety unsubtituted in the β- and γ-positions with an amide (*N*)-linked acyl side chain at the α-position. The acyl side chains consisted of fatty acids at different length (vary from 4 to 18 carbons), degree of saturations, and the presence of substituent which could be either a hydroxyl- or an oxo-group at C3 (carbon 3) positions (Swift *et al*., 1997).

AHL biosynthesis system mostly depends on a family of genes which consist of a gene that encodes the AHL synthase , the '*luxI*' gene and another that encodes for the AHL receptor; the '*luxR*' gene (Parsek *et al.*, 1999; Fuqua and Greenberg, 2002; Taga and Bassler, 2003) The unique AHL generate via LuxI-like enzymes by pairing the

acyl-side chain of a specific acyl-acyl carrier protein (acyl-ACP) to the homocysteine moiety of *S*-adenosylmethionine (SAM) (Fuqua *et al*., 2002; Parsek *et al*., 1999).

The AHLs molecules are either transported out of the cell (for long chain AHLs) or diffuse out from the cells (for short chain AHLs) (Eberl, 1999; Fuqua and Greenberg, 2002). These signaling molecules will later accumulate in the growth medium or a particular environment in which the bacteria colonizes. At the threshold concentration, the AHL signals bind to the LuxR protein which reacts as a receptor for the recognition of the AHLs. Once the AHLs signals bounds to the LuxR protein, a complex is formed which then attach to QS-regulated target genes promoter and activate the transcription of its target genes (Fuqua and Greenberg, 2002). The schematic diagram of LuxI/LuxR QS in Gram negative bacteria is illustrated in Figure 2.1.

Currently, there is more than hundred of Gram-negative bacteria have been recognized to utilize LuxI/LuxR QS for mechanisms in controlling their cellular processes. Each of the species has developed exclusive AHL or AHLs combination that only can be detected by the same members of the species resulting to species specific interactions (Fuqua and Greenberg, 2002; Waters and Bassler, 2005).



Transcription is not activated at low cell density.

Transcription activated at high cell density.

Figure 2.1 LuxI/LuxR-type QS schematic diagram in Gram negative bacteria.

2.1.2 Autoinducer 2 (AI-2) Mediated QS

The AI-2 system was initially identified in *V. harveyi* a bioluminescent bacterial (Bassler *et al.*, 1993). This system is recorded in Gram-positive and Gram-negative bacteria for example *Escherichia coli*, *V. harveyi*, *Salmonella typhimurium*, *Staphyloccus aureus*, and *V. cholera* (Mok *et al.,* 2003). In comparison to the LuxI/LuxR type QS, AI-2 promotes inter-species communication which acts like a bacterial "universal language" used for different species communication (Federle and Bassler, 2003). The AI-2 production are depends on a metabolic enzyme, *luxS* encoded synthase, which convert ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which are precursor for AI-2. The LuxR protein is a cytoplasmic receptor and also functions as a transcriptional activator (Mok *et al.,* 2003; Federle and Bassler, 2003).

2.2 Ecological Role of QS as a Fundamental Mechanism to Control Bacterial Social Activities

Before scientist discover the intercellular communication among bacteria, bacteria were believed to live as individual cells that only involve in finding nutrients and multiplying (De Keivit *et al.,* 2000). However this discovery has lead to another level of realization that bacteria are also competent to synchronized activity that was initially thought to only happen in multi cellular organisms. Bacteria capability to perform as a group for energy conservation is vital for every living organism to maintain their existence. Small, self-generated QS signal molecules has been used by bacterial as tools for intercellular communication. Through the use of these signaling molecules bacteria are capable in regulating their behavior in response to population density (De Keivit *et al.,* 2000). Therefore, microbes live in population have developed the so called density dependent expression in controlling their physiological activities

and maintaining its survival. The only means in knowing the density of a population via QS, or cell-to-cell communication relies on the concept that AIs signal molecules release from a single bacterium into the environment are too low to be detected. However, when higher bacterial density is present, more AIs are release into the environment which allows bacteria to detect a critical cell mass and response to it by activating or repressing the target genes. During QS, the bacterial will cooperate to obtain a group-specific benefit (Li and Tian, 2012).

The expression of *V. fischeri* bioluminescence which is density-dependent is one of the examples of QS roles in regulating bacterial social activities. This phenotype is important for its symbiotic relationship in the light organ of the squid (Kaplan and Greenberg, 1985; Fuqua and Greenberg, 2002). The bioluminescence expression in this species is a respond to secreted signaling molecules (autoinducers) which accumulate in the external environment as the bacterial densities increases (Fuqua *et al.*, 1994). When the autoinducer concentration exceeds a threshold level, a signal transduction cascade is initiated which leads to the production of luciferase; an enzyme that responsible for the bioluminescence. The key finding in this study is when they identified that LuxI and LuxR regulatory protein controlled genes encode for the luciferase enzyme complex which also involved in QS (Fuqua *et al.*, 1994; Fuqua and Greenberg, 2002).

On the other hand, studies conducted on *P. aeruginosa*, a model system in studying biofilm, has elucidate that QS plays vital role during biofilm development and maturation. Biofilm formation is considered as pathogenicity trait during chronic infection such as cystic fibrosis. It is defined by the collection of structured microbial communities that are enclosed by an exopolymetric matrix which attach onto natural or manmade surfaces (Costerton *et al.*, 1999). Microorganisms that are able to form a biofilm have a survival advantage compared to their free-swimming counterparts.

Indeed biofilms have been shown to be highly tolerant to a range of stresses including biocides, protozoan grazing and antibiotics (Costerton *et al.*, 1999; Karren *et al.*, 2004).

Different studies on *P. aeruginosa* biofilm has revealed that the QS deficient *P. aeruginosa* mutant formed biofilms that are more susceptible to death with kanamycin treatment in comparison to the wild type one (Shih and Huang, 2002). In this comparison study, wild- type PA01, single mutants JP1 (*ΔlasI*) and PDO100 (*ΔrhlI*) and double mutant JP2 (*ΔlasI ΔrhlI*) biofilms were grown for 72 hours before medium containing kanamycin was fed to the biofilm. In comparison to PA01 and PDO100, the JP1 and JP2 mutants were more susceptible to kanamycin treatment with the surviving cell fraction for both biofilms significantly decreased suggesting that there is a relationship between antibiotic resistant of a biofilm and QS (Shih and Huang, 2002).

Besides biofilm, swarming motility is also tightly regulated by QS. Motility enables *P. aeruginosa* to colonize niches (Drake and Montie, 1988). There are several types of motility observed in *P. aeruginosa* which are swimming, twitching, and swarming (Drake and Montie, 1988). The latter form of motility occurs on semisolid surfaces, and requires functional flagella as well as pili to facilitate the swarming motion. This phenomenon results in a complex motility pattern which is observable on semisolid media. *P. aeruginosa* swarmer cells differentiates into elongated and hyperflagellated cells by sensing the viscosity of the swarming surface and nutritional signals that enables it to thrive (Harshey, 1994).

A study conducted by Köhler and colleagues exhibited diminished swarming behavior in *P. aeruginosa* that carries mutation in its *las* QS system while *rhl* mutants were unable to swarm completely. In addition to their work, *pilR* and *pilA*, genes encoding type VI pili, were found to play an important role in swarming. Cells that were *pilR* and *pilA* mutants were unable to swarm (Köhler *et al.*, 2000). Swarming was also found to be modulated by rhamnolipid production, a lipopeptide biosurfactant formed

by *P. aeruginosa.* Rhamnolipid has a detergent-like structure and act as wetting agents by lowering the surface tension during swarming motility (Soberon-Chavez *et al.*, 2005). It was later found that the *rhl* QS system of *P. aeruginosa* regulate rhamnolipid production. Cells that were *rhlB* and *rhlC* mutant were observed to have altered swarming patterns which were characterized by the irregularly shaped tendrils. However, the mutants were able to swarm on agar that was supplemented with rhamnolipid-containing spent medium of *P. aeruginosa* (Caiazza *et al.*, 2005). These studies show the tight regulation of swarming as well as rhamnolipid by the QS system of *P. aeruginosa.*

QS also has been documentated to regulate *P. aeruginosa* pyocyanin (PCN) production. Pyocyanin is a blue redox reactive toxic *P. aeruginosa* secondary metabolite. It can be found a large quantities in the sputum of *P. aeruginosa* infected cystic fibrosis (CF) patients (Lau *et al.*, 2004). Only later it was found that pyocyanin affects the ion transport regulation, ciliary beats frequency, mucus secretion in epithelial cells airway and also interferes with gene expression and innate immune mechanism (Rada & Leto, 2013).

As *P. aeruginosa* infection is multi factorial and involving various virulence factors thus making it hard to distinguish PCN role during lung infection. Therefore *in vitro* cell culture systems with purified PCN have been used in many studies examining PCN toxicity. These *in vitro* studies revealed that PCN exhibited a large spectrum of cellular lung damage which maybe the reason to the persistent infection of *P. aeruginosa* in CF patients (Lau *et al.*, 2004). Purified PCN can be acquired by repeated choloroform-distillied water extraction from stationary phase of *P. aeruginosa* overnight culture. The absorption spectrum of PCN is pH-dependent whereby at low pH values extracted PCN appear to be red and blue at high pH values (Rada & Leto, 2013).

Another interesting example of QS roles in regulating bacterial social activities is during the infection of *Erwina carotovora*, phytopathogen that causing soft rot in several plants (Barras *et al.*, 1994). During the infection process *E. carotovora* release various enzymes that degrade plant tissues, including pectate lyases, polygalacturonase, cellulase, and proteases which involves in maceration of plant tissue essential to colonize the host. However low concentration of *E. carotovora* exoenzyme would not bring any damage to the plant tissue, but instead activate the plant phytodefense mechanisms. Therefore, during this time QS were used to ensure no exoenzyme production occurring until adequate bacterial density have been reached for successful tissue destruction and evasion of plant defenses (Jones *et al.*, 1993; Pirhonen *et al.*, 1993). This regulation relies on the LuxRI homologs ExpR and ExpI that control expression of the tissue-macerating enzymes in a cell-density dependent manner (Jones *et al.*, 1993; Pirhonen *et al.*, 1993). More examples of bacteria utilizing AHL QS are summarized in Table 2.1.

Table 2.1 Examples of AHL producing bacteria

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Aeromonas caviae YL12	C4-HSL; C6-HSL	*acaI/acaR*	Chitinolytic activity	Lim Y.L. *et al.*, 2014
Aeromonas hydrophila strain 187	C4-HSL	*ahyI/ahyR*	Exoprotease, Biofilm Formation, Hemolysin protein	Chan X.Y. *et al.*, 2014; Swift S. *et al.*, 1997
Burkholderia cepacia	C8-HSL	CepI/R	Swarming motility, Biofilm formation and siderosphore production	Huber B. *et al.*, 2001; Lewenza *et al.,* 1999; TomLin *et al.*, 2004
Burkholderia sp. C10B	C6-HSL; C8-HSL; C10-HSL; C12-HSL	Unidentified	Biofilm formation, swarming	Goh S.Y. *et al.*, 2014.

Bacteria	**AHLs**	**QS Systems**	**Target Genes and Phenotypes**	**References**
Cedecea neteri SSMD04	C4-HSL	*cneI/cneR*	Lipase activity	Tan K.H. *et al.*, 2015
Chromobacterium violaceum	C6-HSL	CviI/CviR	Biofilm formation, chitinase and violacein production	Stauff and Bassler, 2011
Enterobacter asburiae L1	C4-HSL; C6-HSL	*easI/easR*	Intracellular spreading	Lau Y.Y. *et al.*, 2013; Lau Y.Y *et al.*, 2014
Erwinia carotovora subsp. *carotovora*	3-Oxo-C6-HSL	ExpI/ExpR; CarI/CarR	Exoenzymes, Carbapenem antibiotics production	Bainton *et al.*, 1992; Pirhonen *et al.*, 1993
Pandoraea pnomenusa RB38	C8-HSL	*ppnI/ppnR1; ppnR2*	Lignin degradation, sulphur oxidation	Ee R. *et al.*, 2014
Pandoraea sp E26	C8-HSL	Unidentified	Protease secretion, lung cell invasion.	Chan K.G. *et al.*, 2015
Pantoea stewartii	3-Oxo-C6-HSL	EsaI/EsaR	Adhesion and host colonization	Koutsoudis *et al.*, 2006
Pantoea rodasii ND03	3-oxo-C6-HSL	Unidentified	Phytopathogen	Yunos N.Y.M. *et al.*, 2014
*Pantoea sp.*M009	3-Oxo-C6-HSL	Unidentified	Phytopathogen	Tan W.S. *et al.*, 2014
Pseudomonas aeruginosa	C4-HSL; 3-Oxo-C12-HSL	LasI/LasR RhlI/RhlR	Exoenzymes; biofilms formation; rhamnolipid production; secondary metabolites	Chapon- herve *et al.*, 1997; Passador *et al.*, 1993; Glessner *et al.*, 1999
Pseudomonas chlororaphis	C6-HSL	PhzI/PhzR	Phenzine-1-carboximide production	Chin- A- Woeng T.F.C. *et al.*, 2001
Pseudomonas fluorescens	3- hydroxy-C14-HSL; 3-hydroxy- C6-HSL	MpuI/R and HdtS	Phenazine antibiotics production	Shaw *et al*., 1997; Laue *et al*., 2000

Bacteria	AHLs	QS Systems	Target Genes and Phenotypes	References
Pseudomonas putida	3-Oxo-C10-HSL; 3-Oxo-C12-HSL	PpuI/R	Maturation	Dubern *et al.*, 2006

2.3 QS Inhibition as a Way to Control Bacterial Pathogenicity

In the 21st century, we are currently facing dramatic increase of pathogens which are now resistant to many classes of antibiotics. Infectious disease has become the leading cause of death worldwide (Lohner and Staudegger, 2001). According to the 1996 world health report, "too few drugs that has been developed to replace those that have lost their effectiveness". We are currently facing a risk of another pre-antibiotic era. This alarming phenomenon is caused by excessive and abusive usage of antibiotics during treatment and prevention of bacterial infection in human or animal. This situation is worsened with the fact that bacteria adapt rapidly and become antibiotic resistant by means of simple mutations just through the uptake of the mobile genetic elements such as transposons or plasmids or through the arrangement of endogenous antibiotic resistance loci (Rice *et al.*, 2005).

Therefore, there is an urgency to control the rapid emergence of antibiotic-resistant bacteria. In this situation QS-blocking compound is a promising solution to this problem. As most of the pathogenic bacteria depend on QS systems to control its virulence expression, hence interfering with bacterial QS can be a promising means to manage bacterial infections. Blocking of QS signals can induce attenuation of pathogen's virulence determinants. It is hypothesized that QS inhibition does not promote selective pressure that causes the development of resistance as the bacterial growth is not affected (Parsek *et al.*, 2005).

2.4 Plant-Based Anti-QS Compounds

Plants grow in environment exposed to high bacterial density are believed to developed their own protective mechanisms against infections in order to maintain its existence (Viswanathan P. *et al.*, 2015). Hence, natural products have been explored initially due to their therapeutics values in traditional medical practices. In recent years, researchers have shown great interest in natural products therapeutic roles as well as their biological and ecological function in regulating cell-cell interactions within bacteria. To date, some of the plant-derived biologically active natural products components have inspired the innovation of latest drugs used for the treatments of several diseases. However, due to the nonexistence of plants immune systems against the invaders resulted to the speculation of other defense mechanisms to defeat these pathogens, and the manipulation of QS systems is considered to be one of the modes of defense against phytopathogens (Koh *et al.*, 2013).

Australian macro alga, *Delisea pulchra* is an important discovery of the first plant-derived QS antagonist compounds which came from the marine environment (Manefiel *et al.*, 1999). Studies elucidate that this marine algae produces a furanone that interfere several Gram-negative bacteria AHL regulatory system. This furanone have shown inhibitory activity against fouling animals and bacteria which prevent bacterial colonization and fouling on its surface.

The finding of QS antagonist from biological and non-biological origin has increased tremendously in recent years. Some of these QS antagonist are synthesized chemically and are able to inhibit QS signals, but most of the current existing one has been discovered from plants extracts. These plant-derived active compounds that promote QS inhibitory activities should be regard as safe and non toxic to humans as these plants can be ingested by humans However, toxicity studies on these compounds are still necessary. Among the plant-based anti-QS compounds discovered are catechin

(from *Combretum albiflorum* bark extract) (Vandeputte *et al.*, 2010), Malabaricone C (from *Myristica cinnamomea*) (Chong *et al.*, 2011), *Syzygium aromaticum* (Krishnan *et al.*, 2012), and the bud extract of *Melicope lunu-ankenda* (Tan *et al.*, 2012). More examples of plant derived anti-QS compound (antagonist) are summarized in Table 2.4.

Table 2.4 Plant based antagonist of QS against selected bacteria and pathogens

Source	Antagonist	Inhibition against	Reference
Plant extracts	*Vanilla planifolia* (beans)	*C. violaceum* CV026	Choo *et al.*, 2006
	Garlic (bulbs)	*P. aeruginosa*	Bjarnsholt *et al.*, 2005
	Tremella fuciformis (whole)	*C.violaceum* CV026	Zhu and San, 2008
	Laurus nobilis (flowers, fruits, leaves and bark)	*C. violaceum* ATCC 12427	Al-Hussaini and Mahasneh. 2009
	Syzygium aromaticum (bud) *Melicope lunu-ankenda* (leaves)	*C. violaceum* CV026 *E. coli* [pSB401] *E. coli* [pSB1075] *P.aeruginosa lecA::lux* *P. aeruginosa* PA01	Tan *et al.*, 2012; Krishnan *et al.*, 2012
	Moringa oleifera (fruits and leaves)	*C. violaceum* ATCC 12472	Sreelatha *et al.*, 2009
	Areca catechu (seeds) *Imperata cylindrical* (stem) *Nelumbo nucifera* (leaves) *P. ginseng* (roots) *Panax notoginseng* (flowers and roots) *Prunella vulgaris* (whole) *Prunus armeniaca* (kernel of seed) *Punica granatum* (bark)	*C. violaceum* CV026 *P. aeruginosa* PA01	Song *et al.*, 2010; Koh and Tham, 2011;
	Acacia nilotica (green pod)	*C. violaceum* ATCC 12472	Singh *et al.*, 2009
	Scorzonera sandrasica	*C. violaceum* ATCC 12472 *C. violaceum* CV026	Bosgelmez-Tinaz *et al.*,2007

Source	Antagonist	Inhibition against	Reference
Plant extracts	*Ballota nigra* *Castanea sativa* *Cyclamen hederifolium* *Juglans regia* *Leopoldia comosa* *Lonicera alpigena* *Malva sylvestris* *R.officinalis* *Rosa canina*	*Staphylococcus aureus*	Quave *et al.*, 2008
	Scutellaria baicalensis	*C. violaceum* CV026	Song *et al.*, 2012
	Brassica oleracea *Curcuma longa* *Fragaria* sp. *Ocimum basilicum* *Origanum vulgare* *R. eubatus* *Rosemarinus officinalis* *Rubus idaeus* *Thymus* sp. *V. angustifolium* *Vaccinium macrocarpon* *Vitis* sp. *Zingiber officinale*	*C. violaceum* CV026 *C. violaceum* 31532 *P. aeruginosa* PA01 *E. coli* O157:H7	Vattem *et al.*, 2007
	Orange	*Yersina enterocoliticia*	Truchado *et al.*, 2012
	Chamaesyce hypericifolia (aerial) *Conocarpus erectus* (leaves) *Quercus virginiana* (leaves) *Tetrazygia bicolor* (leaves)	*C. violaceum* ATCC 12472 *C. violaceum* CV026 *Agrobacterium 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
	Capparis spinosa (fruits)	*C. violaceum* CV026 *P. aeruginosa* *E. coli* *Proteus mirabilis* *Serratia marcescens*	Abraham *et al.*, 2011

Source	Antagonist	Inhibition against	Reference
Essential oils	Rosemary Tea tree	*C. violaceum* CV026	Alvarez *et al.*,2012
	Piper bredemeyeri (leaves) *P. brachypodom* (leaves) *P. bogotence* (whole)	*C. violaceum* CV026	Stashenko, 2011
	Elettaria cardamomum *Zingiber officinale* *Lippia alba* *Ocotea* sp. *Swinglea glutinosa* *Myntotachys mollis*	*P. putida* [pRK-C12) *E. coli* [pJBA132]	Jaramillo-Colorado *et al.*, 2012

Plant compounds usually target bacterial QS systems (Figure 2.4) by preventing the synthesizing of bacterial signaling molecules, degradation of the signaling molecules or by interfering the signal receptor. However according to Koh *et al.*, 2013 it is rather rare for a plant to posses anti-signal synthase activity therefore suggesting another way that interferes with the signal reception receptor via competitive or non competitive binding. For competitive binding to occur, the QS-blocker molecules must be similar to the AHL(s)-binding site on the receptor protein to prevent activation of QS regulated genes. As for non competitive binding, the QS-blocker molecules binds to the surface of the receptor protein except for the AHL(s)-binding surface and alter the shape of the active site to prevent future binding of the AHL(s) with its receptor protein. In addition, studies also have revealed that plant-derived anti QS compounds is capable to degrade bacterial signaling molecules and interfere with their communication system which leads to disruption of bacteria virulence factors (Dong *et al*., 2001).

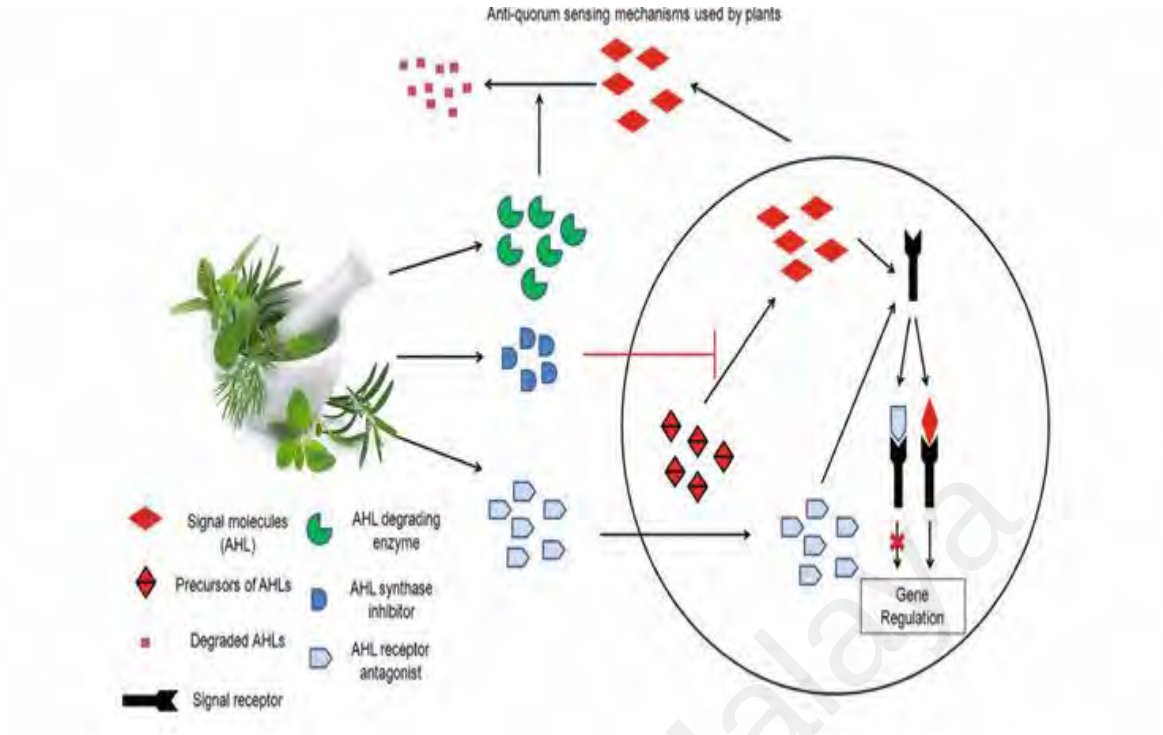


Figure 2.4 Various mechanisms utilized by plants to inhibit QS

2.5 Alkaloids: A Pharmacological Perspective.

Alkaloids are a group of naturally occurring organic compound originates from bacteria, fungi, plants, or animals that contain mostly basic nitrogen atoms. Alkaloids gained its reputation as nature's curse and blessing due to the beneficial effects or harmful poisonous nature of different classes of alkaloids. It have been a key ingredient in the manufacturing of several antibacterial drugs, for example, with the synthesis of quinine leading towards the development of quinolones and the ingenious usage of alkaloids as scaffolds substructures for example the production of Linezolid; an antibiotic for highly resistant gram positive bacterial infections (Bogatcheva *et al.*, 2011; Parhi *et al.*, 2012). Alkaloid can be presence in any parts of a plant; however, specific classes of alkaloids may be concentrated only at specific part of a plant. Up to this day, almost 18 000 alkaloids have been revealed ranging from different sources of plant, funguses, marine animals and insects (Dembitsky *et al.*, 2005).

Among the pharmacological properties of various alkaloids includes anti-tumour (eg. Vinblastine), antimalarial (eg. Quinine), analgesic (eg. Codeine), central nervous system depressant (eg. Morphine), antihypotensive (eg. Ephedrine), antihypertensive (eg. Reserpine), antipyretic (eg. Quinine), and antiemetic (eg. Scopolamine) activities (Dewick, 2001; Evans, 2009; Robbers *et al.*, 1996). All the listed activities are acknowledged and extensively used in both traditional and modern medicine. Examples of alkaloids usage in traditional and modern medicine include usage of quinine-rich cinchona bark for treating malaria and vinblastine in cancer treatment, respectively (Evans, 2009).

2.5.1 Alkaloids and their Ability to Attenuate Bacterial Pathogenicity

In many pathogenic bacteria, the expressions of virulence factors are tightly regulated by QS (Williams *et al.*, 2000). Bacterial pathogenesis is a complicated multistep process, where it starts with the attachment of bacterial cells on the mucous membrane aligning organs or host skin, multiplication and evasion of the hosts' immune system and followed by secretion of toxic proteases as well as inducing inflammation (Williams *et al.*, 2000; Smith & Iglewski, 2003; Yarwood *et al.*, 2004). In many research conducted, several alkaloids have been discovered to inhibit either of the aforementioned process, thus leading to the attenuation of bacterial pathogenicity. One of the ways bacteria ingeniously use to regulate its virulence is by utilizing the transcriptional regulators that are sensitive to environmental changes. For example, the pathogen *V. cholera* transcriptional regulator ToxT responds to the intestinal fatty acids by activating genes that encodes for the cholera toxins and fimbriae for motility. Microarray analyses showed that the alkaloid virstatin inhibited both the mentioned virulence factors and that transcriptional regulator ToxT is the probable target (Yang *et al.*, 2011; Hung *et al.*, 2005).

Gram-positive bacteria employ enzymes known as sortases as an anchor for surface proteins such as internalins and adhesins and host immune system evasion proteins. Recent studies have shown that several classes of alkaloids are able to inhibit sortase at a cellular level. Alkaloids that belong to the class of aaptamine, isoquinolone, and pyrrolidine were able to inhibit the enzyme. Treatment of *S. aureus* with isoaaptamine exhibited decreased attachment to fibronectin, a host cell receptor that is exploited by bacteria for invasion (Jang *et al.*, 2007; Kim *et al.*, 2004; Kudryavtsev *et al.*, 2009).

QS inhibitors have been identified in a few alkaloid classes for example indole, piperidine, steroidal, and 1,3,4-oxadiazole. Compound 37 classified as 1,3,4-oxadiazole were found to inhibit pyocyanin synthesis and 2-heptyl-4-(1H)-quinolone (HHQ; a precursor of PQS) production in *P. aeruginosa,* primarily by targeting the PQS transcriptional regulator, PqsR (Zender *et al.*, 2013). An indole, 7-hydroxyindole, alters the virulence gene expression in *P. aeruginosa.* 7-hydroxyindole inhibited pyocyanin productions, production of the QS signal PQS, rhamnolipid synthesis, as well as a diminished swarming motility of *P. aeruginosa* (Lee *et al.*, 2009). Solenopsin A (piperidine) targets the *rhl* system and inhibits virulence gene transcription. Amongst the virulence factors of *P. aeruginosa* that was inhibited are pyocyanin production, eleastase B, and biofilm formation (Park *et al.*, 2008). Tomatidine, a steroid, was found to inhibit the haemolytic activity of *S. aureus* that is controlled by *agr* system, a well studied QS system in *S. aureus* (Mitchell *et al.*, 2012). The studies conducted show the potential of alkaloids as anti-pathogenic drugs by targeting bacterial QS system.

2.6 Caffeine

The potential of many classes of alkaloids as QS inhibitor, have prompted the research focus onto caffeine. Caffeine is readily found in seeds, leaves and fruits of

some plants at varying quantities. It is classified under the alkaloids group and appear to be white crystalline with bitter taste. Caffeine naturally acts as pesticides that paralyzes and even kills some of the insects that ingest on the plants. The first discovery of caffeine were made by a German chemist Friedlieb Runge in 1819 when he successfully isolated pure caffeine for the first time where it is called as " Kaffebase" which stand for coffee base. There is no exact time that indicates the history of caffeine consumption but it is believed it started as early as 2737 BC when teas were first consumed. Nowadays caffeine is a drug most widely taken by people around the world by infusions extracted of the tea leaves or the seeds of coffee as well as drinks containing products derived from the kola nut. In addition to that, yerba mate, guarana berries as well as guayusa tree are also other sources of caffeine (Mohanpuria, 2010; Nehlig *et al.*, 1992).

Known for its famous stimulant properties, caffeine acted directly to the central nervous system as well as the metabolic systems (Nehlig *et al.*, 1992). It is used medically and also recreationally to restore alertness when drowsiness occurs as well as reduce physical fatigue (Nehlig, 1999). In addition to that, caffeine also has the effect in increasing focus, stimulate faster and clearer flow of thoughts, increase metabolic rate and also improve body coordination (Nehlig, 1999).

Caffeine ingested is absorbed by human small intestine within 45 min of ingestion prior to distribution throughout body tissues (Griffiths and Mumford, 1996). However, the time required for human body to eliminate one-half of total amount of caffeine varies among individuals and are affected by various factors such as age, liver function and hormonal states. In healthy human, the half life of caffeine is measured at the range around 4.9 to 6 hours (Hammami *et al.*, 2010). Caffeine is metabolized in human liver into three primary metabolites which are paraxanthine, theobromine and also theopylline (Arnoud, 1993). As human liver are able to metabolize caffeine and

eliminate it out from the system, caffeine can be considered safe to be consumed at amount less than 250 mg per day.

While it is relatively safe for human consumption, caffeine is found to be toxic to various animals such as dogs and birds (Avery *et al.*, 2005). The increased in toxicity of caffeine in some animals are partly due to their poor liver ability to metabolize caffeine compound. In addition to that, caffeine also has a pronounced effect on various insects, spiders and mollusk (Hollingsworth *et al.*, 2002).

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Bacteria Strains, Plasmid and Oligonucleotides

The growth of *C. violaceum* CV026 was carried out at 28˚C in LB media while *P. aeruginosa* PA01 was cultured in LB media at 37˚C. Incubation in LB broth was done with shaking at 220 rpm.

Table 3.1 Bacterial strains used in this study

Bacterial Strain	Description	Source/Reference
C. violaceum CV026	Mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532 acts as AHL biosensor that will produce a purple pigment in the presence of short chain AHL.	McClean *et al.*, 1997
P. aeruginosa PA01 lecA::lux	Prototroph *lecA::luxCDABE* genomic reporter fusion in PA01	Winzer *et al.*, 2000

3.2 Chemical Reagents

All the chemical reagents used in this study are of the analytical grade (or highest grade) purchased from Bio-Rad Labarotories Ltd. U.S.A.; Merck; Germany; Promega Ltd, U.S.A; Sigma Chemical Corp., U.S.A.; Invitrogen Corp., U.S.A.; BDH Labarotory Supplies, England; and Ajax Pacific Specialitty Chemicals Limited ABN., Australia.

3.3 Equipments

Equipments and instruments that were used during the course of this study included Eppendorf Research micropipettes, Eppendorf mini spin centrifuge machine, Eppendorf thermomixer compact, Sortorius weighing balance, Hirayama autoclave machine, Tecan luminometer (Infinite M200), Exelo water distiller, Merck mili-Q water synthesis, orbital shaker incubator and N-Biotek Inc. shaking incubator.

3.4 Growth Media and Buffer Solutions

Preparations of the growth media and solutions in this study required sterilization by autoclaving at 121˚C, 15 psi for 20 min unless stated otherwise. Sterilization of heat sensitive solutions was done by filter sterilization with syringe (filter pore size of 0.22µm).

3.4.1 Luria-Bertani (LB) Medium

Preparation of Luria-Bertani broth consisted of 1% w/v trytone, 0.5% w/v yeast extract and 1.0% (w/v) NaCl in 1 L of distilled water, Bacto agar was added to broth medium at concentration 1.5% w/v to prepare LB agar (Sambrook *et al.*, 1989). The solutions were then autoclave to sterilize. For extraction of AHL molecules, the LB broth was supplemented with 50mM of 3-(N-Morphalino) propanesulfonic acid (MOPS) prior to inoculation of bacteria. The function of MOPS is to maintain the acidity of the media and to prevent the elevation of media pH above 7.0 to avoid lactonolysois of AHL molecules which will be induced at high pH (Yates *et al.*, 2002).

3.4.2 Phosphate Buffered Saline (PBS)

PBS solution was prepared by mixing 0.23 g of NaH_2PO_4, 1.15 g of Na_2HPO, and 9.0 g of NaCl in 1 L of distilled H2O. The pH of the media was adjusted to the value of 6.5 before autoclave sterilization.

3.4.3 Mueller-Hinton agar (MHA)

MHA agar was prepared by adding 38 g of premix Mueller- Hinton agar from Sigma-Aldrich© into 1 L of distilled water before autoclaved.

3.4.4 Swarming Agar

Swarming agar was prepared by adding glucose (1% w/v), Bacto agar (0.5% w/v), Bacto agar (0.5% w/v), Bacto peptone (0.5% w/v), and yeast extract (0.2% w/v into 1 litre of distilled water before autoclaved.

3.5 Stock Solutions

3.5.1 Synthetic Caffeine

Synthetic Caffeine molecules were obtained from Sigma (St. Louis, MO, USA). Caffeine samples were prepared fresh by dissolving caffeine powder in ultrapure water at 80 ˚C. The aliquots were then sterilized by filtration (filter pore size of 0.25 μm) (Milipore) and diluted to appropriate concentrations using sterilized ultrapure water.

3.5.2 Synthetic (+)-Cathechin

(+)-Catechin was purchased from Sigma and dissolved in 20% (v/v) DMSO before sterilized by filtration (using 0.25 μm filter pore size) and further diluted to appropriate concentrations using sterilized ultrapure water.

3.5.3 Synthetic *N*-Acyl Homoserine Lactones

Synthetic AHL molecules were obtained from Sigma-Aldrich© and Cayman Chemicals. AHLs were dissolved in acetonitrile (ACN) to desired concentrations. AHL stocks were kept at 16˚C for storage.

3.6 Antibacterial Assay

Paper disc diffusion assay was performed according to the Clinical and Laboratory Standard Method with certain modifications (National Committee for Clinical Laboratory Standards (United States), 2006). *C. violaceum* CV026 overnight culture were adjusted to OD_{600nm} 0.1 before spreading 100 µL of the culture on the MHA plates evenly and left to dry for 30 min. Next, sterilized paper discs (10 mm diameter) were placed on the plates with distance of 3 cm between each paper disc. Subsequently, 20 µL of freshly prepared caffeine stocks at final concentration of 0.1, 0.2, 0.3 and 0.4 mg/mL were loaded on each paper disc. Ultrapure water, 20% (v/v) DMSO and 20 µL of (+)-catechin dissolved in 20% DMSO at final concentration of 0.1, 0.2, 0.3 and 0.4 mg/mL were also included as negative controls. DMSO (100%) was used as positive control. The plates were again left to dry for 10 min before incubate at 28 °C for 24 hours. Any growth and inhibition zones were observed and record after that. This assay was repeated in three independent triplicates.

3.7 Screening for Caffeine Anti-Quorum Sensing Activities

3.7.1 *C. violaceum* CV026 Plate Assay

This assay was conducted as described by Krishnan *et al.* with modifications (Krishnan *et al.*, 2012). Firstly, 8 mL *C. violaceum* CV026 overnight culture were

adjusted to OD_{600nm} of 1.2 before adding into 40 mL of warm molten LB agar buffered with 50mM MOPS. Subsequently, synthetic *N*-hexanoylhomoserine lactone (C6-HSL) was added into the agar mixture at the final concentration of 0.12 µg/mL. The agar was gently swirled to mix the media without the formation of bubbles. The molten agar was poured immediately into a petri dish (150 mm diameter). The *C. violaceum* CV026 lawn was then left to solidify for 2 hours.

Next, 4mm diameter wells were made on the solidified agar plate using the end of white pipette tips. Distance between two wells was measured to be approximately 2 cm. Then, 40 µL of freshly prepared caffeine stock with final concentration of 0.1, 0.2 and 0.3 mg/mL were dispensed into each well. (+)-Catechin was used as positive control for QS inhibition while ultrapure water and DMSO served as negative controls. The plate was incubated for 18–24 hours at 28 °C. This assay was repeated in three independent triplicates.

3.7. 2 *C. violaceum* CV026 Violacein Quantification assay

C. violaceum CV026 overnight culture in LB broth was adjusted to an OD_{600} of 0.01 before adding C6-HSL to a final concentration of 0.12 µg/mL. Approximately, 9 mL of the culture were transferred into 50-mL sterile plastic tubes followed by the addition of 1mL caffeine stocks at final concentration of 0.1, 0.2, and 0.3 mg/mL, respectively into each tube. One of the tube was added with only 1 mL ultrapure water to serve as negative control. After 16 h incubation, the treated cells were vortex for 30 sec to re-suspend any pellicle of adherent cells. Next, 1 mL of the treated cells from each 50 mL tubes were transferred into 1.5 mL eppendorf tube and centrifuged at 13,000 rpm for 10 min to precipitate the insoluble violacein. The supernatant was discarded before adding 1 mL of 100% DMSO to rehydrate the pellet. The solution was again vortex vigorously for 30 sec to ensure that the violacein has completely

solubilised before centrifuged at 13,000 rpm for 10 min. Subsequently 200 µL of the violacein-containing supernatants were then added into 96-well flat-bottomed micro plate (SPL Life Sciences, Pocheon-Si, Korea) in triplicate. The absorbance was read using the Tecan Infinite M200 luminometer at a wavelength of 585 nm.

3.7.3 Caffeine Anti-Microbial Quantification Assay Against C. *Violaceum* CV026

Sample preparation for the anti-microbial activities assay are conducted as described in 3.7.2 without the addition of synthetic C6-HSL. Briefly, 9 mL *C. violaceum* CV026 overnight culture OD_{600nm} 0.01 were dispensed into 50mL tube before adding 1 mL of caffeine stocks to a final concentration of 0.1, 0.2, and 0.3 mg/mL, respectively. One of the tubes was added with only 1 m/L ultrapure water to serve as negative control. The culture was incubated for 16h at 28 ˚C. After incubation period, the overnight culture were vortex for 30 sec to re-suspend any pellicle of adherent cells before transferring 200 µL of the culture into 96-well flat-bottomed micro plate (SPL Life Sciences, Pocheon-Si, Korea) in triplicate. The absorbance was read using the Tecan Infinite M200 luminometer at wavelength of 600 nm

3.7.4 AHL Degradation assay

This assay was performed as described by Chan *et al.* with modifications (Chan *et al.*, 2011). Briefly, 10 µL of freshly prepared caffeine stock were added at final concentration of 0.1, 0.2, and 0.3 mg/mL into 2 mL micro centrifuge tubes filled with 1 mL of LB broth with 50 mM MOPS and C6-HSL (0.12 µg/mL). The tubes were incubated overnight at 28 °C with shaking (220 rpm). After 18 hours incubation, extraction of C6-HSL from the mixture was conducted by adding equal volume of ethyl acetate into each tubes before vortex it vigorously. Next, the supernatant which contained ethyl acetate with C6-HSL was transferred into new tubes and left air-dried in the fume hood. After drying, 0.1 mL of phosphate buffered saline (PBS) (10 mM, pH

7.4) was added into each micro centrifuge tubes to rehydrate C6-HSL. Then 10 µL of the mixture was spotted onto sterile paper discs placed on CV026 lawn followed by overnight incubation at 28 °C. This assay was repeated in three independent triplicates.

3.7.5 *P. aeruginosa* PA01 Short Chain AHLs Inhibition Assay

Freshly prepared caffeine stocks (250 µL) at final concentration of 0.1 to 0.3 mg/mL were added into 15 mL of *P. aeruginosa* PA01 overnight culture grown in LB broth buffered with 50 mM MOPS adjusted to OD_{600nm} of 0.1. The mixture was vortex gently before incubated for 16–18 hours at 37 °C (220 rpm). After 16–18 hours incubation, the tubes were vortex for 30 sec to re-suspend any pellicle of adherent cells. Bacterial culture (200 µL) were added into each well of the 96-well flat bottomed micro plate (SPL Life Sciences) in triplicate. The absorbance of each well was read using the Tecan Infinite M200 luminometer at a wavelength of 600 nm. Extraction of the short chain AHLs from the remaining overnight culture was conducted after that as explained in section 3.9. Extracted AHLs (20 µL) was spotted onto sterile paper discs placed on CV026 lawn and incubated overnight at 28 °C for 24 hours. This assay was repeated in three independent triplicates.

3.7.6 *P. aeruginosa* PA01 Swarming Assay

250 µL of caffeine at final concentration of 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL were seeded into 14.75 mL of 60˚C molten swarming agar. The molten swarming agar (15 mL) was poured into the petri dish. The petri dish was then placed into the laminar flow for 30 min to solidify. Finally, 2 µL of *P. aeruginosa* PA01 overnight culture adjusted at OD_{600nm} of 0.1 were inoculated at the centre of the agar and incubated for 16 hours at 37 °C. This assay was repeated in three independent triplicates.

3.7.7 *P. aeruginosa* PA01 Pyocyanin Quantification Assay

P. aeruginosa PA01 overnight culture adjusted to an OD_{600nm} of 0.2. Next, 250 µL of caffeine at final concentration of 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL was added and mix well to the adjusted *P. aeruginosa* PA01 overnight culture (4.75mL) in a polypropylene tube and incubated at 37 °C for 24 h. After the incubation period the 5 mL culture was extracted using 3 mL of chloroform, followed by mixing the chloroform layer with 1 mL of 0.2 M HCL. The pink organic layer extracted were measured it absorbance at 520nm (Essar*et al.*, 1990).

3.7.8 Statistical Analysis

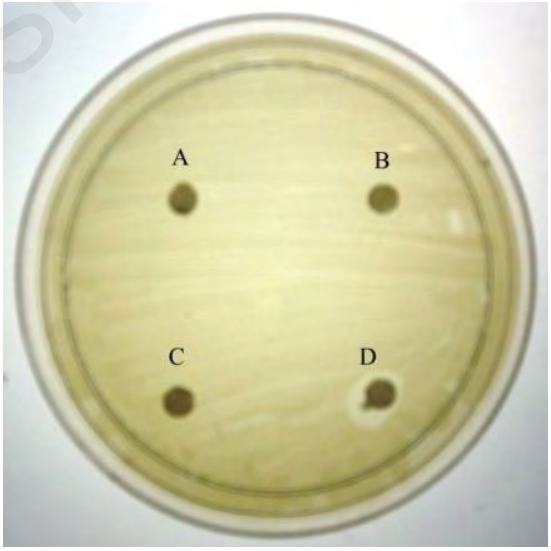
All statistical results in this study represent the average of three independent experiments. The data were analyzed using one-way ANOVA test at $P < 0.05$ using GraphPad Prism 5 statistical software.

CHAPTER 4.0

RESULTS

4.1 Antibacterial Assay

As QS inhibition is focused on the interference of bacterial signaling and not antibacterial activity, it is important to ensure any anti-QS effect is not resulted from antibacterial activity. Therefore, paper disc diffusion assay was performed to test antibacterial activity of caffeine against *C. violaceum* CV026. Result demonstrates no inhibition zones caffeine applied at 0.1-0.4 mg/mL (Figure 4.1 (E), (F), (G) and (H)) and (+)-catechin applied at 0.1-0.4 mg/mL (Figure 4.1 (I), (J), (K) and (L)) suggesting all tested concentrations of caffeine and (+)-catechin showed no antibacterial activity. This indicates that caffeine concentrations used in the study does not inhibit the growth of *C. violaceum* CV026. Visible inhibition zone was only observed on the disc treated with 100% DMSO (positive control).



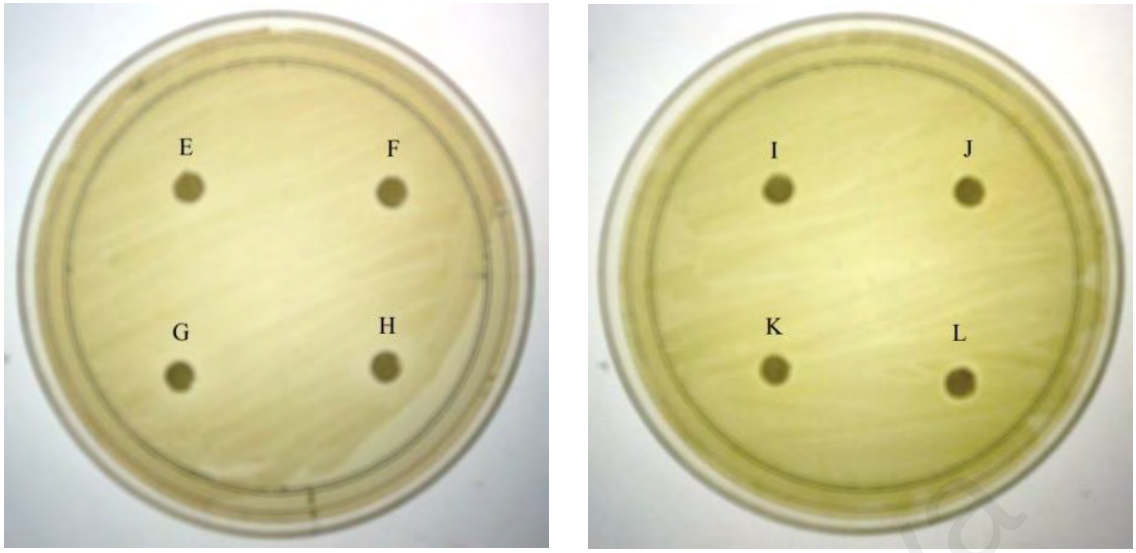


Figure 4.1. Antibacterial assay. (A) Disc without treatment; (B) 20% (v/v) DMSO; (C) Ultrapure water; (D) 100% DMSO; (E) 0.1 mg/mL **caffeine; (F) 0.2 mg/**mL **caffeine; (G) 0.3 mg/**mL **caffeine; (H) 0.4 mg/**mL **caffeine; (I) 0.1 mg/**mL **(+)-catechin; (J) 0.2 mg/**mL **(+)-catechin; (K) 0.3 mg/**mL **(+)-catechin; (L) 0.4 mg/**mL **(+)-catechin.**

4.2 Pre-Screening of Caffeine for Anti-QS Properties

Preliminary screening of anti-QS properties of caffeine was done by using *C. violaceum* CV026 as a biosensor. *C. violaceum* CV026 produce purple pigment with the presence of exogenously supplied short chained AHLs. Any formation of halo zone around the well signified anti-QS existed in the treatments. Observation of purple pigment inhibition of the biosensor was illustrated as in Figure 4.2. All caffeine and (+)-catechin concentration tested (0.1 mg/mL- 0.3 mg/mL) showed halo zone formation with an increased in the size of halo zone formed around the well with any increment of treatment concentrations (Figure 4.2 (D), (E), (F), (G), (H) and (I)). This suggests that caffeine promote QS inhibitory effect at concentration depending manner. Caffeine applied at the concentration of 0.3 mg/mL showed the most prominent formation of halo zone surrounded by purple violoacein background (Figure 4.2 (I))

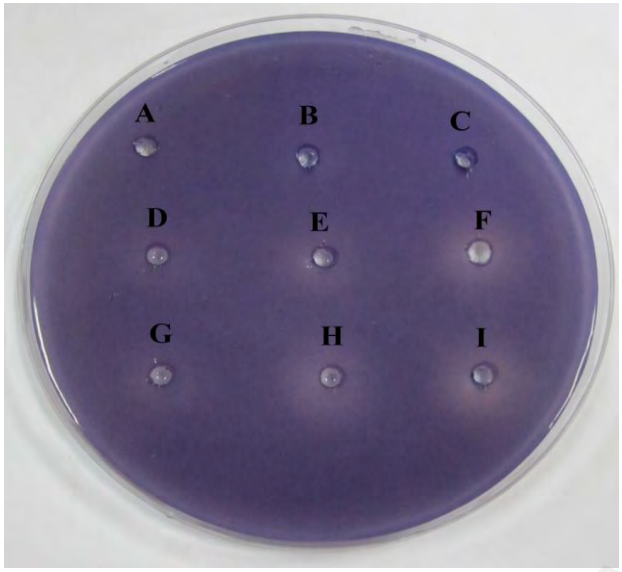


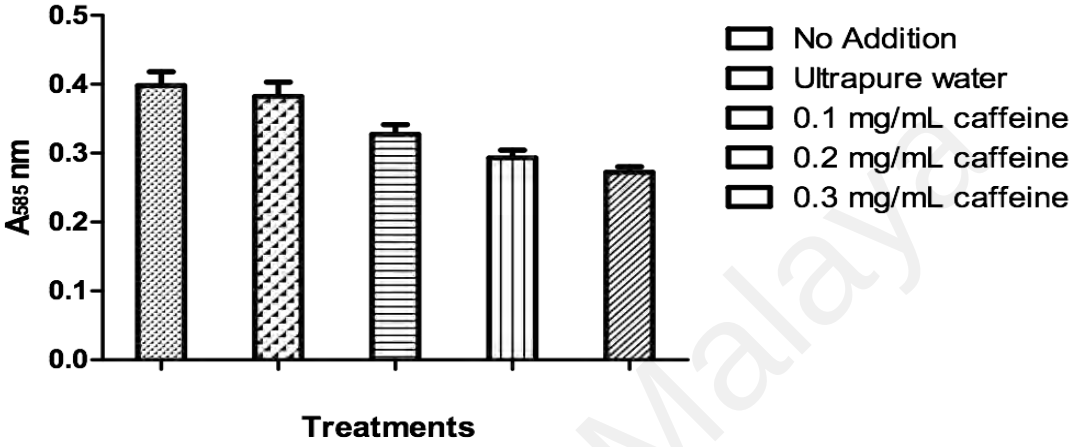
Figure 4.2. Anti-QS properties of caffeine. (A) untreated well; (B) 20% (v/v) DMSO; (C) ultrapure water; (D) 0.1 mg/mL **(+)-catechin; (E) 0. 2 mg**/mL **(+)-catechin; (F) 0.3 mg**/mL **(+)-catechin; (G) 0.1 mg**/mL **caffeine; (H) 0.2 mg**/mL **caffeine; (I) 0.3 mg**/mL **caffeine.**

4.3 Quantification of Anti-QS activity of Caffeine towards *C. Violaceum* CV02*6*

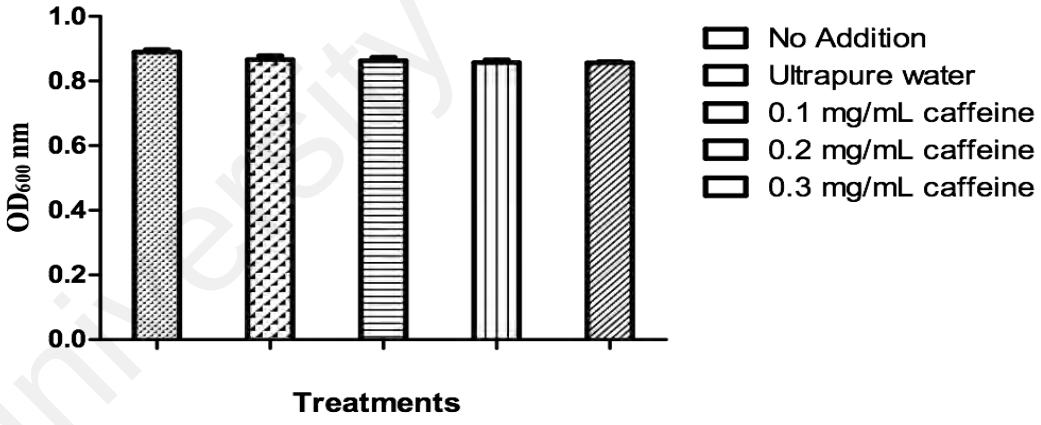
The inhibitory effect of caffeine against bacterial QS was quantified using the tube incubation assay. The violacein produced by treated *C. violaceum* CV026 overnight culture was extracted as described in materials and methods. Ultrapure water and 20% (v/v) DMSO served as negative controls. The violacein production was measured spectrophotometrically and quantified at OD values 585 nm. The result was shown in Figure 4.3(A). Statistical test was also conducted using the ANOVA test and it was found that all tested caffeine concentrations showed a significant inhibition of violacein content at $P \leq 0.05$. Consequently, different degree of inhibitory effect was observed at different concentrations of caffeine suggesting that the inhibitory effect of caffeine is concentration dependent manner.

Statistical test conducted using the ANOVA test revealed that all tested caffeine concentrations at 0.1-0.3 mg/mL showed no significant reduction of OD600 reading at

P. at $P \leq 0.05$ (Figure 4.3(B)). It is confirmed that caffeine used at concentration of 0.1-0.3 mg/mL did not inhibited *C.violaceum* CV026 growth thus the reduction of violacein production is solely due to the anti-QS properties of caffeine and not due to antibacterial effects.



(A)



(B)

Figure 4.3 OD values of the solution at 585 nm (A) and 600nm (B). Statistical significant of each test (n=3) was evaluated by conducting one-way ANOVA test and a P value of P < 0.05 being significant.

4.4 Screening for AHL Degradation

This assay was conducted to determine the possibility that the inhibitions of QS signal is due to the degradation of the QS signal itself by means of caffeine. Reductions on the size of purple pigment suggest the degradation of QS signal. No significant difference on the size of purple pigment formed on the CV026 lawn between C6-HSL treated with ultrapure water and 20% (v/v) DMSO (negative control) with C6-HSL treated with caffeine and also catechin applied at 0.1, 0.2, 0.3 mg/mL, respectively (Figure 4.4). This validates that caffeine and catechin at tested concentrations do not degrade the C6-HSL signal. Negative result was shown on paper disc pipette with PBS solution indicating that there are no traces of short chain AHL in the PBS solution used in this assay (Figure 4.4 (C)).

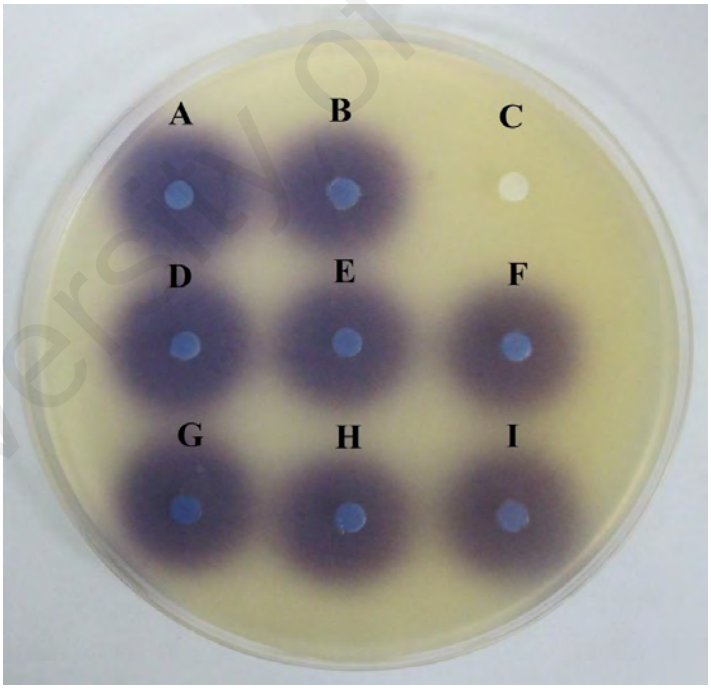


Figure 4.4. C6-HSL degradation by caffeine. Disc (A) C6-HSL treated with ultrapure water (negative control); (B) C6-HSL treated with 20% (v/v) DMSO (negative control); (C) PBS (blank); (D) C6-HSL treated with 0.1 mg/mL caffeine; (E) C6-HSL treated with 0.2 mg/mL caffeine; (F) C6-HSL treated with 0.3 mg/mL caffeine; (G) C6-HSL treated with 0.1 mg/mL (+)-catechin; (H) C6-HSL treated with 0.2 mg/mL (+)-catechin; (I) C6-HSL treated with 0.3 mg/mL (+)-catechin.

4.5 Inhibition of *P. aeruginosa* QS Signal Production

It has been reported that *Pseudomonas* spp. triggered *C. violaceum* CV026 violacein production, suggesting production of short chain AHLs (Chong *et al.*, 2012). A preliminary test was designated to test the inhibition of *P. aeruginosa* PA01 short chain AHLs with caffeine concentrations at 0.1, 0.2, and 0.3 mg/mL. Observation of purple pigmentation of the biosensor was illustrated on Figure 4.5. In general, as the concentration of caffeine treatment increases the intensity of purple pigment (violacein) formed around the *C. violaceum* CV026 wells decreased (Figure 4.5 (D), (E) and (F)). The decreases in the violacein production is due to the decreasing amount of AHLs extracted from *P. aeruginosa* treated with caffeine in comparison to the one untreated. No formation of purple pigmentation was observed on well added with PBS (Figure 4.5 (C)) indicating that there are no detectable short chain AHLs in the PBS solution used in this assay.

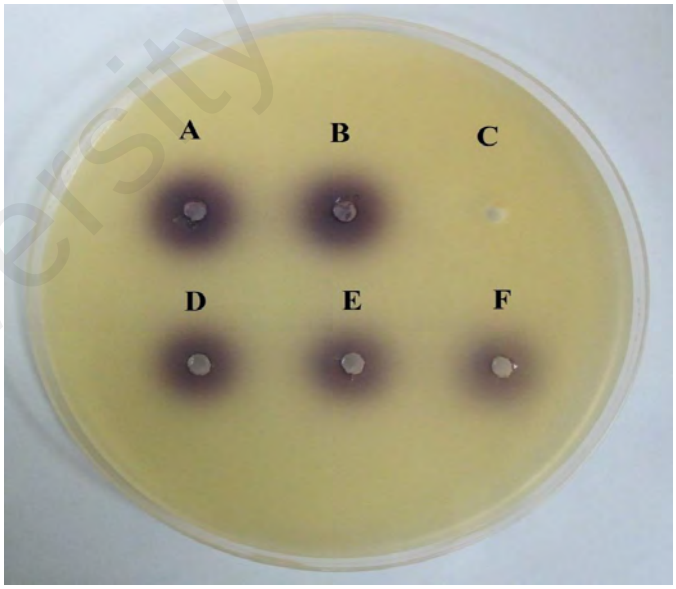
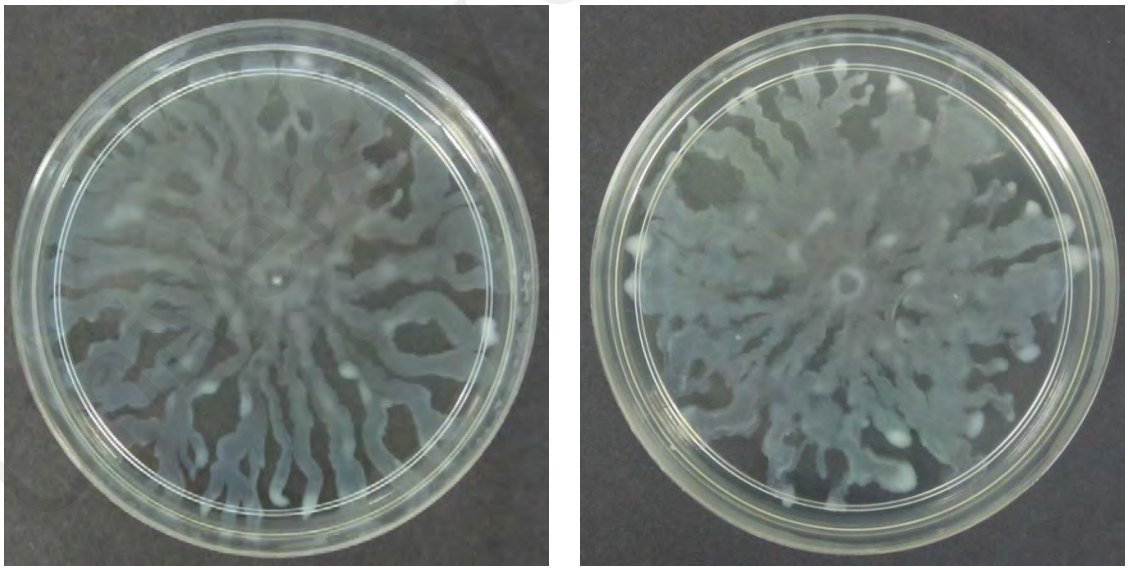


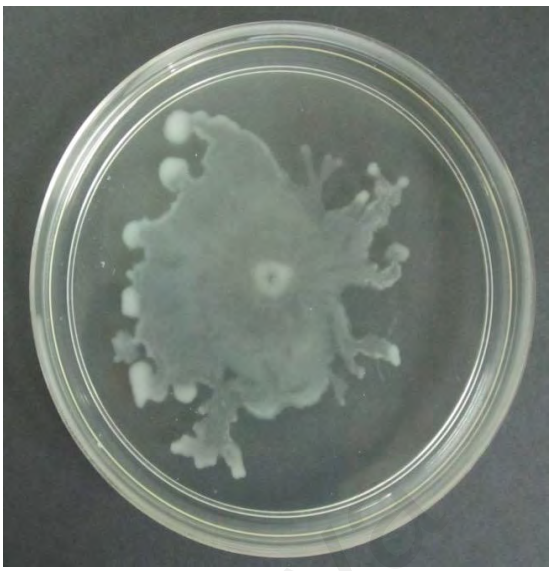
Figure 4.5. Wells added with 40 µL AHL extracted from *P. aeruginosa* culture treated with; (A) ultrapure water (negative control) and (B) 20% (v/v) DMSO (negative control). Well (C) was added with 40µL of PBS solution. Wells (D); (E); and (F) were added with 40 µL AHL extracted from *P. aeruginosa* culture treated with 0.1 mg/mL caffeine; 0.2 mg/mL caffeine and 0.3 mg/mL caffeine respectively.

4.6 Inhibition of *P. aeruginosa* Swarming Motility

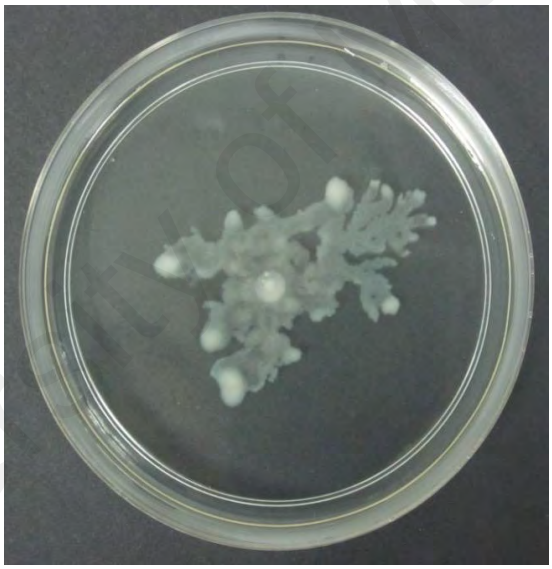
Swarming assay was conducted to verify the application of caffeine in inhibiting *P. aeruginosa* PA01 swarming motility which is QS-mediate. Caffeine was seeded into the swarming agar and ultra pure water was used as negative control. It was observed that after 16h of incubation, *P. aeruginosa* PA01 that has been loaded onto the plate without addition of caffeine (Figure 4.6 (A)) (control) swarmed until the edge of the plate. However, swarming motility is inhibited in swarming agar seeded with caffeine at 0.1 mg/mL (Figure 4.6 (C)), 0.2 mg/mL (Figure 4.6 (D)) and 0.3 mg/mL (Figure 4.6 (E)). In addition, swarming agar seeded with addition of 0.3 mg/mL of caffeine showed observable *P. aeruginosa* PA01 swarming inhibition with formation of short and undefined tendrils and smaller protrusion from its original position (Figure 4.6 (E)).



(A) (B)



(C) (D)



(E)

Figure 4.6. Swarming inhibition assays. Swarming agars of (A) *P. aeruginosa* PA01 supplemented with (B) ultrapure water (v/v, negative control); and caffeine of (C) 0.1 mg/mL (D) 0.2 mg/mL and (E) 0.3 mg/mL.

4.7 *P. aeruginosa* PA01 Pyocyanin Quantification Assay

Pyocyanin assay was conducted to verify the application of caffeine in inhibiting PA01 pyocyanin production which is QS-mediate. Pyocyanin extract was quantified using a spectrophotometer (UV1601, Shidmazu, Kyoto, Japan) at 520 nm. The result was shown in Figure 4.7. Statistical test was also conducted using the ANOVA test and it was found that all tested caffeine concentrations (0.1, 0.2 and 0.3 mg/mL) showed inhibition against pyocyanin production at $P \leq 0.05$. Consequently, different degree of inhibitory effect was observed at different concentrations of caffeine suggesting that the inhibitory effect of caffeine is concentration dependent manner.

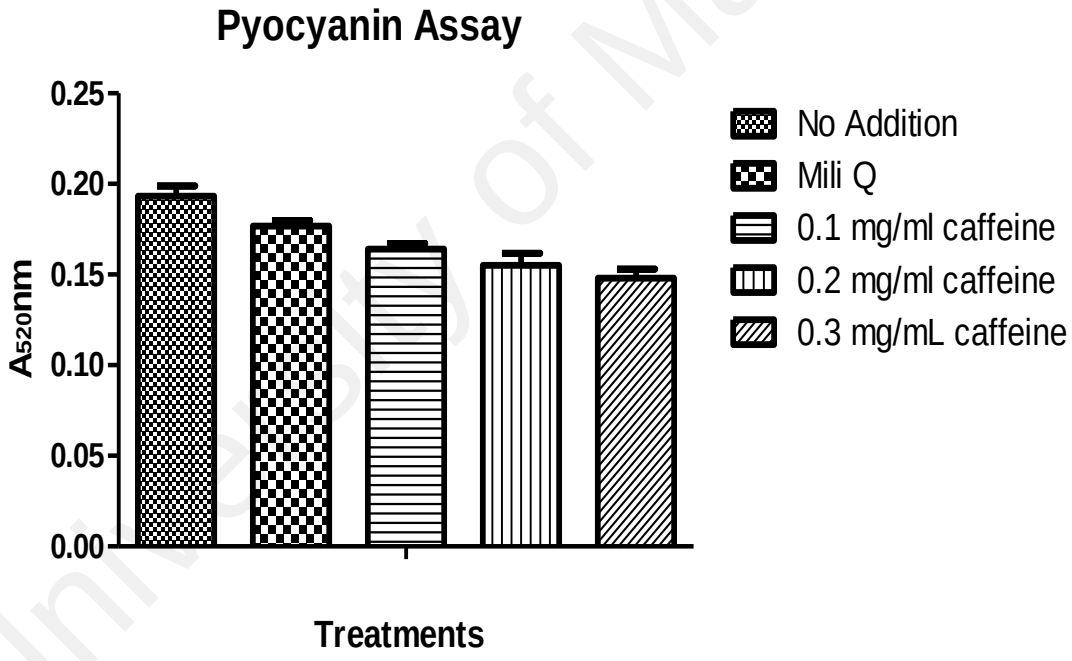


Figure 4.7. Absorbance values of pyocyanin extracts at 520 nm.

CHAPTER 5.0

DISCUSSION

5.1 Caffeine Stocks Preparation

Plants has been used traditionally for the treatments of various disease since thousands of year ago and has become an important source of chemical diversity for the potential identifications of new drugs useful as antimicrobials.

Caffeine (1,3,7-trimethylxanthine, $C_8H_{10}N_4O_2$) is a natural alkaloid or xanthine alkaloid found in coffee beans, tea leaves, cocoa beans, cola nuts and other plants (Nonthakaew A. *et.al*, 2015). It is probably the most frequently ingested pharmacologically active substance in the world, found in common beverages (coffee, tea, soft drinks), products containing cocoa or chocolate, and medications, including headache or pain remedies and over-the-counter stimulants (Nawrot P. *et.al*, 2003). Several studies have shown that caffeine has antimicrobial effect to a wide range of bacterial species for example caffeine at concentration 2.5 mg/mL retards the growth of *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *P. aeruginosa* within a short time (Dash *et al.*, 2008). The main reason caffeine was selected for this study is that despite its antimicrobial properties, as of to date no anti- QS activity of this alkaloids against *C. violaceum* CV026 and *P. aeruginosa* PA01.

As caffeine is commonly found in coffee bean and tea leaves, its concentrations varies and highly depending on several factors, such as freshness, extraction method, types of coffee beans and tea leaves, brewing techniques and plantation area. For instant, fresh green beans of the Arabica genus from Los Altos de Chiapas, Mexico contain 4.00 mg/mL of caffeine compare to the roasted one which contain 9.33 mg/mL of caffeine (Salinas-Vargas *et al.*, 2014). On the other hand, the amount of caffeine in

Arabica fresh coffee was reported to be from 190 to 456 mg/mL (Rodrigues *et al.*, 2007). Dried tea which also provides a significant source of caffeine contains a higher composition of caffeine when compared to the coffee bean (Chu and Juneja, 1997). Unfortunately, the tea brewing method causes reduced caffeine. The caffeine content in tea is also related to the tea type (Rodrigues *et al.*, 2007) and the extraction method. For example after brewing, caffeine in black tea (30.97 mg/mL) was found to be higher than green tea (18.70 mg/mL) and oolong tea (23.89 mg/mL) (Guo *et al.*, 2011).

Caffeine compound used in this study was purchased from Sigma Aldrich instead of using the extraction methods from the coffee beans or tea leaves as caffeine pure compound is readily available in the market. In addition, it is easier to determine the exact concentration of caffeine for analysis purposes. Caffeine stocks were freshly prepared by dissolving Sigma Aldrich caffeine powder in ultrapure water at 80˚C. The aliquots were then filter sterilized using filter pore size of 0.25 μm (Milipore). One of the advantages of using distilled water as solvent is that it is cheap and non toxic. Besides that the ability of caffeine to dissolve in water (180 mg/mL at 80°C and 670 mg/mL at 100°C) is also the reason why distilled water is chosen to prepare caffeine stocks.

5.2 Anti- QS Activity of Caffeine

Antibacterial assay was conducted beforehand as general practice to determine the suitable caffeine concentration to use in this study. As QS inhibition is about interference in bacterial signaling systems to avoid communication within the bacteria without killing the bacteria therefore it is important to ensure that the caffeine concentration used in this study did not promote antimicrobial effect to *C. violaceum* CV026 and *P aeruginosa* PA01. Caffeine at 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL and 0.4 mg/mL concentration were selected in the assay. Result shown that all Caffeine

concentrations tested have no antimicrobial effect to CV026 and *P aeruginosa* PA01.

Based on Caffeine antimicrobial activity on CV026 and *P aeruginosa* PA01 results, three lower Caffeine concentrations which are at 0.1mg/mL, 0.2 mg/mL and 0.3 mg/mL were selected in screening for anti-QS properties. Preliminary screening on caffeine anti-QS properties was conducted using *C. violaceum* CV026, a biosensor that is commonly used to test any presence or inhibition of QS signal molecules. *C. violaceum* CV026 produces the purple pigment violacein in response to the presence of exogenous short chained AHL (Mc. Clean *et al.*, 1997). Therefore in *C. violaceum* CV026 plate assay, formation of any holo zones indicated that caffeine either inhibiting the C6-HSL competitively from binding to *cviR* transcriptional regulator; enzymatically degrading the C6-HSL or removing the C6-HSL by active transport (Teplitsk *et al.*, 2000; Dong *et al.*, 2000; Leadbette & Greenberg, 2000).

All the tested caffeine concentrations formed holo zones on the purple violacein background. These halo white zones formed were closely observed to be opaque and not transparent with different degree of diameter depending on the caffeine concentration. This indicates that the inhibition formed around the well was caused by QS inhibition in concentration depending manner and not due to inhibition of bacterial cell growth. It has also been reported several natural compound that inhibits *C. violaceum* CV026 violacein production including Catechin one of the flavanoids from *Combretum albiflorum* bark extract (Vandeputte *et al.*, 2010) and also Malabaricone C from *M. cinnamomea* (Chong *et al.*, 2010)

Caffeine used at 0.3 mg/mL gives the largest halo zone formation. This was further verified by quantification of the violacein extracted after 16 h of incubation at 28 °C. Quantification of the violacein using luminometer gives a better estimation of inhibition by various caffeine concentrations. The mean value of violacein extracted

from overnight culture treated with caffeine dropped to 0.327 at 0.1 mg/mL; 0.293 at 0.2 mg/mL and 0.273 at 0.3mg/mL while the negative control gave a mean value of 0.398 (Figure 4 a). The extent of violacein reduction is approximately 68% for caffeine at 0.3 mg/mL. On the other hand, study conducted by Musthafa and colleagues (Musthafa *et al.*, 2010) suggest *Ocimum sanctum*, *Manilkara zapota*, *Musa paradiciaca* and *Ananas comosusseems* to be a better QS inhibitor for violacein formation by *C. violaceum* CV026 as these plant extracts caused 94.98%, 90.28%, 89.54% and 89.22% reduction in violacein production respectively.

Caffeine has been shown to inhibit CV026 violacein production, a QS dependent trait. It is not clear how QS has been modulated by caffeine as caffeine could be competing with AHL's from forming AHL-receptor complex; disrupting the AHLs from binding to the receptors by degrading the AHLs or changing the AHLs shape by binding to the AHLs which eventually prevent formation of AHL-receptor complex; changing the structure enzymes that is involved in AHL synthesis (Hong *et al.*, 2012) It is crucial to verify caffeine mechanism in preventing QS.

AHLs degradation assay was conducted in this study to monitor the AHL degradation activity with and without the presence of caffeine. This assay was assessed using C6-HSL at final concentration of 0.12 µg/mL supplied in LB broth with addition of caffeine at final concentration of 0.1, 0.2, and 0.3 mg/mL before incubating it at 28˚C for 18 h. After incubation the remaining C6-HSL was extracted and detected using the *C. violaceum* CV026 AHL biosensor. The results obtained showed the remaining C6-HSL extracted cause violacein production on *C. violaceum* CV026 lawn. This confirmed that all caffeine concentration tested did not degrade or disrupt C6-HSL molecule. In addition, the diameter and intensity of the purple zone formed on *C. violaceum* CV026 lawn (Figure 4) comparatively the same at all tested caffeine

concentration suggesting that caffeine did not degrade or disrupt the shape of AHLs which eventually can prevent formation of AHL-receptor complex.

P. aeruginosa PA01 treated with caffeine at final concentration of 0.1, 0.2, 0.3 mg/mL were cultured in LB broth buffered with 50 mM of MOPS to pH 5.5. The purpose of adding MOPS is to prevent spontaneous AHL degradation induced by pH changes during incubation (Chan *et al.*, 2009). Extraction was carried out during early stationary phase or late exponential phase which is after 18 to 20 hours of incubation with addition of acidified ethyl acetate. The function of acidification of ethyl acetate with acetic acid is to minimize the amount of lactone hydrolosis during extraction and storage (Gould *et al.*, 2006). The remaining AHLs from the treated overnight culture were extracted before spotted onto sterile paper discs placed on CV026 lawn.

Result (Figure 5) show a decreased in the diameter and color intensity of the purple zone formed on the CV026 lawn when spotted with remaining AHLs extracted from *P. aeruginosa* PA01 overnight culture treated with caffeine. The purple zone formed on *C. violaceum* CV026 lawn appears to be lighter and almost opaque when spotted with remaining AHLs from caffeine treatment at 0.3mg/mL compared to the one that left untreated which appear to be in darker shade. By comparing the color intensity and the diameter of the purple zone formed on *C. violaceum* CV026 lawn, this study could suggest that caffeine inhibits *P. aeruginosa* PA01 short chain AHL's production at concentration dependent manner.

5.3 Caffeine inhibits *Pseudomonas aeruginosa* PA01 swarming motility

Swarming motility is a rapid and coordinated migration of a bacterial population in a coordinated manner on a semi-solid surface. It is certainly one possible mode for colonizing its natural environments. Swarming can be divided into three general stages,

firstly the differentiation of vegetative cells into swarmer cells which are usually elongated and hyperflagellated followed by migration of swarmer cell and finally consolidation (Eberl *et al.*, 1999; Kearns, 2010). Study suggests that swarmer cells differentiate from vegetative cells probably by sensing the viscosity of the surface or in response to nutritional signal (Harshey, 1994). Swarming is QS dependent and requires rhamnolipid production, flagella and pili (Köhler *et al.*, 2000; Déziel *et al.*, 2003).

Beside *P. aeruginos; Proteus mirabilis, Serratia marcescens* and *S. liquifaciens* has been recorded to show swarming motility. These bacteria have been extensively used in studies conducted to search for QS antagonist. For example, *P. mirabilis* and *S. marascecens* has been used in a study on *Capparis spinosa* and halogeneted furanones from *D. Pulchra* which have been reported inhibits it swarming motility (Givskov *et al.*, 1996; Abraham *et al.*, 2011).

Swarming motility is usually evaluated using various swarm plate assays in which special swarming agar plates are spot-inoculated at the centre of the agar plates and incubated for several hours. The diameter of the resulting "motility zone" formed by migrating bacteria is then measured. In this study the optimized swarming agar contained of 0.5% (w/v) agar. It is crucial to maintain this agar content in swarming plates as *P. aeruginosa* PA01 swims instead of swarm at agar content lower than 0.5% (w/v). On the other hand agar content higher than 0.5% (w/v) appears to be too dry for the bacteria which resulted to longer time for swarming to occur.

Results obtain from this study found that the diameter formed by migrating *P. aeruginosa* PA01 (motility zone) decreased with caffeine treatments in comparison to the untreated one. This suggests that caffeine inhibits swarming motility of *P. aeruginosa* PA01. As the concentration of caffeine increases the extent of swarming inhibition become more apparent. In agreement to this finding, other plant extract

Malabaricone C from nut meg extract was also been observed to inhibits *P. aeruginosa* PA01 swarming motility (Chong *et al.*, 2011). It is believe that components or extracts that interfere swarming motility has a higher chance in affecting the formation of biofilms, surface-attached bacterial colonies (Verstraetan *et. al*, 2008).

5.4 Caffeine inhibits *Pseudomonas aeruginosa* PA01 pyocyanin production

Pyocyanin (PCN) is a blue redox-active secondary metabolite that is produced by *P. aeruginosa*. PCN can be found in large quantities in sputum from patients with cystic fibrosis; one of the most common fatal genetic disorders among the Caucasian population. The main clinical problem for patients with cystic fibrosis is a progressive loss of pulmonary function resulted from chronic lung infection with mucoid *P. aeruginosa*. This will eventually leads to premature death of over 80% of patients (Goldberg and Pier, 2000; Lau *et al.*, 2004). Studies have found that mutations in *lasR-lasI*, *rhIR-rhiL* and *mvfR-haq* QS systems lead to the loss of pyocyanin production (Cao *et al.*, 2001; Gallagher *et al.*, 2002). Additionally, these QS system is also involved in production of protease, elastase and also rhamnolipids (Lau *et al.*, 2004).

In this assay, pyocyanin was extracted and quantified from *P. aeruginosa* PA01 overnight culture which was left treated with caffeine for 24 h. This assay was assessed by measuring the absorbance of pyocyanin extracted from the overnight culture treated with caffeine at 0.1, 0.2, 0.3 mg/mL and comparing it with the non treated one. The mean value of pyocyanin extracted from non treated overnight culture (negative control) gave a mean value of 0.193 while the one treated with caffeine dropped to 0.164 at 0.1 mg/mL; 0.155 at 0.2 mg/mL and 0.148 at 0.3mg/mL (Figure 7). This shows almost 77% of pyocyanin reduction with caffeine treatment at 0.3 mg/mL. Thus from the result, it is suggested that caffeine inhibits *P. aeruginosa* PA01 pyocyanin production.

Even though in this study caffeine has significantly inhibits pyocyanin production of *P. aeruginosa* PA01, the mechanism on how the inhibition occurs has yet to be discovered. Another downstream study conducted on garlic a QS inhibitors found that *lasA, lasB, rhlAB*, and *chiC* genes were down regulated by garlic extract. These genes are responsible for the pathogenesis of *P. aeruginosa* whereby *lasA* and *lasB* are involved in encoding elastase and protease, *chiC* encodes chitinase and *rhlAB* encodes rhamnolipids and pyocyanin.

5.6 Future work

Future studies would involve testing caffeine anti-QS properties to other pathogenic bacteria species such as *Escherichia coli*, and the *Serratia* species. In addition, downstream study to identify the bacterial genes that is affected with caffeine treatment should be conduct for better understanding anti-QS exerted by caffeine. Besides, it is also important to understand the mechanism as it can be an alternative solution in combating antibiotic-resistant bacteria and this can be done via microarray and transcriptome studies.

CHAPTER 6.0

CONCLUSION

Current study demonstrates the finding of caffeine anti-QS activity against *P. aruginosa* PA01 and *C. violaceum* CV026. Caffeine has shown to inhibit *C. violaceum* CV026 violacein production; *P. aruginosa* PA01 swarming motility of and also *P. aruginosa* PA01 pyocyanin production which relate to bacteria virulence factors. Another important finding from this work is that caffeine seems to be inhibiting QS activity resulted from short chain AHLs. In future, it is vital to establish the exact mechanisms of QS inhibition excreted by caffeine. It is believed that continuous screening of pure compound from plant base for anti-QS activity will be able to yield more natural compound with anti-QS inhibition which can be an alternative source to combat multiple antibiotic resistance bacteria.

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Publications:

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Caffeine as a Potential Quorum Sensing Inhibitor

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Abstract: Quorum sensing enables bacteria to control the gene expression in response to the cell density. It regulates a variety of bacterial physiological functions such as biofilm formation, bioluminescence, virulence factors and swarming which has been shown contribute to bacterial pathogenesis. The use of quorum sensing inhibitor would be of particular interest in treating bacterial pathogenicity and infections. In this work, we have tested caffeine as quorum sensing inhibitor by using *Chromobacterium violaceum* CV026 as a biosensor. We verified that caffeine did not degrade the *N*-acyl homoserine lactones tested. In this work, it is shown that caffeine could inhibit *N*-acyl homoserine lactone production and swarming of a human opportunistic pathogen, namely *Pseudomonas aeruginosa* PA01. To the best of our knowledge, this is the first documentation providing evidence on the presence of anti-quorum sensing activity in caffeine. Our work will allow caffeine to be explored as anti-infective drugs.

Keywords: AHL synthesis inhibition; anti-infective drugs; *Chromobacterium violaceum* CV026; *N*-acyl-L-homoserine lactones (AHL); *Pseudomonas aeruginosa* PA01; swarming; quorum sensing inhibitor; virulence

1. Introduction

Bacteria have developed a form of cell-cell communication system that allows them to communicate. This system is called the quorum sensing (QS), whereby communication within the

bacteria involves the production and sensing of small diffusible signal molecules produce by the bacteria. QS was first found in the marine bioluminescent bacterium *Vibrio fischeri* [1–4]. When threshold level of the signal molecules has been reached, it will mediate bacteria regulation feedback regulatory loop [5,6]. Consequently, QS controlled various phenotypes such as biofilm formation [7–10], bioluminescence [1–4], virulence factors [11] and swarming [12,13] which has been shown contribute to bacterial pathogenesis.

There are three QS systems in bacteria which are: (1) LuxI/LuxR-type QS in proteobacteria which use *N*-acylhomoserine lactones (AHL) as signaling molecules; (2) luxS-encoded autoinducer 2 (AI-2) system that exist in both Gram-positive and Gram-negative bacteria; (3) oligopeptide-two-component-type QS in Gram-positive bacteria [13,14]. As the pathogenicity traits of bacteria are controlled by QS, therefore anti-QS is an alternative measure in combating bacterial pathogenicity. This alternative treatment which doesn't rely on antibiotics and prevent drug-resistance problem is highly desirable.

To date, many studies have been conducted in finding new anti-QS compounds that are analogs to the naturally occurring AHL signals molecules and inhibit the AHL signal receptor proteins. Among the few non bacterial-origin antagonists of QS that have been found are catechin (from *Combretum albiflorum* bark extract) [15], halogenated furanones (from red alga *Delisea pulchra*) [16], Malabaricone C (from *Myristica cinnamomea*) [17], and also the extract of vanilla [18], *Syzygium aromaticum* [19], and *Melicope lunu-ankenda* [20]. However, no study has been conducted to investigate the potential of caffeine as an anti-QS compound.

Caffeine (1,3,7-trimethylxanthine) is one of the few plant products with which the general public is readily familiar, because of its widespread occurrence in beverages such as coffee, tea and various soft drinks [21]. It is classified as an alkaloid, and has a white crystalline appearance and bitter taste. This famous stimulant drug naturally acts as a pesticide that paralyzes and kills certain insects, larvae and beetles [22] that feed on plants. The identity of caffeine remained a mystery until the year 1820 when a German chemist, Friedlieb Runge, managed to isolate pure caffeine for the first time and called it "Kaffebase" meaning a base that exist in coffee. Previous studies have shown that caffeine at a concentration of 2.5 mg/mL retards the growth of *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* within a short time [23]. In addition, it is also reported that the concomitant use of caffeine with amoxicillin potentiates the antibacterial effect of amoxicillin against *Staphylococcus aureus* [24]. Hence, this work aimed to study the potential of caffeine as a potential anti-QS compound.

2. Experimental Section

2.1. Sample Preparation

Caffeine was obtained from Sigma (St. Louis, MO, USA). Caffeine samples were prepared fresh by dissolving caffeine powders in ultrapure water at 80 °C. The aliquots were then sterilized by filtration (using 0.25 μm filter pore size) and diluted to appropriate concentrations using sterilized ultrapure water. (+)-Catechin was purchased from Sigma and dissolved in 20% DMSO before sterilized by filtration (using 0.25 μm filter pore size) and further diluted to appropriate concentrations using sterilized ultrapure water.

2.2. Bacterial Strains and Culture Condition

Chromobacterium violaceum CV026 (Mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532 Hg^R, *cvil*::Tn*5* *xyl*E, Kan^R, plus spontaneous Str^R) was used in this study [25]. *C. violaceum* CV026 is an AHL biosensor that will produce a purple pigment in the presence of s short chain AHL. CV026 was cultured in Luria-Bertani (LB) broth (1% w/v peptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, per 100 mL distilled water) buffered with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) to pH 6.8. *C. violaceum* CV026 culture was incubated at 28 °C with shaking (220 rpm) while *Pseudomonas aeruginosa* PA01 was routinely cultured at 37 °C.

2.3. Antibacterial Assay

A paper disc diffusion assay to assess the antibacterial activity of caffeine was performed according to the Clinical and Laboratory Standard Method with certain modifications [26]. *C. violaceum* CV026 was incubated for 16–18 hours before the OD_{600nm} was adjusted to 0.1. 100 µL of the bacteria culture were then spread on the MHA plates and left to dry for 30 minutes. Paper disc (10 mm diameter) were placed on the plates and 20 µL of caffeine dissolved in ultrapure water at final concentration of 0.1, 0.2, 0.3 and 0.4 mg/mL were loaded on the discs. Ultrapure water, 20% DMSO and 20 µL of (+)-catechin dissolved in 20% DMSO at final concentration of 0.1, 0.2, 0.3 and 0.4 mg/mL were also included as negative controls. DMSO (100%) was used as positive control. The plates were incubated at 28 °C for 24 hours and observed for any growth and inhibition zones. This assay was repeated in three independent triplicates.

2.4. Screening of Caffeine for Anti-QS Properties

This assay was conducted as described by Krishnan *et al.* [19] with modifications. Briefly, *C. violaceum* CV026 lawn was prepared by adding 8 mL of overnight culture adjusted to OD_{600nm} of 1.2 into 40 mL of warm molten LB agar buffered with 50mM MOPS. *N*-hexanoylhomoserine lactone (C6-HSL) was also added into the agar mixture at the final concentration of 0.12 µg/mL. The agar was gently mixed and poured immediately into a Petri dish (150 mm diameter) and left to solidify for 2 hours. Wells (4 mm diameter) were made on the solidified agar plate. Caffeine with final concentration of 0.1, 0.2 and 0.3 mg/mL were loaded into each well (40 µL per well). (+)-catechin was used as positive control for QS inhibition while ultrapure water and DMSO served as negative controls. The plate was incubated for 18–24 hours at 28 °C to check for the violacein inhibition. Halo zone formation on the purple background suggested that caffeine exhibited anti-QS property. This assay was repeated in three independent triplicates.

2.5. Anti-QS Activity of Caffeine against C. Violaceum *CV026*

C. violaceum CV026 overnight culture grown in LB broth was adjusted to an OD_{600} of 0.01 followed by the addition of C6-HSL to a final concentration of 0.12 µg/mL. Then, 10 mL of the aliquots were transferred into 50-mL sterile plastic tubes followed by addition of caffeine solution to a final concentration of 0.1, 0.2, and 0.3 mg/mL, respectively. Ultrapure water was included as negative control. The mixture was vortex for 5 seconds and incubated for 16–18 hours at 28 °C with gentle

shaking. Bacterial cultured without addition of C6-HSL but treated with caffeine (same concentrations and treated as described above) was prepared. After 16–18 hours incubation, the tubes were then vortex for 30 seconds to re-suspend any pellicle of adherent cells. Then, 200 µL of bacterial culture with various treatments were then added into 96-well flat-bottomed microplate (SPL Life sciences) and violacein concentration was monitored at 585 nm. Experiments were done in triplicate. The optical density of the culture was read using an Infinite M200 luminometer (Tecan, Mannerdorf, Switzerland) at wavelength 600 nm.

2.6. Quantification of Violacein

The production of violacein was quantified as previously described [18]. In summary, tubes containing overnight culture and treatment were vortex for 30 seconds to re-suspend any pellicle of adherent cells after 16–18 hours incubation. Bacterial culture (1 mL) of each tube was centrifuged at 13,000 rpm for 10 minutes to precipitate the insoluble violacein. The cultures supernatant was discarded and 1 mL of 100% DMSO was added to the pellet. The solution was then vortex vigorously for 30 seconds to ensure that the violacein has completely solubilized and centrifuged at 13,000 rpm for 10 minutes to remove the cells. Then, 200 µL of the violacein-containing supernatants were then added into 96-well flat-bottomed microplate (SPL Life Sciences, Pocheon-Si, Korea) in triplicate. The absorbance was read using the Tecan Infinite M200 luminometer at a wavelength of 585 nm.

2.7. Screening of N-Hexanoyl Homoserine Lactone Degradation by Caffeine

This assay was performed as described by Chan *et al.* [27] with modifications. Briefly, caffeine was added at final concentration of 0.1, 0.2, and 0.3 mg/mL into 2 mL microcentrifuge tubes filled with 1 mL of LB broth with 50 mM MOPS and C6-HSL (0.12 µg/mL). The tubes were incubated overnight at 28 °C with shaking (220 rpm). After 16–18 hours incubation, extraction of C6-HSL from the mixture was conducted by adding equal volume of ethyl acetate into each tubes and vortexed vigorously. After which, the supernatant (containing ethyl acetate with C6-HSL) was transferred into new tubes and air-dried in a fume hood. After drying, 0.1 mL of phosphate buffered saline (PBS) (10 mM, pH 7.4) was added into each microcentrifuge tubes to rehydrate C6-HSL. For the detection of C6-HSL degradation, 10 µL of the mixture was spotted onto sterile paper discs placed on CV026 lawn and incubated overnight at 28 °C. Decreased violacein production (purple zone) indicated C6-HSL degradation. This assay was repeated in three independent triplicates.

2.8. Inhibition of AHLs Synthesis by Pseudomonas aeruginosa *PA01*

Briefly, 250 µL of caffeine at final concentration of 0.1 to 1.0 mg/mL were added into 15 mL of *P. aeruginosa* PA01 overnight culture (OD_{600nm} of 0.1) grown in LB broth buffered with 50 mM MOPS. The mixture was vortex gently before incubated for 16–18 hours at 37 °C (220 rpm). After 16–18 hours incubation, the tubes were vortex for 30 seconds to re-suspend any pellicle of adherent cells. Bacterial culture (200 µL) were added into each well of the 96-well flat-bottomed microplate (SPL Life Sciences) in triplicate. The absorbance of each well was read using the Tecan Infinite M200 luminometer at a wavelength of 600 nm. Extraction of the short chain AHLs from the remaining

overnight culture was conducted after that as explained in previous section. The extracted AHLs (20 μL) was spotted onto sterile paper discs placed on CV026 lawn and incubated overnight at 28 °C for 24 hours. Decreased violacein production (purple zone) indicated the inhibition of *Pseudomonas aeruginosa* PA01 QS signal. This assay was repeated in three independent triplicates.

2.9. Bacterial Growth

P. aeruginosa PA01 growth was studied using methods reported by Hayouni and colleagues [28] with modifications. Briefly, overnight cultures of *P. aeruginosa* PA01, were diluted to OD_{600nm} of 0.1. The overnight bacterial culture and appropriate concentration of caffeine were placed in a 96-well flat-bottomed microplate (SPL Life Sciences) at a final volume of 200 μL in each well. The optical density OD_{600nm} were determined every 30 minutes for 24 hours using the Tecan Infinite M200 luminometer. The growth of *P. aeruginosa* PA01 was determined by plotting the OD_{600nm} against time.

2.10. Inhibition of Pseudomonas aeruginosa *PA01 Swarming Motility*

To verify application of caffeine to inhibit QS-mediated virulence, we used swarming assay using *P. aeruginosa* PA01 as selected pathogen. Swarming agar was freshly prepared using the following composition: glucose (1% w/v), Bacto agar (0.5% w/v), Bacto peptone (0.5% w/v) and yeast extract (0.2% w/v) [29]. Then, 250 μL of caffeine at final concentration of 0.1 mg/mL, 0.2 mg/mL and 0.3 mg/mL were seeded into 14.75 mL of the molten swarming agar, mixed well gently and dispensed onto Petri dishes. Then, 2 μL of *P. aeruginosa* PA01 overnight culture (OD_{600nm} of 0.1) were inoculated at the centre of the agar and incubated for 16 hours at 37 °C. Reduction in *P. aeruginosa* PA01 swarming motility indicates anti-QS properties of caffeine. This assay was repeated in three independent triplicates.

2.11. Statistical Analysis

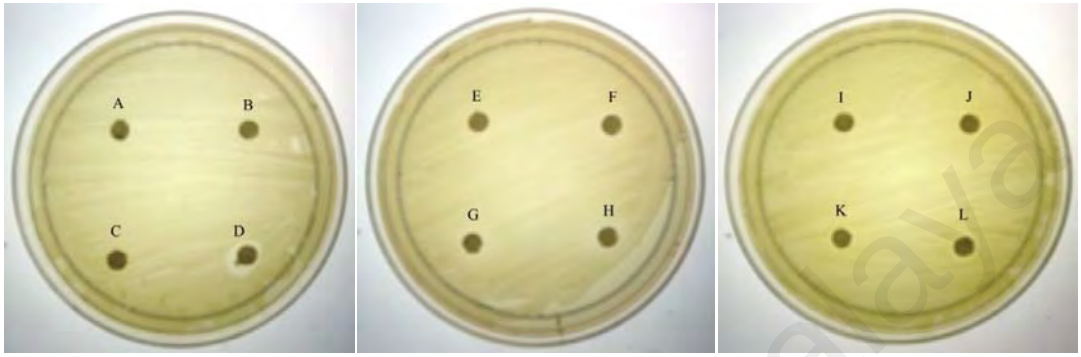
All statistical results in this study represent the average of three independent experiments. The data were analyzed using one-way ANOVA test at $P < 0.05$ using GraphPad Prism 5 statistical software.

3. Results and Discussion

3.1. Antibacterial Assay

As QS inhibition is focused on the interference of bacterial signaling and not antibacterial activity, it is important to ensure any anti-QS effect is not resulted from antibacterial activity. Therefore, paper disc diffusion assay was performed to test antibacterial activity of caffeine against *C. violaceum* CV026. Our result (Figure 1) shown that there was no inhibition zones observed suggesting all tested concentrations of caffeine showed no antibacterial activity. This indicates that caffeine at these concentrations does not inhibit the growth of *C. violaceum* CV026. Visible inhibition zone was only observed on the disc treated with 100% DMSO (positive control).

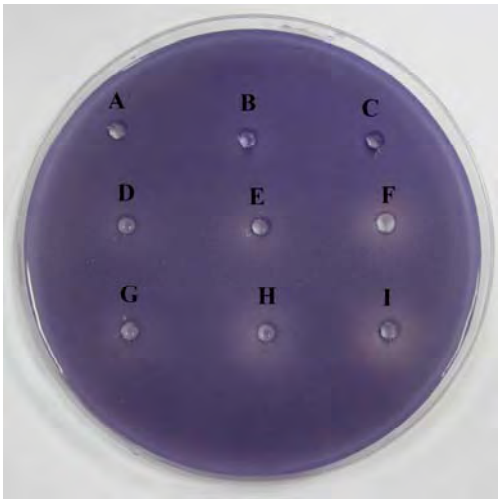
Figure 1. Antibacterial assay. (**A**) Disc without treatment; (**B**) 20% DMSO; (**C**) Ultrapure water; (**D**) 100% DMSO; (**E**) 0.1 mg/mL caffeine; (**F**) 0.2 mg/mL caffeine; (**G**) 0.3 mg/mL caffeine; (**H**) 0.4 mg/mL caffeine; (**I**) 0.1 mg/mL; (**J**) 0.2 mg/mL (+)-catechin; (**K**) 0.3 mg/mL (+)-catechin; (**L**) 0.4 mg/mL (+)-catechin. None of the tested caffeine concentrations showed growth inhibition zones except for paper disc treated with 100% DMSO (positive control). This assay was conducted in three independent triplicates.



3.2. Screening of Caffeine for Anti-QS Properties

The preliminary screening of anti-QS properties of caffeine was done by using *C. violaceum* CV026 as biosensor. Our results have shown that the increased amount of caffeine shown increased inhibition suggesting that higher the concentration of caffeine, the stronger inhibition of *C. violaceum* CV026 responding to C6-HSL (Figure 2).

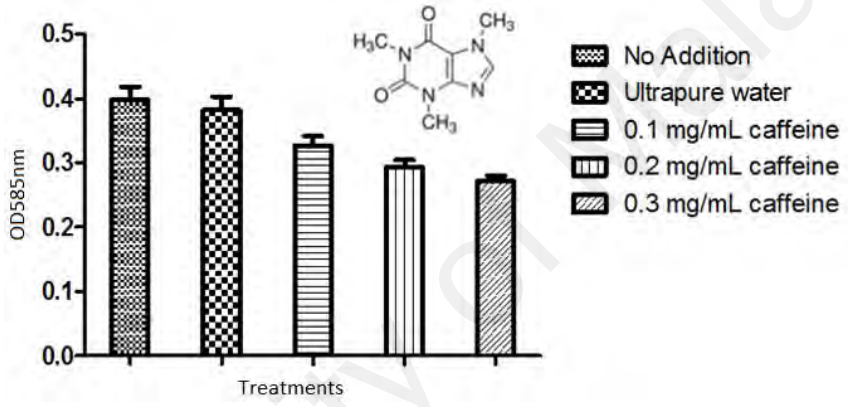
Figure 2. Anti-QS properties of caffeine. (**A**) untreated well; (**B**) 20% DMSO; (**C**) ultrapure water; (**D**) 0.1 mg/mL (+)-catechin; (**E**) 0. 2 mg/mL (+)-catechin; (**F**) 0.3 mg/mL (+)-catechin; (**G**) 0.1 mg/mL caffeine; (**H**) 0.2 mg/mL caffeine; (**I**) 0.3 mg/mL caffeine. Result showed that caffeine promoted QS inhibitory effect in concentration dependent manner. This assay was conducted in three independent triplicates.



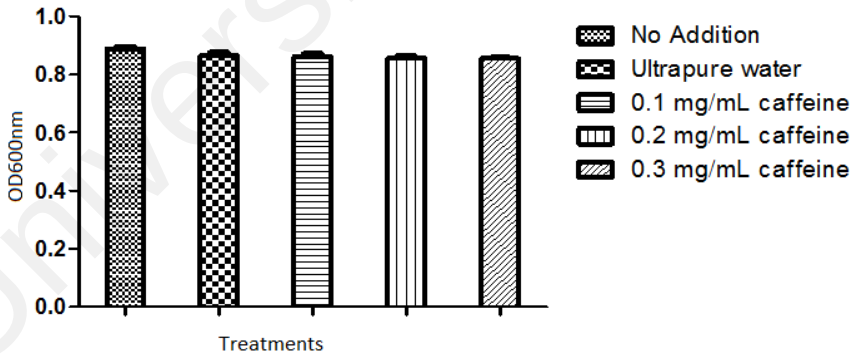
3.3. Caffeine Shown Anti-QS Properties but not Antibacterial Activity Using C. Violaceum *CV026 Bioassay*

The violacein produced by treated *C. violaceum* CV026 overnight culture was extracted as described in materials and methods. Ultrapure water and 20% DMSO served as negative controls. The result was shown in Figure 3. Statistical test was also conducted using the ANOVA test and it was found that all tested caffeine concentrations showed a significant inhibition of violacein content ($P < 0.05$).

Figure 3. Caffeine inhibits CV026 violacein production by anti-QS. The violacein production was measured spectrophotometrically as described in Materials and Methods and quantified by reading the OD values of the solution at (**a**) 585nm and (**b**) bacterial growth at 600 nm.The statistical significant of each test (n = 3) was evaluated by conducting one-way ANOVA test and a P value of $P < 0.05$ being significant. Inset: Structure of caffeine.



(**a**)



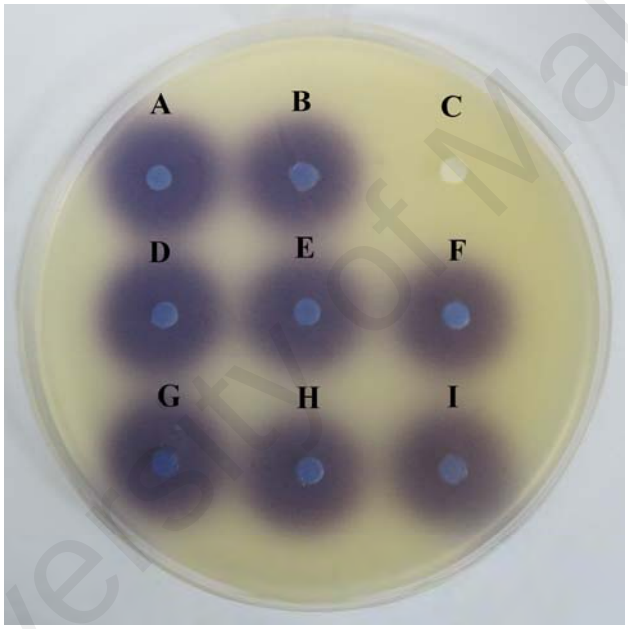
(**b**)

To confirm the anti-QS effect of caffeine was not due to any antibacterial activity, we monitored the bacterial growth at OD_{600nm}. No significant antibacterial activity was shown by caffeine ($P < 0.05$) (Figure 3(b)). The result confirmed that caffeine at concentrations of 0.1, 0.2, and 0.3 mg/mL did not inhibit the growth of *C. violaceum* CV026. This confirmed that the reduction of the violacein production is due to anti-QS effects and not anti-bacterial properties.

3.4. Caffeine did not Degrade C6-HSL

This assay was conducted to confirm the anti-QS property of caffeine were not due to the degradation of the C6-HSL. Figure 4 showed that caffeine and (+)-catechin at the tested concentrations did not degrade C6-HSL.

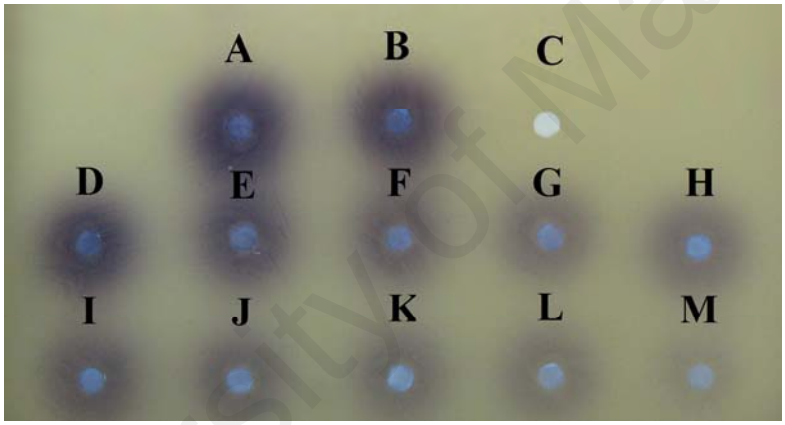
Figure 4. Caffeine did not degrade C6-HSL. Disc (**A**) C6-HSL treated with ultrapure water (negative control); (**B**) C6- HSL treated with 20% DMSO (negative control); (**C**) PBS; (**D**) C6-HSL treated with 0.1 mg/mL caffeine; (**E**) C6-HSL treated with 0.2 mg/mL caffeine; (**F**) C6-HSL treated with 0.3 mg/mL caffeine; (**G**) C6-HSL treated with 0.1 mg/mL (+)-catechin; (**H**) C6-HSL treated with 0.2 mg/mL (+)-catechin; (**I**) C6-HSL treated with 0.3 mg/mL (+)-catechin. The result showed that both caffeine and (+)-catechin have no effect on C6-HSL. This assay was conducted in three independent triplicates.



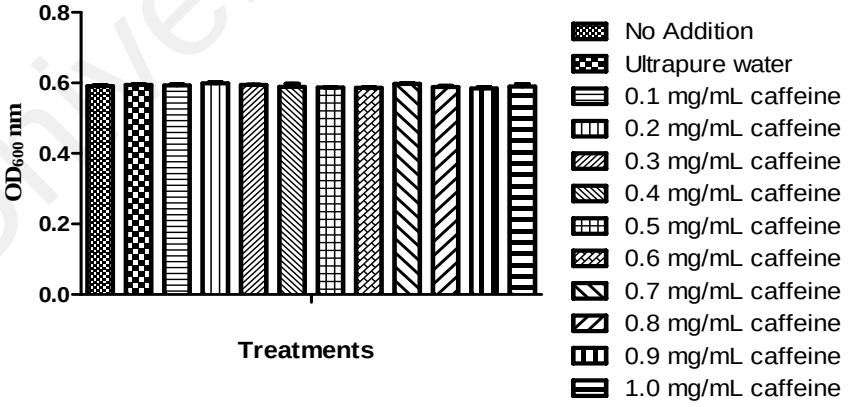
3.5. Caffeine Inhibited P. aeruginosa *PA01 Short Chain AHLs Production*

Our previous results have shown that caffeine did not degrade C6-HSL. We then further investigated the inhibition of *P. aeruginosa* PA01 AHL signal production by caffeine. It has been reported that *Pseudomonas* spp. triggered CV026 violacein production, suggesting production of short chain AHLs [30]. With this knowledge, a preliminary test was designated to test the inhibition of short chain AHLs produced by *P. aeruginosa* PA01 with various concentrations of caffeine ranging from 0.1 to 1.0 mg/mL. Our result shown a decreasing in the intensity of purple pigment (violacein) formed on the CV026 lawn is directly proportionate to the concentration of caffeine applied. When caffeine was applied at the concentration of 1.0 mg/mL, only trace amount of AHLs production by *P. aeruginosa* PA01 was observed (Figure 5(a)) and this is not due to antibacterial effects (Figure 5(b)).

Figure 5. (**a**) Caffeine inhibited *P. aeruginosa* PA01 QS signal production. Filter paper A and B were added with 20 µL AHL extracted from *P. aeruginosa* PA01 culture (A); *P. aeruginosa* PA01 culture treated with ultrapure water (B). In (C) only PBS buffer (20 µL) was spotted on the CV026 lawn. Filter paper (D); (E); (F); (G); (H); (I); (J); (K); (L); and (M) were added with 20 µL AHLs extracted from *P. aeruginosa* PA01 culture treated with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL caffeine, respectively. This assay was conducted in three independent triplicates and representative data are shown. This result shows that caffeine interfered AHLs production of *P. aeruginosa* PA01 in a concentration dependent manner as evident. (**b**) OD_{600nm} reading of *P. aeruginosa* PA01 overnight culture treated with various concentrations of caffeine. The statistical significant of each test (n = 3) was evaluated by conducting one-way ANOVA test and a P value of $P < 0.05$ being significant. Our result indicated no significant differences between the treatments and the control at $P < 0.05$ suggesting that caffeine at these concentrations did not exhibit any antibacterial activity in *P. aeruginosa* PA01.



(**a**)



(**b**)

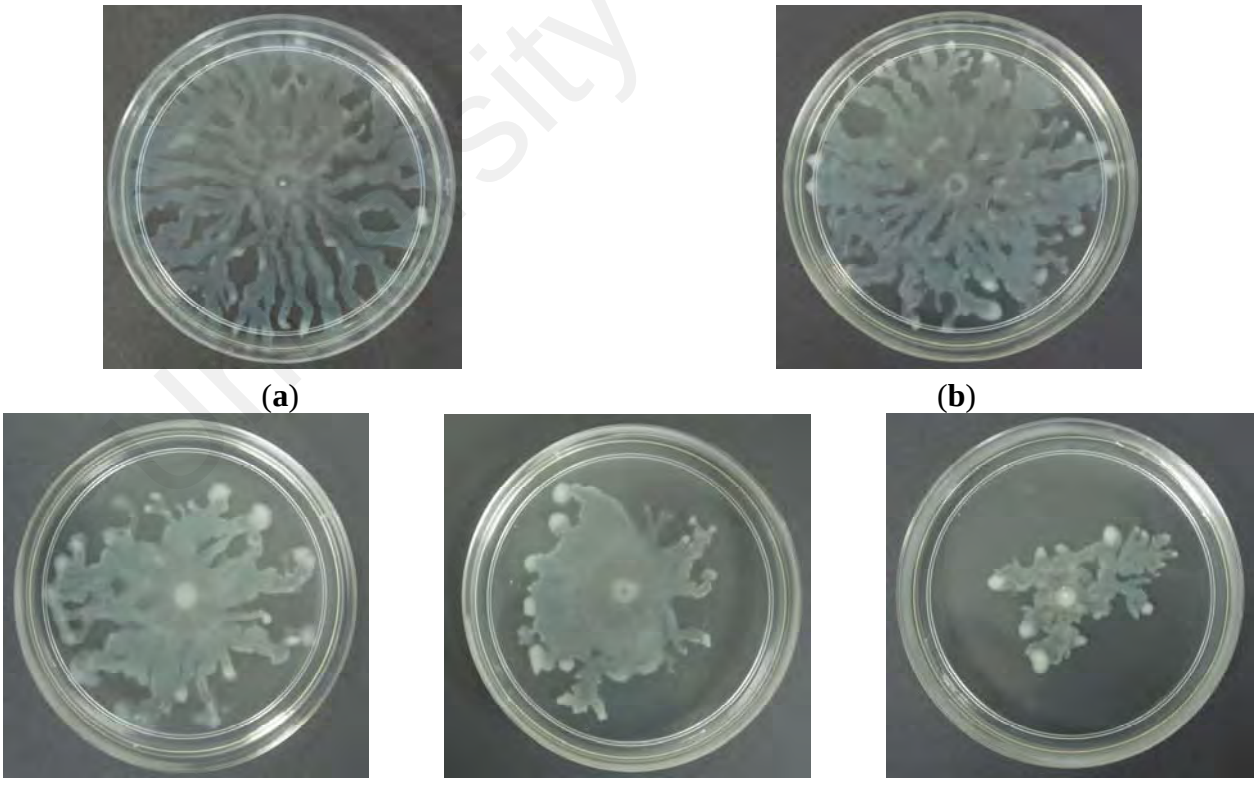
To ensure caffeine (at various concentrations) showed anti-QS instead of antibacterial activity, we monitored the growth of *P. aeruginosa* PA01 and the data was analyzed using one-way ANOVA test. Our result showed no significant differences between the treatments and the control ($P < 0.05$). This indicated that caffeine at concentrations ranging from 0.1 to 1.0 mg/mL did not inhibit the growth of *P. aeruginosa* PA01 hence confirmed that the reduction of the AHLs production is due to the

inhibition of *P. aeruginosa* PA01 short chain AHL production. Similar results were obtained when the growth of *P. aeruginosa* PA01 was further monitored for duration of 24 hours (data not shown).

3.6. Caffeine Inhibited Pseudomonas aeruginosa *PA01 Swarming Motility*

Swarming is a complex type motility that is defined by rapid and coordinate translocation of a bacterial population across a semi-solid surface [29]. A bacterial swarming motility was shown by the observation of long and hyperflagellated cells formed on the swarming plate. In addition to flagella and pili, swarming of *P. aeruginosa* also requires the production of two biosurfactants; rhamnolipids and 3-hydroxyalkonics acids [31]. Study conducted has found out that the production of rhamnolipids in *P. aeruginosa* PA01 is controlled by QS [32]. In this study we investigated the ability of caffeine to inhibit *P. aeruginosa* PA01 swarming activity. Caffeine was seeded into the swarming agar and ultra-pure water was used as negative control. Our results (Figure 6) indicated that caffeine at 0.3 mg/mL concentration showed observable inhibition against the swarming of *P. aeruginosa* PA01 with formation of bacterial colony of short and undefined tendrils.

Figure 6. Swarming inhibition assays. Swarming agars of *P. aeruginosa* PA01 (**a**); supplemented with ultrapure water (**b**); (v/v, negative control); and caffeine of 0.1 mg/mL (**c**); 0.2 mg/mL (**d**); and 0.3 mg/mL (**e**). Images shown are *P. aeruginosa* PA01 swarming patterns and inhibition effects after 16 hours of incubation at 37 °C. This assay was conducted in three independent triplicates.



(**a**) (**b**)

(**c**) (**d**) (**e**)

4. Conclusions

In summary, our results have demonstrated caffeine as a potential QS inhibitor. We also verified that caffeine inhibited swarming and AHL production of *P. aeruginosa* PA01. It is confirmed that caffeine did not degrade AHLs, but rather inhibited its production. At this point, few questions remained to be answered for instance, can caffeine inhibit the AHL synthesis by changing the structures of the AHL synthase, or competitive and/or non-competitive binding to the AHL synthase and/or AHL receptors by blocking AHLs from forming AHL-receptor complex? Further work will have to be done to address these issues.

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