ANTI-QUORUM SENSING PROPERTIES OF *MELICOPE LUNU-ANKENDA*, *PIPER BETLE*, *GNETUM GNEMON* AND *PIPER NIGRUM*

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2013

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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KUALA LUMPUR

2013

ABSTRACT

Quorum sensing (QS) is a communication tool of bacteria that is involved in the regulation of bacterial virulence determinants, therefore making it an interesting target to attenuate pathogens. In this study, the anti-QS activities of four Malaysia's edible, local plants, namely, *Melicopelunu-ankenda*, *Piper betle*, *Gnetumgnemon* and *Piper nigrum* were explored by testing the hexane, chloroform and methanol extracts of the plants on *Chromobacteriumviolaceum* CV026, *Escherichia coli* [pSB401], *E. coli* [pSB1075],*Pseudomonas aeruginosa* PA01 and *P. aeruginosa*PA01*lecA::lux.* Qualitative studies of violacein inhibition of *C. violaceum* CV026 showed that most of the extracts except for the methanol extract of *P. betle* and chloroform and methanol extracts of *G. gnemon*inhibited the violacein productionin the presence of QS signals. On the other hand, quantitation assay on the violacein produced by *C. violaceum* CV026 showed that *M. lunu-ankenda* chloroform and methanol extracts, all three extracts of *P. betle* and *P. nigrum* significantly inhibited the violacein synthesis. In the presence of QS signals, bioluminescence produced by *E. coli* [pSB401] was inhibited by all of the extracts while only *P. betle* hexane extract, *G. gnemon* chloroform and methanol extracts inhibited bioluminescence of *E. coli* [pSB1075]. All three extracts of *M. lunu-ankenda* and *P. betle* as well as chloroform extract of *G. gnemon* inhibited pyocyanin synthesis of *P. aeruginosa*PA01. *lecA* expression was significantly disrupted by the chloroform extracts of both *G. gnemon* and *P. betle* as well as the methanol extracts of *P. betle*. Swarming motility of *P. aeruginosa* PA01 was inhibited by the extracts of *M. lunu-ankenda* (chloroform) and *P. betle* (methanol). Fractionation of chloroform extract of *M. lunu-ankenda* leads to a total of 14 fractions. These 14 fractions were then tested on *E.*

coli [pSB401], *P. aeruginosa*PA01*lecA::lux* and swarming motility of *P. aeruginosa* PA01. None of the fractions exhibited inhibition against *lecA*expression. Fraction 4 and Fraction 11-14 showed inhibition against bioluminescence synthesis by *E. coli* [pSB401] while for swarming motility assay, only Fraction 5 and Fraction 11 showed observable inhibition. At this point of time, although the chemical nature of the anti-QS compounds from *M. lunu-ankenda*, *P. betle*,*G. gnemon* and *P. nigrum* are currently unknown, this study proves that local Malaysian plants could serve as leads in the search for anti-QS compounds.

ABSTRAK

Pengesanan kuorum (QS) merupakan satu alat komunikasi bakteria yang terlibat dalam pengaturan penentu kebisaan bakteria, dengan itu menjadikan QS sebagai satus asaran yang menarik untuk melemahkan pathogen tersebut.Dalam kajian ini, aktiviti-aktiviti anti-QS empat tumbuh-tumbuhan Malaysia yang boleh dimakan dan endemik, iaitu *Melicopelunu-ankenda*, *Piper betle*, *Gnetumgnemon* dan *Piper nigrum* telah diterokadengan menguji heksana, klorofom dan methanol ekstrak tumbuh-tumbuhan tersebut ke atas *Chromobacteriumviolaceum* CV026 , *Escherichia coli* [pSB401], *E. coli* [pSB1075], *Pseudomonas aeruginosa* PA01 dan*P. aeruginosa*PA01*lecA:: lux*. Kajian kualitatif perencatan violacein *C. violaceum* CV026 menunjukkan bahawa kebanyakan ekstrak kecuali ekstrak methanol *P. betle* sertaekstrak kloroform dan methanol *G. gnemon* menghalang pengeluaran violacein itu.Selain itu, esei pengkuantitian violacein yang dihasilkan oleh *C. violaceum* CV026 menunjukkan bahawa ekstrak kloroform dan methanol *M. lunu-ankenda*, ketiga-tigaekstrak *P. betle* dan *P. nigrum* Berjaya menghalang pengeluaran violacein dengan ketara. Bioluminasi yang dihasilkan oleh *E. coli* [pSB401] telah dihalang pengeluarannya oleh semua ekstrak manakala hanya heksanaekstrak *P. betle*, ekstrak kloroform dan methanol *G. gnemon*berjayamenghalangpengeluaranbioluminasioleh*E. coli* [pSB1075]. Ketiga-tigaekstrak*M. lunu-ankenda*dan*P. betle* serta ekstrak kloroform *G. gnemon*dapatmenghalangsintesispyocyanin*P. aeruginosa* PA01. Pengeluaran *leçA* nyata diganggu oleh ekstrak kloroform kedua-dua*G. gnemon* dan *P. betle* serta ekstrak methanol *P. betle*. Motiliti berkerumun *P. aeruginosa* PA01 telah dihalang oleh ekstrak*M. lunu-ankenda* (klorofom) dan *P. betle* (methanol).Pemecahan klorofom

ekstrak *M. lunu-ankenda* membawa kepada sejumlah 14 pecahan. Pecahan sebanyak 14 ini kemudiannya diuji pada *E. coli* [pSB401], *P. aeruginosa* PA01*lecA::lux* dan motility berkerumun *P. aeruginosa* PA01.Tiada pecahan yang mempamerkan sebarang perencatan terhadap ungkapan *lecA*. Pecahan 4 dan Pecahan 11-14 menunjukkan perencatan terhadap sintesis bioluminasi oleh *E.coli* [pSB401] manakala bagi esei motility berkerumun, hanyaPecahan 5 danPecahan 11 menunjukkan perencatan yang ketara. Pada masa ini, walaupun sifat kimiasebatian anti-QS dari *M. lunu-ankenda*, *P. betle*, *G. gnemon* dan*P. nigrum* kini tidak diketahui, kajian ini membuktikan bahawa tumbuh-tumbuhan tempatan Malaysia boleh dijadikan sebagai sumber pencarian untuk sebatian anti-QS.

ACKNOWLEDGEMENTS

First of all, I would like to offer my sincerest gratitude to my supervisor, Dr. Chan KokGan who has supported me throughout my MSc with his patience and guidance. His great ideas, mentorship and encouragement have enabled me to complete my project within the time frame given and have publications. Secondly, a million thanks to my labmates, Thiba, Teik Min, XinYue, KarWai, Yee Meng, See-Too, JianWoon and Joanita for their opinions, suggestions, friendliness, knowledge and guidance whenever I needed a helping hand. I would like to acknowledge with much appreciation to Ms. Yin Wai Fong for always assisting me with the administration paper work.

I would also like to thank YayasanKhazanah for providing me with the scholarship that enabled me to pursue my MSc. To Amy Kok, VenessaTye, Phan Li Ting, Tan Wan Xin and Adrian Leong, thank you for being my friends of more than a decade. Special thanks to my family members for always being there for me with encouragement and love always. Without the support from my parents (Mr. Tan PengSeng and Madam Yio Kim Lian) and my siblings (Tan I Yeing, Tan Chun Sia, Tan Khuey Chen, Tan Chia Huey, Tan Chia Li and Tan Kwok Wei), I would not be where I am today.

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LIST OF ABBREVIATIONS

QS	Quorum Sensing
DNA	Deoxyribonucleic acid
AHL	*N*-acyl-homoserine lactone
C4-HSL	*N*-butanoylhomoserine lactone
C8-HSL	*N*-octanoylhomoserine lactone
C6-HSL	*N*-hexanoylhomoserine lactone
3-OC6-HSL	*N*-3-oxohexanoyl-L-homoserine lactone
3-OC12-HSL	*N*-3-oxododecanoyl-L-homoserine lactone
%	Percentage
PQS	*Pseudomonas* quinolone signal
µM	Micromolar
HAA	3-(3-hydroxyalkanoyloxy)alkanoic acid
cm	Centimetre
mm	Millimetre
m	Metre
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
˚C	Degree Celsius
psi	Pounds per square inch
min	Minute
h	Hour
µm	Micrometre
w/v	Weight per volume

ACN	Acetonitrile
mg	Milligram
ml	Millilitre
µg	Microgram
µl	Microlitre
nm	Nanometre
v/v	Volume per volume
DMSO	Dimethyl Sulfoxide
RLU	Relative Light Unit

CHAPTER 1

INTRODUCTION

Quorum sensing (QS) is a system used by the bacteria to control the gene expression in response to cell density. QS is involved in many regulation of physiological functions such as bioluminescence, biofilm formation, sporulation, exchange of DNA and secretion of virulence factors, some may which contributes to bacterial pathogenesis (Henke & Bassler, 2004). Usage of antibiotics to counter bacterial infections has led to a rise in antibiotic-resistant pathogenic bacteria and this phenomenon pose as a global threat for public health management. As such, an alternative bacterial treatment that does not require inhibition or killing of bacterial growth is highly desirable as the evolutionary pressure to develop resistance towards these kinds of alternative treatment is much lower. One of the available alternative treatments is by using anti-QS molecules, which can inhibit the virulence factors secreted by the infectious bacteria (Chong *et al*., 2011; Krishnan*et al*., 2012; Tan*et al*., 2012).

Malaysia has large flora diversity and four plants have been chosen to be tested for their anti-QS activities. *Melicopelunu-ankenda* (locally known as “tenggekburung”), *Piper betle* (locally known as “sireh”), *Gnetumgnemon* (locally known as “melinjau”) and *Piper nigrum* (locally known as “lada”) will be extracted with hexane, chloroform and methanol in order to obtain crude extracts with compounds of different polarity. These crude extracts will be tested on anti-QS assays involving bioluminescence, violacein, pyocyanin, swarming and *lecA* expression. Fractionation of the active crude

extract will be carried out using Agilent Technologies 1260 Infinity Series HPLC system (Agilent Technologies, Germany). Then, fractions shall be tested on some anti-QS bioassays to determine which fraction contains the active compounds that have anti-QS abilities. The anti-QS compounds from these four Malaysia local plants may be a new class of non-bacterial origin antagonist and more of the local plants should be screened for anti-QS activities.

The objectives of this study are as follows:

1. To screen and identify plant samples that show anti-QS activities.
2. To attenuate QS-mediated virulence of *P. aeruginosa* PA01.
3. To fractionate the plant extract that gives significant anti-QS results and identify which fraction that has the active compounds.

CHAPTER 2

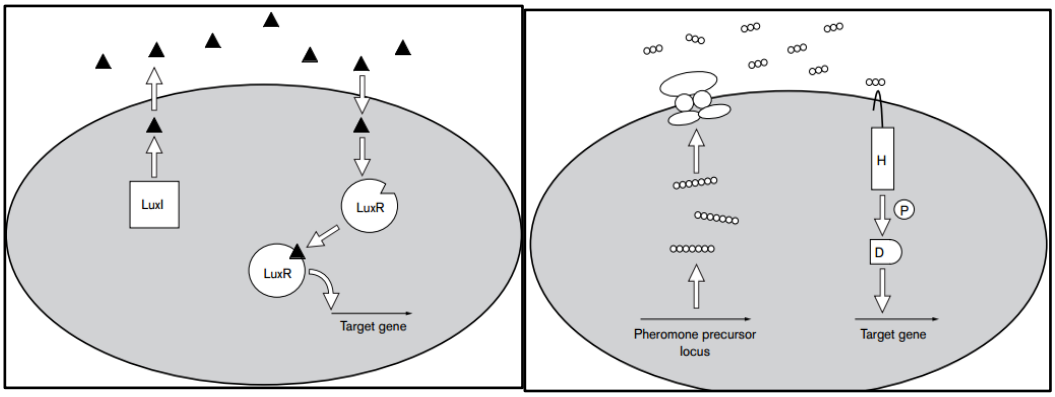
LITERATURE REVIEW

2.1 Quorum Sensing

Bacteria are constantly exposed to multiple environmental challenges such as fluctuation in nutrient availability, temperature, pH, osmolarity and other forms of stimuli. Eventually, bacteria develop complex systems that enable them to achieve homeostasis in these harsh environments. QSis a cell-to-cell communication tool used by the bacteria to produce extracellular signaling molecules which enables it to monitor its population density. As the population density increases, the amount of extracellular signaling molecules in the environment increases as well. When the concentration of these signaling molecules reaches a certain threshold level, the bacteria specific target gene will be activated (Henke & Bassler, 2004; Miller & Bassler, 2001; Waters & Bassler, 2005).

The genes that are activated as a result of QS leads to the expression of physiological processes such as bioluminescence, swarming, swimming, twitching, biofilm formation, production of antibiotics, plasmid conjugal transfer, pyocyanin and elastase production (Whitehead*et al*., 2001; Daniels*et al*., 2004; Parsek & Greenberg, 2005; Antunes*et al*., 2010). QS was first discovered from *Vibrio fischeri*, a bioluminescent marine bacteria. *V. fischeri* colonizes the light organ of *Euprymna scolopes* and when the density of the bacteria is high, QS induces genes expression required for luminescence.Gram-negative and gram-positive bacteria use different kind signaling molecules where the former utilizes *N*-acyl-homoserine lactones (AHLs)

which are from a group of fatty acid derivatives while the latter uses post-translationally processed peptides for QS (Figure 2.1). In Gram-negative bacteria, the AHLs in the environment will diffuse freely into the cell and then binds to specific receptor which acts as a transcriptional activator. The binding will then cause an allosteric regulation of proteins to happen, that will lead to activation of genes including those responsible for AHLs synthesis. As for the post-translationally processed peptides used by the Gram-positive bacteria, these peptides do not diffuse into the cell, but they will bind to the specific receptors that can be found on the membrane. This will then cause a cascade activation of signal transduction pathway that leads to change in genes regulations. The *agr* gene of the staphylococcal bacteria is an example QS system which exists in Gram-positive bacteria (Novick & Geisinger, 2008; Waters & Bassler, 2005).



(a) (b)

Figure 2.1: The general QS system in (a) Gram-negative bacteria and (b) Gram-positive bacteria (Bassler, 1999).

2.2 AHL Biosensors

AHL biosensors such as *C. violaceum* CV026,*Escherichia coli* [pSB401] and *E. coli*[pSB1075] relies on exogenous AHLs for QS to occur as these bacteria lack of the synthase gene requires for AHLs synthesis. *C. violaceum* CV026 is a gram negative bacterium that produces violacein, a purple pigmented compound in the presence of short chain AHLs ranging from *N*-butanoyl homoserine lactone (C4-HSL) to *N*-octanoyl homoserine lactone (C8-HSL) with different level of sensitivity. It was found that *C. violaceum* CV026 responds best to *N*-hexanoyl homoserine lactone (C6-HSL) (McClean *et al*., 1997). *E. coli* [pSB401] and *E. coli* [pSB1075] produces luminescence in the presence of *N*-3-oxohexanoyl-L-homoserine lactone (3-OC6-HSL)and *N*-3-oxododecanoyl-L-homoserine lactone (3-OC12-HSL) respectively(Winson *et al*., 1998).

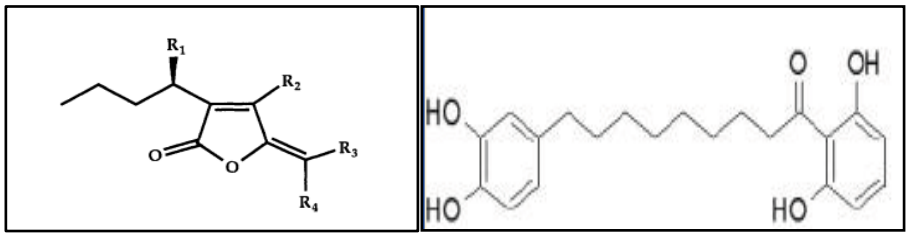
These biosensors are used to determine whether the plant extracts or compounds possess anti-QS properties. In the study conducted by Zhu and Sun, violacein formation by *C. violaceum* CV026 was quantified after incubation with *Tremella fuciformis* extract and it was found that the violacein formed was significantly reduced. Thus, it was concluded in this study that compounds extracted from *T. fuciformis* could inhibit a QS-regulated behavior in *C. violaceum*CV026(Zhu & Sun, 2008).

2.3 Anti- Quorum Sensing

In an environment where resources are limited and bacteria have to compete for these resources, the bacteria which have the capability to disrupt QS may have higher chances for survival over other species that relies on QS (Waters & Bassler, 2005). Disruption of QS or anti-QS can be achieved via a few ways. Firstly, competitive inhibition between signaling molecules for the receptor as well as degradation of signaling molecules by enzymes can cause anti-QS to happen. For example, enzymes involved in AHLs degradation include AHLs lactonase and acylase (Kalia*et al*., 2011). Besides that, by targeting the enzymes responsible for the production of these signaling molecules, bacterial QS can be inhibited. This involves inhibition of the enzymes responsible for the production of acyl chain, *S*-adenosylmethionine synthase and LuxI homolog proteins. AHLs are derivatives of *S*-adenosylmethionine. LuxI-based enzymes bind the acyl group from a specific acylated acyl carrier protein to the methionine on *S*-adenosylmethionine. Lactonization of the intermediate results in the synthesis of AHLs (Hong*et al*., 2012; Parveen & Cornell, 2011; Bassler, 2002). Besides that, blocking AHLs from forming AHL-receptor complex can also prevent QS from happening (Koch *et al*., 2005).

Since interference with QS showed much potential in controlling undesirable microbial activity and many opportunistic pathogenic bacteria rely on QS to regulate their virulence expression, various works has been carried out to identify compounds that have anti-QS activity. Compounds which can inhibit QS are known as QS inhibitors. QS inhibitors do no kill or inhibit bacterial growth and thus reduced the evolutionary pressure of the bacteria to develop resistance (Issac *et al*., 2011).

The first QS inhibitor compound, halogenated furanones (Figure 2.2 (a))was isolated from *Delisea pulchra*, a marine red alga which can be found in Australia. These furanones have structures which are similar to AHLs and has been shown to be able to inhibit QS expression through the displacement of AHLs from the receptor (Manefield *et al*., 1999). By displacing the AHLs from its receptor competitively, *D. pulchra* is able to promote the rate of proteolytic degradation without inhibiting the bacterial growth(Manefield *et al*., 2002). Isolated furanones interferes with *Serratia liquefaciens* swarming activity while synthetic derivatives of the halogenated furanones has been shown to interfere with biofilm production in *P. aeruginosa*(Hentzer *et al*., 2002; Rasmussen *et al*., 2000).Plants derived extracts and compounds have been extensively tested for anti-QS due to their traditional pharmacological values. Besides, active compounds with anti-QS activities derived from plants are deem safe as these plants are normally eaten by humans. Malabaricone C (Figure 2.2 (b)) is a pure compound isolated from the bark of nutmeg tree, *Myristica cinnamonea* that has anti-QS activity. Malabaricone C inhibited the production of the purple pigment, violacein by *Chromobacterium violaceum* CV026 in the presence of QS signal, pyocyanin and biofilm synthesis in *P. aeruginosa* PA01 (Chong *et al*., 2011)



(a) (b)

Figure 2.2: Structure of (a) halogenated furanones with structural variations and (b) malabaricone C (Adapted from Manefield *et al*., 1999; Chong *et al*., 2011).

2.4 *Pseudomonas aeruginosa*

P. aeruginosa is the most common Gram-negative bacterium that has been linked to cause nosocomial infections, particularly in immunocompromised patients. *P. aeruginosa*has been reported to be responsible for nosocomial pneumonia cases,hospital-acquired urinary tract infections, surgical wound infections and bloodstream infections (Micek *et al*., 2005; Mikuniya *et al*., 2007; Sadikot *et al*., 2005).

Neutropenic cancer and bone marrow transplant patients are more vulnerable to be exposed to *P. aeruginosa* infections. At the same time, *P. aeruginosa*is also one of the most typical and lethal pathogens responsible for ventilator-associated pneumonia intubated patients, with direct attributed death rates reaching 38% (Dunn & Wunderink, 1995; Hauser *et al*., 2002; Parkins*et al*., 2010; Paterson, 2006). The ability of *P. aeruginosa* to express these diverse and severe infections is due to a wide range of virulence factors. Collectively, these virulence factors are causing extended tissue damages in not only humans, but also other mammals (Van Delden & Iglewski, 1998).

P. aeruginosa cell surface virulence factors such as flagella, pili, lipopolysaccharide and alginate are responsible for movement, progression, adhesion and colonization towards surface contact with other bacteria (Kipnis*et al*., 2006). Found on the cell surface of *P. aeruginosa*, the flagella are made of protein complex body which forms a filamentous polar appendage. These filamentous polar appendages assist *P. aeruginosa* motility through a propeller or screw-like motion while swimming (Feldman *et al.*, 1998; Toutain*et al*., 2007). The flagella not only function in locomotion of *P. aeruginosa* as they also participate in pathogenesis and cause inflammation which leads to interleukin-8 production (Adamo*et al*., 2004; DiMango*et al*., 1995).

Twitching motility of *P. aeruginosa* is due to the retractile properties of pili or frimbriae. Airways can be colonized quickly through this characteristic and asialoGM1 plays an important role in pili during adhesion phase (Burrows, 2012; Gupta*et al*., 1994). Therapeutic drugs have been targeting flagella and pili of *P. aeruginosa* as these characteristics seem to be playing a crucial role in the bacterial virulence (Döring & Pier, 2008). One of the components in lipopolysaccharide is known as Lipid A, whereby Lipid A can cause activation of many pro-inflammatory systems (Kipnis *et al*., 2006).

In the year 2000, the completion of the *P. aeruginosa*PA01 genome sequence gave a global view into the basis for its flexibility and resilience towards antibiotics resistance. The large genome of *P. aeruginosa*PA01 contains a comparatively large number of genes that are thought to encode regulators of gene expression(Stover *et al*., 2000). Regulation of the genes that are encoding the virulent factors is heavily dependent on the signaling system that consists of at least two categories of LuxR-LuxI homologues.

P. aeruginosa consists of *las*(LasI and LasR) and *rhl* (RhlI and RhlR) QS system. The cell-to-cell signaling system described in *P. aeruginosa* was firstly shown in regulation of LasB elastase and thus, named the *las* system (Passador*et al*., 1993). LasI (signal synthase) of *P. aeruginosa*is responsible for the synthesis of 3-OC12-HSL and LasR is the corresponding transcriptional activator (Schuster &Greenberg, 2006). When 3-OC12-HSL binds to the signal receptor, LasR, transcription of certain specific genes is activated. This activation has been shown to require 3-OC12-HSL-dependent multimerization of LasR (Kiratisin*et al*., 2002).

The *las*system coordinates the virulence expression of LasA elastase, LasB elastase, endotoxin A and alkaline protease in *P. aeruginosa*(Williams & Cámara, 2009).The expression of LasI is very sensitive to concentration of LasR-3-OC12-HSL complex. An increment in LasI synthesis will eventually lead to a rapid increase in 3-OC12-HSL expression and subsequently, increase the formation of LasR-3-OC12-HSL activator complex. This autoregulatory loop showed that the activation of *las*-dependent virulence factor expression is correlated closely with 3-OC12-HSL availability in the surrounding environment (Pesci*et al*., 1997; Seed*et al*., 1995; Venturi, 2005).This autoinduction hierarchy of *las* system has been shown to activate the *xcpP* and *xcpR* genes that encode proteins of the *P. aeruginosa* secretory pathway. These genes are involved in the Type II secretion system to discharge extracellular proteins in *P. aeruginosa* surrounding environment. There are six types of secretion systems that have been described in Gram-negative bacteria and of the six secretion system*P. aeruginosa* displays five of those systems (Bleves *et al*., 2010; Chapon-Hervé *et al*., 1997).

Another signaling system in *P. aeruginosa* is known as the *rhl* system due to its ability to control the production of rhamnolipid. The *rhl* system consists of the signal synthase RhlI and the signal receptor RhlR. RhlI produces C4-HSL, which will bind to RhlR, causing the activation of certain gene expression (Kievit*et al*., 2006; Pesci *et al*., 1997).It was found that RhlR requires C4-HSL for transcriptional activation but not necessary for dimerization(Ventre *et al*., 2003). C4-HSL interacts with RhlR in order to activate the expression of *rhl*AB, which is the operon encoding a rhamnosyltransferase required for the production of rhamnolipid biosurfactant. When the rhamnolipid biosurfactant is present, it enables *P. aeruginosa* cells to swarm across semi-solid

surfaces (Déziel*et al*., 2003; Ochsner & Reiser, 1995; Soberon-Chavez*et al*., 2005). Functions controlled by *rhl* QS systems include expression of alkaline protease, pyocyanin, hydrogen cyanide, lectins and elastase (Juhas*et al*., 2005).

The *las* and *rhl* systems had been shown to be involved in the expression of alkaline protease and elastase and subsequent studies were conducted to define the relationship between the two systems. The two QS systems has been shown to be coordinating each other in a hierarchical way whereby the LasI-LasR system positively controlling the activity of RhlI-RhlR system (Rasmussen & Givskov, 2006).

In 1999, Pesci and his collegues found another type of *P. aeruginosa* signaling molecules known as 2-heptyl-3-hydroxy-4-quinolone or *Pseudomonas* quinolone signal (PQS). This molecule was found to be involved in the gene expression of *lasB* which encodes for LasB elastase (Pesci *et al*., 1999).PQS has a high affinity for lipid-rich membranes of bacterial cells as it is a hydrophobic molecule with low solubility in water(Lépine*et al*., 2003). As PQS synthesis and expression is mediated by *las* and *rhl* systems respectively, these three systems should be inter-related to one another. Expression of *lasB* was further amplified by the combined effect of C4-HSL and PQS as compared to with either C4-HSL or PQS alone (Diggle*et al*., 2006).

Pyocyanin is a QS-regulated trait and it is a blue redox-active secondary metabolite produced by *P. aeruginosa*. It can be found in large quantities in the sputum of cystic fibrosis patients that had been infected by *P. aeruginosa.* Pyocyanin not only affects regulation of ion transport, frequency of ciliary beats and secretion of mucus in airway epithelial cells by altering the cytosolic concentration of calcium but also

interferes with gene expression and innate immune mechanism (Rada & Leto, 2013). Pyocyanin production involves a series of complicated steps which are assisted by the gene products encoded by two*phzABCDEFG* operons and by the *phzH*(encodes putative phenazine-specific methytransferase),*phzM*(encodes flavin-containing monooxygenase) and *phzS* genes, which modifies the precursors into tricyclic compounds (Mavrodi *et al*., 2001).

The final steps in pyocyanin synthesis are catalyzed by *phzM* and *phzS* operons. Pyocyanin production is positively regulated by the LysR-like transcriptional activator MvfR (PqsR) through the synthesis of PQS molecules, with additional regulatory signals from the LasR-LasI, RhlR-RhlR, GacA-GacS and Vfr regulatory system. The LysR transcription factor, MvfR regulates the production of quinolones and in return, the PQS positively regulates the expression of *rhl*system(Cao *et al*., 2001; Gallagher*et al*., 2002; McKnight*et al*., 2000).

It has been a difficult to discern the role of pyocyanin during lung infection as *P. aeruginosa* infection is multifactorial and genes involved have pleiotropic characteristics. To overcome this problem, many studies used *in vitro* cell culture systems with purified pyocyanin to determine the toxicity level of pyocyanin. Through these studies, it was shown that an extensive range of cellular damage was caused by pyocyanin and this maybe the causal factor that contributes to the persistency of *P. aeruginosa* in the lungs of cyctic fibrosis patients. Purified pyocyanin can be obtained through repeated chloroform-distilled water extraction cycles from the stationary phase of *P. aeruginosa* cultures.Absorption spectrum of pyocyanin is pH-dependent and at low

pH values, extracted pyocyanin is red while in high pH values, they are blue (Lau *et al*., 2004; Rada & Leto, 2013).

In A549 human alveolar epithelial cell line and human bronchial epithelial cells, it was shown that pyocyanin inactivates the activity of catalase but have no effect against manganese superoxide dismutase or copper-zinc superoxide dismutase. Pyocyanin not only inhibits catalase functions in cell-free system, but it also lowers the transcription of the genes encoding catalase (O'Malley *et al*., 2003).A certain concentration of pyocyanin recovered from the sputum of cystic fibrosis patients has been shown to induce apoptosis of neutrophils. Results showed that the rate of apoptosis activation is concentration and time dependent whereby within five hours of exposure to 50 µM of pyocyanin, the apoptosis induction increased 10 fold. Since the apoptosis caused by similar concentration of pyocyanin only affects neutrophils and not monocyte-derived macrophage or airway epithelial cells, it was concluded that these form of apoptosis induction are cell-type dependent and this discovery was found to be interesting(Usher *et al*., 2002). On top of that, an *in vivo* study which compares the neutrophils clearance in mice affected with wild-type and pyocyanin-deficient strains of *P. aeruginosa* showed that mice affected with wild-type strains has a much higher percentage of neutrophils apoptosis. *P. aeruginosa*, through pyocyanin production, significantly reduced the acute inflammatory response by accelerating apoptosis of neutrophils. By doing so, *P. aeruginosa* has a higher chance of survival (Allen *et al*., 2005).

Swarming is a one of the possible QS-regulated phenotypes in *P. aeruginosa*. Swarming consists of a flagella-driven movement of differentiated swarmer cells (hyperflagellated, elongated, multinucleated) which enables bacteria to span over a semisolid surface as biofilms (Daniels *et al*., 2004). QS regulation of swarming motility allows the optimal dissemination of bacterial cells when a population is getting too large to inhabit a single niche or when the nutrients in the environment no longer suits their needs (Daniels *et al*., 2004; Déziel *et al*., 2003).

Swarming of *P. aeruginosa* is induced over a semisolid surface (0.5-0.7% agar). It was found that cells that are isolated from the swarm edge and the center possess two polar flagella (Kohler*et al*., 2000). Swarming is dependent on flagella and type IV pili whereby these two components facilitate the swarming motion (Overhage*et al*., 2008). In swarming motility of *P. aeruginosa*, rhamnolipid function as wetting agents by reducing the surface tension.Rhamnolipid is rhamnose-containing glycolipid biosurfactant and it has a detergent-like structure (Soberon-Chavez *et al*., 2005).The loss of lung biosurfactant may be the cause of atelectasis that has been associated with chronic and acute *P. aeruginosa* infection. In addition, rhamnolipid was also found to be inhibiting the mucociliary transport as well as the ciliary function of human respiratory epithelium (Abdel-Mawgoud*et al*., 2010; Read *et al*., 1992).

In general, the two more abundant rhamnolipids are rhamnosyl-beta-hydoxy-decanoyl-beta-hyroxydecanoate (a mono-rhamnolipid) and rhamnosyl-rhamnosyl-beta-hydroxydecanoyl-beta-hydroxydecanoate as revealed by mass spectrometry (Soberon-Chavez *et al*., 2005). The *rhlAB* operon, an *rhlIR*-mediated target gene, catalyzes the synthesis of mono-rhamnolipid (L-rhamnosyl-beta-hydoxydecanoyl-beta-

hyroxydecanoate) from dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) moieties from various lengths (Ochsner & Reiser, 1995). HAAs are the rhamnolipid anabolic precursor in rhamnolipid biosynthesis and they display evident surface-active characteristics. It was found that *rhlA* is required for the production of HAA. The same study also observed that swarming requires the expression of *rhlA* but does not necessitate rhamnolipid production because HAA alone can act as surfactants(Déziel *et al*., 2003).

Besides needing rhamnolipid biosurfactant for swaming motility, *P. aeruginosa* also need FlhF, a signal recognition particle-like protein. FlhF is required for the transcription of certain class of genes in flagella and thus absence of FlhF will cause disruption in the movement (Murray & Kazmierczak, 2006). To add on, type IV pili assists the flagella in surface propagation and it could be involved in the sensing of viscosity of the surface and sensing a signal for initiation of swarming. In the absence of flagella and type IV pili, *P. aeruginosa* moves in sliding motion instead of swarming motility (Köhler*et al*., 2000; Murray & Kazmierczak, 2008).

Biofilms are made of colonies of microorganisms embedded in a matrix of extracellular polymeric substances, in which they accumulate rapidly in watery favorable physiological conditions surfaces (Kuehn *et al*., 1998).*P. aeruginosa*forms biofilm for defense purposes (protect itself from hostile environment in the host), colonization (isolation to a nutrient-rich area) and utilization of cooperative advantages through the community of bacteria. Biofilms are able to resist the shear forces produced by blood flow and the washing action of saliva. On the other hand, the bacteria kept within the biofilms can withstand deprivation of nutrients, changes of pH, oxygen

radicals, disinfectants and antibiotics better than planktonic organisms. The important trait that further strengthens biofilm resistance is the sticky matrix which may contain DNA and other polymers, but in general, it is predominantly composed of exopolysaccharides (Jefferson, 2004). The development of adhesins and motility factors are suppressed once biofilms formation has been established. This suggests that the prominent role of adhesins, pili and flagella is for initial attachment, but once the development of the biofilm has surpass this stage, the proteins are no longer needed and their expressions are inhibited (Lejeune, 2003).

Exotoxin A, which is produced by most *P. aeruginosa*strains can cause clinical infections. The exotoxin A catalyzes adenosine diphosphate-ribosylation and inactivation of elongation factor 2, which could lead to inhibition of protein biosynthesis and cell death. Exotoxin A that has been purified was found to be highly lethal for mice and this supports the major role of exotoxin A as the systemic virulence factor of *P. aeruginosa*(Van Delden & Iglewski, 1998).

During *P. aeruginosa* infection, proteases play a major role and the type of proteases that are produced by *P. aeruginosa* includes LasB elastase, LasA elastase and alkaline protease(Van Delden & Iglewski, 1998). *P. aeruginosa* ability to destroy protein elastin during acute infection is a major virulence determinant. Elastin makes up a major part of human lung tissue and it is responsible for lung expansion and contraction. Consequently, the blood vessel relies on elastin due to its resilience, thus making elastin an important component in blood vessels. LasB elastase and LasA elastase synergistically causes elastolytic activity (Galloway, 2006).

LasB elastase is a zinc metalloprotease that acts on a number of proteins including elastin and it is highly efficient. LasB has a proteolytic activity of approximately 10 times that of *P. aeruginosa* alkaline protease and an activity toward casein of approximately four times that of trypsin (Galloway, 2006). On the other hand, LasA elastase is a serine protease that acts in a concerted action with LasB elastase to degrade elastin. LasA elastase nicks elastin and caused elastin to be sensitive to degradation by other proteases such as LasB elastase, alkaline proteases and neutrophil elastase (Van Delden & Iglewski, 1998).

2.5 *Melicope lunu-ankenda*

Figure 2.3: *M. lunu-ankenda* (Gaertn.) T. G. Haertly plant voucher that has been deposited into Herbarium of University Malaya.

M. lunu-ankenda (Gaertn.) T. G. Haertly (Figure 2.3) is locally known as 'tenggek burung'. It is a medium-sized tree, with trifoliate leaves and small, greenish white flowers. The roots, roots bark, stem wood, leaves and flowers are used in Indian traditional medicine for fever, as tonic and for improving complexion (Johnson *et al*., 2010).

The young leaves of *M. lunu-ankenda*are usually eaten raw as ‘ulam’ by some local Malaysians and it was said to be able to reduce hypertension and revitalize the body. The leaves extract of *M. lunu-ankenda* were found to contain different mixtures of hydrocarbons and squalenes, fatty acids and esters. The major compound that was isolated was a geranylated coumaric acid (Ramli*et al*., 2004). Previous study had isolated three phenylethanones, five furoquinoline alkaloids, bergapten and lupeol from the root bark of *M. lunu-ankenda*. Some of the phenylethanones isolated showed fungicidal activity (Kumar*et al*., 1990). Past studies has shown that the extract of *M. lunu-ankenda*exhibits antioxidant, antipyretic, analgesic and anti-inflammatory properties (Johnson *et al*., 2010).

2.6 *Piper nigrum*

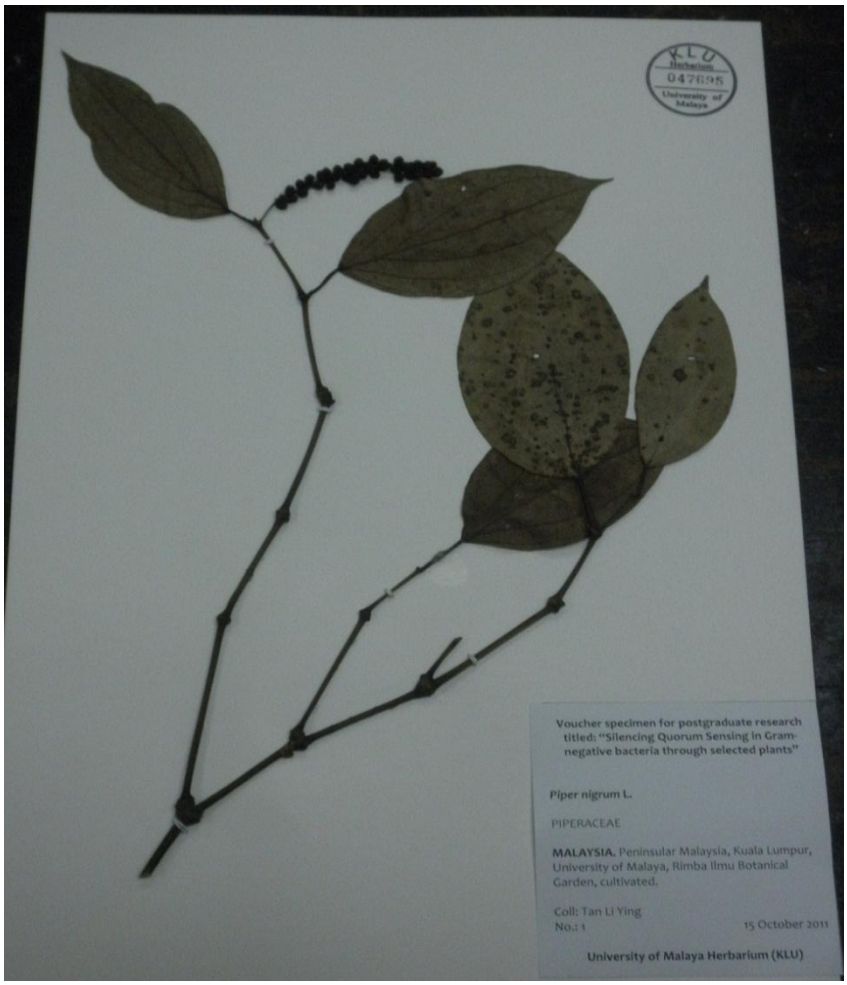


Figure 2.4: *P. nigrum* L. plant voucher that has been deposited into Herbarium of University Malaya.

P. nigrum is in the family of Piperaceae and it is a flowering vine. It is usually cultivated for the fruits (peppercorn) and when the peppercorns fully mature, they are dark red in color. *Piper* species are widely distributed in the tropical and subtropical regions of the world and have multiple applications in different folk medicines. In traditional Chinese medicine, many species are used to treat inflammatory diseases (Stöhr*et al*., 2001).*P. nigrum*is a natural spice widely used in the Ayurvedic medicine. It is used in treatment for asthma, cough, diabetes and heart problem(Chatterjee *et al*.,

2007). Due to the medicinal benefits of *P. nigrum* compounds, dozens of studies has been performed to identify the compounds available in *P. nigrum*. The types of compounds that can be found in *P. nigrum* include piperidine and pyrrolidine alkamides. Piperine which exists in *P. nigrum* is able to act as an analgesic, antipyretic and actifeedant (Parmar *et al*., 1997). Research also showed that piperine can act as a suitable template for the development of therapeutics drugs for trypanocidal infection treatment (Ribeiro *et al*., 2004). Eugenol, myristicin, safrole, monoterpenes, oxygenated sesquiterpenes and β-caryophyllene to name a few, are among the many compounds of *P. nigrum* that has been identified over the years (Jirovetz *et al*., 2002)

PP

2.7 *Piper betle*

Figure 2.5:*P. betle* L. plant voucher that has been deposited into Herbarium of University Malaya.

P. betle belongs to the family of Piperaceae (same as *P. nigrum*) and it is a perennial dioecious, semi woody climber. The stems of *P. betle* are swollen at the nodes and papillose when young. The stems will then grow to form a glabrous morphology. The leaves (heart-shaped) of *P. betle* have a petiole of approximately 1-2 cm long and 1.2-1.8 mm thickness when dry and glabrous at maturity. Cultivation of *P. betle* predominantly takes place in Sri Lanka, India, Malay Peninsula, Philippine and East

Africa. The fundamental part of the leaves of *P. betle* is the betel oil. Betel oil has a clove like flavor and comprise of terpenes and phenols. Betel leaves are consumed among the Southeast Asians population and it is usually taken with areca nut, slaked lime or tobacco (Arambewela *et al*., 2005).

Betel oil is a volatile oil and it has high safrol content. Besides that, eugenol, allyl diacetoxy benzene and chavibitol acetate were also found in betel oil. Studies have shown that the extract of *P. betle* leaves possess antidiabetic, gastroprotective, wound healing, hepatoprotective, antiallergic and antioxidant charateristics (Arambewela *et al*., 2005; Dasgupta & De, 2004; Milton *et al*., 2012; Wirotesangthong*et al*., 2008). Betel juice can also be given to children as a treatment for cough and admistered on to the eye for treating night blindness in adults. On top of that, betel juice is also used to treat inflammation of the mucus membrane in the airways or the body cavities (Arambewela *et al*., 2005; Jayaweera, 1982).

2.8 *Gnetum gnemon*

Figure 2.6: *G. gnemon* L. plant voucher that has been deposited into Herbarium of University Malaya.

G. gnemon(family of Gnetaceae) is commonly found in Southeast Asia and is used as food in Indonesia. It is a medium-size tree and can grow up to 15-20 m tall. *G. gnemon* is an aboreal dioecious plant. The leaves are evergreen and when mature, it forms a glossy dark green color. The strobilus has a large seed and this seed can be eaten.*G. gnemon* is a very popular food in Indonesia and the seeds are ground into flour

and deep-fried as crackers while the leaves are used for vegetable dishes (Iliya *et al*., 2003; Kato*et al*., 2009). The acetone and methanol extracts of *G. gnemon* roots yield four stilbene derivatives, namely gnemonols K and L (resveratrol trimers), M (isorhapontigenin dimer) and gnemonoside K (glucoside of resveratrol trimer); 11 known stilbenoids; a lignan. Some of these compounds have antioxidant properties (Iliya *et al*., 2003).

On the other hand, isolation of C-glycosylflavones was first reported from the leaves of *G. gnemon* in 1978(Wallace & Morris, 1978). Besides being traditionally used in the treatment of arthritis, bronchitis and asthma, *G. gnemon* compounds were shown to be able to lower the blood sugar level, cause apoptosis of colon cancer cells and have anti-inflammatory activity (Kato *et al*., 2009)

2.9 High Performance Liquid Chromatography (HPLC)

Chromatography refers to the separation of the elements of a sample partition between two phases, namely the stationary phase and the mobile phase (gas, liquid or supercritical fluid). Chromatography can be divided into difference categories such as adsorption, partition and ion-exchange chromatography. Liquid chromatography (LC) is a technique in adsorption chromatography in which the mobile phase is in liquid form and it can divided further into normal phase LC and reverse phase LC based on the polarity of the mobile phase. Traditional LC consists of a glass column that has been filled with slurry of porous solid material. Samples to be separated are firstly dissolved in appropriate solvents and applied directly to the top of the column(Chromatography, 2013; McMaster, 2007).

The adsorbent (stationary phase) is usually made of aluminium oxide or silica gel. Slurry of the adsorbent that has been mixed with the mobile phase is poured into the column and a layer of sand is placed at the top of the column. Samples (dissolved in solvents) to be separated is distributed evenly at the top of the column and as the mobile phase passes through the column, compounds with weaker adsorption will move at a faster rate as compared to the compounds with stronger adsorption. By changing the polarity of the mobile phases, separation of complex mixtures can be done. The type of mobile phases to be used is dependent on the compounds in the samples to be separated. Polarity of solvents normally used in LC in increasing order is as follows: petroleum ether > carbon tetrachloride > hexane > carbon disulfide > benzene > toluene > methylene chloride > chloroform > diethyl ether > ethyl acetate > acetone > ethanol > methanol > water (General principles of chromatography, 1997; McMaster, 2007).

HPLC instrument was first constructed by Csaba Horvath in 1964 and current improved HPLC has higher resolving power, faster separation, able to perform efficient qualitative and quantitative measurements and isolation and automation of methods and data analysis as compared to traditional LC. Analytical HPLC typically uses column of smaller size, lower flow rate, lower injection sample volume and concentration but better resolution as compared to preparative HPLC. Scale-up from analytical to preparative HPLC enable us to quantitate the desired separated compounds. Main components of HPLC are reservoir, degasser, pumps, autosampler/manual injector, column, guard column, detector and fraction collector. Mobile phases is placed in the reservoir and for analytical analysis, acids are added into the mobile phase to produce better chromatographic results (Gupta & Shanker, 2008).

HPLC pumps ensure consistent delivery and flow rate of the mobile phases throughout the whole separation process. An autosampler is able to work without operator attendance once the settings has been made and sample will be injected into the system and continuously separated without hassle. Generally, HPLC columns consist of silica bonded-phase columns. Carbon-18 (C-18) column are made of silica packing that has been attached to octyldecyl carbon units, making it to have high affinity to nonpolar compounds(Comparison guide to C18 reversed phase HPLC columns, 2008; McMaster, 2007).

Detectors of HPLC should not react with the mobile phases (no peak should be detected) and should be able to detect a wide range of compounds. Among the type of detectors available for HPLC systems include ultraviolet/visible light spectroscopy; refractive index; flurorescence; electrochemical; evaporative light scattering; conductivity; mass; infrared detectors (Various detectors used in the high performance liquid chromatography with comparison to their sensitivity, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemical Reagents

Chemical reagents used in this study were purchased from Merck, Germany; Sigma Chemical Corp., USA; BDH Laboratory Supplies, England; and Ajax Pacific Specialty Chemicals Limited ABN., Australia.

3.2 Growth Media and Agar

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

3.2.1 Luria-Bertani (LB) Medium

Luria-Bertani broth (LBB) was prepared by adding 1.0 % w/v trytone, 1.0 % w/v NaCl and 0.5 % w/v yeast extract into 1 litre of distilled water. On the other hand, LB agar was prepared by adding Bacto agar to a final concentration of 1.5 % w/v into the LBB. Supplementation of antibiotics was done after autoclave sterilization of the medium.

3.2.2 Swarming Agar

Swarming agar was prepared by adding glucose (1% 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 and autoclaved.

3.3 Stock Solutions

3.3.1 Synthetic *N*-acyl-homoserine Lactones

Synthetic AHL molecules were obtained from Sigma-Aldrich© and Cayman Chemicals. Resuspension of the powder was performed using acetonitrile (ACN) to the desired concentration.

3.3.2 Kanamycin and Chloramphenicol Stock Solution

Kanamycin and chloramphenicol stock solution was prepared by dissolving in sterile distilled water and ethanol respectively resulting in the final concentration of 100 mg/ml. The antibiotic stock was filter sterilized into new sterile microcentrifuge tubes followed by storage of the aliquots at -20 ˚C until further usage.

3.3.3 Preparation and Dilution of Plants Stock Solution

The dried crude extract was weight 10 mg and dissolved in 1 ml of absolute dimethyl sulfoxide (DMSO) to prepare a 10 mg/ml sample stock solution which was then kept in a 1.5 ml microcentrifudge tube. The tube which contained the stock solution was mixed well by vortex machine. Once labeled, the sample tube was stored at -20˚C freezer. Prior to be used, the plants extract was diluted with sterile distilled water to desired concentration.

3.4 Bacterial Strains

Table 3.1: Bacterial strains used in this study.

Strain	Description	Source/Reference
C. violaceum CV026	Double mini-Tn*5* mutant derived from ATCC 31532, Kan^R, Hg^R, *cvil*::Tn*5xyl*E, plus spontaneous Str^R AHL biosensor, produces violacein pigment only in the presence of exogenous AHL	(McClean *et al*., 1997)
P. aeruginosa PA01 *lecA::lux*	Prototroph *lecA::luxCDABE*genomic reporter fusion in PA01	(Winzer *et al*., 2000)
E. coli [pSB401]	*luxRluxl'*(*Photobacterium fischeri*[ATCC7744])::*luxCDABE* (*Photorhabduslumi nescens* [ATCC 29999]) fusion; pACYC184-derived, Tet^R, AHL biosensor producing bioluminescence	(Winson *et al*., 1998)
[pSB1075]	*lasRlasl'* (*P. aeruginosa* PAO1)::*luxCDABE*(*P.luminescens* [ATCC 29999]) fusion in pUC18 Amp^R, AHL biosensor	(Winson *et al*., 1998)

	producing bioluminescence	

Bacteria were grown in LBBin shaking (220 rpm) incubator. *C. violaceum*CV026 was cultured in 28 ˚C, while *P. aeruginosa*strains at 37 ˚C. *C. violaceum*CV026growth medium was supplemented with kanamycin (30 µg/ml) and chloramphenicol (30 µg/ml).

3.5 Identification and Processing of Plant Samples

Plant samples used in this study were purchased from the farmer's market in Petaling Jaya, Selangor.Voucher specimen of each plant samples was deposited in the Herbarium of University of Malaya for proper identification. Fresh plant samples were washed, cut into small pieces and dried in hot air oven (45 ˚C) for 3 days.

3.6 Extraction of Dried Samples by Infusion

Dried samples(Leaves of *M. lunu-ankenda*, *P. betle* and *G. gnemon*; Seeds for *P. nigrum*) were blended to fine powder and submerged sequentially in solvent (ratio 1:10

w/v), namely, hexane, chloroform and methanol. Infusions were filtered using Whatman No. 1 filter paper to obtain filtrate which contained solvent and secondary metabolites extracted from the dried samples. Filtrates were concentrated in vacuum using a rotary evaporator to yield crude extract with traces of solvent. First, a little extraction was transferred into a round bottom flask and the mouth of the round bottom flask was locked to the condenser part of the rotary evaporator. The extraction was heated in 45-50 ˚C water bath in the electric heating mantle. The solvent evaporated and passes through the side arm into the upper reflux condenser, then condensed into liquid and dripped to another round bottom flask placed at the other side. The process was continued until concentrated extract was collected, and then transferred into a sample bottle. The concentrated extract was left to dry through natural evaporation process until no traces of solvent smell were left. Solvent-extracted crude extract was placed in a desiccator or a fume cupboard.

3.7 Screening for Anti-Quorum Sensing Activities of the Plant Samples

3.7.1 *C. violaceum* CV026 Plate Assay

Overnight culture of *C. violaceum* CV026 cells (15 ml) were added into 200 ml of molten LB agar. Then,C6-HSL of 0.25 µg/ml was added into the LB agar that has been seeded with *C. violaceum* CV026 cells. Immediately after that, the *C. violaceum* CV026 agar suspension was poured into petri dishes and allowed to solidify for 30 min. Once the agar has solidified, wells were made on the agar by using sterile pipette tips. Then, 30 µl of plant extract was placed in the well and the plates were incubated for 24 h at 28 ˚C(Blosser & Gray, 2000).

3.7.2 *C. violaceum* CV026 Violacein Quantification Assay

The optical densityat 600 nm (OD_{600nm}) of the overnight culture of *C.violaceum* CV026 was adjusted to 1.2 prior to be used. Den C6-HSL of 0.125 µg/ml was supplemented into the culture. In each well, approximately 90 µl of *C. violaceum* CV026 cells was placed in it, followed by the addition of 10 µl of plant crude extract. Later, the 96-well plate was placed in a shaking incubator of 28 ˚C. After incubation of 16 h, the treated cells were placed in a 60 ˚C oven with an open lid to allow evaporation of the LBB. Once the wells were dried completely, DMSO of 100 µl was placed into each well. Then, the 96-well plate was placed in a shaker until all the violacein was solubilized. The absorbance of each well was read at 590 nm using using DYNEX MRX Elisa reader (Chantilly, VA, USA)(Martinelli *et al*., 2004).

3.7.3 Quantification of Bioluminescence from *E. coli* [pSB401] and *E. coli* [pSB1075]

Bioluminescence expression was quantified using a Tecanluminometer(InfiniteM200,Mannerdorf,Switzerland). Briefly, overnight culture of *E. coli* biosensors cells was diluted to an OD_{600nm} of 0.1. Then, 230 µl of *E. coli* biosensors cells and 20 µl of plant extract were added into the well of 96-well microtitre plate. The bioluminescence and OD_{495nm} were determined every 30 min for 24 h by the luminometer. Expression of bioluminescence was given as relative light unit (RLU)/OD_{495nm} against time(Winzer *et al*., 2000).

3.7.4 *P. aeruginosa* PA01 Pyocyanin Quantification Assay

Overnight culture of *P. aeruginosa* PA01 was adjusted to an OD_{600nm} of 0.2. Then, 250 µl of plant extract was added and mixed well with *P. aeruginosa* PA01 cells (4.75 ml) in a polypropylene tube and incubated at 37 ˚C for 24 h. The 5 ml culture was extracted with 3 ml of chloroform, followed by mixing the chloroform layer with 1 ml of 0.2 M HCl. The absorbance of the pink extracted organic layer was then measured at 520 nm (Essar*et al*., 1990).

3.7.5 Quantification of *P. aeruginosa* PA01 *lecA* Expression

P. aeruginosa PA01 *lecA* expression was quantified using a Tecanluminometer (Infinite M200,Mannerdorf, Switzerland). Briefly, overnight culture of *P. aeruginosa* PA01 *lecA::lux* was diluted to an OD_{600nm} of 0.1. Then, 230 µl of *P. aeruginosa* PA01 *lecA::lux* and20 µl of plant extract were added into the well of 96-well microtitre plate. The bioluminescence and OD_{495nm} were determined every 30 min for 24 h by the luminometer. *P. aeruginosa* PA01 *lecA::lux* expression was given as RLU/OD_{495nm}against time(Winzer *et al*., 2000).

3.7.6 *P. aeruginosa* PA01 Swarming Assay

Molten swarming agar (10 ml) was poured into the petri dish. The petri dish was then placed in the laminar flow for 30 min. Then, the solidified swarming agar was overlaid with 4.75 ml of molten swarming agar that has been supplemented with 250 µl of plant extract. The 15 ml thickness agar was then air blown for another 30 min in the laminar flow. Finally, overnight culture of *P. aeruginosa* PA01 of 2 µl was inoculated in the centre of the agar and incubated for 16 h at 37 ˚C.

3.7.7 Statistical Tests

All assays were performed on triplicate basis and the significance of the data was tested using ANOVA test ($P< 0.05$) using GraphPad Prism software (Version 5.00).

3.8 Fractionation of Active Plant Extract (Chloroform Extract of *M. lunu-ankenda*) using Agilent Preparative HPLC

Fractionation of active crude extract was carried out using an Agilent Technologies 1260 Infinity Series HPLC system (Agilent Technologies, Germany) that consists of two preparative pumps, an autosampler, a diode-array detector (DAD) and a fraction collector. Crude extract to be fractionated was diluted with ACN before injected into the system. Sample of 1500 µl was injected into the HPLC system and was applied onto a C18 preparative cartridge (Agilent PrepHT XDB-C18, 21.2 mm × 250 mm, 7 µm particle size) with an attached guard column (Agilent ZORBAX XDB-C18, 5 µm particle size).The preparative system was run on an isocratic profile of ACN-water (30:70, v/v) for 40 min at a constant flow rate of 10 ml/min and the spectrum was monitored at 210 nm. Fractions were collected from the 4^{th} min till the 40^{th} min of the run (Table 3.2).

Fractions collected were concentrated via rotary evaporator and once dried, fractions were weight 5 mg and dissolved in 0.5 ml of DMSO to prepare a 10 mg/ml sample stock solution which was then kept in a 1.5 ml microcentrifudge tube. The tube which contained the stock solution was mixed well by vortex machine. Once labeled, the sample tube was stored at -20˚C freezer for next usage. Prior to be used, the plants extract was diluted with sterile distilled water to desired concentration. The fractions were tested on swarming assay, *P. aeruginosa*PA01 *lecA::lux* and *E. coli* [pSB401] to determine which fraction contains active compounds.

Table 3.2: Collection time for the separated fractions.

Time (min)	Fractions
4^{th}- 5^{th}	1
5^{th}- 6th	2
6^{th}- 7^{th}	3
7^{th}- 8^{th}	4
8^{th}- 9^{th}	5
9^{th}- 10^{th}	6
10^{th}- 11^{th}	7
11^{th}- 12^{th}	8
12^{th} – 13^{th}	9
13^{th} – 18.5^{th}	10
18.5^{th} – 21^{st}	11
21^{st} – 23^{rd}	12
23^{rd} – 31^{st}	13
31^{st} – 40^{th}	14

CHAPTER 4

RESULTS

4.1 Plant Samples Identification and Depositions of Voucher Specimens

Plant samples that have been deposited in the Herbarium of University of Malaya were each given an identification number (Table 4.1).

Table 4.1: List of plant samples and their respective voucher specimen number.

Sample	Voucher specimen number
M. lunu-ankenda	047697
P. betle	047696
G. gnemon	047698
P. nigrum	047695

4.2 *C. violaceum* CV026 Plate Assay

After incubation for 24 h, the data was documented using a camera. *The C. violaceum* CV026 lawn turned purple in the presence of exogenously supplied short chain AHLs. Plant extracts that caused formation of halo zone around the well signified that anti-QS compounds existed in the plant sample.

In general, as the concentration of the extract increases, the size of the halo zone formed around the well increased as well. All of the plant extracts (Figure 4.1 and Figure 4.2) showed halo zone formation except for the methanol extract of *P. betle* (Figure 4.1 (f)), chloroform and methanol extracts of *G. gnemon* (Figure 4.2 (b) and (c)). The extracts which showed the most prominent formation of halo zone surrounded by purple violacein background were the chloroform and methanol extracts of *P. nigrum*(Figure 4.2 (e) and (f)).

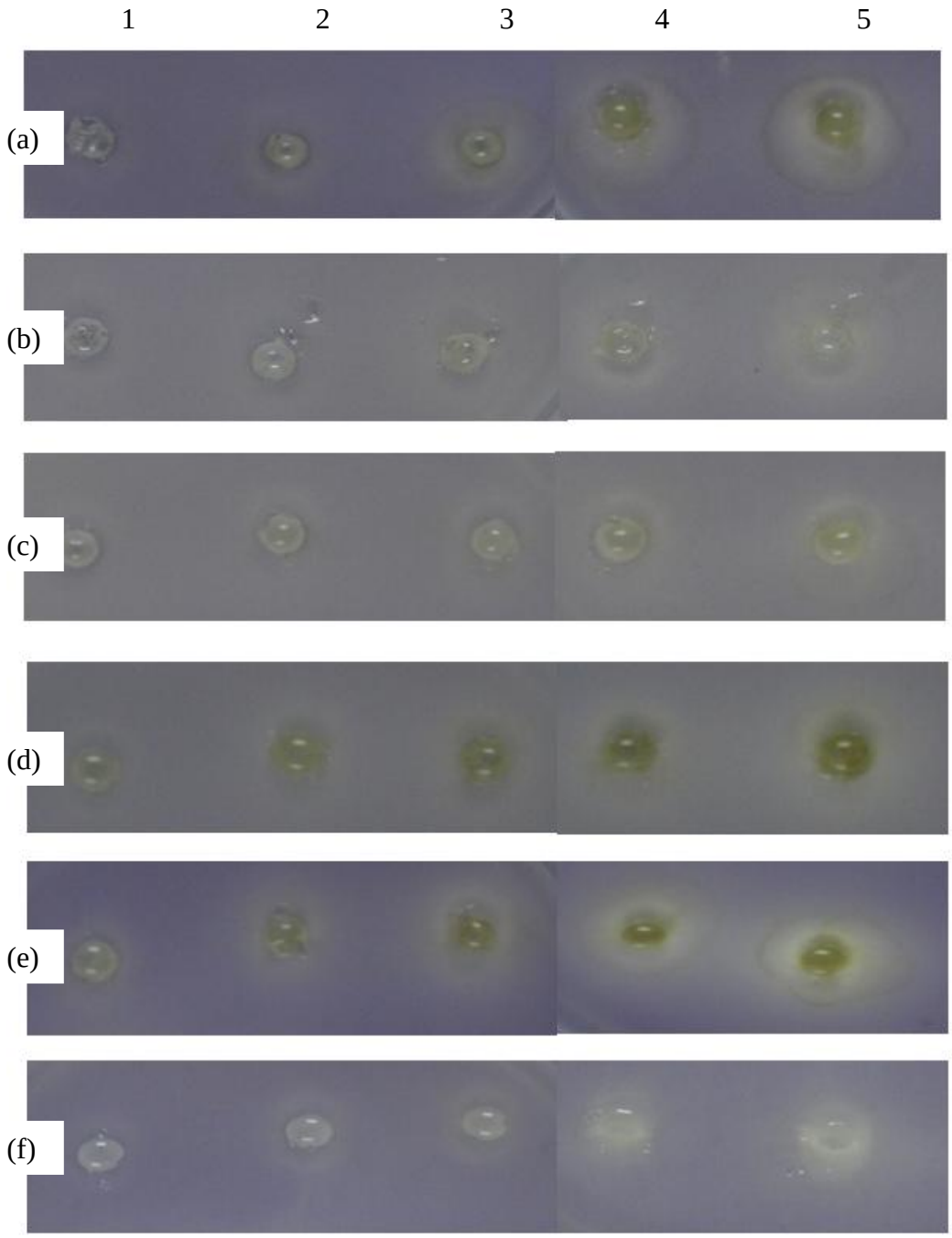


Figure 4.1: (a) *M. lunu-ankenda*-Hexane extract; (b) *M. lunu-ankenda*-Chloroform extract; (c) *M. lunu-ankenda*-Methanol extract; (d) *P. betle*-Hexane extract; (e) *P. betle*-Chloroform extract; (f) *P. betle*-Methanol extract.

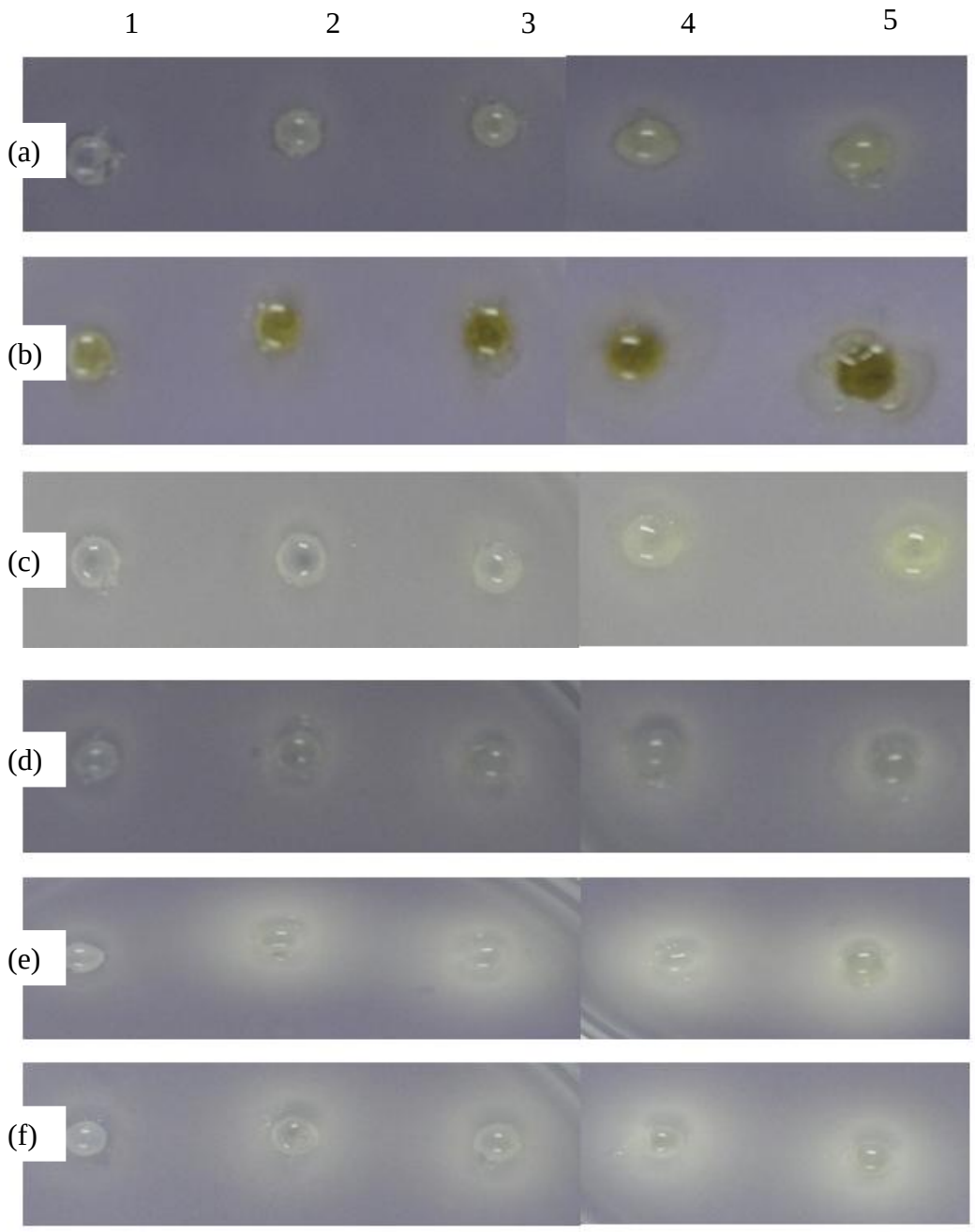


Figure 4.2: (a) *G. gnemon*-Hexane extract; (b) *G. gnemon*-Chloroform extract; (c) *G. gnemon*-Methanol extract; (d) *P. nigrum*-Hexane extract; (e) *P. nigrum*-Chloroform extract; (f) *P. nigrum*-Methanol extract.

4.3 *C. violaceum* CV026 Violacein Quantification Assay

Violacein formed by *C. violaceum* CV026 was quantified by measuring the amount of violacein that has solubilized in the DMSO using DYNEX MRX Elisa reader (Chantilly, VA, USA). The triplicate results obtained were used to calculate the standard error as seen on the bar graphs (Figure 4.3 and 4.4). Extracts that caused significant reduction in violacein productions were *M. lunu-ankenda* chloroform (Figure 4.3 (b)) and methanol extracts (Figure 4.3 (c)) and all three extracts of *P. betle* (Figure 4.3 (d-f)) and *P. nigrum* (Figure 4.4 d-f).

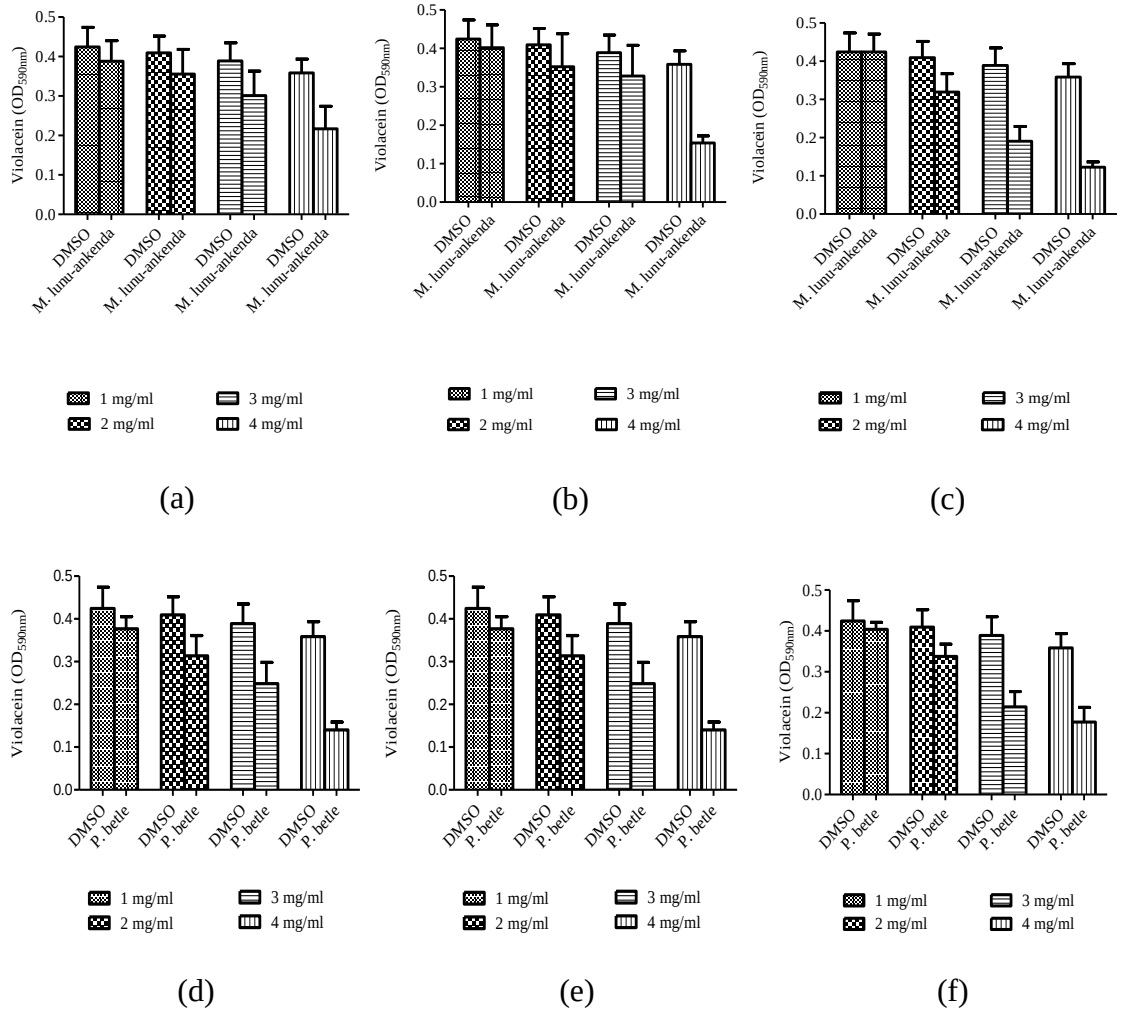


Figure 4.3: (a) *M. lunu-ankenda*-Hexane extract; (b)*M. lunu-ankenda*-Chloroform extract; (c) *M. lunu-ankenda*-Methanol extract; (d) *P. betle*-Hexane extract; (e) *P. betle*- Chloroform extract; (f) *P. betle*-Methanol extract.

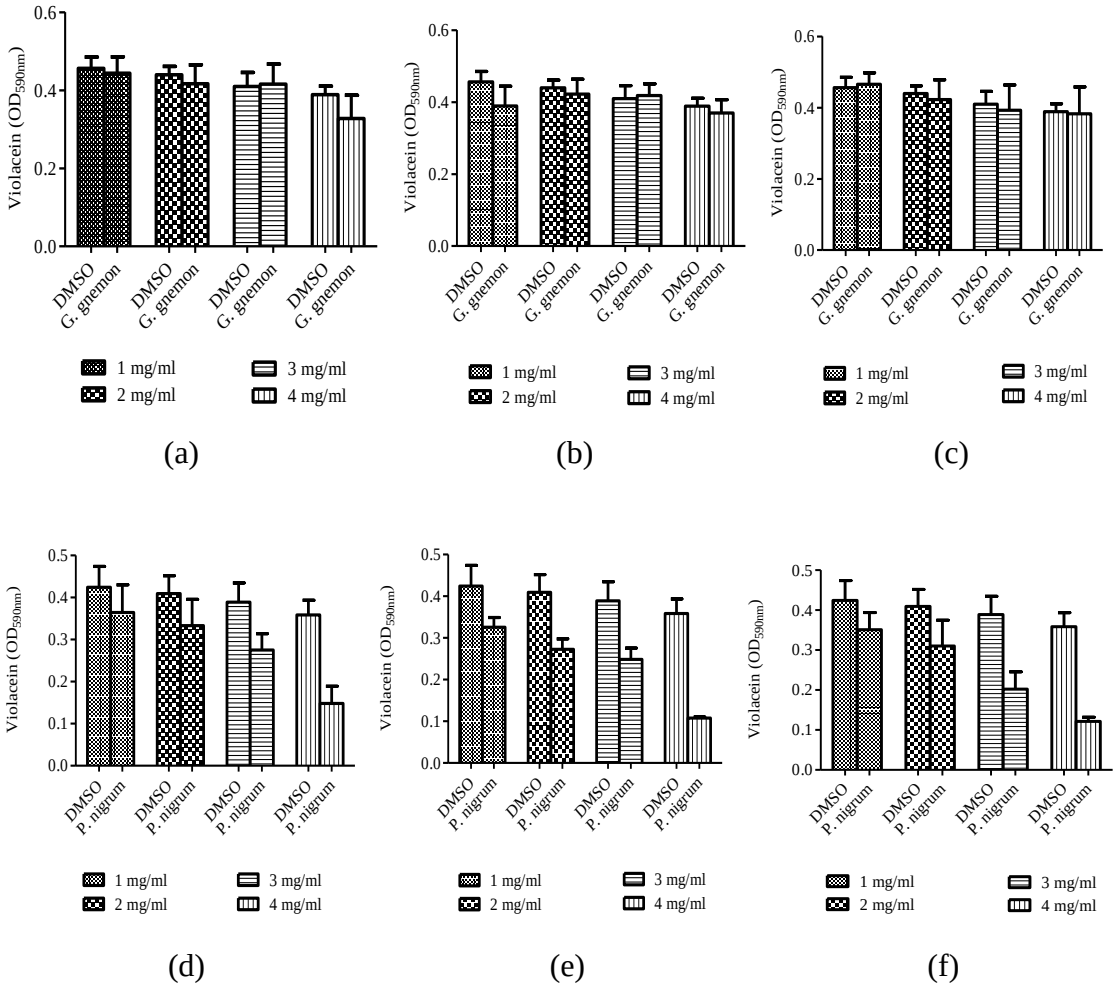


Figure 4.4: (a) *G. gnemon*-Hexane extract; (b) *G. gnemon*-Chloroform extract; (c) *G. gnemon*-Methanol extract; (d) *P. nigrum*-Hexane extract; (e) *P. nigrum*-Chloroform extract; (f) *P. nigrum*-Methanol extract.

4.4 Quantification of Bioluminescence from *E. coli* [pSB401]

Bioluminescence of *E. coli* [pSB401] was quantified using a Tecan luminometer(InfiniteM200,Mannerdorf,Switzerland). Plant extracts that contain anti-QS compounds will cause the bioluminescence reading to be reduced significantly. The graphs drawn were based on triplicate reading and the error bars were shown on the graphs as well. Surprisingly, all of the extracts significantly inhibited the bioluminescence of *E. coli* [pSB401] (Figure 4.5- Figure 4.10).

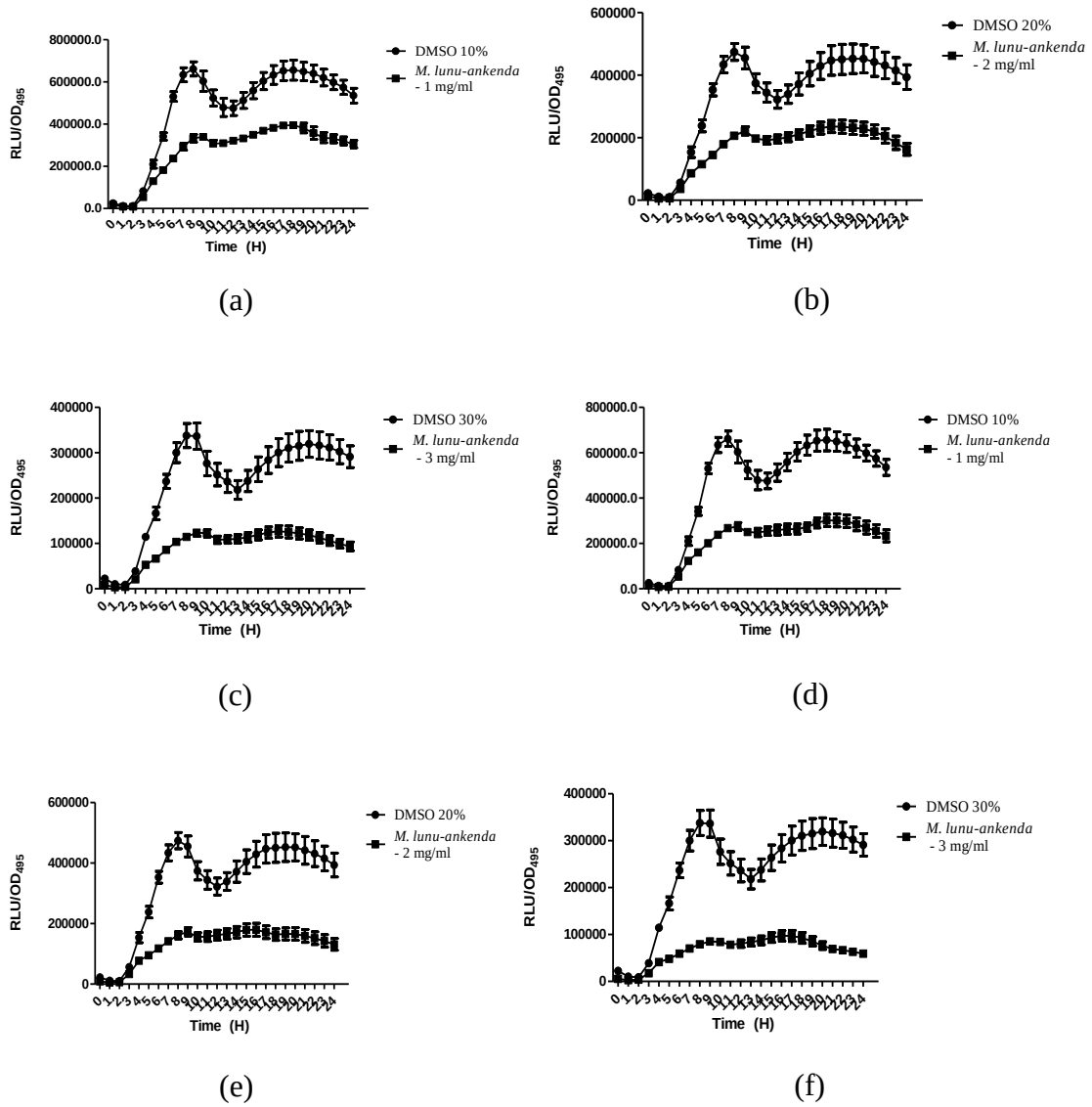


Figure 4.5: (a) *M. lunu-ankenda*-Hexane extract (1 mg/ml); (b) *M. lunu-ankenda*-Hexane extract (2 mg/ml); (c)*M. lunu-ankenda*-Hexane extract (3 mg/ml); (d) *M. lunu-ankenda*-Chloroform extract (1 mg/ml); (e) *M. lunu-ankenda*-Chloroform extract (2 mg/ml); (f)*M. lunu-ankenda*-Chloroform extract (3 mg/ml).

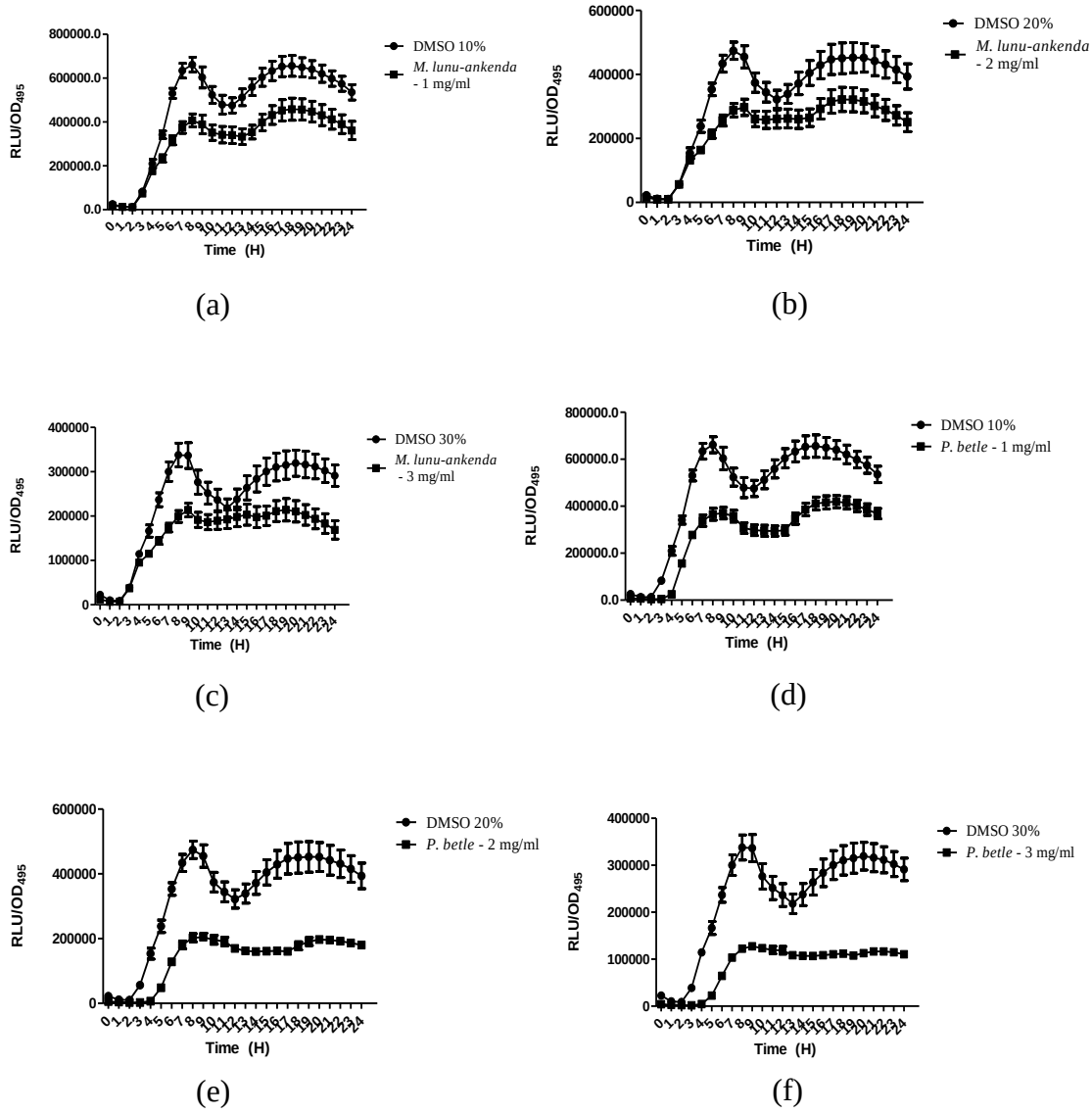


Figure 4.6: (a) *M. lunu-ankenda*-Methanol extract (1 mg/ml); (b) *M. lunu-ankenda*-Methanol extract (2 mg/ml); (c)*M. lunu-ankenda*-Methanol extract (3 mg/ml); (d) *P. betle*-Hexane extract (1 mg/ml); (e) *P. betle*-Hexane extract (2 mg/ml); (f) *P. betle*-Hexane extract (3 mg/ml).

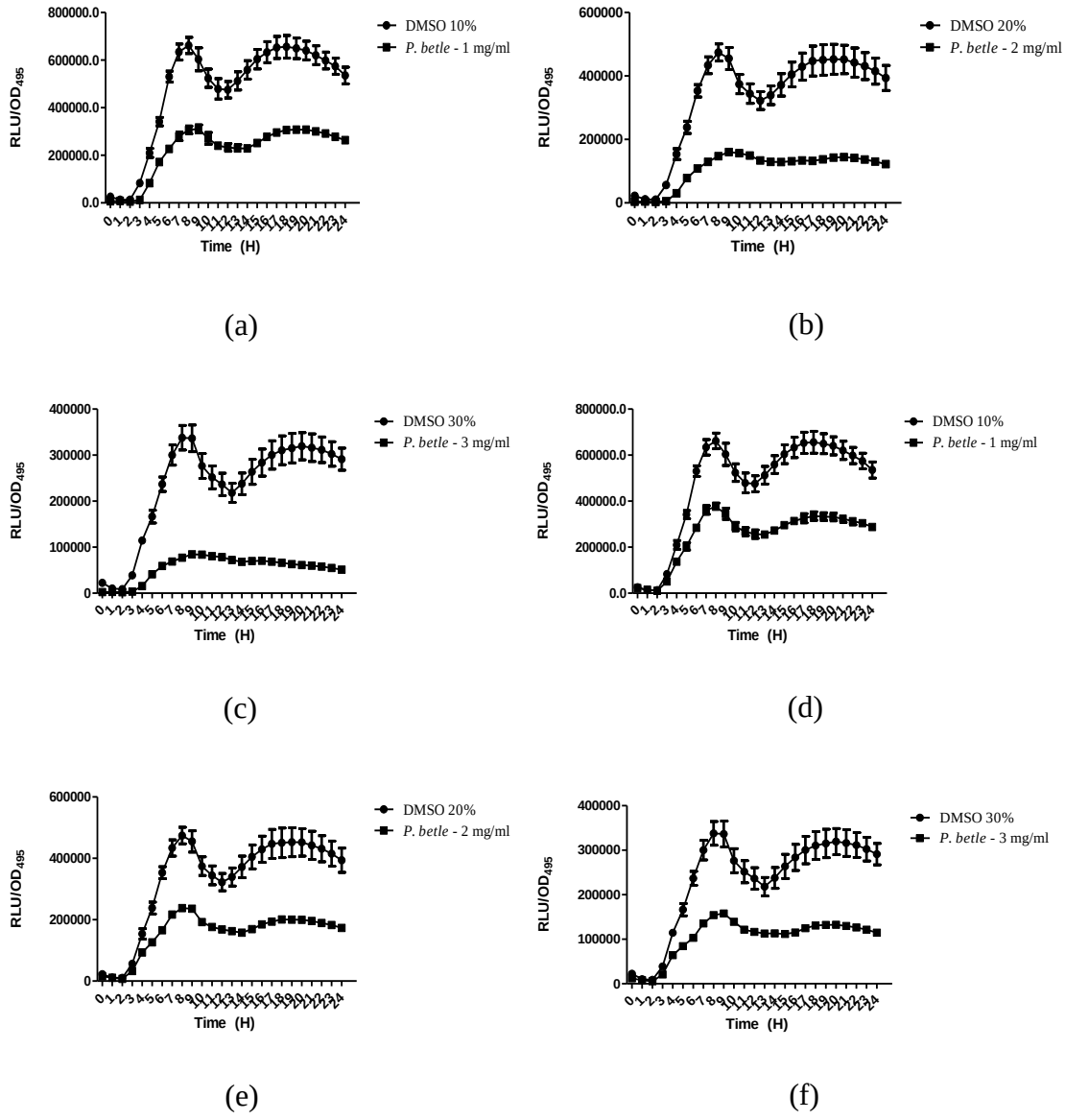


Figure 4.7: (a) *P. betle*-Chloroform extract (1 mg/ml); (b) *P. betle*-Chloroform extract (2 mg/ml); (c) *P. betle*-Chloroform extract (3 mg/ml); (d) *P. betle*-Methanol extract (1 mg/ml); (e) *P. betle*-Methanol extract (2 mg/ml); (f) *P. betle*-Methanol extract (3 mg/ml).

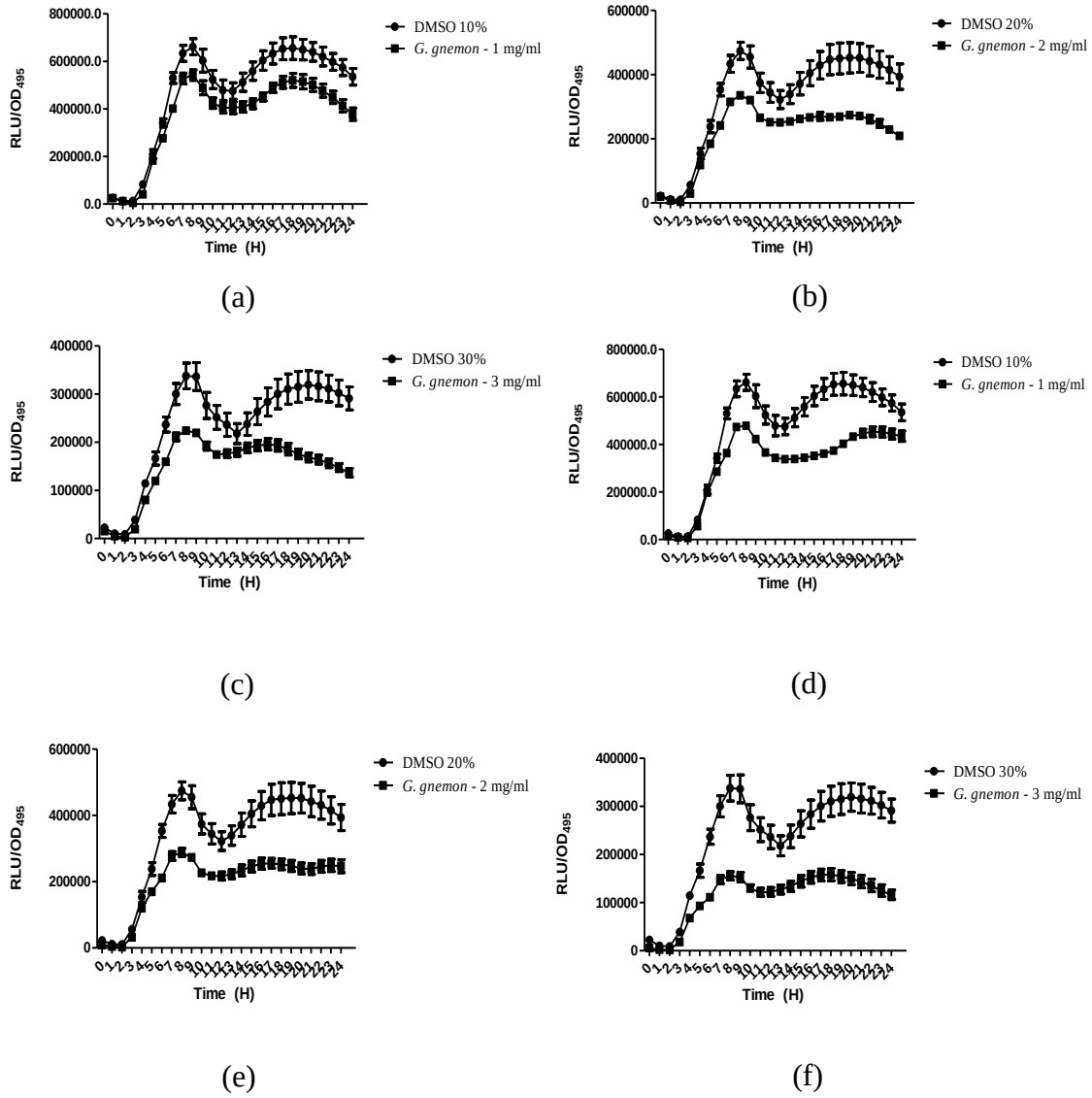


Figure 4.8: (a) *G. gnemon*-Hexane extract (1 mg/ml); (b) *G. gnemon*-Hexane extract (2 mg/ml); (c) *G. gnemon*-Hexane extract (3 mg/ml); (d) *G. gnemon*-Chloroform extract (1 mg/ml); (e) *G. gnemon*-Chloroform extract (2 mg/ml); (f) *G. gnemon*-Chloroform extract (3 mg/ml).

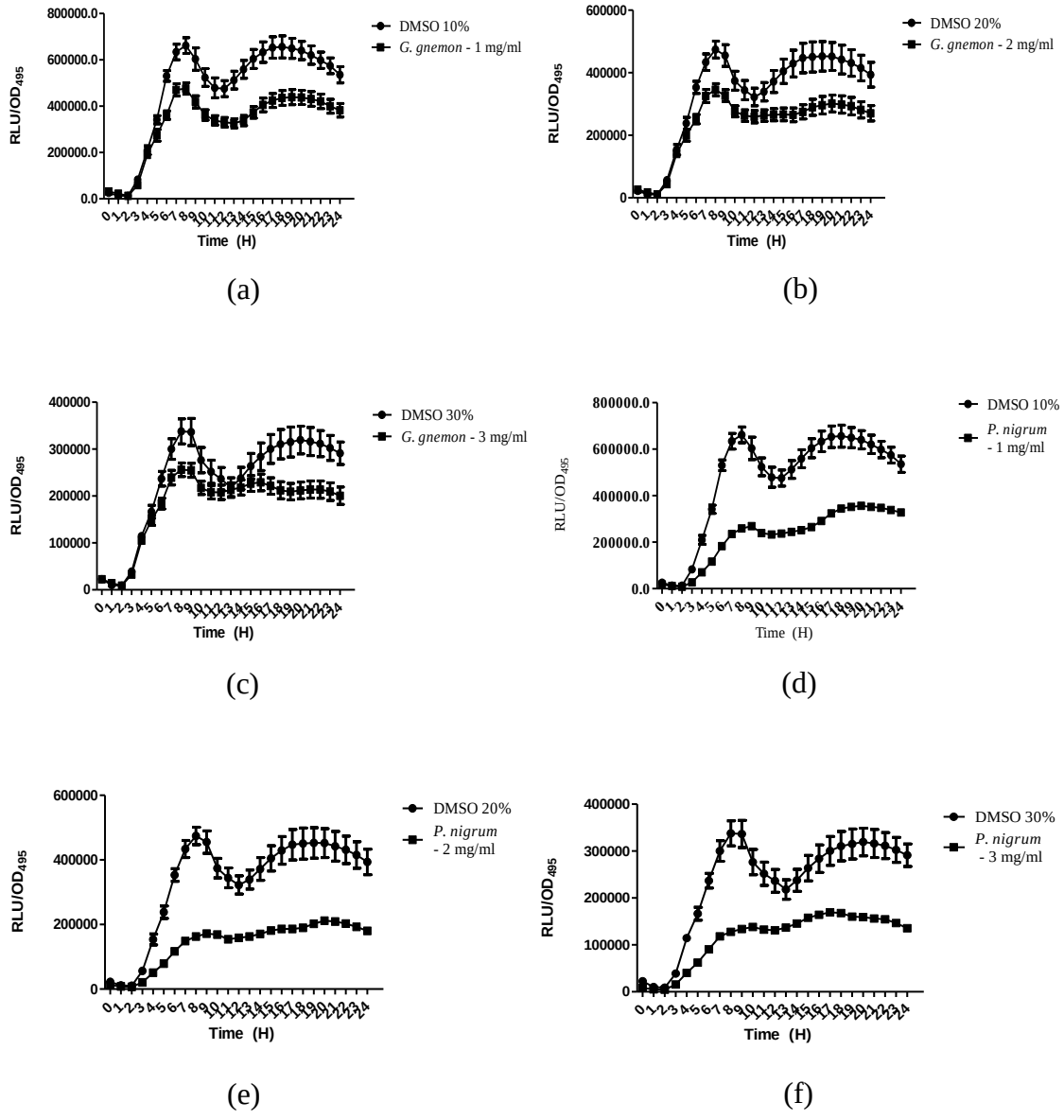


Figure 4.9: (a) *G. gnemon*-Methanol extract (1 mg/ml); (b) *G. gnemon*-Methanol extract (2 mg/ml); (c) *G. gnemon*-Methanol extract (3 mg/ml); (d) *P. nigrum*-Hexane extract (1 mg/ml); (e) *P. nigrum*-Hexane extract (2 mg/ml); (f)*P. nigrum*-Hexane extract (3 mg/ml).

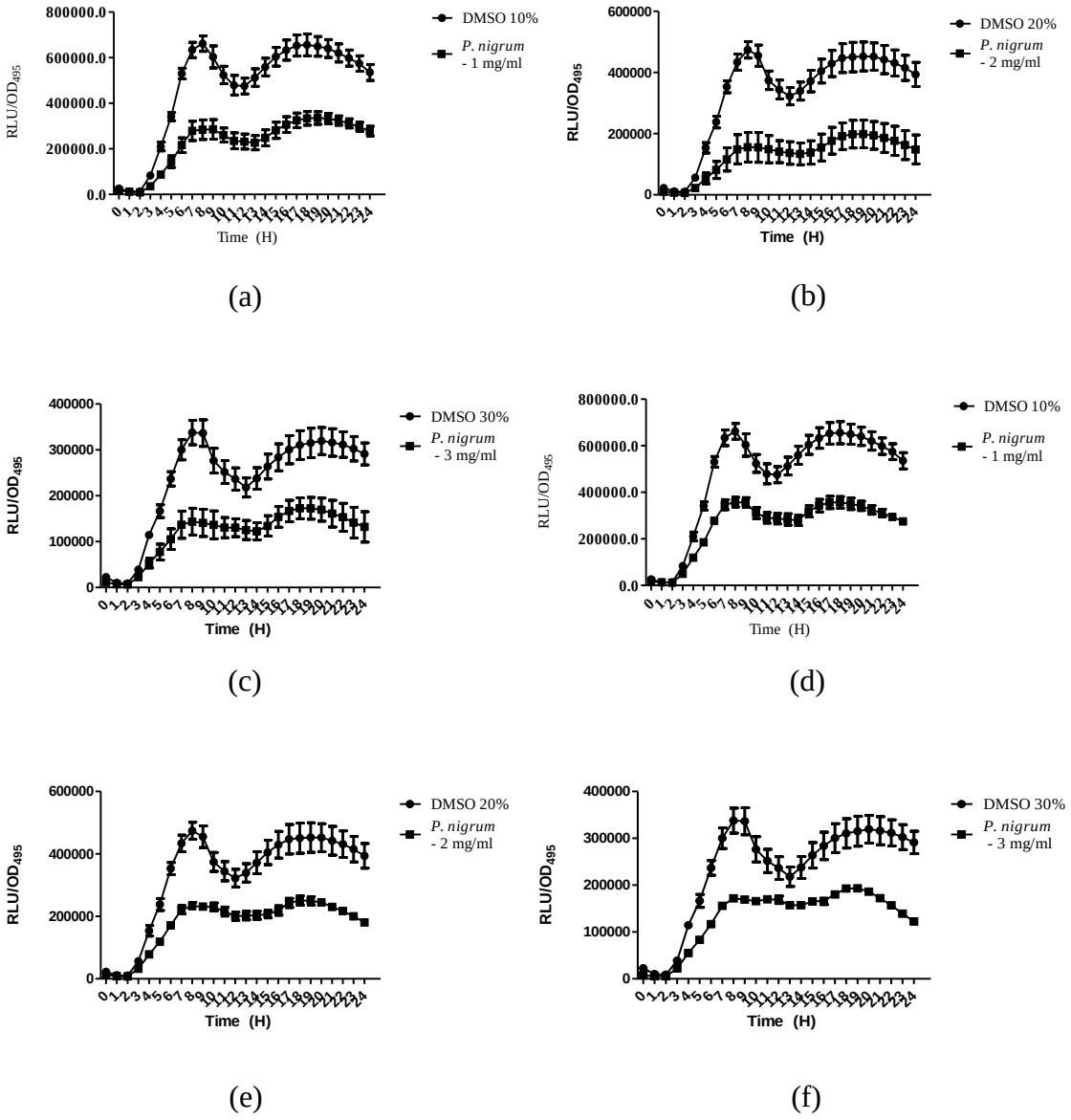


Figure 4.10: (a) *P. nigrum*-Chloroform extract (1 mg/ml); (b) *P. nigrum*-Chloroform extract (2 mg/ml); (c)*P. nigrum*-Chloroform extract (3 mg/ml); (d) *P. nigrum*-Methanol extract (1 mg/ml); (e) *P. nigrum*-Methanol extract (2 mg/ml); (f)*P. nigrum*-Methanol extract (3 mg/ml).

4.5 Quantification of Bioluminescence from *E. coli* [pSB1075]

After 24 h, the readings were collected from the luminometer(InfiniteM200,Mannerdorf,Switzerland) and the graphs of RLU/OD_{495} against time (h) were plotted. Hexane, chloroform and methanol extracts of each plant were tested against *E. coli* [pSB1075] in three concentrations namely 1, 2 and 3 mg/ml. Plant extracts that showed significance inhibition of bioluminescence from *E. coli* [pSB1075] were *P. betle*-Hexane extract (Figure 4.12 (d-f)), *G. gnemon*-Chloroform (Figure 4.14 (d-f)) and methanol extracts (Figure 4.15 (a-c)) while all the extracts of *M. lunu-ankenda* (Figure 4.11 and Figure 4.12 (a-c)), chloroform and methanol extracts of *P. betle* (Figure 4.13), hexane extract of *G. gnemon* (Figure 4.14 (a-c)) and all extracts of *P. nigrum* (Figure 4.15 (d-f) and Figure 4.16) did not showed any significant inhibition.

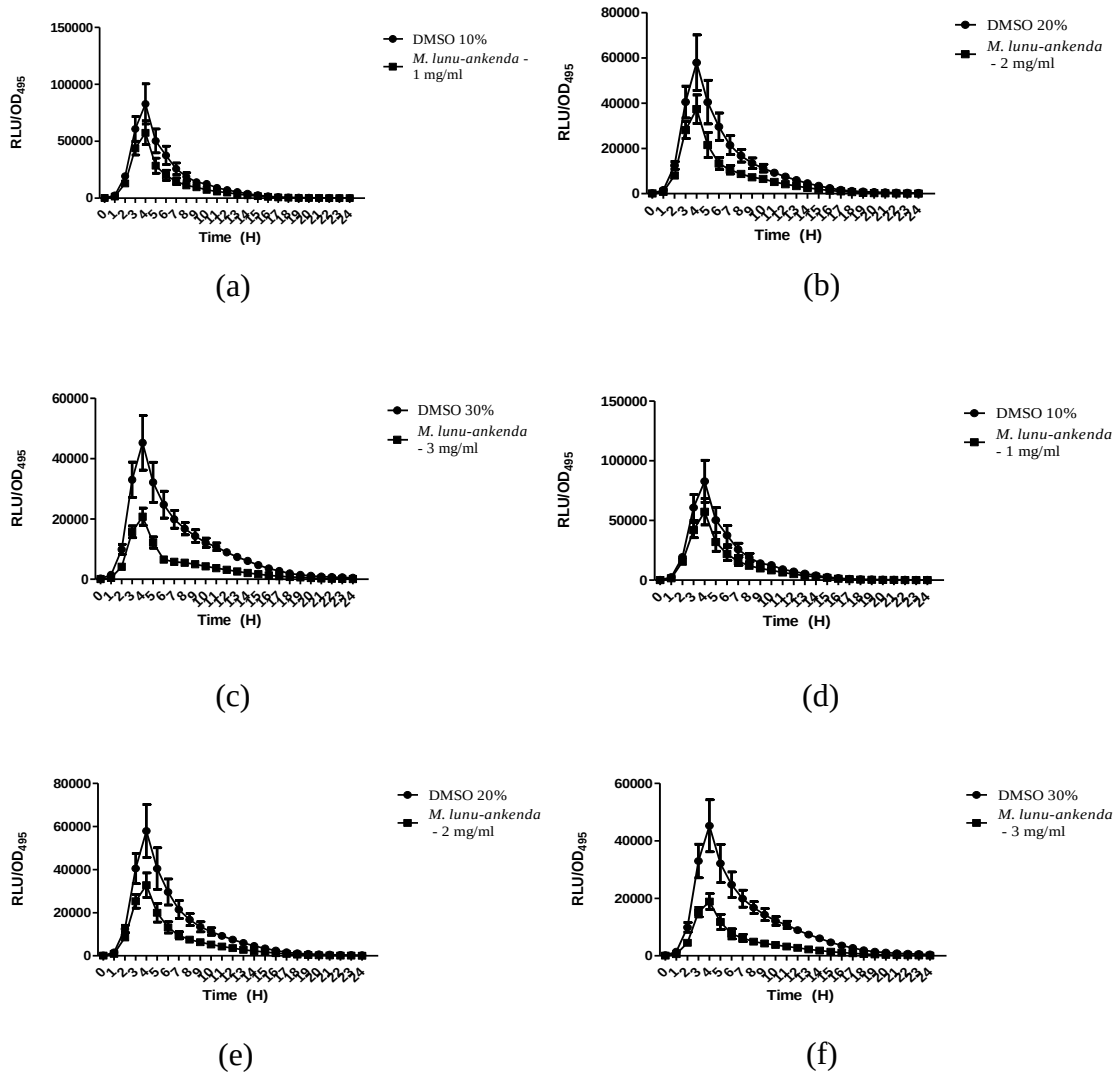


Figure 4.11: (a) *M. lunu-ankenda*-Hexane extract (1 mg/ml); (b) *M. lunu-ankenda*-Hexane extract (2 mg/ml); (c)*M. lunu-ankenda*-Hexane extract (3 mg/ml); (d) *M. lunu-ankenda*-Chloroform extract (1 mg/ml); (e)*M. lunu-ankenda*-Chloroform extract (2 mg/ml); (f)*M. lunu-ankenda*-Chloroform extract (3 mg/ml).

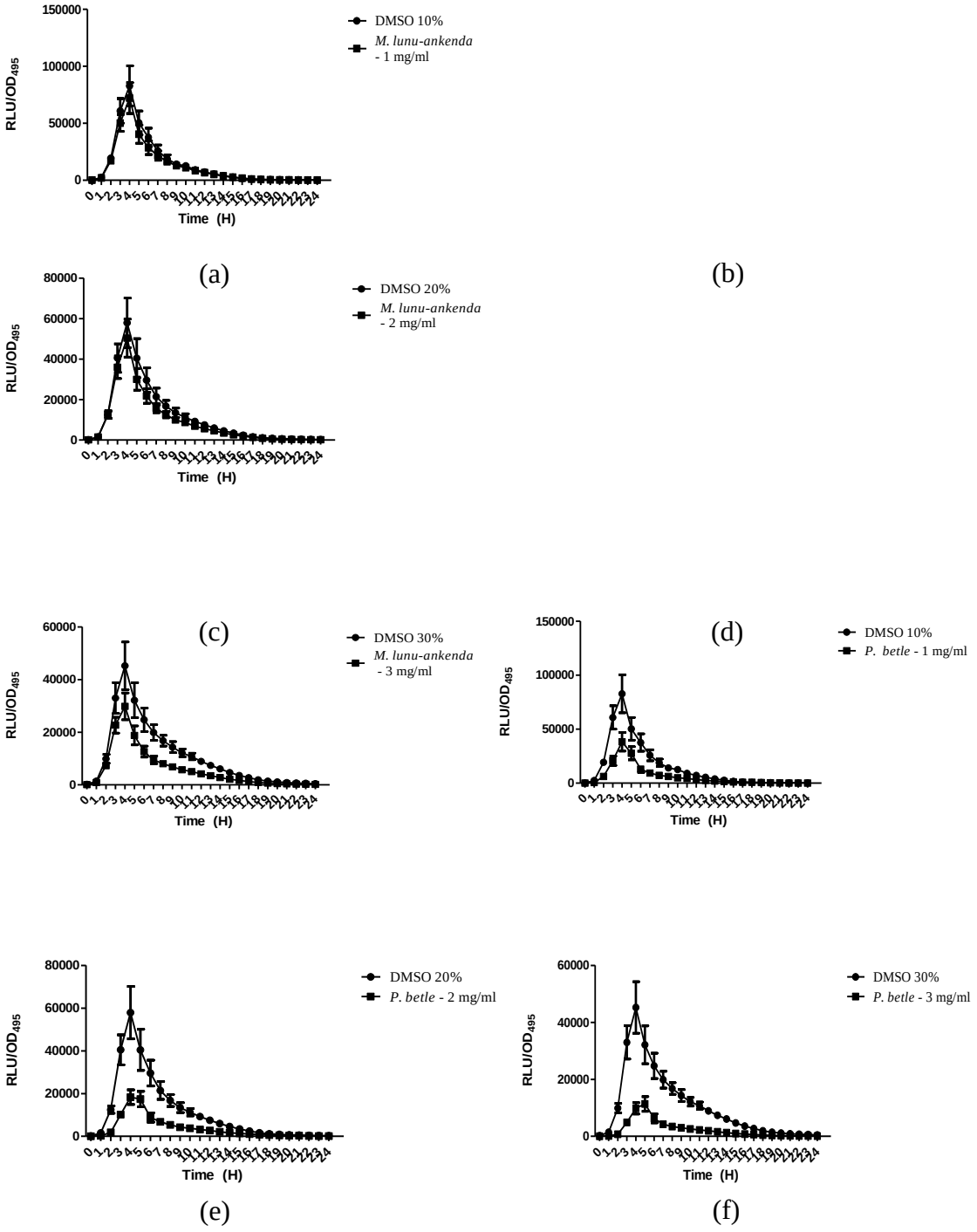


Figure 4.12: (a) *M. lunu-ankenda*-Methanol extract (1 mg/ml); (b) *M. lunu-ankenda*-Methanol extract (2 mg/ml); (c)*M. lunu-ankenda*-Methanol extract (3 mg/ml); (d) *P. betle*-Hexane extract (1 mg/ml); (e) *P. betle*-Hexane extract (2 mg/ml); (f) *P. betle*-Hexane extract (3 mg/ml).

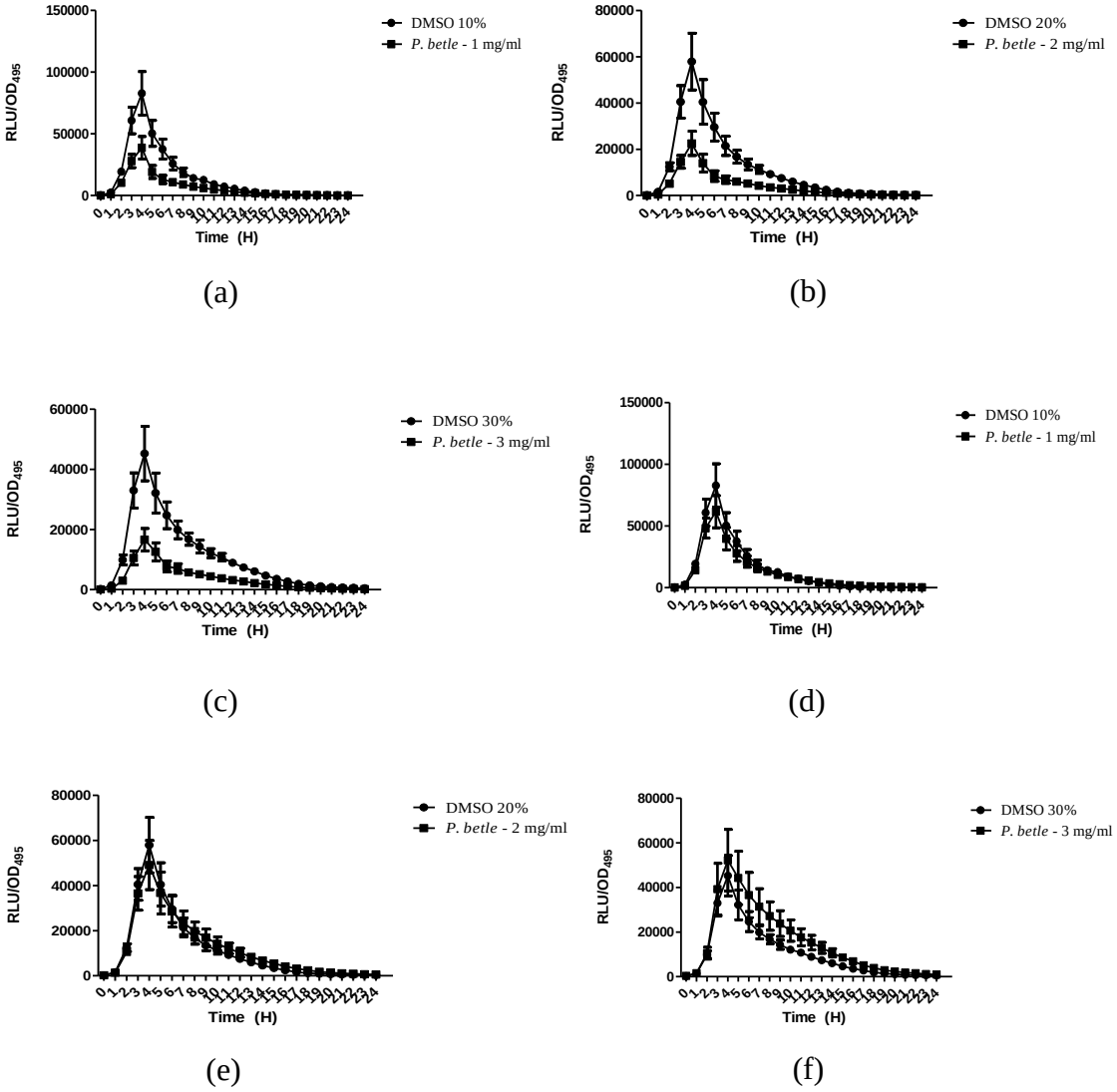


Figure 4.13: (a) *P. betle*-Chloroform extract (1 mg/ml); (b) *P. betle*-Chloroform extract (2 mg/ml); (c) *P. betle*-Chloroform extract (3 mg/ml); (d) *P. betle*-Methanol extract (1 mg/ml); (e) *P. betle*-Methanol extract (2 mg/ml); (f) *P. betle*-Methanol extract (3 mg/ml).

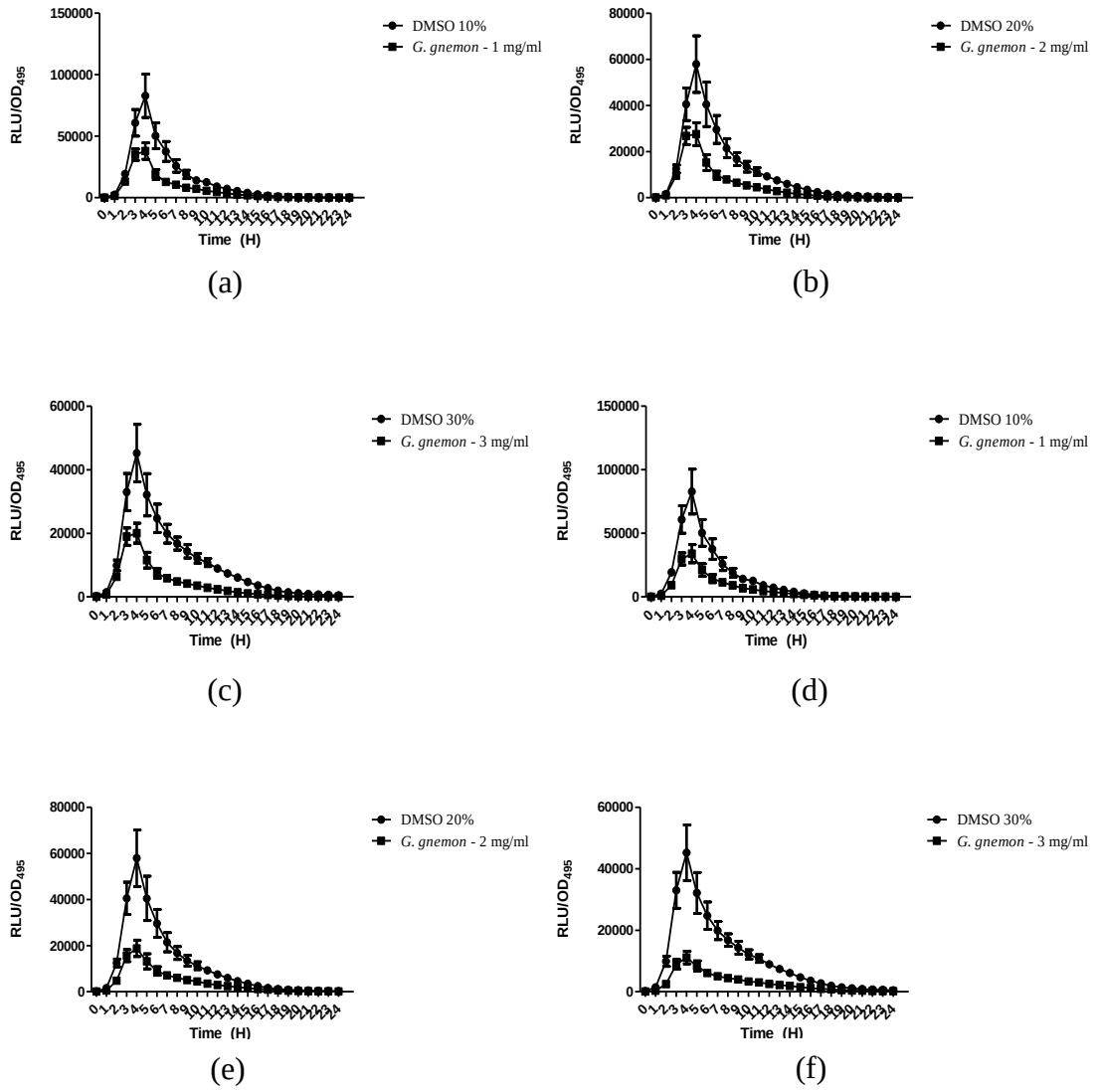


Figure 4.14: (a) *G. gnemon*-Hexane extract (1 mg/ml); (b) *G. gnemon*-Hexane extract (2 mg/ml); (c) *G. gnemon*-Hexane extract (3 mg/ml); (d) *G. gnemon*-Chloroform extract (1 mg/ml); (e) *G. gnemon*-Chloroform extract (2 mg/ml); (f) *G. gnemon*-Chloroform extract (3 mg/ml).

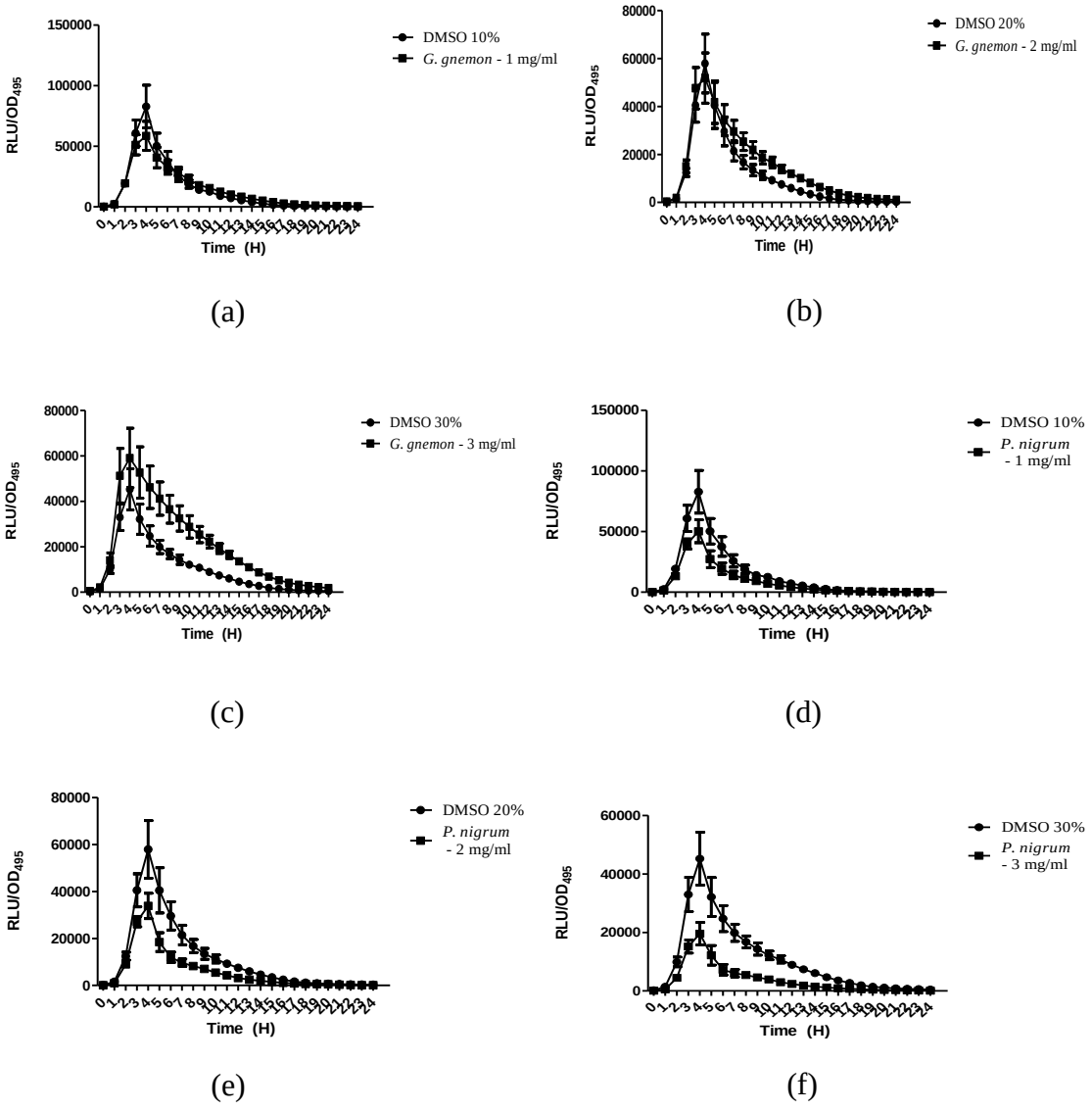


Figure 4.15: (a) *G. gnemon*-Methanol extract (1 mg/ml); (b) *G. gnemon*-Methanol extract (2 mg/ml); (c) *G. gnemon*-Methanol extract (3 mg/ml); (d) *P. nigrum*-Hexane extract (1 mg/ml); (e) *P. nigrum*-Hexane extract (2 mg/ml); (f)*P. nigrum*-Hexane extract (3 mg/ml).

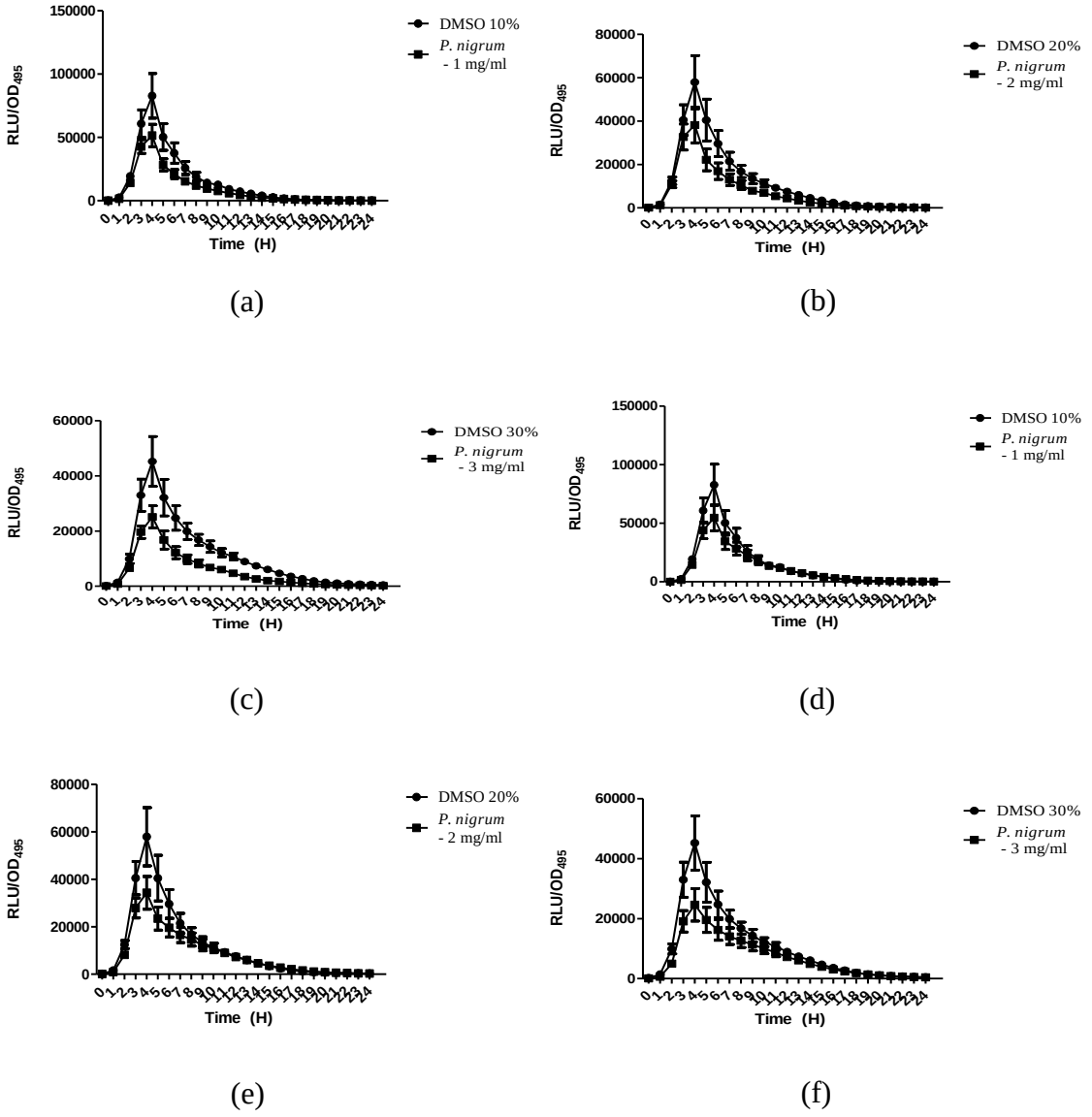


Figure 4.16: (a) *P. nigrum*-Chloroform extract (1 mg/ml); (b) *P. nigrum*-Chloroform extract (2 mg/ml); (c)*P. nigrum*-Chloroform extract (3 mg/ml); (d) *P. nigrum*-Methanol extract (1 mg/ml); (e) *P. nigrum*-Methanol extract (2 mg/ml); (f)*P. nigrum*-Methanol extract (3 mg/ml).

4.6 *P. aeruginosa* PA01 Pyocyanin Quantification Assay

Pyocyanin extracted was quantified using a spectrophotometer (UV1601, Shidmazu, Kyoto, Japan)at 520 nm. It was found that all the extracts of *M. lunu-ankenda* (Figure 4.17 (a-c)) and *P. betle* (Figure 4.17 d-f)) showed significant inhibition against pyocyanin production. Chloroform extract of *G. gnemon* (Figure 4.18 (b)) was the only extract of *G. gnemon* that inhibited pyocyanin synthesis of *P. aeruginosa* PA01 significantly. On the other hand, extracts that did not showed any significant inhibition against pyocyanin production were the hexane and methanol extract of *G. gnemon* (Figure 4.18 (a) and (c)) and all extracts of *P. nigrum* (Figure 4.18 (d-f)).

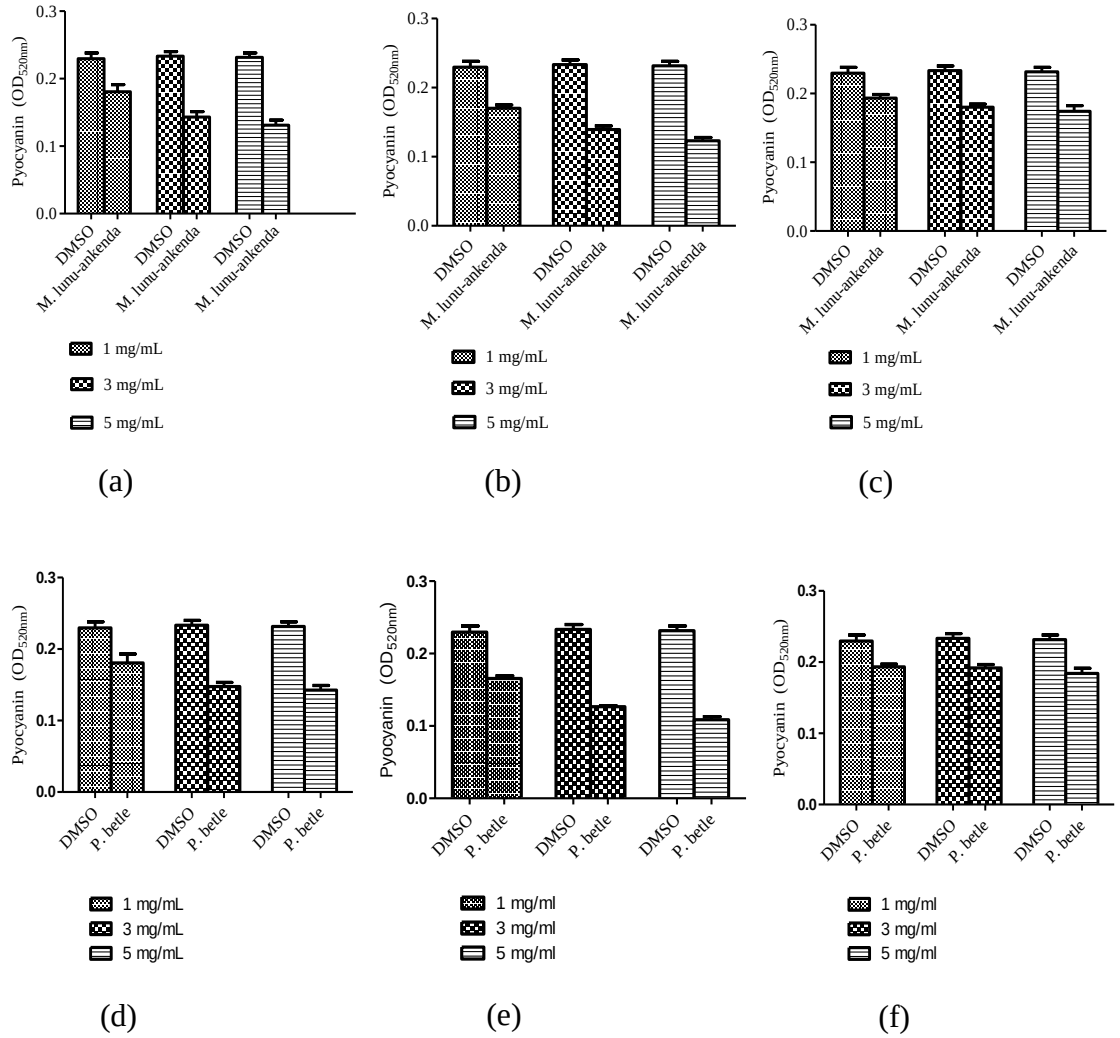


Figure 4.17: (a) *M. lunu-ankenda*-Hexane extract; (b) *M. lunu-ankenda*-Chloroform extract; (c) *M. lunu-ankenda*-Methanol extract; (d) *P. betle-Hexane* extract; (e) *P. betle*- Chloroform extract; (f) *P. betle*- Methanol extract.

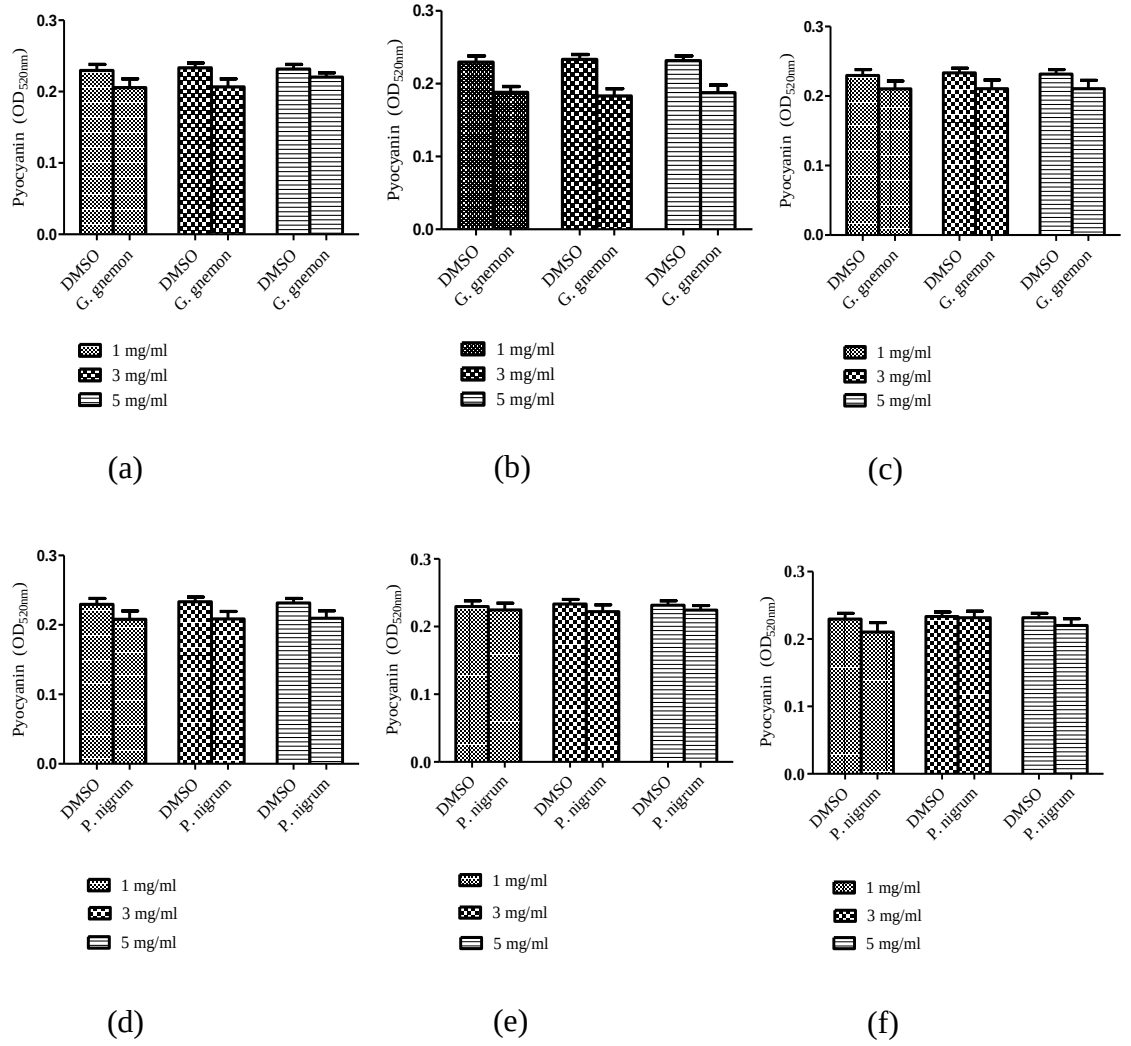


Figure 4.18: (a) *G. gnemon*-Hexane extract; (b) *G. gnemon*-Chloroform extract; (c) *G. gnemon*-Methanol extract; (d) *P. nigrum*-Hexane extract; (e) *P. nigrum*-Chloroform extract; (f) *P. nigrum*-Methanol extract.

4.7 Quantification of *P. aeruginosa* PA01 *lecA* Expression

Quantification of the *lecA* expression of *P. aeruginosa* PA01 was enabled using a luminometer (InfiniteM200,Mannerdorf,Switzerland)that detects bioluminescence due to the construction of *P. aeruginosa* PA01 *lecA::lux*. Similar to the bioluminescence assay of *E. coli* [pSB401] and *E. coli* [pSB1075], hexane, chloroform and methanol extracts of *M. lunu-ankenda*(Figure 4.19 (a-f) and Figure 4.20 (a-c)), *P. betle*(Figure 4.20 (d-f) and Figure 4.21 (a-f)), *G. gnemon*(Figure 4.22 (a-f) and Figure 4.23 (a-c)) and *P. nigrum*(Figure 4.23 (d-f) and Figure 4.24 (a-f)) were tested against *P. aeruginosa* PA01 *lecA::lux*at three ascending concentration, namely 1, 2 and 3 mg/ml. Of the 12 extracts, the chloroform and methanol extracts of *P. betle* (Figure 4.21 (a-f)) and chloroform extracts of *G. gnemon* (Figure 4.22 (d-f)) significantly reduced *lecA* expression of *P. aeruginosa*PA01 *lecA::lux*.

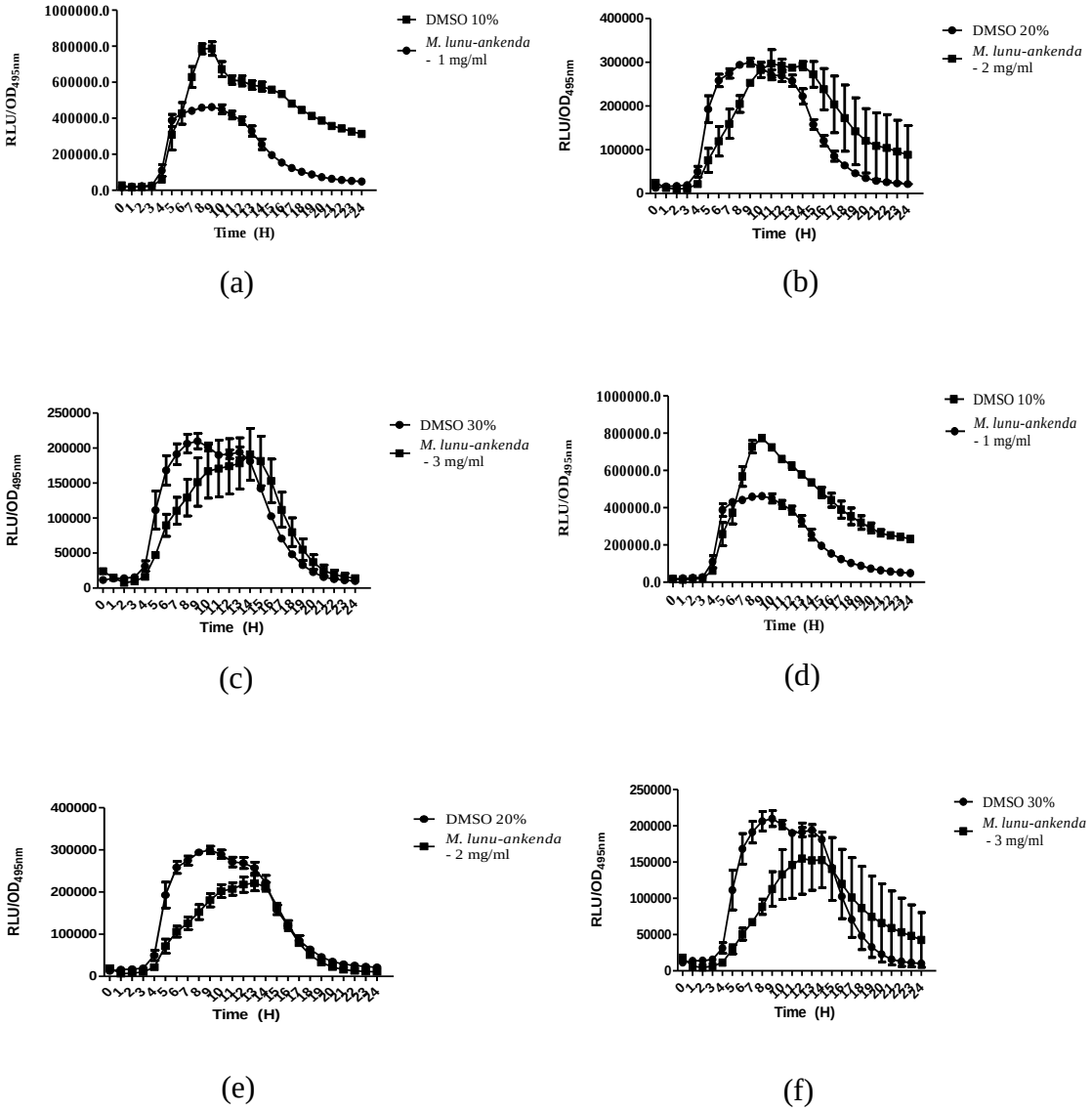


Figure 4.19: (a) *M. lunu-ankenda*-Hexane extract (1 mg/ml); (b) *M. lunu-ankenda*-Hexane extract (2 mg/ml); (c)*M. lunu-ankenda*-Hexane extract (3 mg/ml); (d) *M. lunu-ankenda*-Chloroform extract (1 mg/ml); (e) *M. lunu-ankenda*-Chloroform extract (2 mg/ml); (f)*M. lunu-ankenda*-Chloroform extract (3 mg/ml).

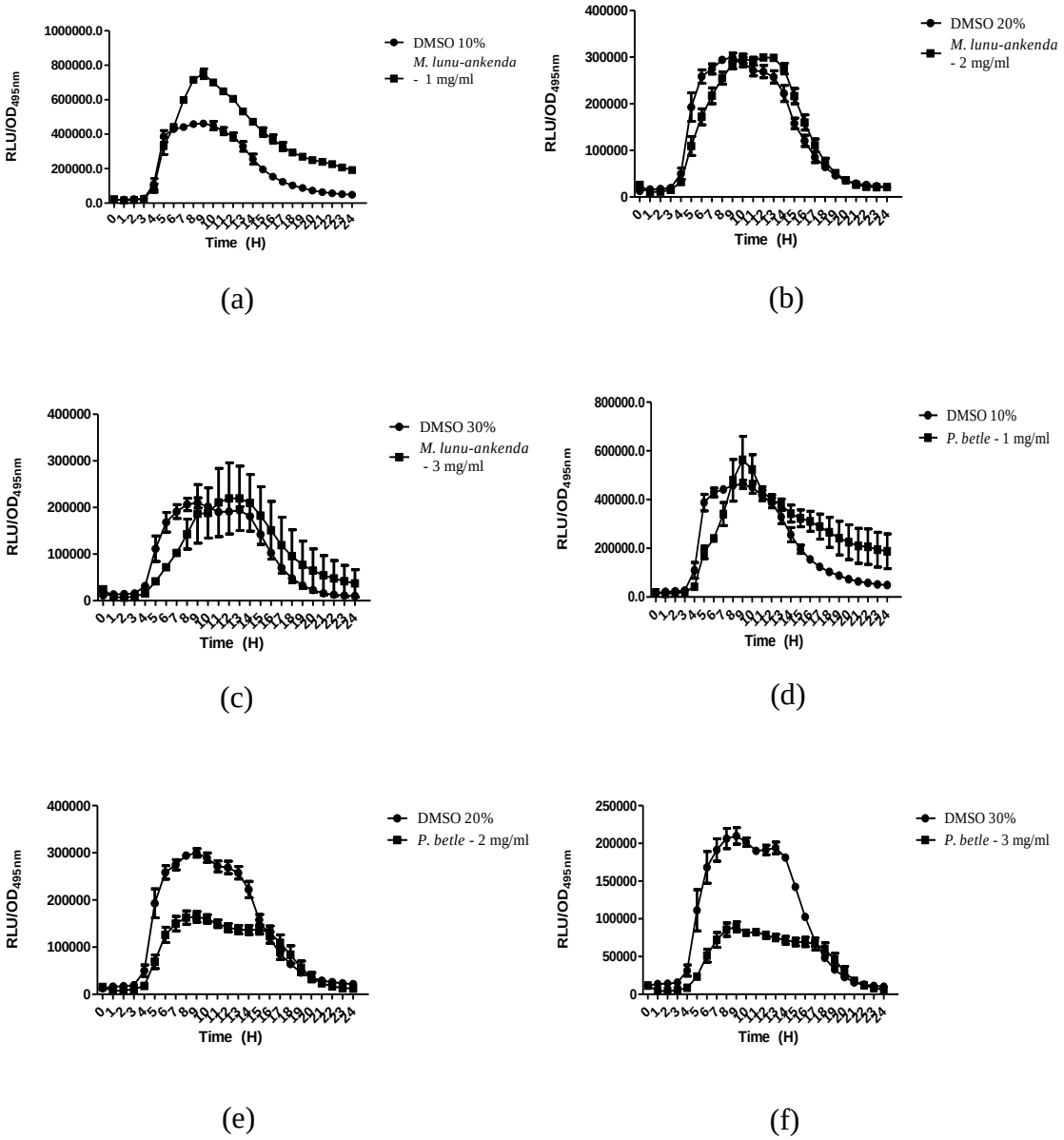


Figure 4.20: (a) *M. lunu-ankenda*-Methanol extract (1 mg/ml); (b) *M. lunu-ankenda*-Methanol extract (2 mg/ml); (c)*M. lunu-ankenda*-Methanol extract (3 mg/ml); (d) *P. betle*-Hexane extract (1 mg/ml); (e) *P. betle*-Hexane extract (2 mg/ml); (f)*P. betle*-Hexane extract (3 mg/ml).

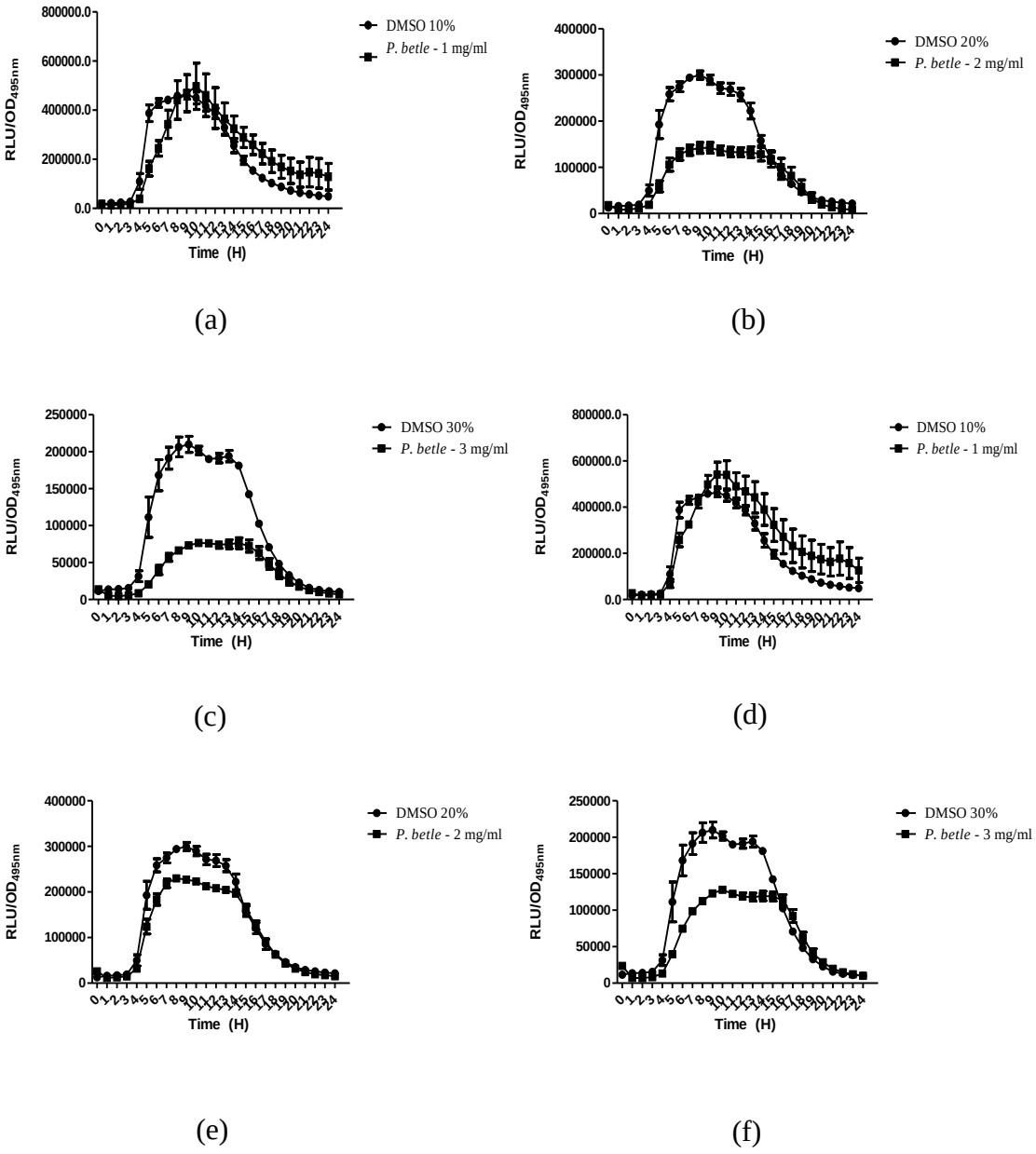


Figure 4.21: (a) *P. betle*-Chloroform extract (1 mg/ml); (b) *P. betle*- Chloroform extract (2 mg/ml); (c) *P. betle*-Chloroform extract (3 mg/ml); (d) *P. betle*-Methanol extract (1 mg/ml); (e) *P. betle*- Methanol extract (2 mg/ml); (f) *P. betle*-Methanol extract (3 mg/ml).

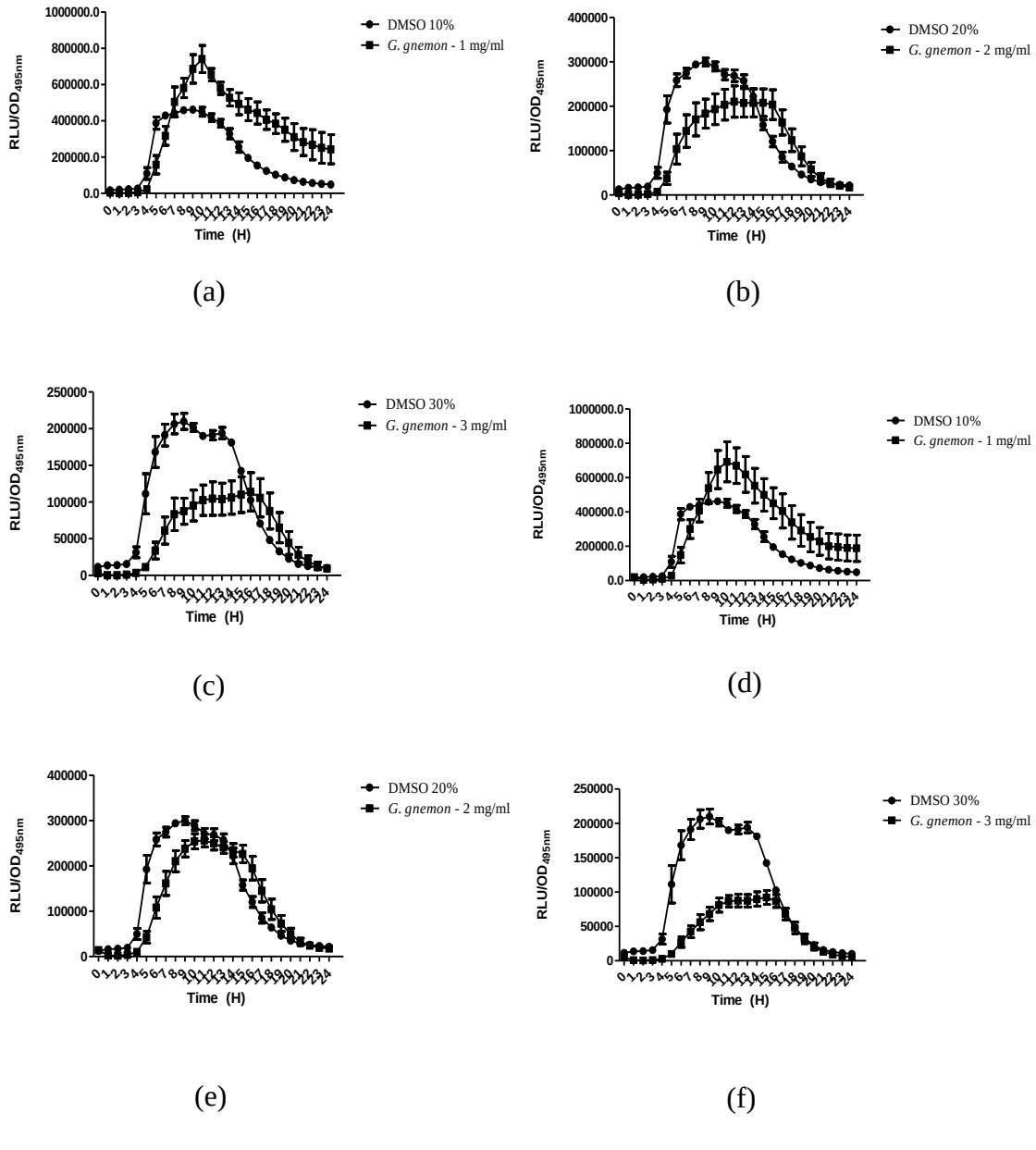


Figure 4.22: (a) *G. gnemon*-Hexane extract (1 mg/ml); (b) *G. gnemon*-Hexane extract (2 mg/ml); (c) *G. gnemon*-Hexane extract (3 mg/ml); (d) *G. gnemon*-Chloroform extract (1 mg/ml); (e) *G. gnemon*-Chloroform extract (2 mg/ml); (f) *G. gnemon*-Chloroform extract (3 mg/ml).

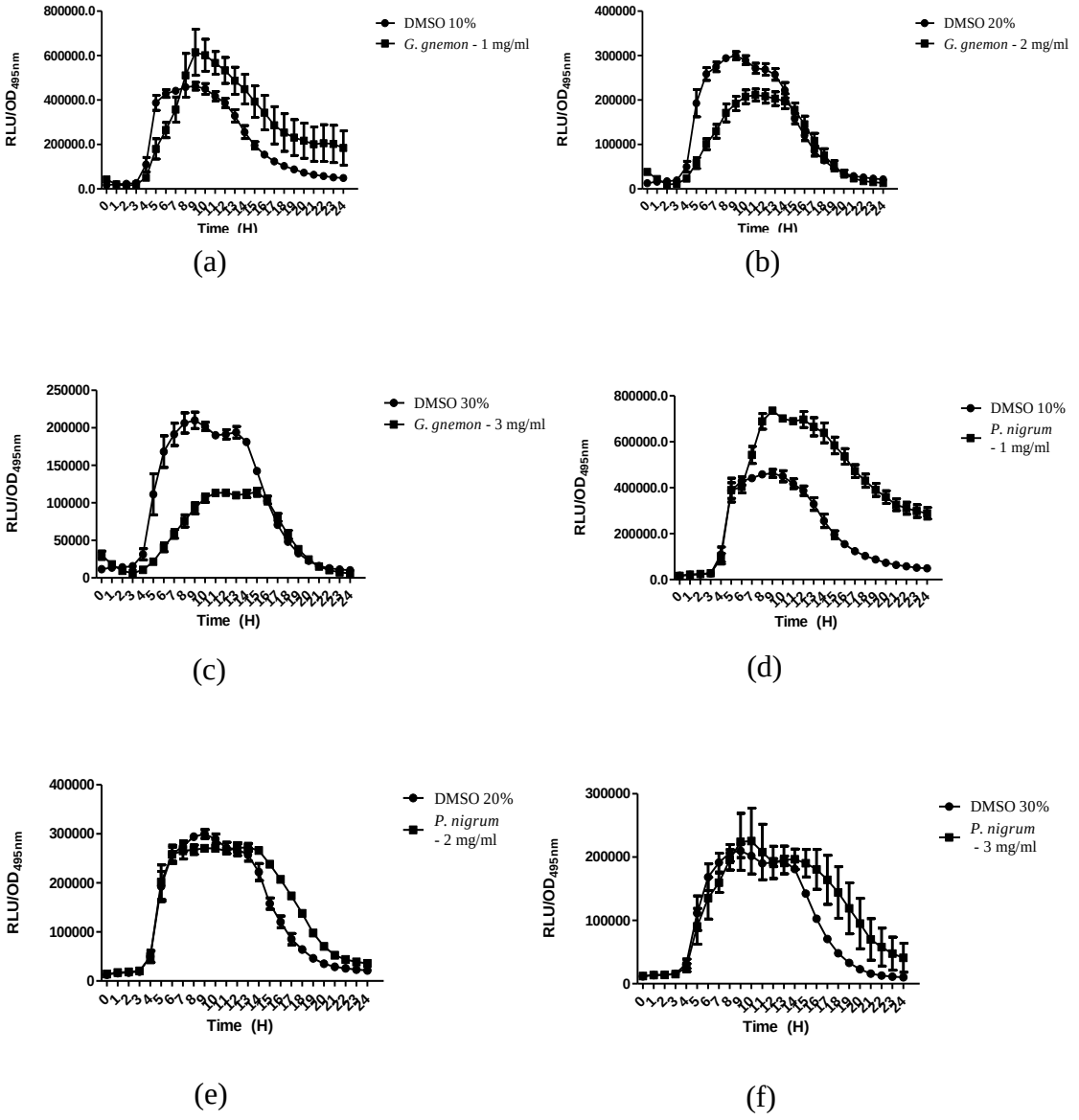


Figure 4.23: (a) *G. gnemon*-Methanol extract (1 mg/ml); (b) *G. gnemon*-Methanol extract (2 mg/ml); (c) *G. gnemon*-Methanol extract (3 mg/ml); (d) *P. nigrum*-Hexane extract (1 mg/ml); (e) *P. nigrum*- Hexane extract (2 mg/ml); (f)*P. nigrum*-Hexane extract (3 mg/ml).

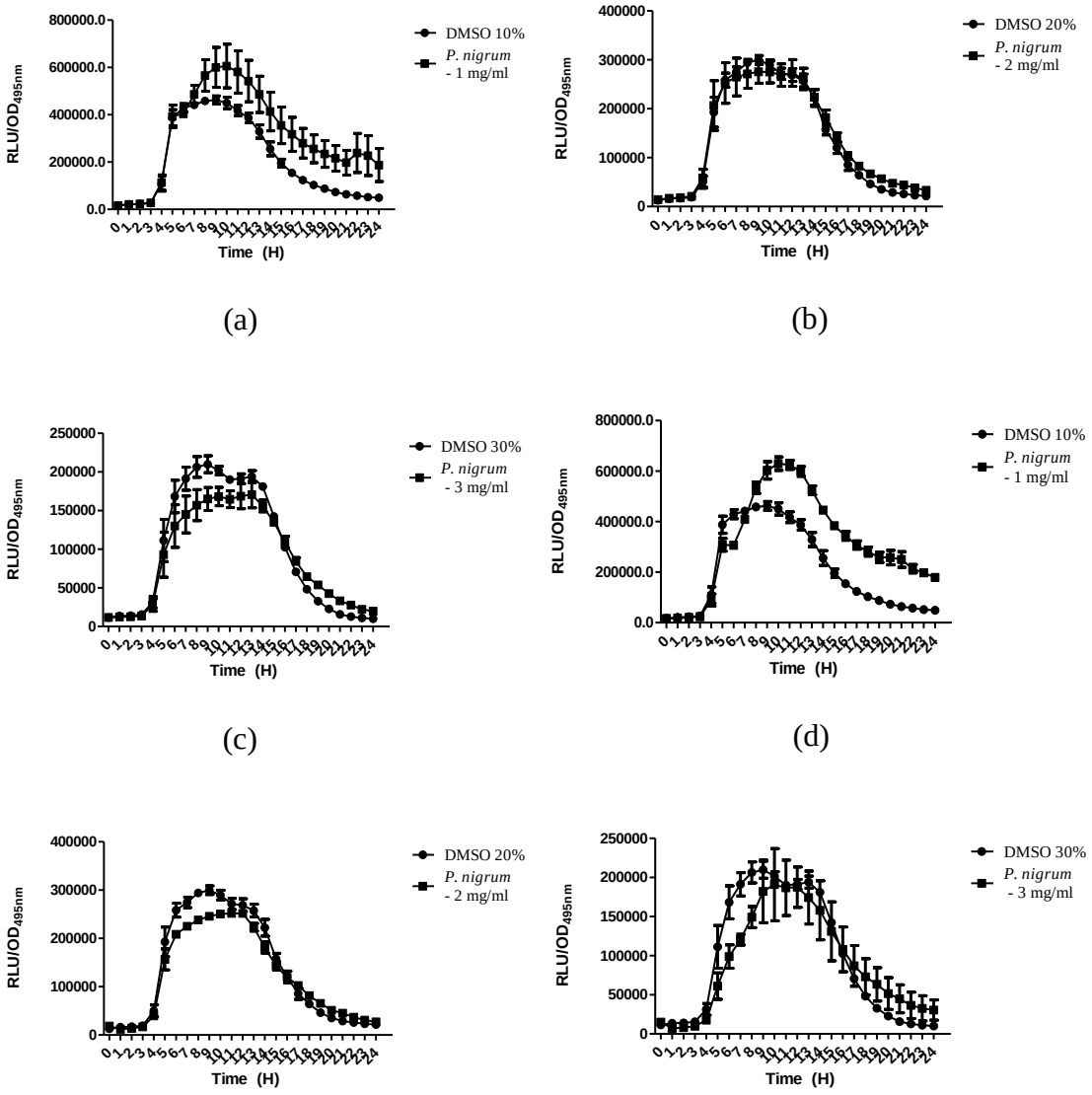


Figure 4.24: (a) *P. nigrum*- Chloroform extract (1 mg/ml); (b) *P. nigrum*-Chloroform extract (2 mg/ml); (c)*P. nigrum*-Chloroform extract (3 mg/ml); (d) *P. nigrum*-Methanol extract (1 mg/ml); (e) *P. nigrum*-Methanol extract (2 mg/ml); (f)*P. nigrum*-Methanol extract (3 mg/ml).

4.8 *P. aeruginosa* PA01 Swarming Assay

P. aeruginosa PA01 that has been loaded onto the control plate (without addition of plant extracts) swarmed until the edge of the plate after 16 h of incubation. Hexane, chloroform and methanol extract of *M. lunu-ankenda* (Figure 4.25), *P. betle* (Figure 4.26), *G. gnemon* (Figure 4.27) and *P. nigrum* (Figure 4.28) were seeded into the swarming agar and the extent of swarming inhibition exerted by these extracts was compared with the control plate. *M. lunu-ankenda*-Chloroform (Figure 4.25 (f-h)) and *P. betle*-Methanol (Figure 4.26 (i-k)) extracts display observable inhibition against swarming motility of *P. aeruginosa* PA01. Agar that has been seeded with these two extracts has successfully inhibited the swarming motility of *P. aeruginosa* PA01 as the bacteria did not swarm until the edge of the plate and just formed a smaller protrusion from its original position as compared with other plant extracts.

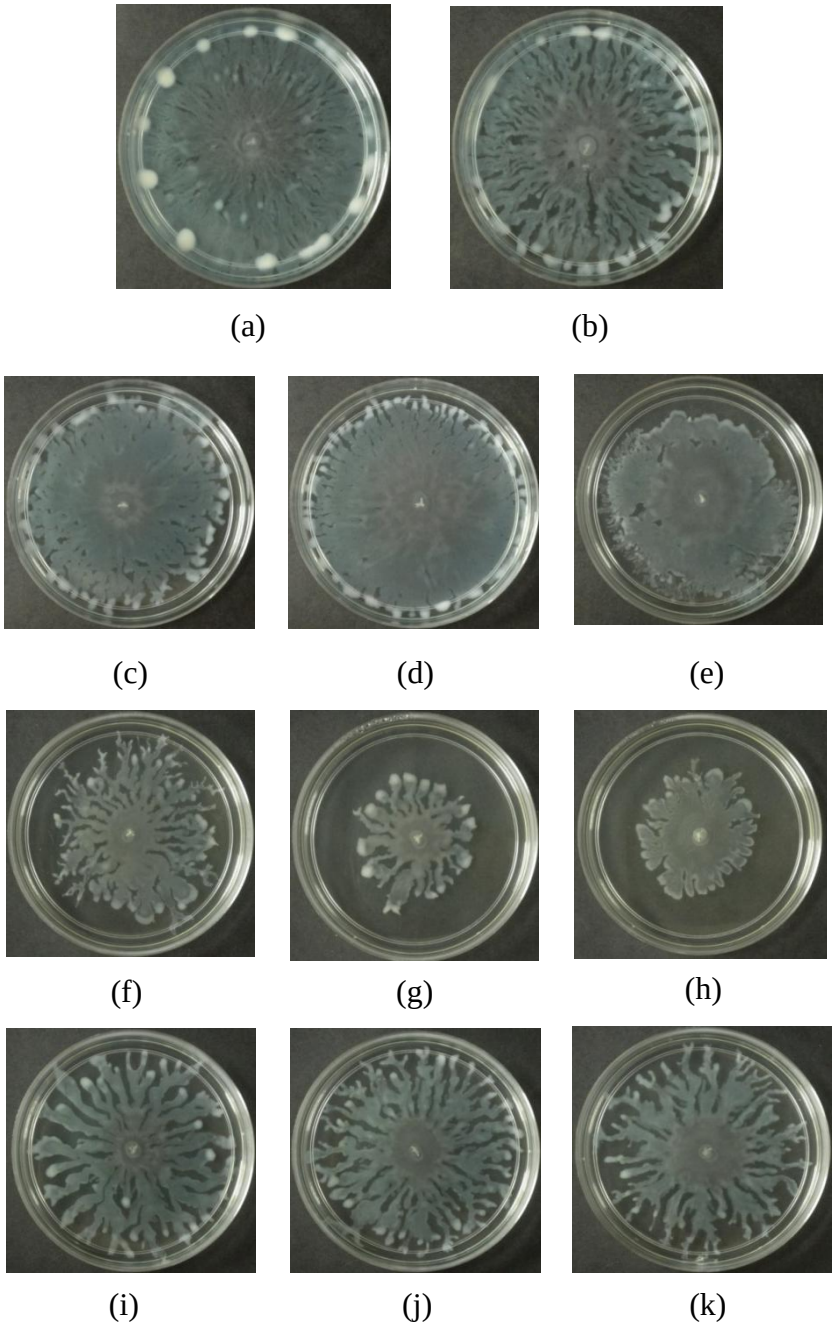


Figure 4.25: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 30% (v/v, negative control);*M. lunu-ankenda*-Hexane extracts of (c) 1 mg/ml, (d) 2 mg/ml and (e) 3 mg/ml; *M. lunu-ankenda*-Chloroform extracts of (f) 1 mg/ml, (g) 2 mg/ml and (h) 3 mg/ml; and *M. lunu-ankenda*-Methanol extracts of (i) 1 mg/ml, (j) 2 mg/ml and (k) 3 mg/ml.

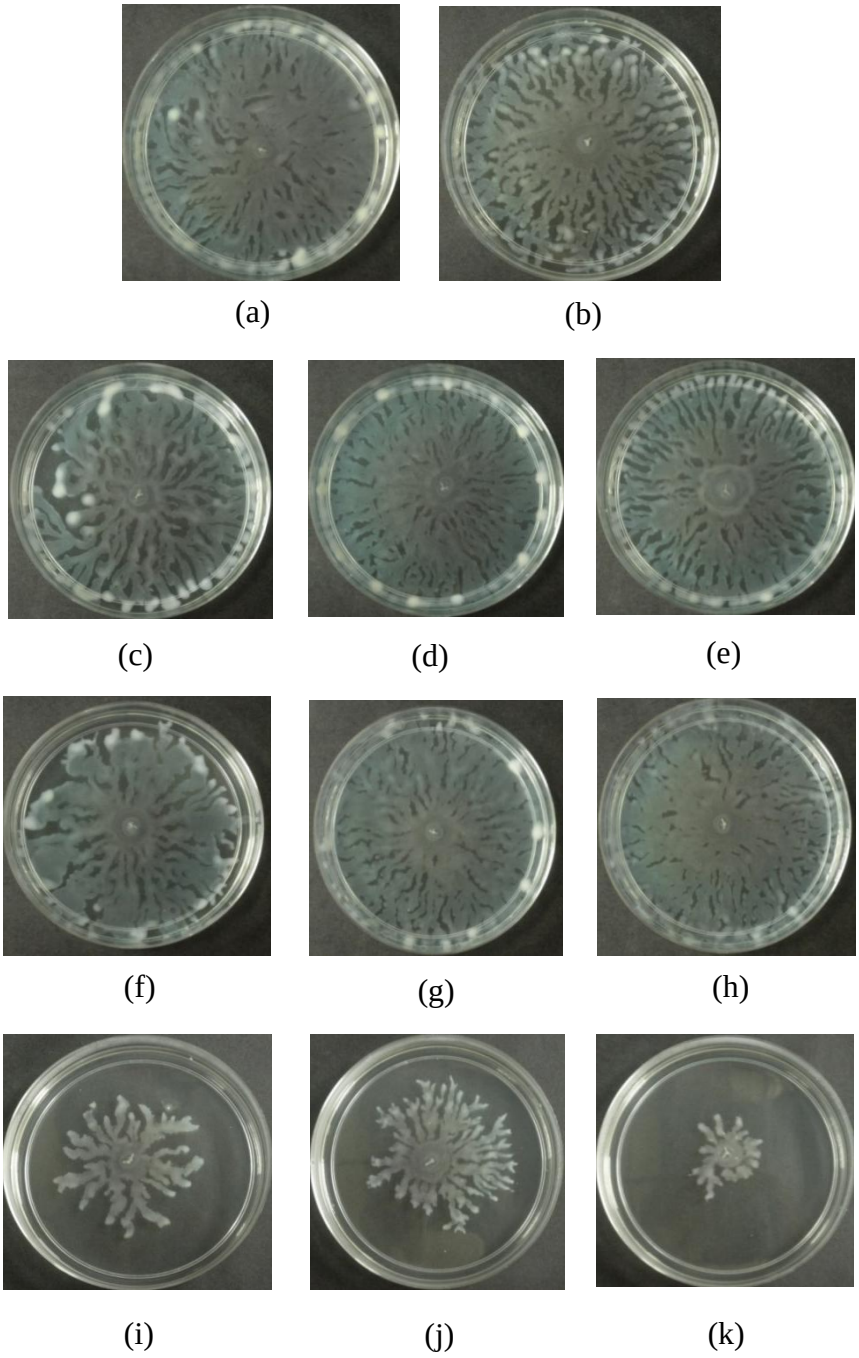


Figure 4.26: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 30% (v/v, negative control);*P. betle*-Hexane extracts of (c) 1 mg/ml, (d) 2 mg/ml and (e) 3 mg/ml; *P. betle*-Chloroform extracts of (f) 1 mg/ml, (g) 2 mg/ml and (h) 3 mg/ml; and *P. betle*-Methanol extracts of (i) 1 mg/ml, (j) 2 mg/ml and (k) 3 mg/ml.

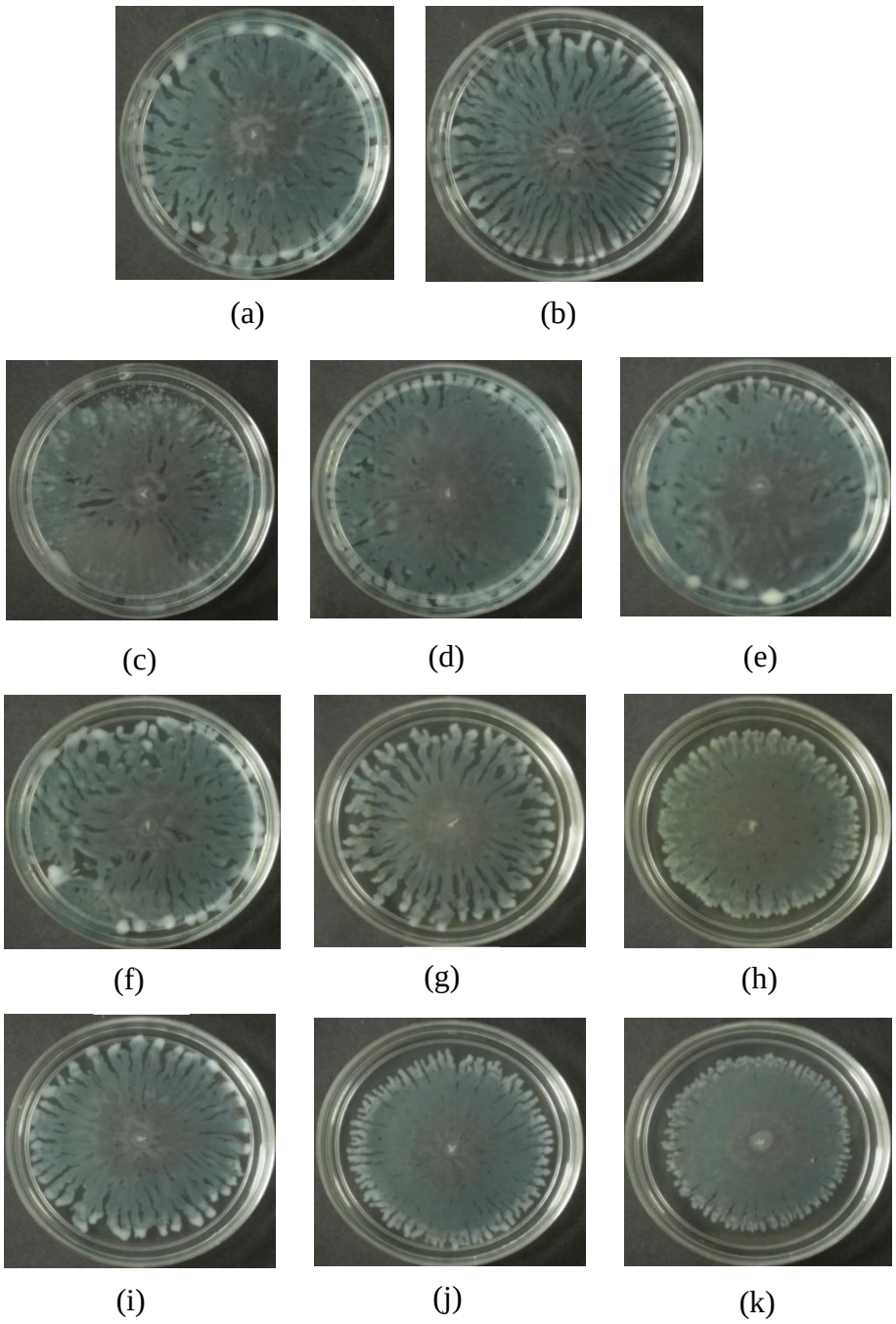


Figure 4.27: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 30% (v/v, negative control); *G. gnemon*-Hexane extracts of (c) 1 mg/ml, (d) 2 mg/ml and (e) 3 mg/ml; *G. gnemon*-Chloroform extracts of (f) 1 mg/ml, (g) 2 mg/ml and (h) 3 mg/ml; and *G. gnemon*-Methanol extracts of (i) 1 mg/ml, (j) 2 mg/ml and (k) 3 mg/ml.

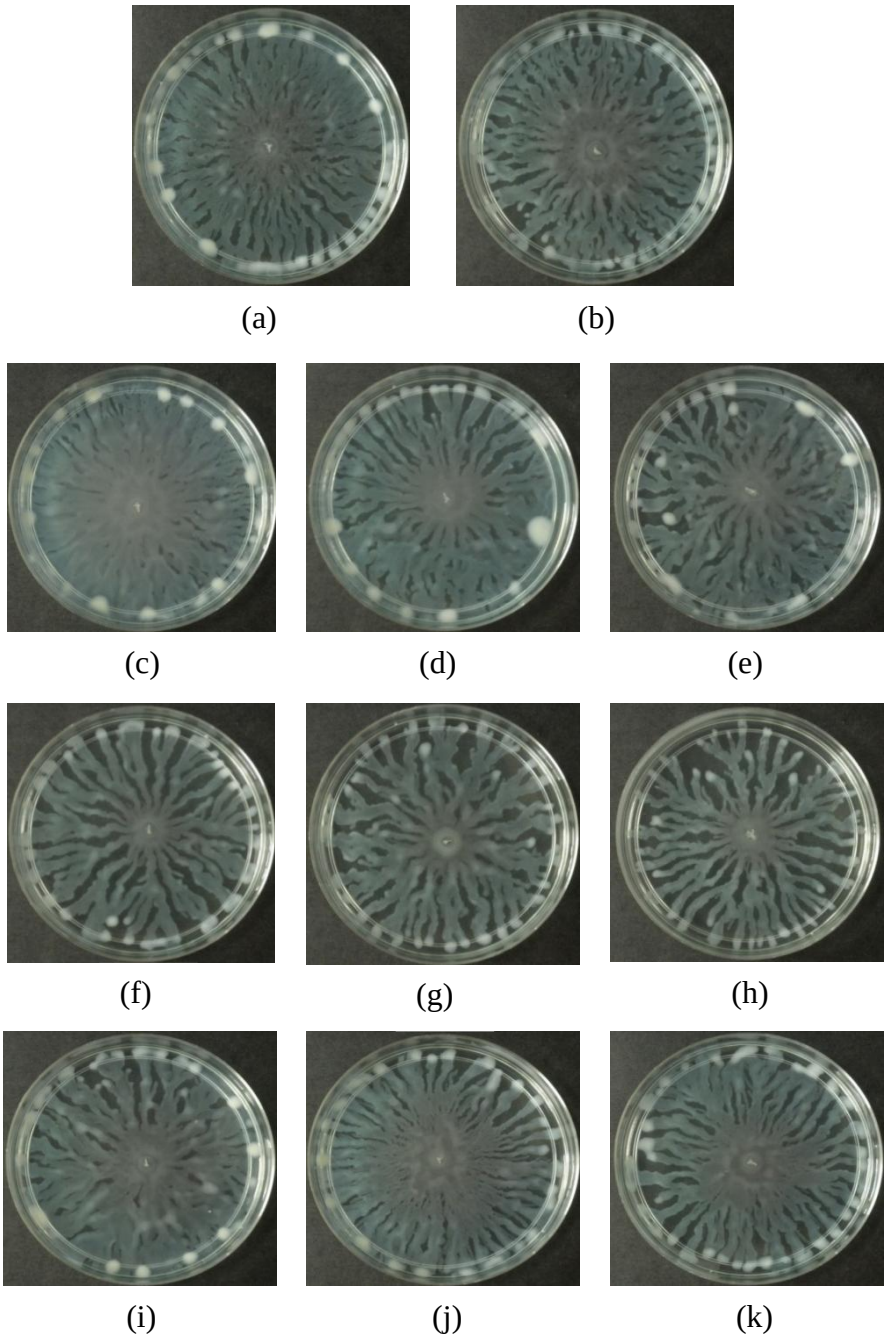


Figure 4.28: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 30% (v/v, negative control); *P. nigrum*-Hexane extracts of (c) 1 mg/ml, (d) 2 mg/ml and (e) 3 mg/ml; *P. nigrum*-Chloroform extracts of (f) 1 mg/ml, (g) 2 mg/ml and (h) 3 mg/ml; and *P. nigrum*-Methanol extracts of (i) 1 mg/ml, (j) 2 mg/ml and (k) 3 mg/ml.

4.9 Fractions Collected from *M. lunu-ankenda* Chloroform Extract

A total of 14 fractions were collected over the course of 40 min at 210 nm (Figure 4.29). No collection was made from minute 0-4 as no peak was observed.

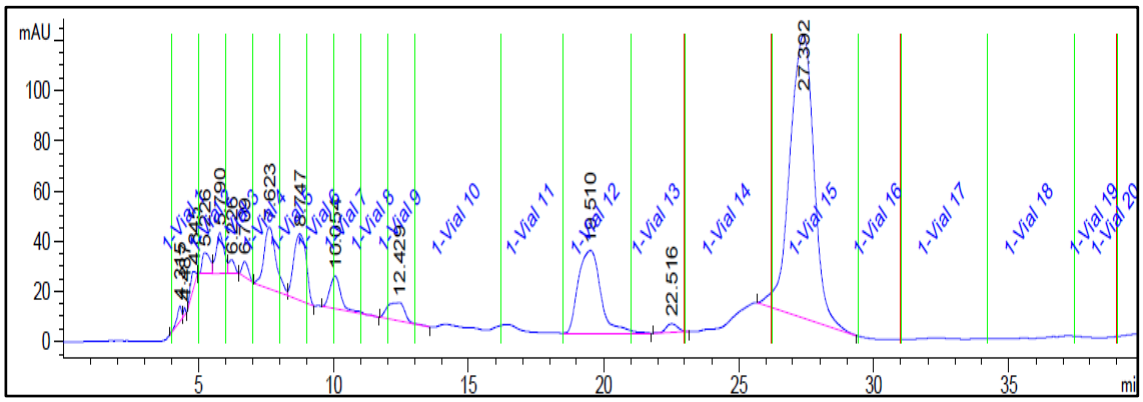


Figure 4.29: Chromatogram of *M. lunu-ankenda* chloroform extract after 40 min.

4.10 Bioluminescence Assay of *P. aeruginosa*PA01 *lecA::lux* on Fractions of *M. lunu-ankenda* Chloroform Extract

The fractions collected from *M. lunu-ankenda* were tested against *P. aeruginosa* PA01 *lecA::lux* at three concentrations, namely 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml (Figure 4.30- Figure 4.36). None of the fraction of *M. lunu-ankenda*showed significant inhibition against *P. aeruginosa* PA01 *lecA::lux.*

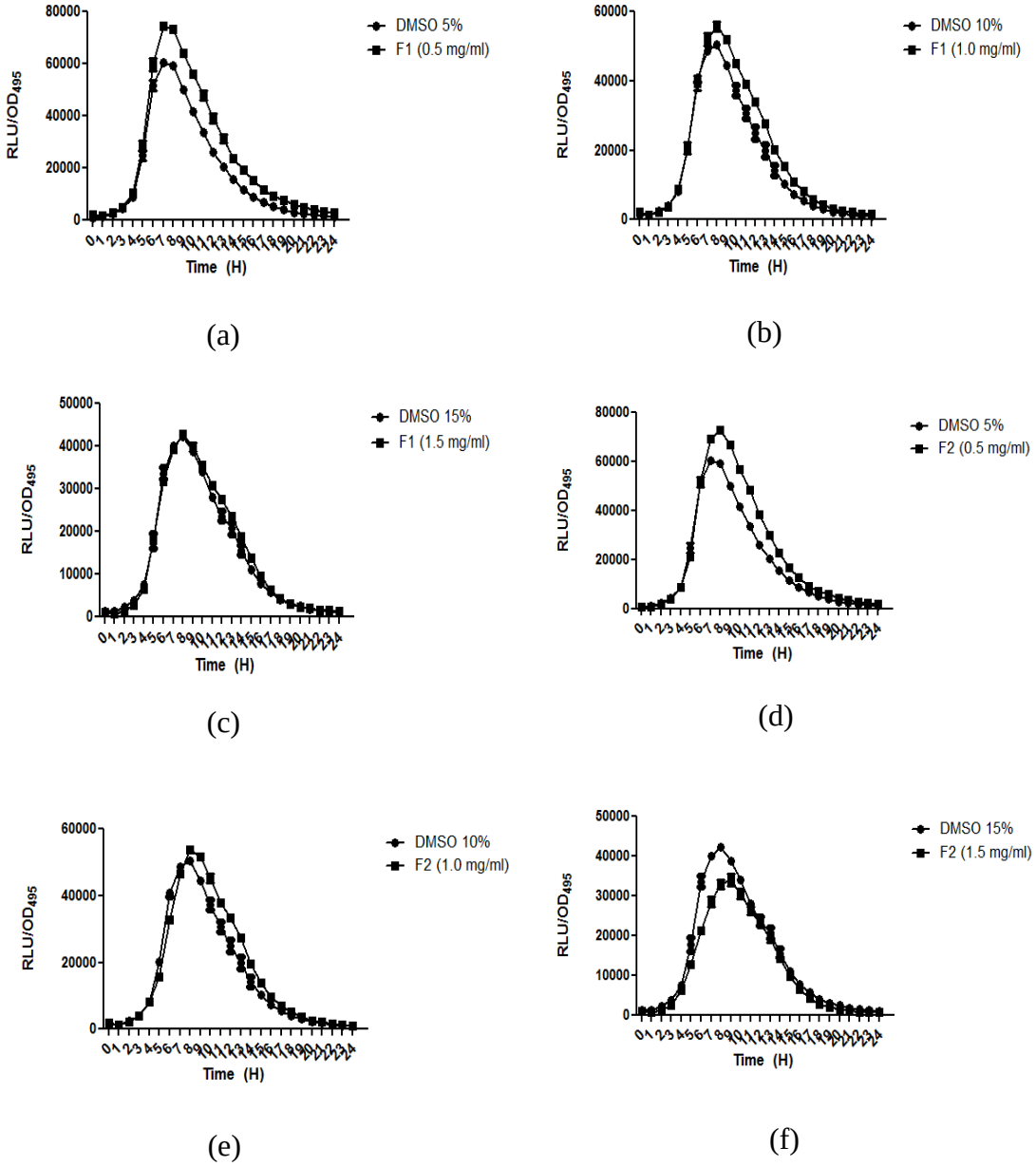


Figure 4.30: (a) Fraction 1 (0.5 mg/ml); (b) Fraction 1 (1.0 mg/ml); (c) Fraction 1 (1.5 mg/ml); (d) Fraction 2 (0.5 mg/ml); (e) Fraction 2 (1.0 mg/ml); (f) Fraction 2 (1.5 mg/ml).

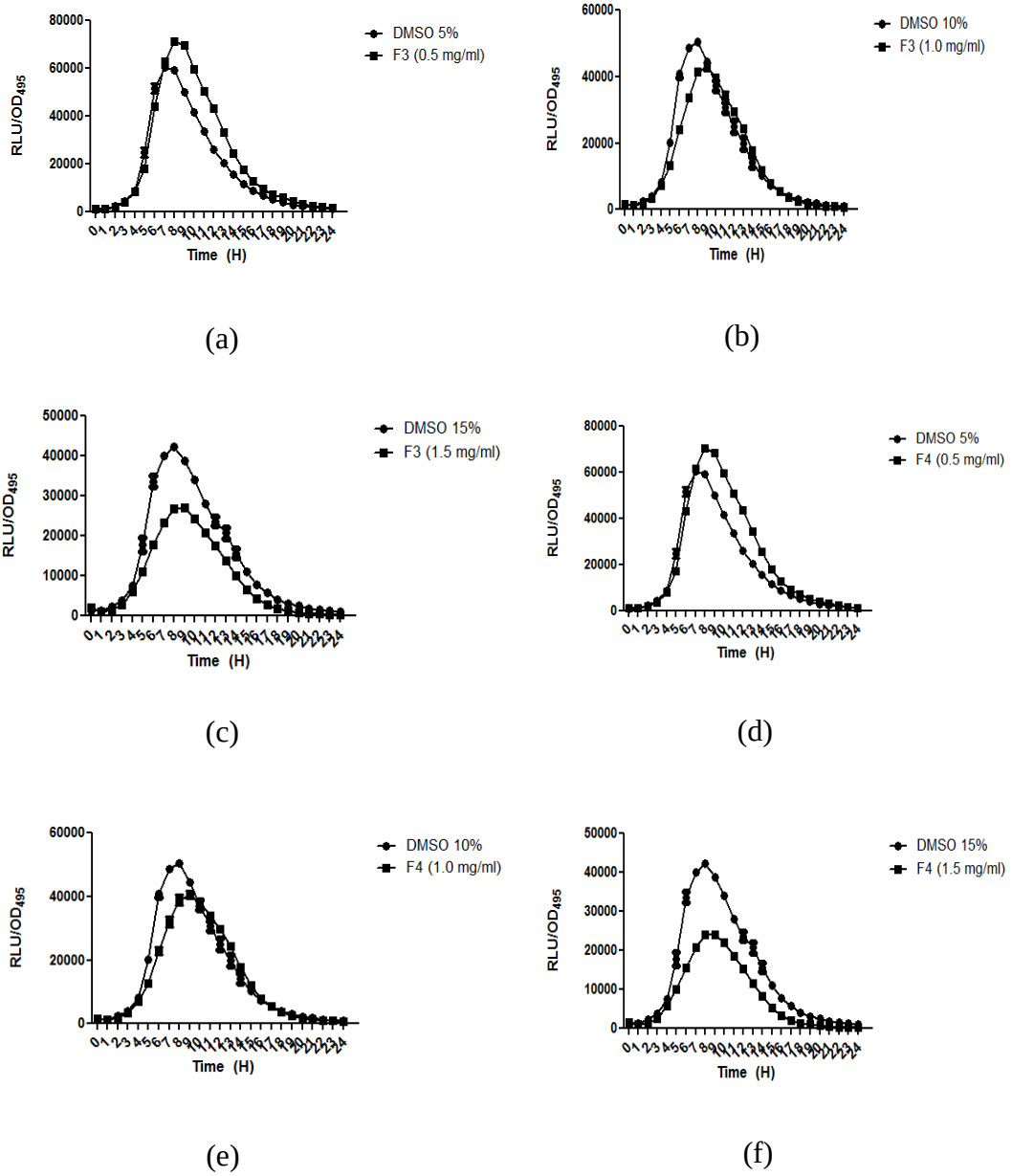


Figure 4.31: (a) Fraction 3 (0.5 mg/ml); (b) Fraction 3 (1.0 mg/ml); (c) Fraction 3 (1.5 mg/ml); (d) Fraction 4 (0.5 mg/ml); (e) Fraction 4 (1.0 mg/ml); (f) Fraction 4 (1.5 mg/ml).

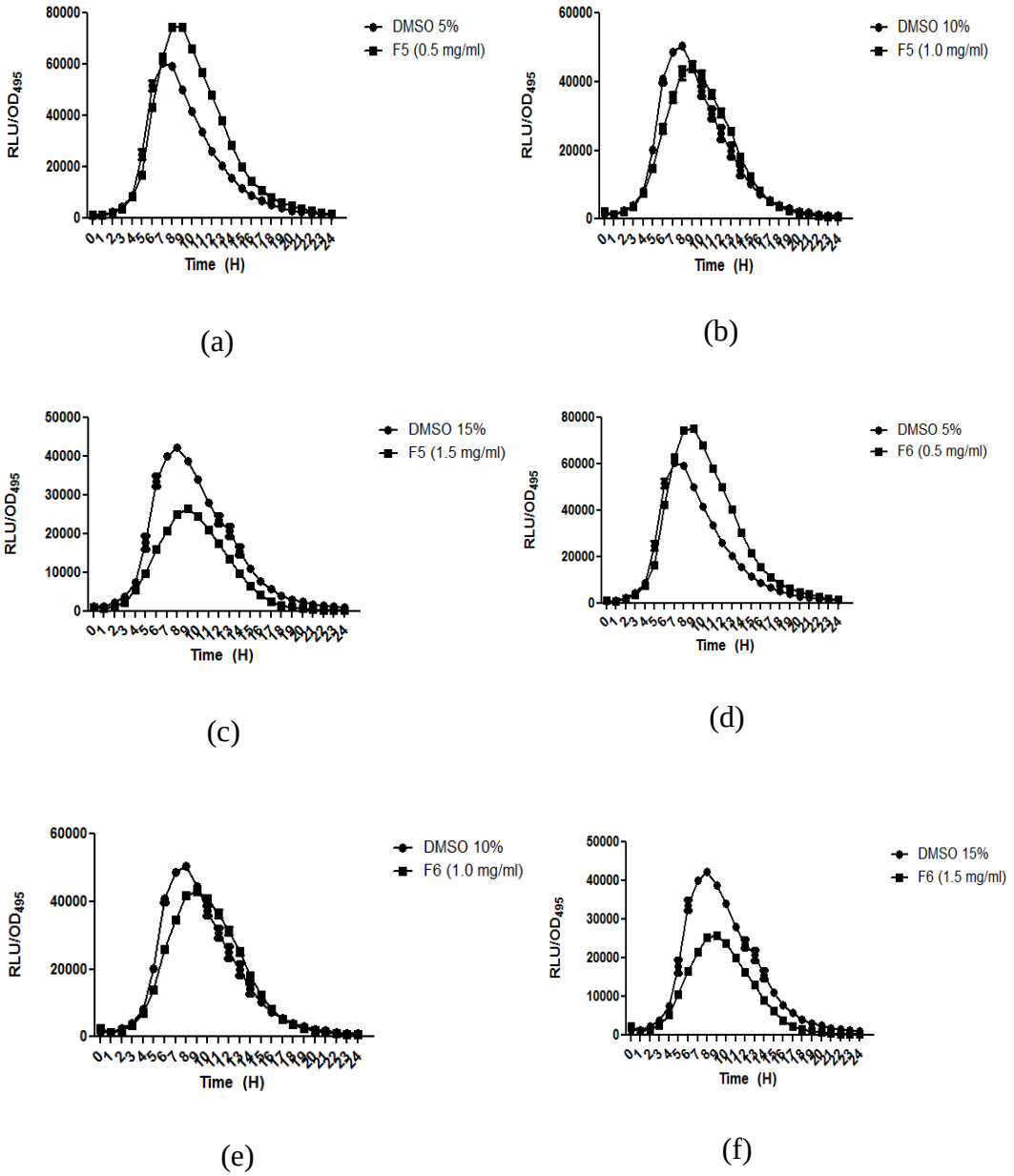


Figure 4.32: (a) Fraction 5 (0.5 mg/ml); (b) Fraction 5 (1.0 mg/ml); (c) Fraction 5 (1.5 mg/ml); (d) Fraction 6 (0.5 mg/ml); (e) Fraction 6 (1.0 mg/ml); (f) Fraction 6 (1.5 mg/ml).

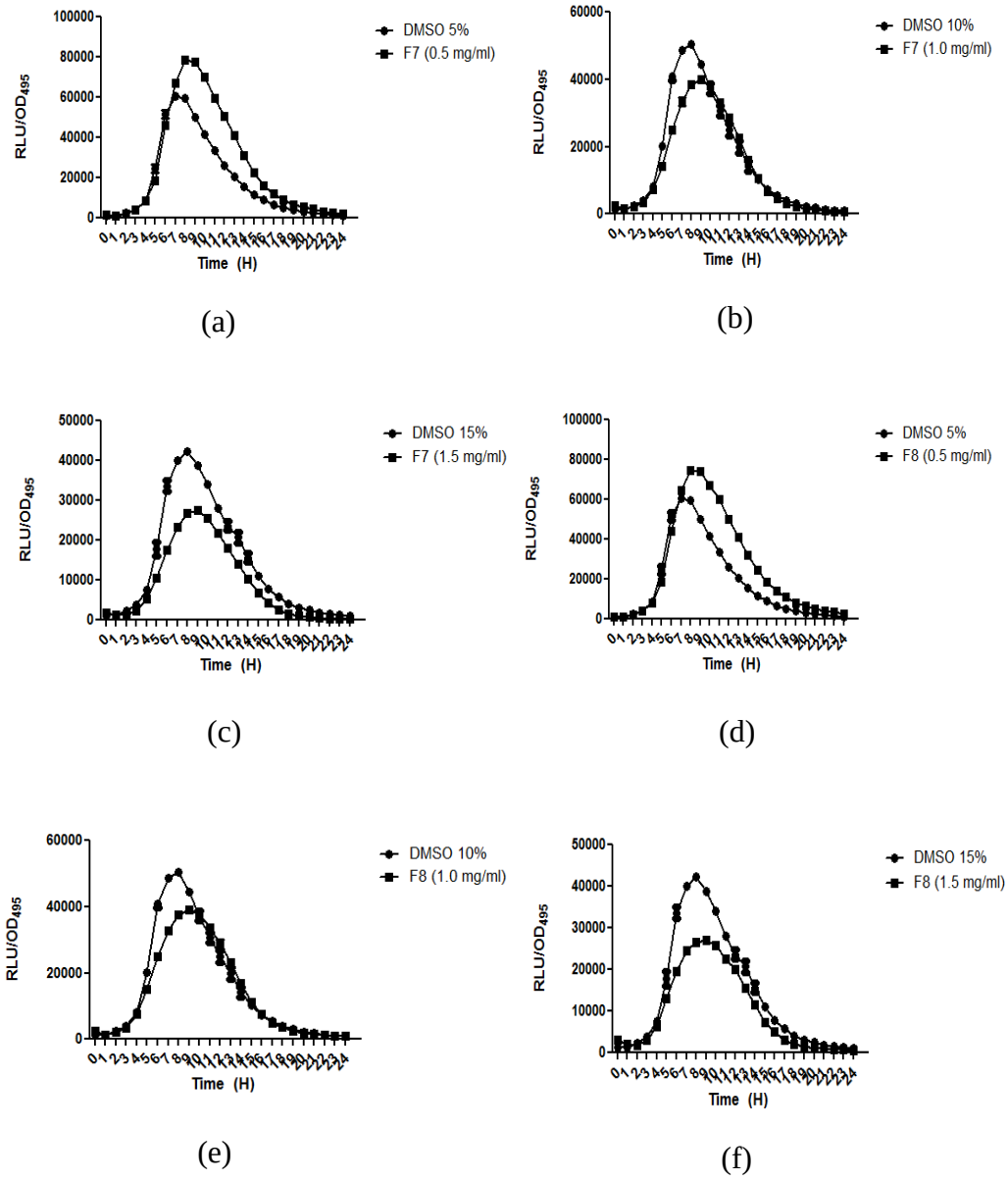


Figure 4.33: (a) Fraction 7 (0.5 mg/ml); (b) Fraction 7 (1.0 mg/ml); (c) Fraction 7 (1.5 mg/ml); (d) Fraction 8 (0.5 mg/ml); (e) Fraction 8 (1.0 mg/ml); (f) Fraction 8 (1.5 mg/ml).

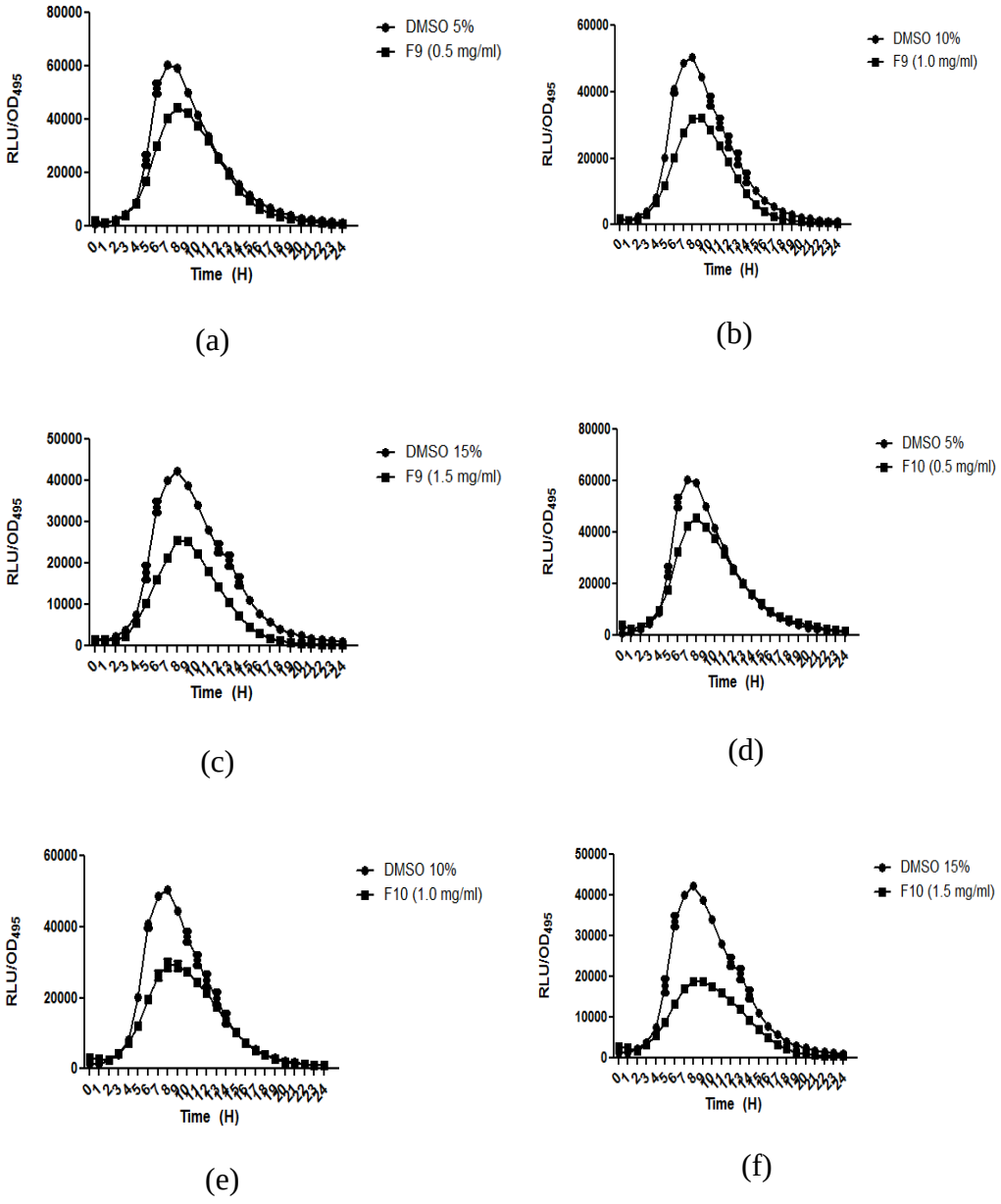


Figure 4.34: (a) Fraction 9 (0.5 mg/ml); (b) Fraction 9 (1.0 mg/ml); (c) Fraction 9 (1.5 mg/ml); (d) Fraction 10 (0.5 mg/ml); (e) Fraction 10 (1.0 mg/ml); (f) Fraction 10 (1.5 mg/ml).

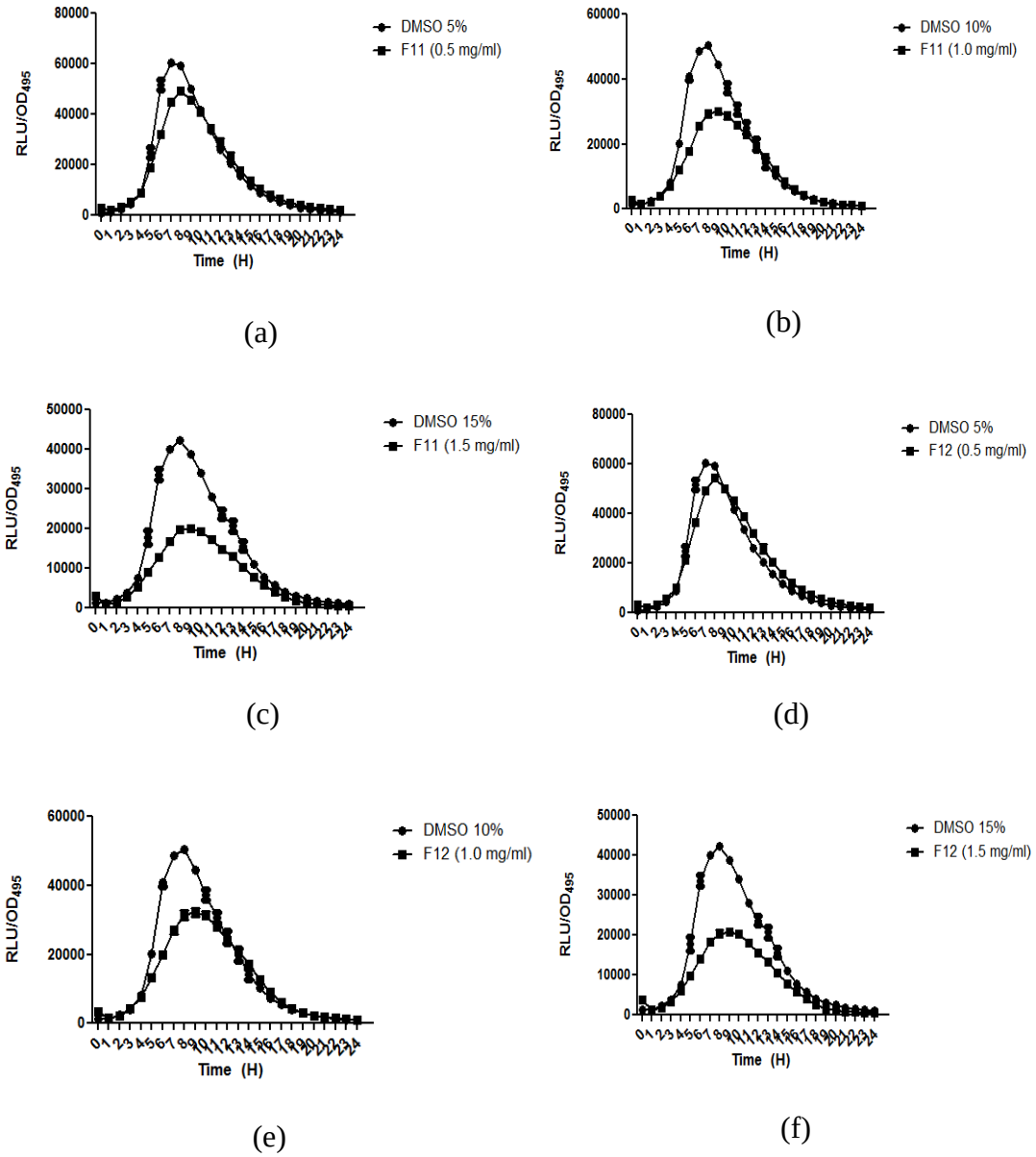


Figure 4.35: (a) Fraction 11 (0.5 mg/ml); (b) Fraction 11 (1.0 mg/ml); (c) Fraction 11 (1.5 mg/ml); (d) Fraction 12 (0.5 mg/ml); (e) Fraction 12 (1.0 mg/ml); (f) Fraction 12 (1.5 mg/ml).

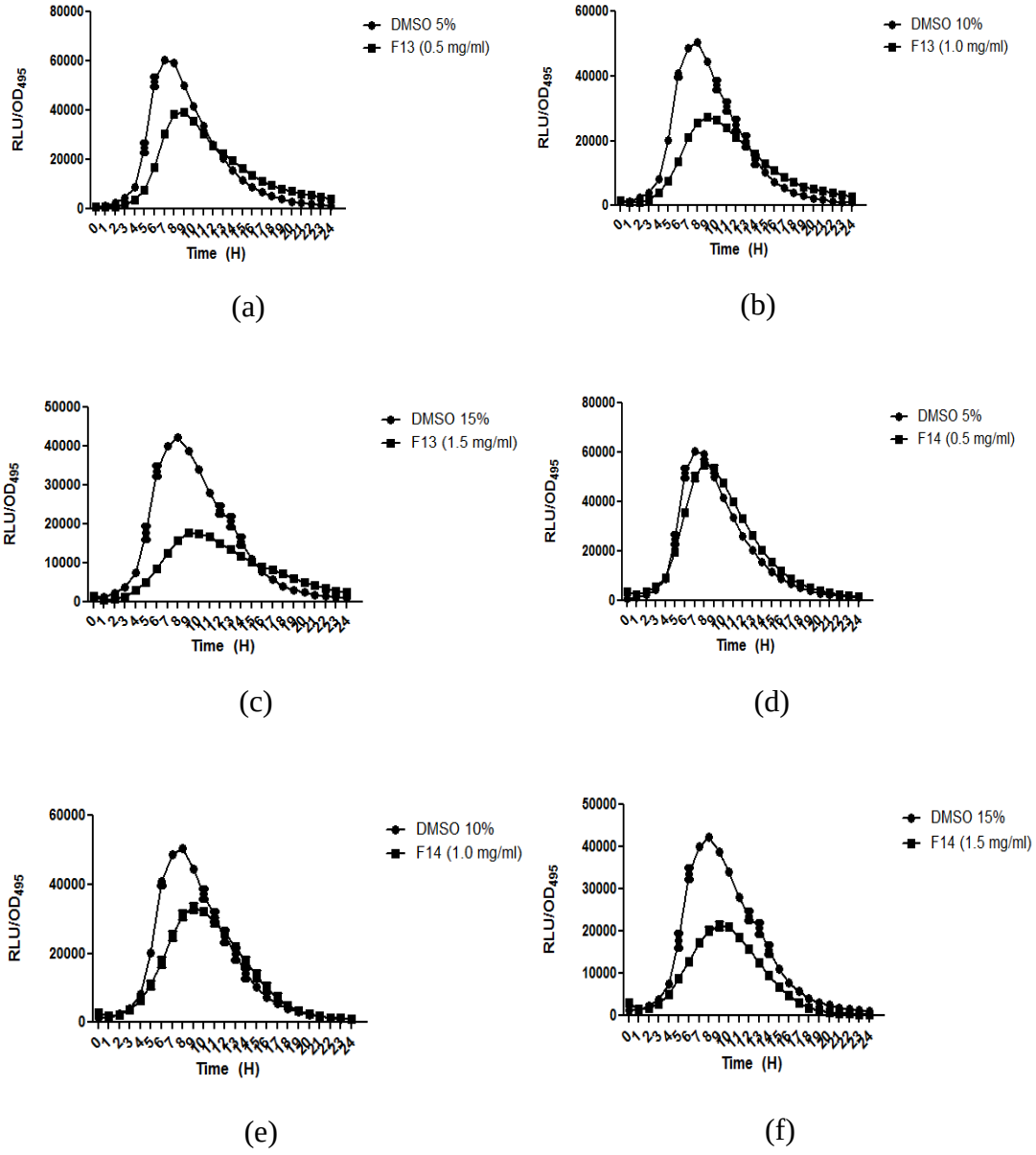


Figure 4.36: (a) Fraction 13 (0.5 mg/ml); (b) Fraction 13 (1.0 mg/ml); (c) Fraction 13 (1.5 mg/ml); (d) Fraction 14 (0.5 mg/ml); (e) Fraction 14 (1.0 mg/ml); (f) Fraction 14 (1.5 mg/ml).

4.11 Bioluminescence Assay of *E. coli* [pSB401] on Fractions of *M. lunu-ankenda* Chloroform Extract

Of the 14 fractions of *M. lunu-ankenda*(Figure 4.37- Figure 4.43) that has been tested against *E. coli* [pSB401], only Fraction 4 at 1.5 mg/ml (Figure 4.38 (f)), Fraction 11 at 1.0 mg/ml and 1.5 mg/ml (Figure 4.42 (b) and (c)), Fraction 12 at 1.5 mg/ml (Figure 4.42 (f)), Fraction 13 at all concentrations (Figure 4.43 (a-c)) and Fraction 14 at 1.5 mg/ml (Figure 4.43 (f)) gave significant inhibition.

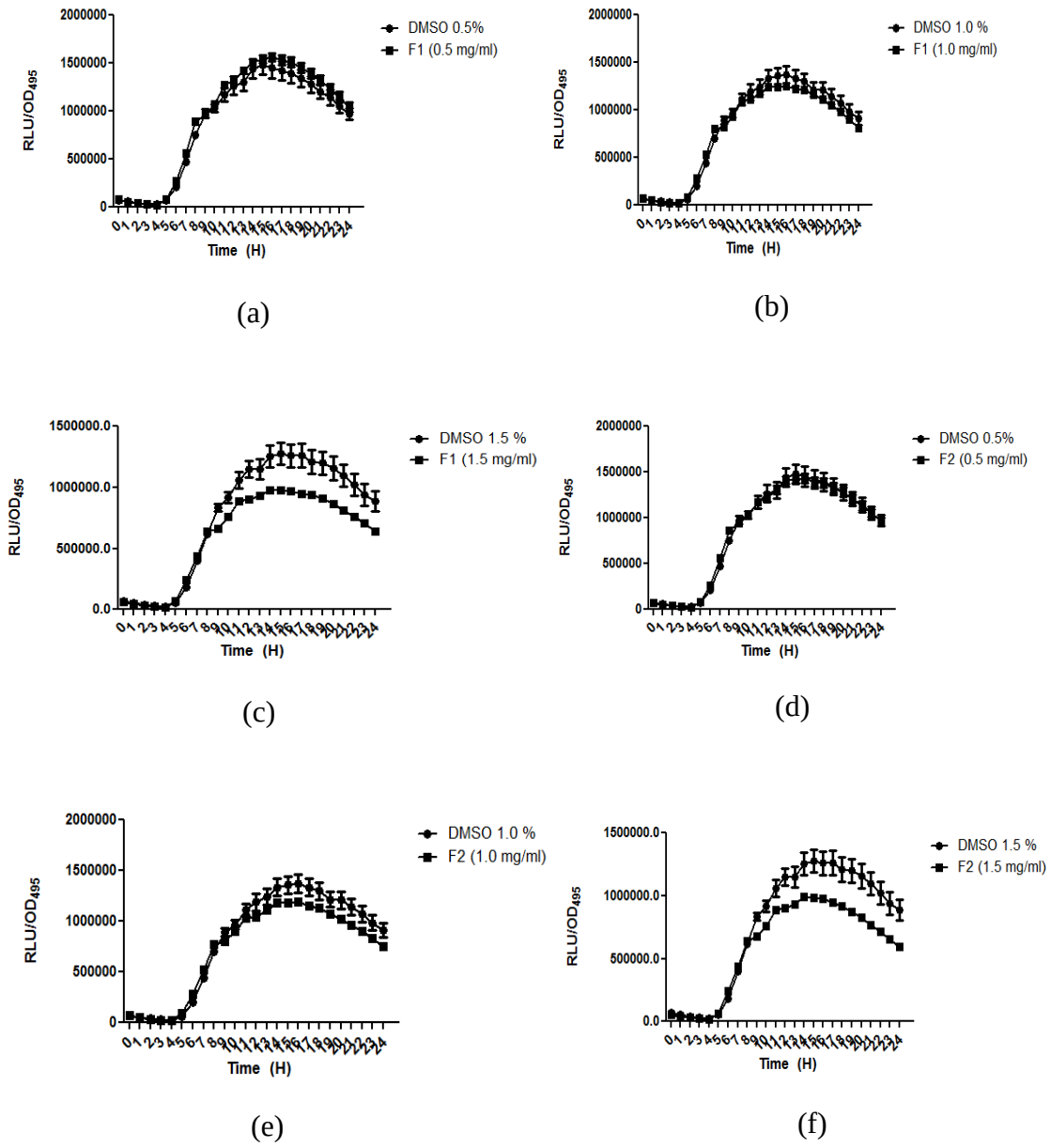


Figure 4.37: (a) Fraction 1 (0.5 mg/ml); (b) Fraction 1 (1.0 mg/ml); (c) Fraction 1 (1.5 mg/ml); (d) Fraction 2 (0.5 mg/ml); (e) Fraction 2 (1.0 mg/ml); (f) Fraction 2 (1.5 mg/ml).

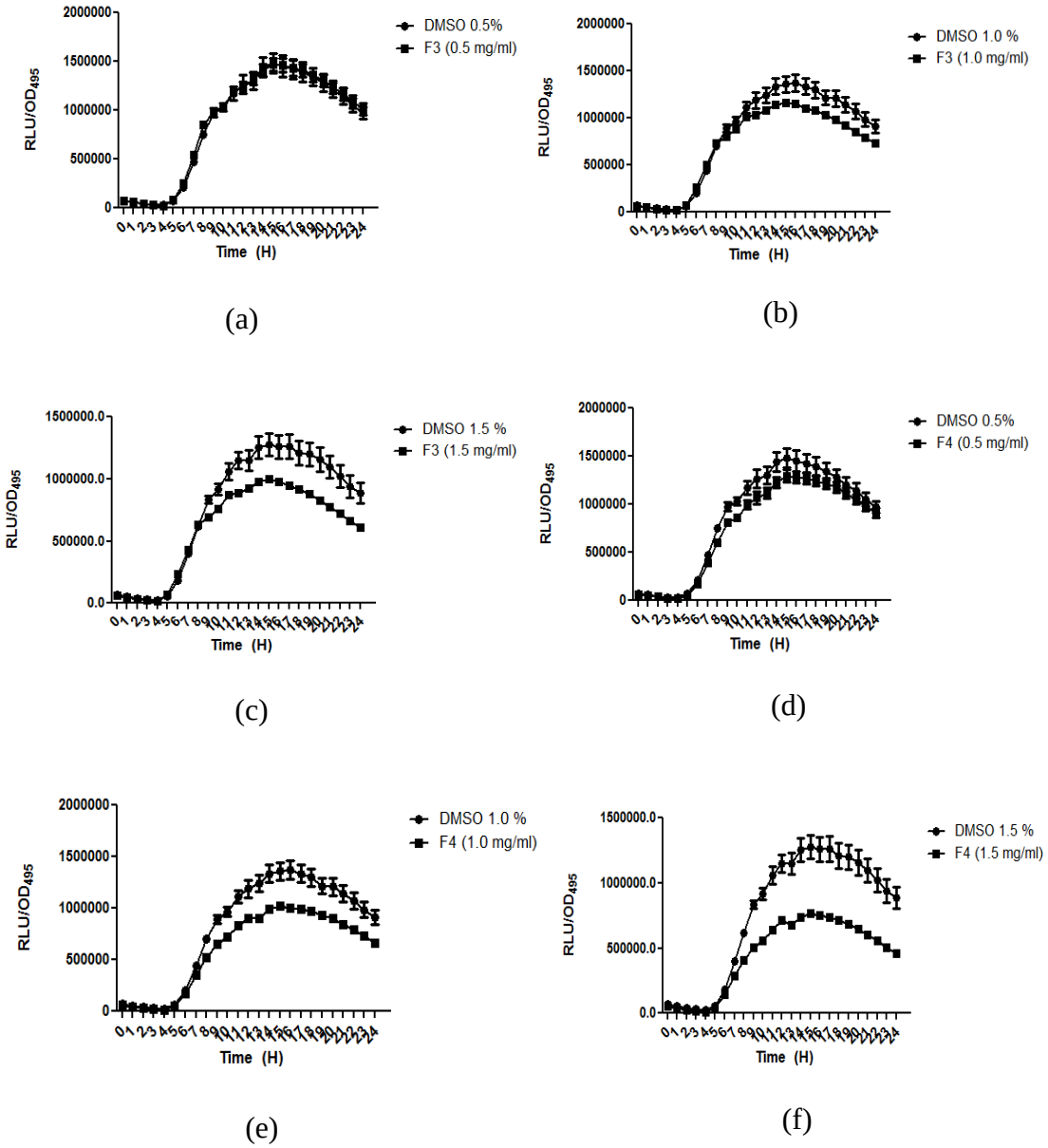


Figure 4.38: (a) Fraction 3 (0.5 mg/ml); (b) Fraction 3 (1.0 mg/ml); (c) Fraction 3 (1.5 mg/ml); (d) Fraction 4 (0.5 mg/ml); (e) Fraction 4 (1.0 mg/ml); (f) Fraction 4 (1.5 mg/ml).

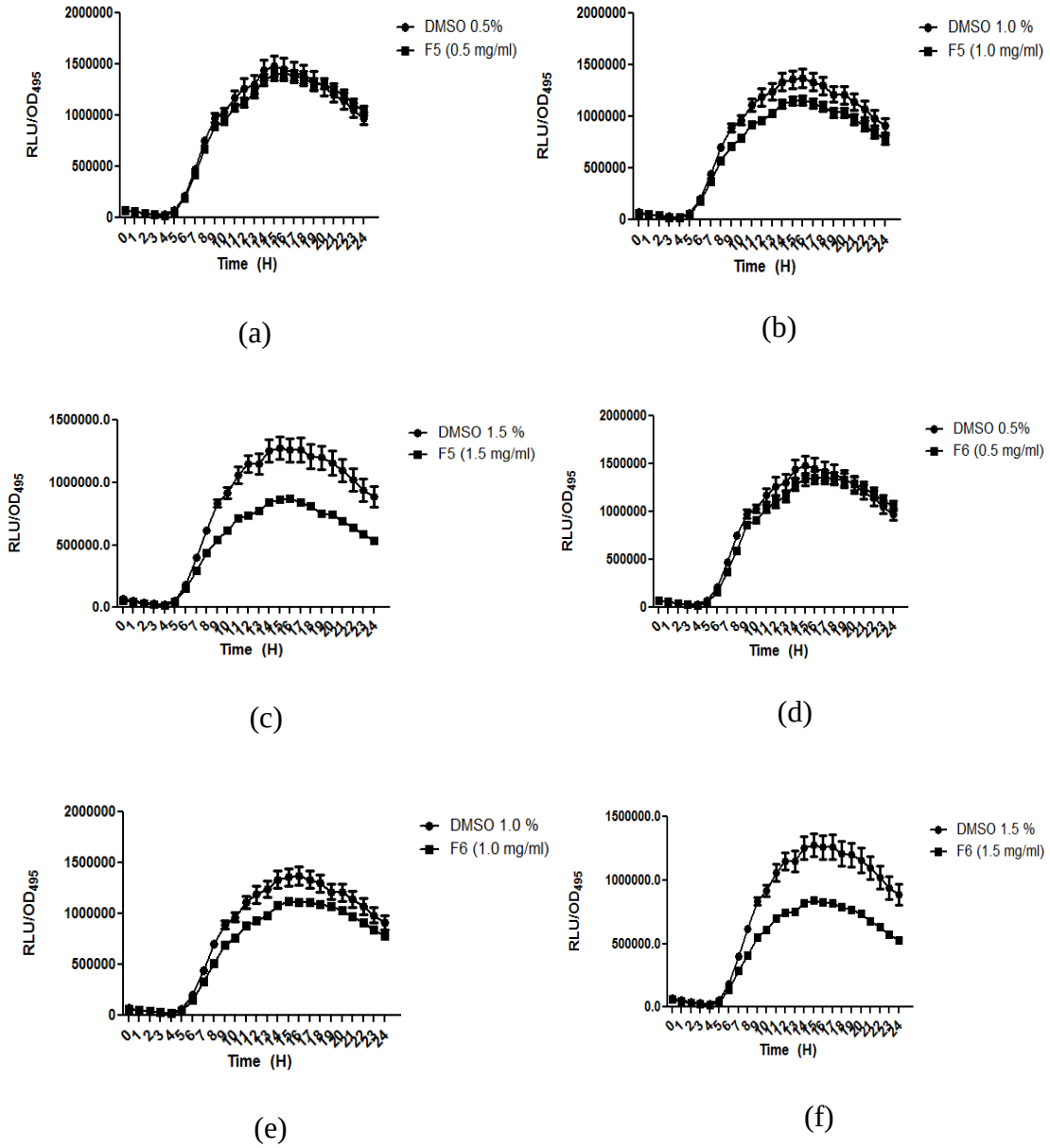


Figure 4.39: (a) Fraction 5 (0.5 mg/ml); (b) Fraction 5 (1.0 mg/ml); (c) Fraction 5 (1.5 mg/ml); (d) Fraction 6 (0.5 mg/ml); (e) Fraction 6 (1.0 mg/ml); (f) Fraction 6 (1.5 mg/ml).

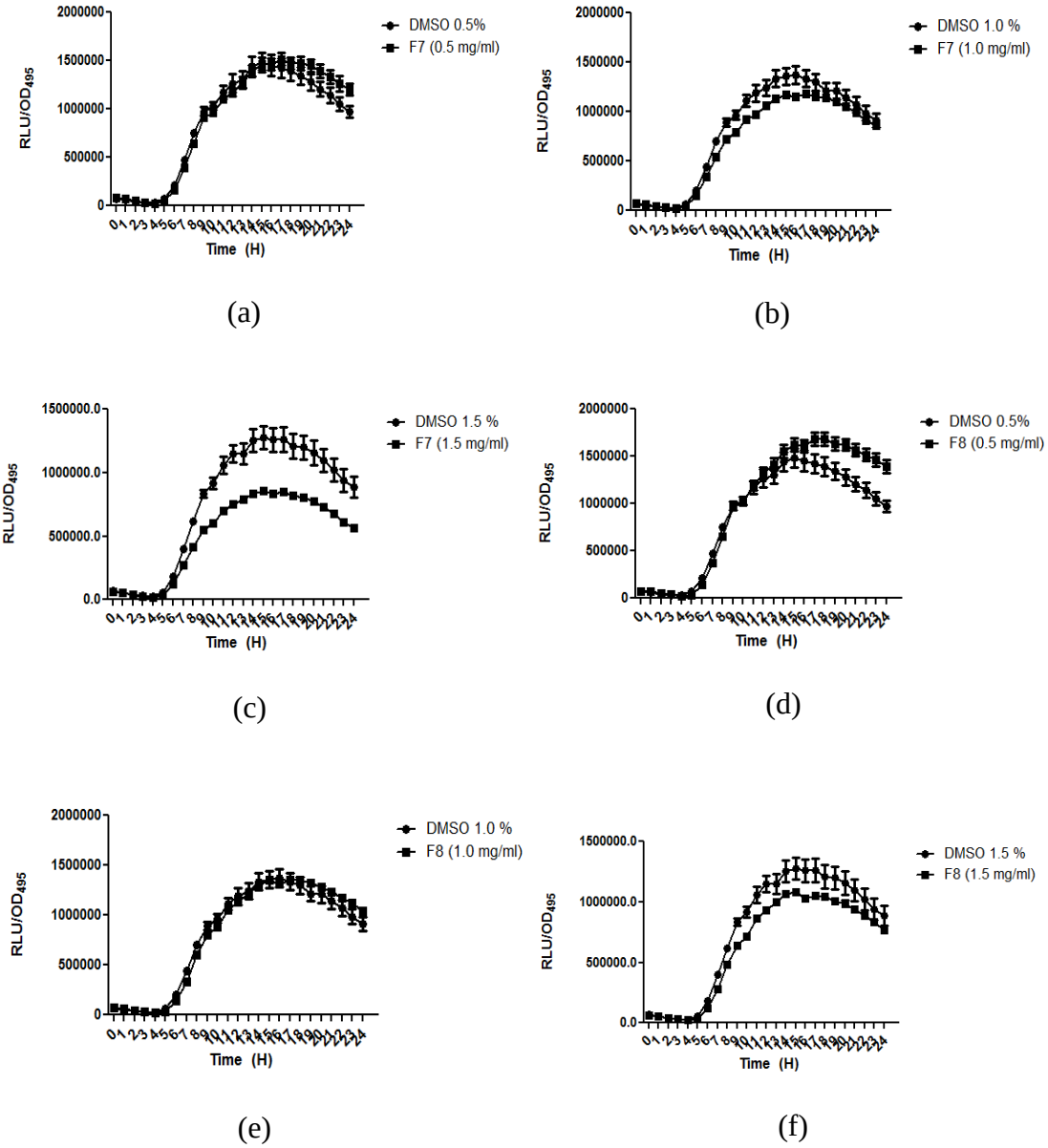


Figure 4.40: (a) Fraction 7 (0.5 mg/ml); (b) Fraction 7 (1.0 mg/ml); (c) Fraction 7 (1.5 mg/ml); (d) Fraction 8 (0.5 mg/ml); (e) Fraction 8 (1.0 mg/ml); (f) Fraction 8 (1.5 mg/ml).

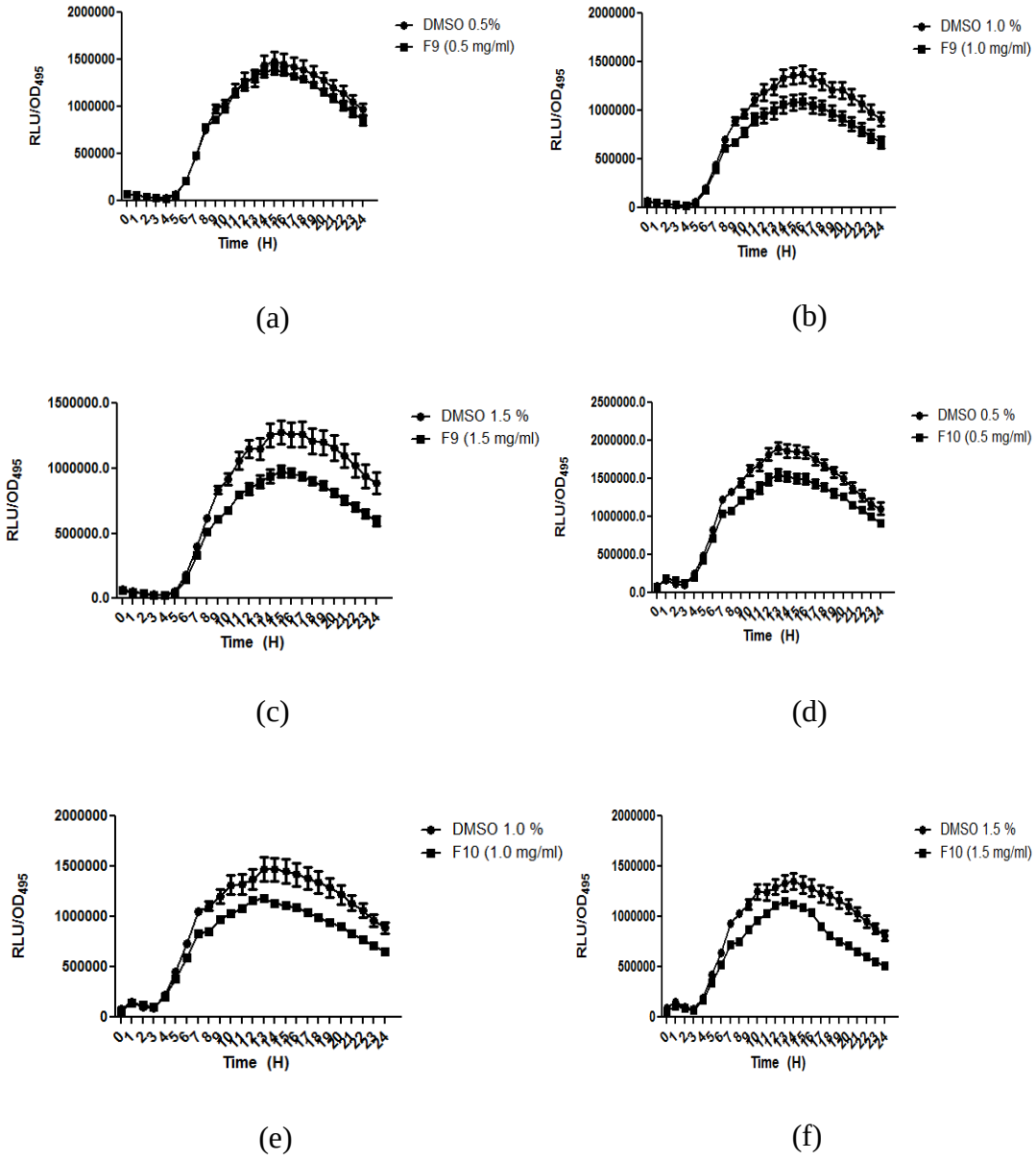


Figure 4.41: (a) Fraction 9 (0.5 mg/ml); (b) Fraction 9 (1.0 mg/ml); (c) Fraction 9 (1.5 mg/ml); (d) Fraction 10 (0.5 mg/ml); (e) Fraction 10 (1.0 mg/ml); (f) Fraction 10 (1.5 mg/ml).

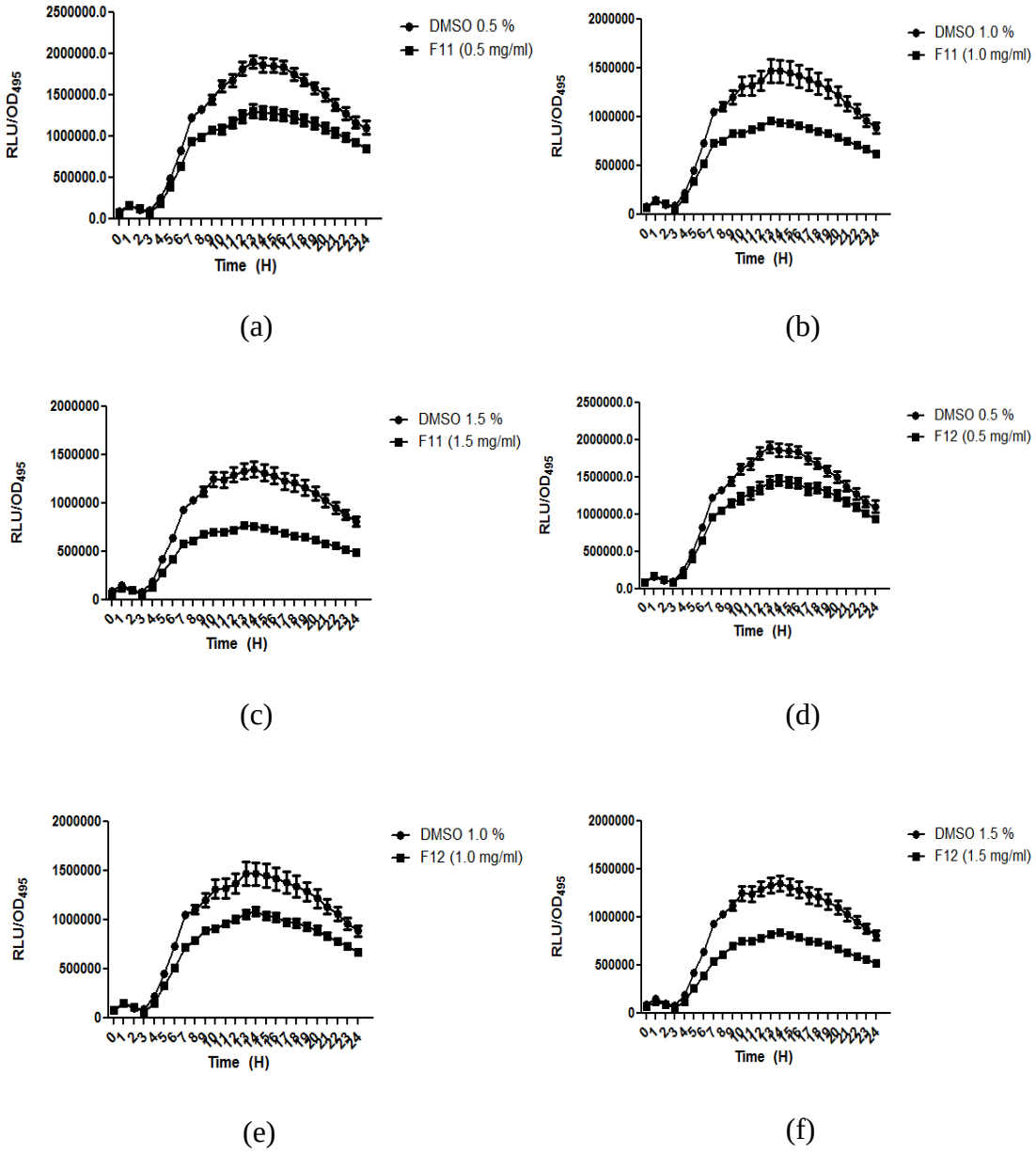


Figure 4.42: (a) Fraction 11 (0.5 mg/ml); (b) Fraction 11 (1.0 mg/ml); (c) Fraction 11 (1.5 mg/ml); (d) Fraction 12 (0.5 mg/ml); (e) Fraction 12 (1.0 mg/ml); (f) Fraction 12 (1.5 mg/ml).

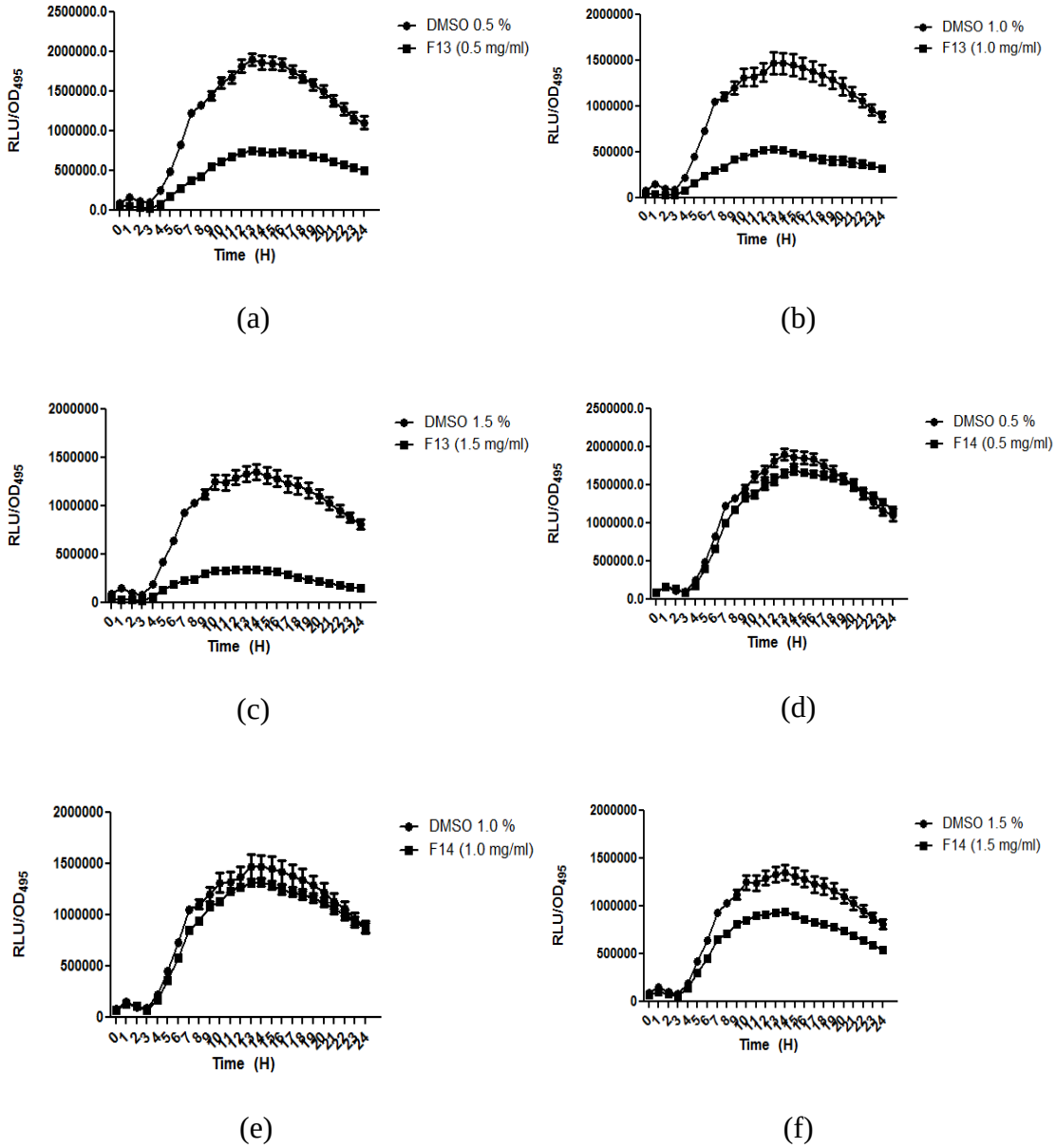


Figure 4.43: (a) Fraction 13 (0.5 mg/ml); (b) Fraction 13 (1.0 mg/ml); (c) Fraction 13 (1.5 mg/ml); (d) Fraction 14 (0.5 mg/ml); (e) Fraction 14 (1.0 mg/ml); (f) Fraction 14 (1.5 mg/ml).

4.12 Anti-Swarming Properties of Fractions of *M. lunu-ankenda* Chloroform Extract

Of the 14 fractions of *M. lunu-ankenda*chloroform extracts (Figure 4.44-Figure 4.48), only two fractions showed significant inhibition. Fraction 5 (Figure 4.45 (f-h)) and Fraction 11 (Figure 4.47 (f-h)) significantly inhibited the swarming motility of *P. aeruginosa* PA01 because at 0.5 mg/ml, there was no observable swarming of the bacteria.

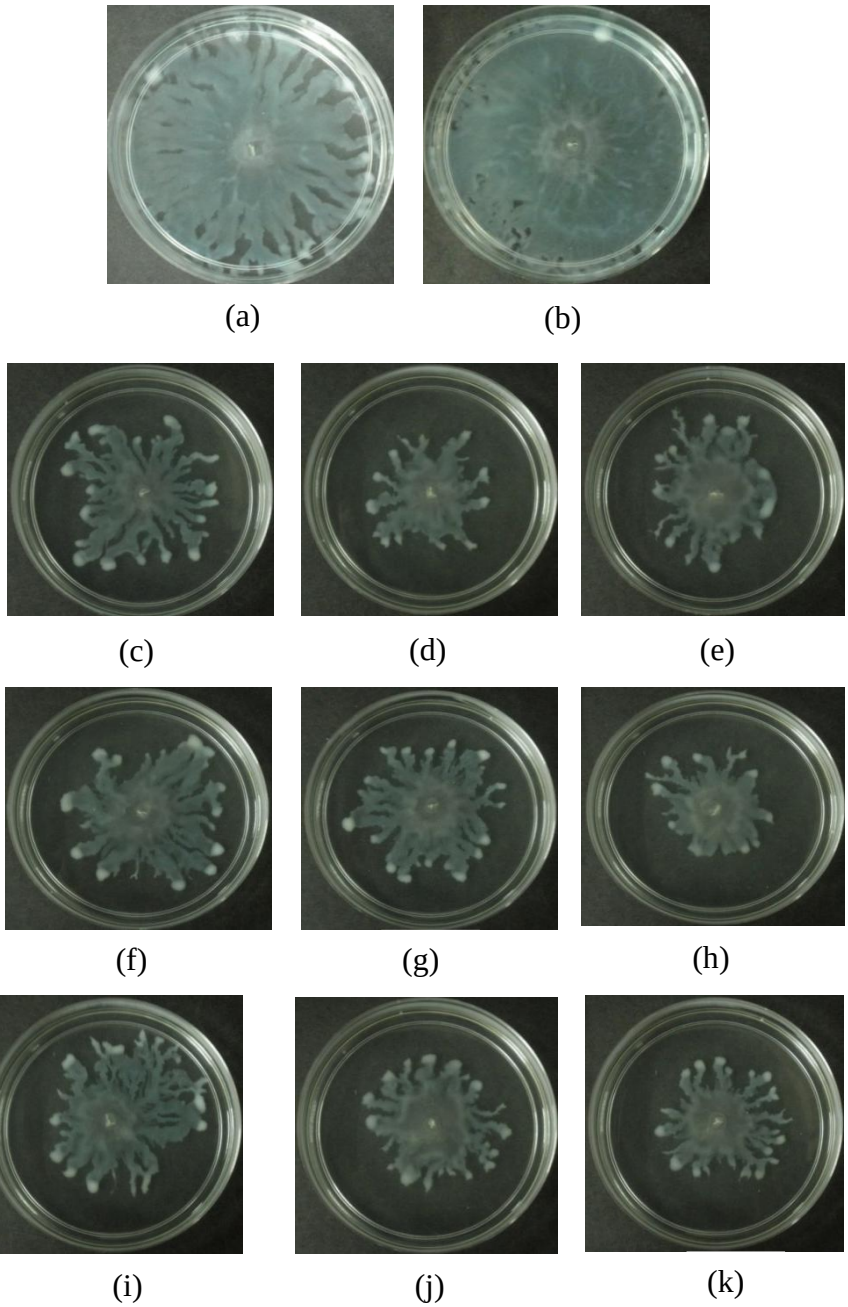


Figure 4.44: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 15 % (v/v, negative control);*M. lunu-ankenda*-Chloroform extracts of Fraction 1 (c) 0.5 mg/ml, (d) 1 mg/ml and (e) 1.5 mg/ml; Fraction 2 (f) 0.5 mg/ml, (g) 1 mg/ml and (h) 1.5 mg/ml; and Fraction 3 (i) 0.5 mg/ml, (j) 1 mg/ml and (k) 1.5 mg/ml.

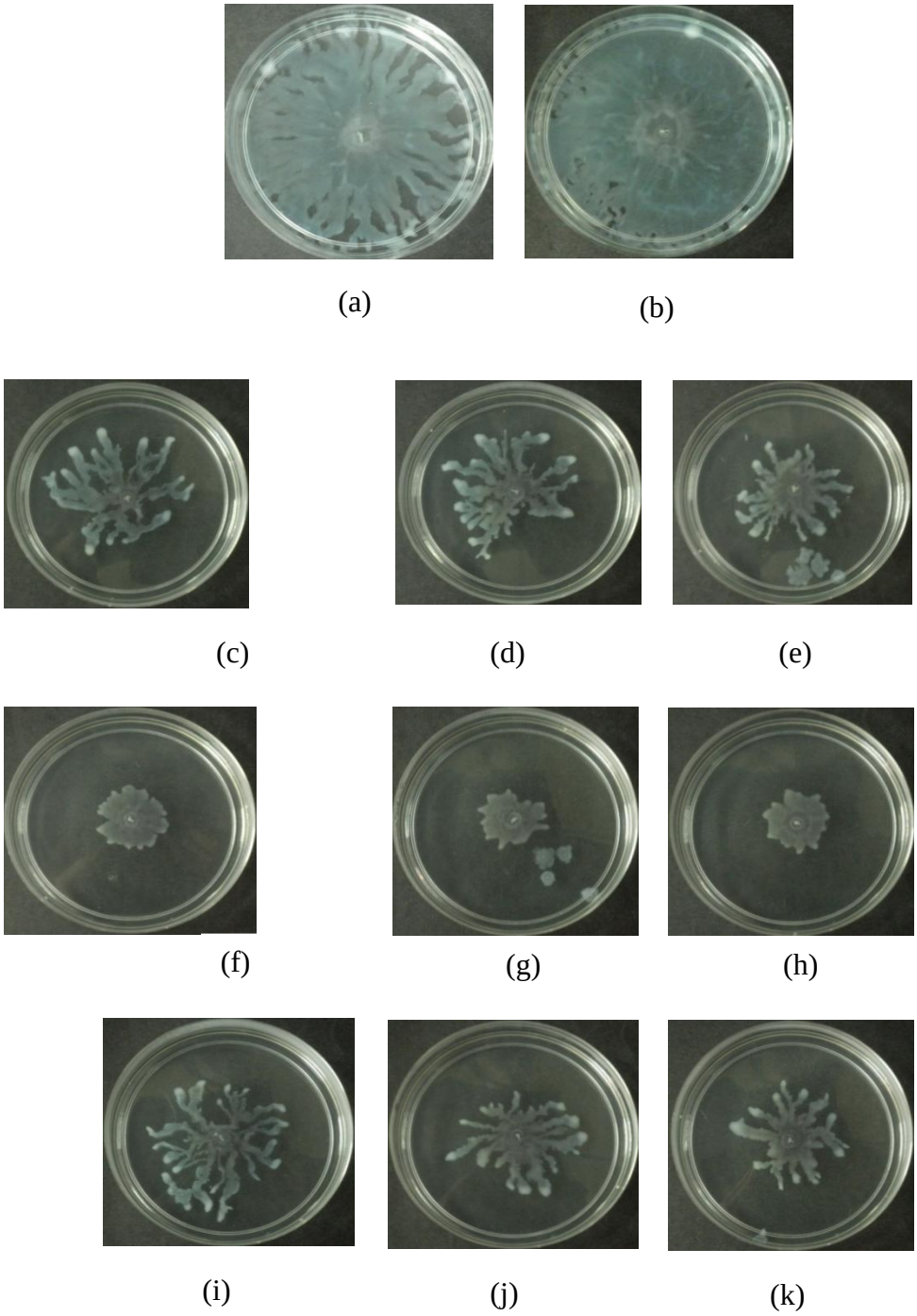


Figure 4.45: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 15 % (v/v, negative control);*M. lunu-ankenda*-Chloroform extracts of Fraction 4 (c) 0.5 mg/ml, (d) 1.0 mg/ml and (e) 1.5 mg/ml;Fraction 5 (f) 0.5 mg/ml, (g) 1 mg/ml and (h) 1.5 mg/ml; and Fraction 6 (i) 0.5 mg/ml, (j) 1 mg/ml and (k) 1.5 mg/ml.

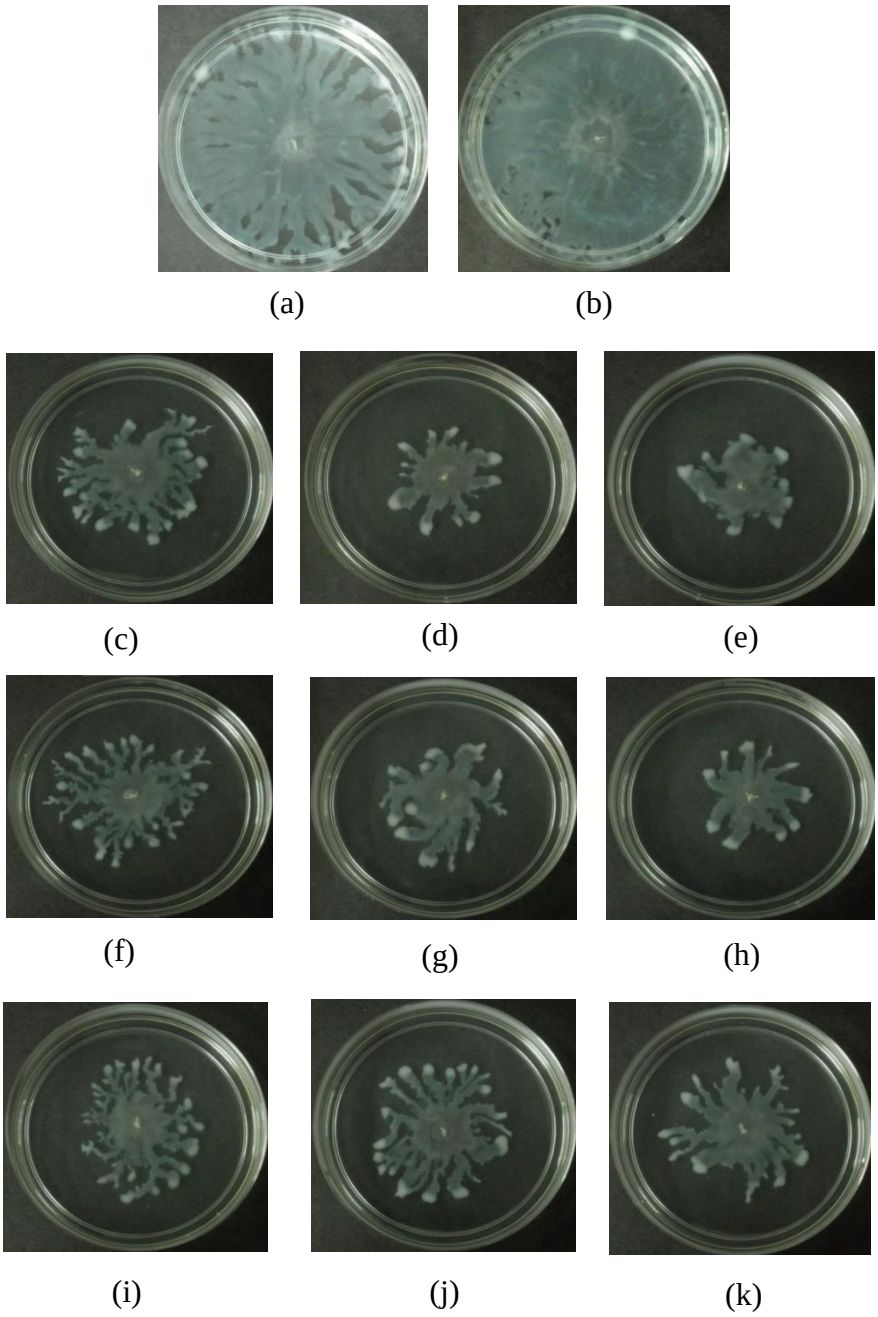


Figure 4.46: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 15 % (v/v, negative control); *M. lunu-ankenda*-Chloroform extracts of Fraction 7 (c) 0.5 mg/ml, (d) 1.0 mg/ml and (e) 1.5 mg/ml;Fraction 8 (f) 0.5 mg/ml, (g) 1 mg/ml and (h) 1.5 mg/ml; and Fraction 9 (i) 0.5 mg/ml, (j) 1 mg/ml and (k) 1.5 mg/ml.

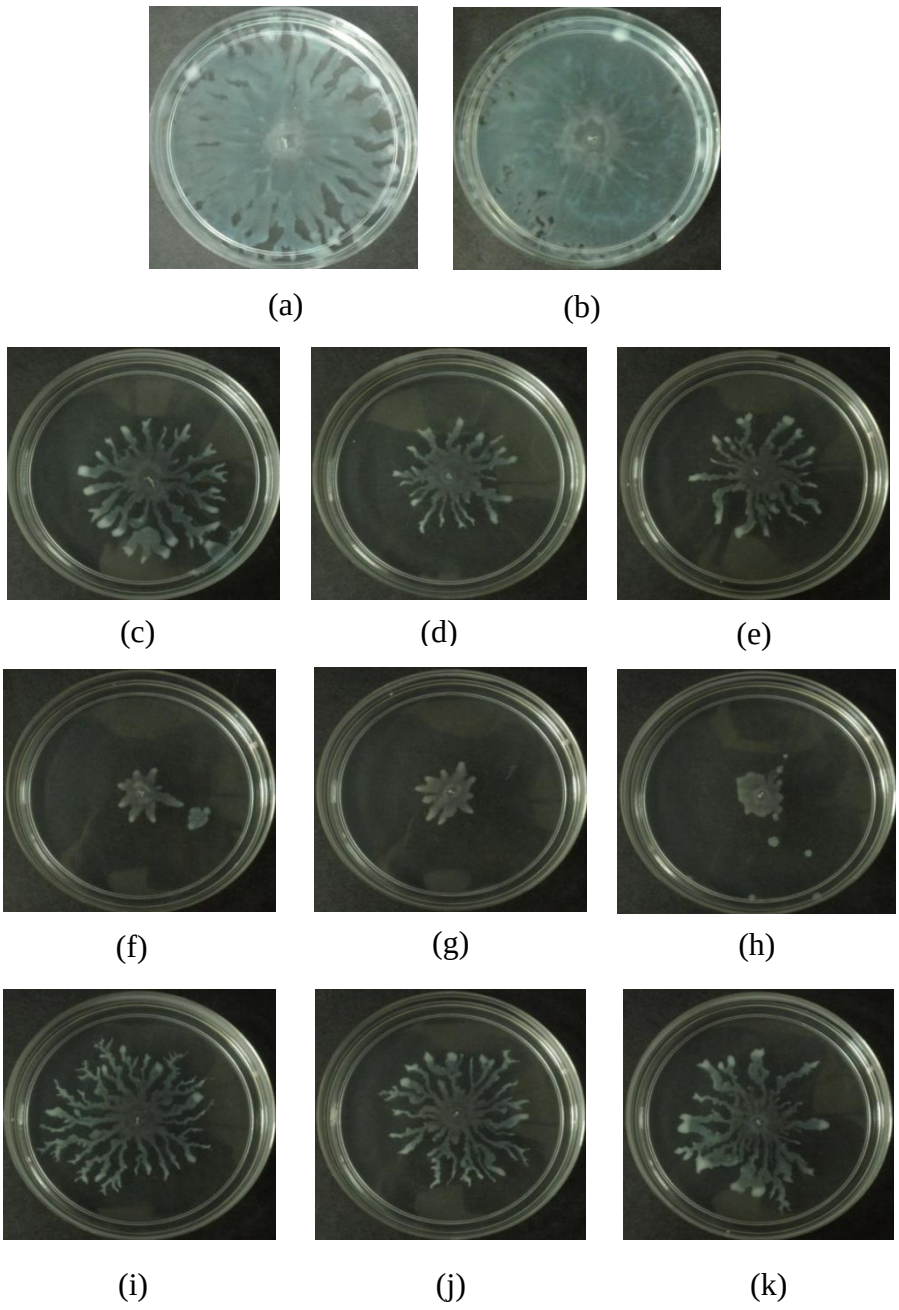


Figure 4.47: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 15 % (v/v, negative control);*M. lunu-ankenda*-Chloroform extracts of Fraction 10 (c) 0.5 mg/ml, (d) 1.0 mg/ml and (e) 1.5 mg/ml; Fraction 11 (f) 0.5 mg/ml, (g) 1 mg/ml and (h) 1.5 mg/ml; and Fraction 12 (i) 0.5 mg/ml, (j) 1 mg/ml and (k) 1.5 mg/ml.

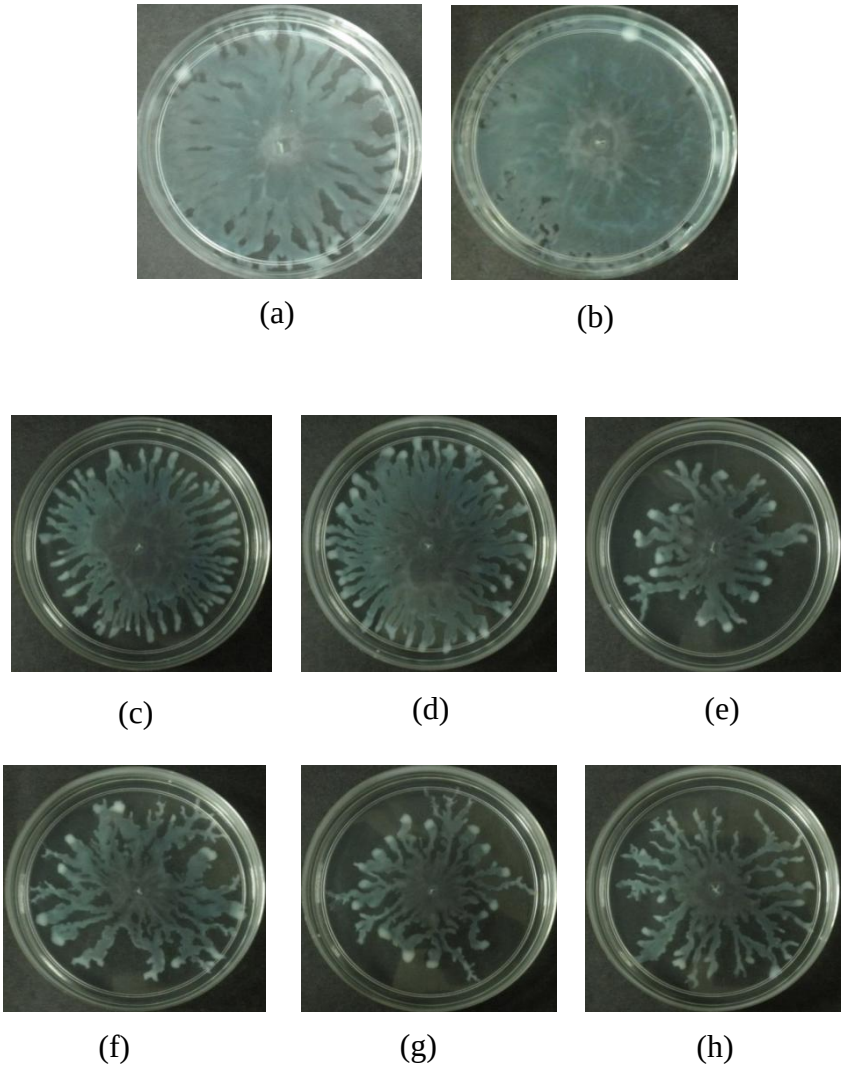


Figure 4.48: Swarming agars of (a) *P. aeruginosa* PA01that has been supplemented with (b) DMSO 15 % (v/v, negative control);*M. lunu-ankenda*-Chloroform extracts of Fraction 13 (c) 0.5 mg/ml, (d) 1.0 mg/ml and (e) 1.5 mg/ml; and Fraction 14 (f) 0.5 mg/ml, (g) 1 mg/ml and (h) 1.5 mg/ml.

4.13 Summary of the Assays and Results Obtained

Data collected from *M. lunu-ankenda*, *P. nigrum*, *P. betle* and *G. gnemon* on the seven assays were summarized in Table 4.2 and 4.3, while Table 4.4 is the summary of the data collected from the 14 fractions of *M. lunu-ankenda*-Chloroform extract.

Table 4.2: Summary of all the assays and results that has been collected from *M. lunu-ankenda* and *P.nigrum*.(√)indicatesthattheplantextracthasanti-QSpropertieswhileplantextractswith (-) has no significant inhibition against QS.

Assays	*M. lunu-ankenda*-Hexane	*M. lunu-ankenda*-Chloroform	*M. lunu-ankenda*-Methanol	*P. nigrum*-Hexane	*P. nigrum*-Chloroform	*P. nigrum*-Methanol
C. violaceum CV026 plate assay	√	√	√	√	√	√
C. violaceum CV026 violacein quantification assay	-	√	√	√	√	√
E. coli[pSB401] bioluminescence quantification assay	√	√	√	√	√	√
E. coli[pSB1075] bioluminescence quantification assay	-	-	-	-	-	-
P. aeruginosa PA01 pyocyanin quantification assay	√	√	√	-	-	-
P. aeruginosa PA01 lecA expression quantification	-	-	-	-	-	-
P. aeruginosa PA01 swarming assay	-	√	-	-	-	-

Table 4.3: Summary of all the assays and results that has been collected from *P. betle* and *G. gnemon*.(√)indicatesthattheplantextracthasanti-QSpropertieswhileplantextractswith (-) has no significant inhibition against QS.

Assays	*P. betle*- Hexane	*P. betle*- Chloroform	*P. betle*- Methanol	*G.gnemon* - Hexane	*G.gnemon*- Chloroform	*G.gnemon* - Methanol
C. violaceum CV026 plate assay	√	√	-	√	-	-
C. violaceum CV026 violacein quantification assay	√	√	√	-	-	-
E. coli[pSB401] bioluminescence quantification assay	√	√	√	√	√	√
E. coli[pSB1075] bioluminescence quantification assay	√	-	-	-	√	√
P. aeruginosa PA01 pyocyanin quantification assay	√	√	√	-	√	-
P. aeruginosa PA01 lecA expression quantification	-	√	√	-	√	-
P. aeruginosa PA01 swarming assay	-	-	√	-	-	-

Table 4.4: Summary of all the assays and results that has been collected from *M. lunu-ankenda*- Chloroform extract fractions. (√)indicatesthattheplantextracthasanti-QSpropertieswhileplantextractswith (-) has no significant inhibition against QS.

Fractions	*E. coli* [pSB401] bioluminescence quantification assay	*P. aeruginosa* PA01 lecA expression quantification	*P. aeruginosa* PA01 swarming assay
1 (0.5 mg/ml)	-	-	-
1 (1.0 mg/ml)	-	-	-
1 (1.5 mg/ml)	-	-	-
2 (0.5 mg/ml)	-	-	-
2 (1.0 mg/ml)	-	-	-
2 (1.5 mg/ml)	-	-	-
3 (0.5 mg/ml)	-	-	-
3 (1.0 mg/ml)	-	-	-
3 (1.5 mg/ml)	-	-	-
4 (0.5 mg/ml)	-	-	-
4 (1.0 mg/ml)	-	-	-
4 (1.5 mg/ml)	√	-	-
5 (0.5 mg/ml)	-	-	√
5 (1.0 mg/ml)	-	-	√
5 (1.5 mg/ml)	-	-	√
6 (0.5 mg/ml)	-	-	-
6 (1.0 mg/ml)	-	-	-
6 (1.5 mg/ml)	-	-	-
7 (0.5 mg/ml)	-	-	-
7 (1.0 mg/ml)	-	-	-
7 (1.5 mg/ml)	-	-	-
8 (0.5 mg/ml)	-	-	-
8 (1.0 mg/ml)	-	-	-
8 (1.5 mg/ml)	-	-	-

Table 4.4, continued.

9 (0.5 mg/ml)	-	-	-

9 (1.0 mg/ml)	-	-	-
9 (1.5 mg/ml)	-	-	-
10 (0.5 mg/ml)	-	-	-
10 (1.0 mg/ml)	-	-	-
10 (1.5 mg/ml)	-	-	-
11 (0.5 mg/ml)	-	-	√
11 (1.0 mg/ml)	√	-	√
11 (1.5 mg/ml)	√	-	√
12 (0.5 mg/ml)	-	-	-
12 (1.0 mg/ml)	-	-	-
12 (1.5 mg/ml)	√	-	-
13 (0.5 mg/ml)	√	-	-
13 (1.0 mg/ml)	√	-	-
13 (1.5 mg/ml)	√	-	-
14 (0.5 mg/ml)	-	-	-
14 (1.0 mg/ml)	-	-	-
14 (1.5 mg/ml)	√	-	-

CHAPTER 5

DISCUSSIONS

5.1 Identification of Plant Samples and Preparation of Plant Crude Extracts

The plant samples that had been purchased were identified by the curator of the University of Malaya Herbarium in order to obtain the accurate genus and species name for each sample. Voucher specimens (Figure 2.3-Figure 2.6) that had been deposited into the Herbarium can be searched easily thru the identification numbers (Table 4.1) given to each specimen.

The technique used for sample extraction in this study is known as infusion. Three solvents with different degree of polarity were used to extract various secondary metabolites from *M. lunu-ankenda, P. betle, G. gnemon* and *P. nigrum*. Methanol is the most polar solvent used in this study followed by chloroform with intermediated polarity and the least polar of the three solvents used is hexane. Types of compound commonly extracted by hexane includes waxes, fats, fixed oils and volatile oils while for chloroform, it includes alkaloids, aglycones and volatile oils. Sugars, amino acids and glycosides can be extracted by methanol(Houghton & Raman, 1998).

M. lunu-ankenda, P. betle, G. gnemon and *P. nigrum* were known to contain essential oils (volatile oils) (Berry, 1980; Garg & Jain, 1992; Johnson *et al*., 2010; Sumathykutty *et al*,, 1999). A popular method to obtain volatile oils is the steam distillation method. There are several ways to execute steam distillation processes, for example by mixing the plant material with water and then heat it to boiling point (distillation with water). The vapours will be collected and once condensation occurs, the oil will be separated from the water. Though the steam distillation method requires

relatively simple equipment and no filtration step is needed to separate the oil from the plant material, it is not suitable to be use in the case where the extracted compounds contains heat sensitive or easily oxidized components (Houghton & Raman, 1998). Therefore, hexane is used in this study to extract the non-polar compounds.

The plant samples obtained were washed to remove any foreign particle and soil before being placed in the oven for drying. The purpose of washing is to ensure that these particles do not cause any contamination. The drying oven has been set to 45 ˚C as this is the optimum temperature for efficient plant drying and at the same time, will not cause protein components of the plant to undergo denaturation. Solvents with different polarity were used in this study so that secondary metabolites with varying polarity can be obtained. After submerging the plant materials in the solvents, the materials were filtered and filtrate obtained was subjected to rotary evaporation. This is to ensure that all of the solvents are totally eliminated from the crude extract in the end. Besides that, total elimination of extraction solvents will also ensure that any bioactivity tested is not influence by the solvents.

Plants crude extract stocks were prepared by dissolving the extract in DMSO. DMSO is usually used to solubilize poorly soluble drugs in permeation assays. One of the advantages of using DMSO is that it helps to remove the possibility of microbial contamination in the crude extract. Besides that, the ability of DMSO to solubilize a wide range of compounds is also the reason why it was chosen to dissolve the crude extracts (Dimethyl Sulphoxide (DMSO), a Dipolar Aprotic Reaction Solvent, 2007).

5.2 Anti-Qs Activity of *M. lunu-ankenda, P. betle, G. gnemon* and *P. nigrum* Crude Extracts

Malaysia biodiversity is rich with many local plants that have yet to be tested for their anti-QS properties. The plant samples used in this study are available locally. *M. lunu-ankenda, P. betle* and *G. gnemon*leaves can be bought fresh from the market while the seeds of *P. nigrum* were obtained in dried form. The main reason these plants were selected for this study is that as of to date, no anti-QS activity by these four plants against *E. coli* [pSB401], *E. coli* [pSB1075], *C. violaceum* CV026, *P. aeruginosa* PA01 and *P. aeruginosa* PA01 *lecA::lux* were reported although their medicinal values have been published (Johnson *et al*., 2010; Pamar *et al*., 1997; Milton *et al*., 2012; Dasgupta & De, 2004; IIliya *et al*., 2003). The leaves of *M. lunu-ankenda* and *G. gnemon*are consumed by the locals as salad while the *P. betle* leaves are one of the ingredients found in betle quid. Peppercorns of *P. nigrum* are used as spices in many local cooking. *M. lunu-ankenda*, *P. betle*, *G. gnemon* and *P. nigrum*are also traditionally used to treat hypertension, bad breath, asthma and stomach discomfort respectively.

M. lunu-ankenda, *P. betle*, *G. gnemon* and *P. nigrum* crude extracts has shown significant inhibition in the anti-QS assays performed in this study. To our knowledge, this is the first report of anti-QS activities from these plants. Data collected from this study showed that the crude extracts of *M. lunu-ankenda* significantly inhibited bioluminescence production by *E. coli* [pSB401], violacein formation by *C. violaceum* CV026, pyocyanin and swarming motility of *P. aeruginosa* PA01 while the extracts of *P. betle* showed similar assays inhibition with the addition of inhibiting the QS activities from *E. coli* [pSB1075] and *P. aeruginosa* PA01 *lecA::lux*. On the other hand, *G.*

gnemon has anti-QS activities against *E. coli* [pSB401], *E. coli* [pSB1075], pyocyanin production by *P. aeruginosa* PA01 and *P. aeruginosa* PA01 *lecA::lux*. *P. nigrum* crude extracts only inhibits QS activities by *E. coli* [pSB401] and *C. violaceum* CV026.

C. violaceum,a Gram negative bacterium that was first reported as a pathological strain when studies showed that this bacterium is the cause of infections in fetal water buffaloes in the Philippines (Duran & Menck, 2001). *C. violaceum* is commonly found in soil and water, particularly in the tropical and subtropical areas(Teoh *et al*., 2006). *C. violaceum* synthesizes the purple pigment violacein, a QS-mediated trait regulated by C6-HSL. *C. violaceum* CV026, on the other hand, is a transposon mutant strain of *C. violaceum* that is unable to synthesize C6-HSL. Thus, *C. violaceum* CV026 can only produce violacein in the presence of exogenously supplied short chain AHLs (Choo*et al.*, 2006; McClean *et al*., 1997).Violacein is a strong antioxidant and acts by protecting the bacteria membrane against oxidative stress (Konzen *et al*., 2006).

In *C. violaceum* CV026 plate assay, formation of halo zone indicated that the plant samples is either inhibiting the C6-HSL competitively from binding to its transcriptional regulator, *cviR*; degrading the C6-HSL enzymatically, or removing the C6-HSL via active transport (Bauer & Teplitski, 2000; Dong *et al*., 2000; Leadbetter & Greenberg, 2000). All of the crude extracts with the exception of *P. betle* methanol extract, *G. gnemon* chloroform and methanol extracts have halo formation on the violacein background. The halo, white zone formed was opaque and not transparent indicating that the inhibition formed around the well was caused by QS inhibition and not due to inhibition of bacterial cell growth. In agreement to this finding, other plant extracts such as vanilla, *T. fuciformis, Conocarpus erectus, Quercus virgiana* and pea

seedlings have been found to possess anti-QS activity against bioreporter strain *C. violaceum* CV026. (Adonizio *et al*., 2006; Bauer & Teplitski, 2000; Choo *et al*., 2006; Teplitski *et al*., 2000; Zhu & Sun, 2008).

The extracts that give the most prominent halo zone formation are the *P. nigrum* chloroform (Figure 4.2 (e)) and *P. nigrum*methanol (Figure 4.2 (f)) extracts. This was further verified by the violacein quantified after 16 h of incubation in 28 ºC. Quantification of the violacein gives a better picture of the extent of inhibition by the plant extracts. Violacein in *P. nigrum* chloroform extracts dropped to 0.108 at 4 mg/ml while the control gave a value of 0.359 (Figure 4.4 (e)) and the extent of violacein reduction is approximately 70 %. *Ocimum sanctum*, *Manilkara zapota*, *Musa paradiciaca* and *Ananas comosus*seems 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 formation respectively in a study conducted by Musthafa and colleagues(Musthafa *et al*., 2010). Besides plants, fungal extracts can also inhibit violacein production in *C. violaceum* CV026. The medicinal mushroom, *Phellinus igniarius* has a special life cycle which requires it to produce bioactive compounds throughout its growth and metabolism. The freeze-dried mushroom was found to be inhibiting violacein production in *C. violaceum* CV026 after violacein quantification and measurement by the microplate reader (Zhu *et al*., 2012).

E. coli [pSB1075] (with the *lasR* receptor) produces optimum luminescence in the presence of exogenous 3-OC12-HSL while *E. coli* [pSB401] (with the *luxR* receptor) respond well to 3-OC6-HSL. Both of these biosensors were initially constructed to ease the identification of the AHLs signal produced by other bacteria using methods such as

cross streaking or conditioned medium assays. *E. coli* [pSB1075] was constructed through the fusion of *P. aeruginosalasRI* regulatory region to the *P. luminescens luxCDABE*region while for *E. coli* [pSB401], its construction involves the fusion of *luxRluxI* region of *V. fischeri* with the *P. luminescens luxCDABE*region (Winson *et al*., 1998). The assay was then modified to suit the need of this study by quantitating the extend of bioluminescence produced at the end of the 24 h cycle in the Tecan luminometer. The positive control well consist of the biosensor with the addition of exogenous AHLs while the treated well contains biosensor, AHLs and plants extracts. The bioluminescence quantitated from the positive controlled well will be compared with the treated well. The results obtained from *M. lunu-ankenda, P. betle, G. gnemon* and *P. nigrum* crude extracts showed that all of the extracts significantly inhibited *E. coli* [pSB401] (Figure 4.5-Figure 4.10) while*P. betle*-Hexane extract (Figure 4.12 (d-f)), *G. gnemon*-Chloroform (Figure 4.14 (d-f)) and Methanol extracts (Figure 4.15 (a-c)) inhibited *E. coli* [1075]. By screening the crude extracts with these biosensors, it gives a better picture on which type of QS system (long or short chain AHLs) that can be inhibited by the plant samples. Data collected from this study could suggest that the plant extracts may interrupt QS that depends on short chain AHLs more efficiently as compared to QS system that produce long chain AHLs.

Similarly, clove (*Syzygium aromaticum*), a type Ayurveda spice which was traditionally used for treatment of asthma and allergies was extracted with a series of solvents. The chloroform and methanol extracts was found to be significantly inhibiting bioluminescence production of *E. coli* [1075]. Besides using *E. coli* [pSB401] and *E. coli* [1075] that has *lux*-based nature for screening, fluorescence-based biosensors can

also be used for the same purpose. *Pseudomonas putida* F117 (pKR-C12) was used for detection of long chain AHLs while *E. coli* MT102 (pJBA 132) was used for the detection of short chain AHLs in a study conducted to evaluate the anti-QS properties of essential oils from Colombian plants. The results obtain from this study showed that *Lippia alba* possess promising inhibitory against *E. coli* MT102 (pJBA 132) (Jaramillo-Colorado *et al*., 2012; Krishnan *et al*., 2012).

Pyocyanin, an exoproduct of *P. aeruginosa* PA01 was extracted and quantitated after cultured with plant crude extracts for 24 h. Data collected from this study showed that indeed Malaysian local plants have anti-QS properties. The assay was conducted based on the absorbance of pyocyanin in acidic medium at 520 nm. Generally, pyocyanin can be found in great quantity in the sputum of cystic fibrosis patients and it is also highly permeable to the biological membranes. *P. aeruginosa* causes developing loss of pulmonary function which leads to premature death in the majority of cystic fibrosis patient(Goldberg & Pier, 2000; Lau *et al*., 2004). It was found that mutations in *lasR-lasI, rhlR-rhlI* and the *mvfR-haq* QS systems caused loss in pyocyanin production(Cao *et al*., 2001; Gallagher *et al*., 2002). These QS systems are also involved in production of among others, rhamnolipids, proteases and elastase (Lau *et al*., 2004).

Pyocyanin formation involved a multifactorial system and though extracts of *M. lunu-ankenda, P. betle* and *G. gnemon* crude extracts could significantly inhibit its production in this study, the mechanism on how the inhibition occurs has yet to be established. As for QS inhibitors such as garlic, a downstream study was conducted to find out which genes of *P. aeruginosa* were affected by the extracts. It was found that *lasA, lasB, rhlAB* and *chiC* genes were downregulated by garlic extract. These genes are

essential for the pathogenesis of *P. aeruginosa*. *lasA* and *lasB* are involved in encoding elastase and protease, *rhlAB* encodes rhamnolipids and pyocyanin while *chiC* encodes chitinase (Rasmussen *et al*., 2005).

S. aromaticum, Myristica cinnamomea, A. comosus and *Musa paradiciaca*are among the few types of plants found to possess compounds that can inhibit pyocyanin production besides *M. lunu-ankenda*, *P. betle* and *G. gnemon*(Chong *et al*., 2011; Krishnan *et al*., 2012; Musthafa *et al*., 2010). Catechin, a type of flavonoid found in *Combretum albiflorum* was found to be able to reduce pyocyanin production in *P. aeruginosa* PA01 (Vandeputte *et al*., 2010).

The name *P. aeruginosa* PA01 *lecA::lux* means that a *luxCDABE* gene region was cloned to the region of *lecA* in *P. aeruginosa* PA01 in such a way every time *lecA* gene is activated or expressed, the *lux* region will also be activated, thus bioluminescence is detected (Winzer *et al*., 2000). *lecA* produced by *P. aeruginosa* is a known adhesion and cytotoxic lectin which binds hydrophobic galactosides with high specificity and affinity. Study has shown that *lecA* expression can be found in biofilm-grown cells and the results suggested that *lecA* expressed may aid *P. aeruginosa* biofilm structure development under various environmental conditions (Diggle *et al*., 2006). Results obtain from this study found that only three out of the total 12 extracts from *M. lunu-ankenda, P. betle, P. nigrum* and *G. gnemon* showed significant inhibition against *lecA* expresison. It is, however a very promising results considering the pathogenicity that can be caused by *lecA*. To the best of our knowledge, *S. aromaticum* is the only plant other than *G. gnemon* and *P. betle* to cause inhibition against of *lecA* expression (Krishnan *et al*., 2012).

Swarming motility of *P. aeruginosa* can be divided into three general stages, firstly, differentiation of vegetative cells into swarmer cells, followed by migration of swarmer cell populations and finally consolidation(Eberl *et al*., 1999; Kearns, 2010).Swarming is a bacterial surface translocation process which is QS-dependent and requires flagella and pili. Other types of surface translocation characteristic used by the bacteria include swimming, gliding, twitching, sliding and darting(Köhler *et al*., 2000; Sharma & Anand, 2002). Swarming usually occurs on semi-solid agar and this motion enables the cell to colonize the surrounding surfaces(Sharma & Anand, 2002).

The optimized swarming agar in this study comprised of 0.5% (w/v) agar. When the agar content was lower than 0.5%, *P. aeruginosa* PA01 swims instead of swarm. Subsequently, when the agar was content higher than 0.5%, it was too dry for the bacteria and the swarming motion took longer than 20 h to occur. The chloroform extract of *M. lunu-ankenda*and the methanol extract of *P. betle*might be exerting its inhibition of swarming during the migration of swarmer cells or causing biofilm dispersal. As the concentration of the crude extract increases, extend of swarming inhibition become more apparent. Similar observation has also been observed in malabaricone C, a compound purified from nut meg extract (Chong *et al*., 2011).

Extracts that showed inhibition against swarming has a higher chances of inhibiting biofilm regulation as swarming is involved in the biofilm formation through mass translocation of cells. In consistence with our finding, *Areca catechu*, *Panax ginseng* and *Prunus armeniaca* are among the traditional Chinese medicinal plants that were found to contain compounds with anti-swarming activity against *P. aeruginosa*. The extracts from the seeds of *A. catechu* has the highest area of colony reduction as compare with the control (Issac *et al*., 2011; Koh & Tham, 2011).

P. aeruginosa is not the only bacteria which exhibits swarming motility. *Proteus mirabilis*, *Serratia marcescens* and *S. liquifaciens* also exhibits swarming motility. These bacteria have been used by studies in search of QS antagonists. Extracts from *Capparis spinosa*and halogenated furanones from *D. pulchra*were reported to be inhibiting swarming motility in *P. mirabilis* and *S. marascecens*(Givskov *et al*., 1996; Gram *et al*., 1996; Issac *et al.*, 2011). The halogenated furanones from *D. pulchra* was the first compound discovered from non-bacteria origin to exhibit anti-QS properties. The furanones were found to be binding to the LuxR proteins competitively and this in turn increased their rate of proteolytic degradation without the need to kill the bacteria (Givskov *et al*., 1996; Manefield *et al*., 1999; Manefield *et al*., 2002).

It is not clear at which level QS has been modulated by these extracts because the extracts could be competing or disrupting the AHLs binding to the receptors by degradation of AHLs; blocking AHLs from forming AHL-receptor complex; changing the structures of the enzymes that is involved AHLs synthesis(Hong *et al*., 2012). Variation in the results between the crude extracts of the same plants showed that each crude extracts contains different compounds with varying degree of inhibitions. This is

further explored in the following steps of this study, which involves fractionation of the crude extracts that showed anti-QS in the assays performed.

5.3 Bioassay-Guided Fractionation of *M. lunu-ankenda* Chloroform Extract

Fractionation of *M. lunu-ankenda* chloroform extract was performed using Agilent Technologies 1260 Infinity Series HPLC system. This crude extract was selected to be fractionated because it inhibited most of the anti-QS assays and to the best of our knowledge, fractionation of the chloroform extract of *M. lunu-ankenda* has yet to be reported.

In most practice, before performing fractionation on a preparative catridge, the sample will be analyzed using an analytical column. We tried to employ this practice but the preparative system that we have did not have a degasser and as a consequence, bubble was introduced into the analytical column. Thus, instead of performing a scale-up fractionation from analytical to preparative scale, the latter was choosen for the fractionation optimization of *M. lunu-ankenda* chloroform extract. After approximately three weeks, the best method for fractionation of *M. lunu-ankenda* chloroform extract was established and a full run was conducted to begin the collection of the plant fractions. Mixtures of acetonitrile and ultrapure water coupled with the usage of C18 column makes the mode of fractionation used as reversed-phase preparative HPLC.

Fractions were collected based on the peaks observed in the chromatogram at absorbance of 210 nm starting from the 4^{th} min. The crude extracts fraction peaks were best observed at 210 nm. Alkaloids and agylcones are the group of compounds which can be extracted by chloroform. Majority of alkaloids are basic and they have medium

polarity. Aglycones are the non-sugar moiety of glycosides. Generally, aglycones can be obtained from glycosides through hydrolysation of glycosides in aqueous medium (Houghton & Raman, 1998). Thus, the compounds from the chloroform crude extract of *M. lunu-ankenda* may consist of mixtures of both alkaloids and aglycones. To the best of our knowledge, phytochemical studies of various parts of *M. lunu-ankenda* can be found from the year 1985 till present (Kumar *et al*., 1990; Manandhar *et al*., 1985; Mohan *et al*., 1985; Rastogi & Mehrotra, 1990). Monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, eviodione and leptonol was found in the leaves oil of *M. lunu-ankenda* through gas chromatographic analysis. Leptonol and evodione have shown anti-inflammatory, anti-pyretic and anti-oxidant properties (Johnson *et al*., 2010). Stem wood of *M. lunu-ankenda* contains a type of alkaloid known as evolitrine which has anti-feedant activity (Lal *et al*., 2005).

The crude extract has shown positive inhibition against *E. coli* [pSB401] and swarming motility of *P. aeruginosa* PA01. After testing the fractions on these two assays, we found that the fractions were able to inhibit those QS traits at a lower concentration as compared to the crude extract. Fraction 4 and Fraction 11-Fraction 14 gave significant inhibition against *E. coli* [pSB401] while for swarming motility, Fraction 5 and Fraction 11 gave positive inhibition. The results that we obtain from the bioassays performed on those collected fraction proved that the fractionation method in this study which employs isocratic elution of mobile phases was successful. Not all of the fractions gave significant inhibition, which suggests that the compounds that were exerting the inhibition have been categorized accurately into their respective fractions. Inactive compounds that were masking the active compounds activity may have been

fractionated into a different vial, thus the active fractions was able to inhibit at a lower concentration.

Though the crude extract did not have any significant inhibition against *P. aeruginosa* PA01 *lecA::lux*, the fractions were tested for this bioassay as well. Since the mixture of crude extract has been fractionated, it is worthwhile to determine whether there are any active compounds that could be inhibited by other unwanted compounds in the crude extract previously. From the results obtain, we concluded that the chloroform extracts of *M. lunu-ankenda* did not contain active compounds that can cause inhibition against *P. aeruginosa* PA01 *lecA::lux*as none of the fractions showed significant inhibition against this bacteria. If *M. lunu-ankenda* chloroform extracts contains active compounds that could have inhibited the *lecA* expression, one or some of the fractions would have given positive results though the crude did not inhibited the expression. Fractions that were eluded later was said to have a higher affinity to the column. Thus, fractions that were collected earlier were the most polar while the fractions eluded at a later time were the least polar.

5.4 Future Work

The identity of the compounds from the active fractions has yet to be established. Gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) are among the two machines that can be used to identify the compounds that are responsible for those significant inhibitions. In addition, it is also important for us to know what mechanisms that were inhibited by those active compounds as it might be possible to overcome the problems posed by emerging antibiotic-resistant bacteria. Continuous screening of new Malaysia's local plant for anti-QS activity is also vital as massive amount of flora in Malaysia has yet to be tested for anti-QS. This massive flora diversity may one day provide us with an effective new library of anti-QS compounds.

CHAPTER 6

CONCLUSION

The current work represents the finding of anti-QS activity from *M. lunu-ankenda*, *P. nigrum*, *G. gnemon* and *P. betle* against *P. aeruginosa* PA01, *P. aeruginosa* PA01 *lecA::lux*, *C. violacaeum* CV026, *E. coli* [pSB401] and *E. coli* [pSB1075]. The results from the present work showed that anti-QS compounds are not only found among bacteria, but can also be found from non-bacteria origins. One important finding from this work is that most of the plant extracts seems to be inhibiting the anti-QS activity resulted from short chain AHLs. Before establishing the exact mechanism of inhibition exerted by these plant samples, it is vital to determine the compound responsible for the activity. It is believed that these samples will be able to provide a new class of anti-QS compounds and continuous screening of local Malaysia plant for anti-QS activity will be able to yield more new plants with anti-QS inhibitions.

PUBLICATIONS AND CONFERENCES ATTENDED

Publications:

Tan, L. Y., Yin, W. –F., & Chan, K. –G. (2012). Silencing quorum sensing through extracts of *Melicope lunu-ankenda*. *Sensors,12*(4), 4339-4351.

Tan, L. Y., Yin, W. –F., & Chan, K. –G. (2013). *Piper nigrum*, *Piper betle* and *Gnetum gnemon*- natural food source with anti-quorum sensing properties. *Sensors*, *13*(3), 3975-3985.

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

Conferences attended:

Poster presenter-

Tan Li Ying, Yin Wai Fong and Chan Kok Gan (2011).Anti-quorum sensing properties of *Piper nigrum*.The 16th Biological Sciences Graduate Congress, National University of Singapore, Singapore.

Oral presenter-

Tan Li Ying and Chan Kok Gan (2012).Anti-quorum sensing properties of *Melicope lunu-ankenda*, *Piper nigrum*, *Piper betle* and *Gnetum gnemon*.II International Conference on Antimicrobial Research 2012, Lisbon, Portugal.

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