

**INHIBITION OF BIOFILM DEVELOPMENT BY USING *Solanum melongena*
FRUIT EXTRACTS AGAINST SOME CLINICALLY IMPORTANT
PATHOGENS**

CHAI CHIA JIAN

**THIS DISSERTATION IS SUBMITTED IN FULFILLMENT OF THE
REQUIREMENT FOR THE DEGREE OF BACHELOR OF
BIOTECHNOLOGY (HONOURS)**

**FACULTY OF HEALTH AND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI MALAYSIA**

NON-PLAGIARISM DECLARATION

By this letter I declare that I have written this thesis completely by myself, and that I have used no other sources or resources than the ones mentioned.

I have indicated all quotes and citations that were literally taken from publications, or that were in close accordance with the meaning of those publications, as such. All sources and other resources used are stated in the references.

Moreover I have not handed in a thesis similar in contents elsewhere.

In case of proof that the thesis has not been constructed in accordance with this declaration, the Faculty of Health and Life Sciences has the right to consider the research thesis as a deliberate act that has been aimed at making correct judgment of the candidate's expertise, insights and skills impossible.

I acknowledge that the assessor of this item may, for the purpose of assessing this item,

- reproduce this assessment item and provide a copy to another member of the University; and/or,
- communicate a copy of this assessment item to a plagiarism checking service (which may then retain a copy of the assessment item on its database for the purpose of future plagiarism checking).

In case of plagiarism the examiner has the right to fail me and take action as prescribed by the rules regarding Academic Misconduct practiced by INTI International University.

Chai Chia Jian

NAME

I15007400

I.D. NUMBER

Chai Chia Jian

SIGNATURE

20th December 2017

DATE

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged, and completed under the supervision of Dr. Geetha Subramaniam.

Chai Chia Jian

Dr. Geetha Subramaniam

I.D. NUMBER: I15007400

(SUPERVISOR)

DATE: 20th December 2017

(CO-SUPERVISOR if any)

ACKNOWLEDGEMENT

'I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me' said Isaac Newton. This world is grand and there lies an ocean of undiscovered findings that are waiting for eager and curious minds. During these 10 weeks of laboratory work, I found myself as a small fish in this deep ocean full of undetermined truth. I faced a lot unexpected challenges and the intense learning has enriched my life experiences, not only in terms of scientific knowledge but most importantly my mental virtues. Splendid encouragement and motivation from the surrounding people have given me strength to accomplish this project. First and foremost, I would like to sincerely thank my beloved supervisor, Dr. Geetha Subramaniam for her great guidance and valuable suggestions throughout these weeks. Her vision, passion and wisdom have been inspiring me not to be just a better researcher but also a better person with high dignity. Furthermore, I would like to show my sincere gratitude to all the lecturers especially Ms. Lalita Ambigai Sivasamugham, Dr. Yuka Hara and Dr. Ong Ghim Hock who have supported me by contributing their precious time and professionalism. Not forgetting my senior, Himashi Imanda Gurudeniya and my friend, Chia Zheng Yang who have held out their hands in friendship to provide me countless help and motivation during this project. Last but not least, I would like to express my greatest affection and sincere appreciation to my family who has always been there for me in the good and bad moments.

ABSTRACT

Antibiotic resistance has been a global issue because generally-prescribed antibiotics are no longer effective against the resistant bacteria. One of the factors that have contributed to antibiotic resistance is the formation of biofilms. Common nosocomial pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococci faecalis* and *Staphylococcus aureus* are capable of forming biofilms on dry hospital surfaces, indwelling medical devices and other areas of healthcare institutions. This causes difficulties in treatment of bacterial infections and further increases the rate of mortality of patients. Thus, there is an immediate need to discover alternative biotherapeutic agents with significant antibacterial and anti-biofilm properties. This study determined the potential role of *Solanum melongena* in helping to overcome antibiotic resistance and biofilm development in four clinically important pathogens namely *A. baumannii*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. Fruit extracts of *S. melongena* were prepared by using three different organic solvents including acetone, ethanol and methanol in order to compare their effectiveness in inhibiting the growth of the bacterial strains mentioned above. Phytochemical screening was also done to qualitatively determine the phenolic compounds that were contained in the three different plant extracts. Both acetone and ethanolic extracts contained flavonoids, alkaloids and tannins while only flavonoids and tannins were found in methanolic extract. Agar well diffusion assay was carried out to determine antibacterial activity of *S. melongena* fruit extracts against the four bacterial strains. *P. aeruginosa* was the only bacteria tested to be susceptible towards methanolic and ethanolic extracts whereas other bacterial isolates were resistant towards all three extracts. Subsequently, both methanolic and ethanolic fruit extracts were used in microtiter plate biofilm assay to evaluate their anti-biofilm properties on *P. aeruginosa*. However, biofilm size observed was not reduced by the fruit extracts. Further investigation has to be carried out to determine the effect of *S. melongena* in inhibiting biofilm development by testing it on a wider range of bacteria.

TABLE OF CONTENT

	Page
NON-PLAGIARISM DECLARATION	ii
DECLARATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
TABLE OF CONTENT	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATION	xi
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	3
2.1 Bacterial Strains	3
2.1.1 Gram-negative Bacteria	3
2.1.1.1 <i>Acinetobacter baumannii</i>	3
2.1.1.2 <i>Pseudomonas aeruginosa</i>	3
2.1.2 Gram-positive Bacteria	4
2.1.2.1 <i>Enterococcus faecalis</i>	4
2.1.2.2 <i>Staphylococcus aureus</i>	5
2.2 Biofilm Development	5
2.2.1 Antibiotic Resistance of Biofilms	8
2.3 <i>Solanum melongena</i>	8
2.3.1 Medicinal Properties of <i>Solanum melongena</i>	9
2.3.2 Antibacterial Properties of <i>Solanum melongena</i>	9
2.3.3 Extraction Method	10
2.4 Biofilm Assay	11
3 MATERIALS & METHODS	13
3.1 Media & Reagent Preparation	13
3.2 Preparation of Pure Bacterial Culture	13
3.3 Confirmation Tests	13
3.3.1 Gram Staining	13
3.3.2 Physiological Characterization of Gram-positive & Gram-negative Bacteria	14
3.4 Collection and Preparation of Plant Materials	14
3.5 Phytochemical Screening of Plant Extracts	16
3.6 Antimicrobial Assay Using Agar Well Diffusion Method	16
3.7 Anti-biofilm Assay	17

3.8	Data Analysis Through ANOVA	17
4	RESULTS	19
4.1	Confirmation Tests	19
4.1.1	Gram Staining	19
4.1.2	Physiological Characterization of Gram-positive Bacteria	20
4.1.2.1	Catalase Test	20
4.1.2.2	Growth on Mannitol Salt Agar (MSA)	20
4.1.3	Physiological Characterization of Gram-negative Bacteria	21
4.1.3.1	Indole Test	21
4.1.3.2	Oxidase Test	21
4.1.3.3	Triple Sugar Iron (TSI) Test	22
4.2	Phytochemical Screening of Plant Extracts	23
4.3	Antimicrobial Assay Using Agar Well Diffusion Method	24
4.4	Anti-Biofilm Assay	29
5	DISCUSSION	31
5.1	Confirmation Tests	31
5.1.1	Gram Reaction	31
5.1.2	Physiological Characterization of Gram-positive Bacteria	32
5.1.2.1	Catalase Test	32
5.1.2.2	Growth on Mannitol Salt Agar (MSA)	32
5.1.3	Physiological Characterization of Gram-negative Bacteria	33
5.1.3.1	Indole Test	33
5.1.3.2	Oxidase Test	33
5.1.3.3	Triple Sugar Iron (TSI) Test	34
5.2	Phytochemical Screening of Plant Extracts	34
5.3	Antimicrobial Assay Using Agar Well Diffusion Method	36
5.4	Anti-Biofilm Assay	39
6	CONCLUSION AND RECOMMENDATIONS	42
	REFERENCES	44
	APPENDICES	66

LIST OF TABLES

Tables		Page
2.1	Phytochemical composition of round and oval varieties of <i>Solanum melongena</i> (Agoreyo et al., 2014).	10
3.1	Physiological characterization of Gram-positive and Gram-negative bacteria (Acharya, 2012a; 2012b; 2013a; 2013b; 2013c).	14
3.2	Phytochemical screening of plant extracts.	16
3.3	Zone diameter interpretive standards of imipenem for different bacteria tested (CLSI, 2017).	17
4.1	Summary of confirmatory tests carried out on four different bacterial strains (Holt, Krieg, Sneath, Staley & Williams, 1994).	22
4.2	Phytochemical screening of <i>S. melongena</i> fruit extracts prepared from three different organic solvents.	23
4.3	Diameter of inhibition zone formed around each well which containing different agents tested on different bacterial strains.	25
4.4	Interpretation on susceptibility and resistance of bacteria towards imipenem in this study.	26

LIST OF FIGURES

Figure		Page
2.1	Process of biofilm development (Garnett & Matthews, 2012).	7
2.2	<i>Solanum melongena</i> (Pinterest, 2017).	9
3.1	Procedure of gram staining (Hugon et al., 2013)	14
3.2	Distribution of wells in agar plate. (I) Methanolic fruit extract (II) Methanol (III) Ethanolic fruit extract (IV) Ethanol (V) Acetone fruit extract (VI) Acetone (VII) Neem leaves extract (VIII) Imipenem disc.	17
3.3	Procedure of anti-biofilm assay.	18
3.4	Addition of antimicrobial agents in respective wells of round-bottom 96-wells plate.	18
4.1.1a	Example of Gram-positive cocci shown as purple spherical cells under magnification of 1000X.	19
4.1.1b	Example of Gram-negative bacilli shown as pink, rod-shaped cells under magnification of 1000X.	19
4.1.2.1a	Formation of bubbles in catalase positive <i>S. aureus</i> .	20
4.1.2.1b	Absence of bubble formation in catalase negative <i>E. faecalis</i> .	20
4.1.2.2	Yellow colonies of <i>S. aureus</i> (left) and <i>E. faecalis</i> (right) on MSA	20
4.1.3.1	Yellow layer of Kovac's reagent on <i>P. aeruginosa</i> (left) and <i>A. baumannii</i> (right) indicated them as indole negative.	21
4.1.3.2a	Development of purple colour indicated <i>P. aeruginosa</i> to be oxidase positive.	22
4.1.3.2b	Absence of purple colour indicated <i>A. baumannii</i> to be oxidase negative.	22
4.1.3.3	Red slant and butt in tubes cultured with <i>P. aeruginosa</i> (left) and <i>A. baumannii</i> (right).	22

LIST OF FIGURES

Figure		Page
4.2	Example of agar well diffusion of <i>A. baumannii</i> : methanolic extract (top left); methanol (top right); ethanolic extract (bottom left); ethanol (bottom right).	25
4.3	Example of agar well diffusion of <i>A. baumannii</i> : acetone (top left); acetone extract (top right); neem leaves extract (bottom left); imipenem disc (bottom right).	25
4.4	Example of agar well diffusion of <i>P. aeruginosa</i> : ethanol (top left); ethanolic extract (top right); methanol (bottom left); methanolic extract (bottom right).	25
4.6	Antimicrobial effect of <i>S. melongena</i> fruit extracts against <i>A. baumannii</i> .	26
4.7	Antimicrobial effect of <i>S. melongena</i> fruit extracts against <i>P. aeruginosa</i> .	27
4.8	Antimicrobial effect of <i>S. melongena</i> fruit extracts against <i>S. aureus</i> .	28
4.9	Antimicrobial effect of <i>S. melongena</i> fruit extracts against <i>E. faecalis</i> .	28
4.10	Comparison of antimicrobial effect of different <i>S. melongena</i> fruit extracts against different bacteria. "AB"= <i>A. baumannii</i> ; "PA"= <i>P. aeruginosa</i> ; "SA"= <i>S. aureus</i> ; "EF"= <i>E. faecalis</i> .	29
4.11	Antibiofilm property of <i>S. melongena</i> methanolic fruit extract against <i>P. aeruginosa</i> biofilm. "C"= Diluted bacterial culture; "M(P)"= Methanolic fruit extract; "N"= Neem leaves extract; "T"= Tetracycline; "M"= Methanol; "LB"= LB broth; "AA"= 30% (v/v) Acetic acid.	30
4.12	Antibiofilm property of <i>S. melongena</i> ethanolic fruit extract against <i>P. aeruginosa</i> biofilm. "C"= Diluted bacterial culture; "E(P)"= Ethanolic fruit extract; "N"= Neem leaves extract; "T"= Tetracycline; "E"= Ethanol; "LB"= LB broth; "AA"= 30% (v/v) Acetic acid.	30

LIST OF ABBREVIATIONS

%	percent
°C	degree Celsius
μL	microlitre
μm	micrometer
A ₆₂₀	absorbance reading at 620 nm
ANOVA	analysis of variance
CFU/ mL	colony-forming units per millilitre
EPS	extracellular polymeric substances
FeCl	ferric chloride
g	gram
H ₂ O ₂	hydrogen peroxide
H ₂ S	hydrogen sulphide
H ₂ SO ₄	sulphuric acid
HCl	hydrochloric acid
LB	lysogeny broth
MBL	metallo-beta-lactamases
mg	milligram
mL	millilitre
mm	millimeter
NaOH	sodium hydroxide
nm	nanometer
PBPs	penicillin-binding proteins
PBS	phosphate-buffered saline
pH	power of hydrogen
QS	Quorum sensing
rpm	revolutions per minute
UK	United Kingdom
USA	United States of America
v/v	volume per volume

CHAPTER 1

INTRODUCTION

Health care institutions provide patients with medical and surgical treatment for certain diseases with the help of medical specialists and sophisticated equipment (World Health Organization, 2017). However, pathogens such as *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Acinetobacter baumannii* which have been identified as common nosocomial pathogens have caused more severe infections and prolonged hospitalization among patients (Khan, Ahmad & Mehboob, 2015). Such clinically important pathogens are also capable of forming biofilms that further lead to chronic infections as a result of regulating the expression of virulence genes through quorum sensing (Waters, Lu, Rabinowitz, & Bassler, 2008). As a consequence, patients require prolonged therapy and higher dosage of antibiotics to prevent the initial health issue from becoming a potentially fatal illness. There is a high possibility for the symptoms to recur even after repeated treatment since biofilm-causing bacteria have shown high resistance to disinfectants and antibiotics as well as enhanced tolerance to the phagocytic action of immune systems (Hoiby, Bjarnsholt, Givskov, Molin & Ciofu, 2010). These resistance factors can be due to extracellular polymers and specific enzymes in the bacterial biofilms which not only prevent the penetration of antimicrobial agents, but also cause the inactivation of these drugs (Chadha, 2014). The resistance can be acquired through horizontal gene transfer or mutation in previously susceptible bacteria (Tenover, 2006).

The issue of drug resistance in bacterial biofilms has raised concerns globally due to the increased risk of mortality in patients with prolonged hospitalization and indwelling devices. Hence, there is an immediate need to discover alternatives with significant antibacterial and antibiofilm properties. Herbaceous plants are applied widely in medical field and are being used as starting material to formulate allopathic medicine (Palombo, 2011). Several compounds including flavonoids and tannins have been extracted from the plants and proved to have properties such as antibacterial, antioxidant, anti-inflammatory and others (Akter et al., 2016).

Solanum melongena, commonly known as eggplant, is ordinarily cultivated and consumed by every farm household and many families throughout the world (Knapp, Vorontsova & Prohens, 2013). Several studies have been done on this economically important crop and have shown that every part of this plant has significant antimicrobial, antifungal, antitumor and other properties due to the presence of compounds such as solanine, siloxane and linoleic acid (Al-Janabi & Al-Rubeey, 2010; Gao, Wang & Ji, 2006; Sitap, Tilawale, Nadaf & Ghosh, 2015; George Daye Mandy, Anthony & Kingsley, 2014): However, there is a lack of knowledge in its role of inhibiting bacterial biofilm development.

Based on these perspectives, this study was undertaken to determine the antibacterial activity against some clinically important bacteria, namely *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Staphylococcus aureus* using fruit extracts of *Solanum melongena*. In addition, the efficacy of the fruit extracts of *S. melongena* in inhibiting the development of biofilms by these pathogens was studied. This study was also carried out to compare the effectiveness of *S. melongena* fruit extracts prepared using three different organic solvents which were acetone, ethanol and methanol in both the antimicrobial and antibiofilm assays.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacterial Strains

2.1.1 Gram-negative Bacteria

2.1.1.1 *Acinetobacter baumannii*

A.baumannii is a non-motile, Gram-negative bacterium that grows under aerobic condition (Kurcik-Trajkovska, 2009). It colonizes several parts of human body including the hand, forehead, throat and other parts (Almasaudi, 2016). However, it is also an opportunistic pathogen that mostly affects patients who have a weakened immune system and require prolonged medical and surgical treatment (Montefour et al., 2008). It is isolated in high concentrations from the mucous membrane of the oropharynx and respiratory tract as well as from the skin of affected individuals in intensive care units (Howard, O' Donoghue, Feeney & Sleator, 2012). Hospital-associated infections caused by these bacteria can be transmitted through nursing staff and hospital equipment. These infections include urinary tract infections, endocarditis, meningitis, bacteremia, pneumonia as well as skin and soft tissue infections (McConnell, Actis & Pachón, 2012). Before 1970s, these infections could be cured using antibiotics such as sulphonamides and beta-lactams (Gonzalez-Villoria & Valverde-Garduno, 2016). However, *A. baumannii* has currently become a more resilient pathogen due to its ability to acquire genes conferring antibiotic resistance (Peleg, Seifert & Paterson, 2008).

2.1.1.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacterium that can be ubiquitously isolated from river or lake waters (Mena & Gerba, 2009). Quorum sensing (QS), which is a system responsible for the regulation of several virulence traits, acts as a key factor in the pathogenicity of *P. aeruginosa* (Williams & Cámara, 2009). This opportunistic

pathogen causes community- and healthcare-associated infections such as cystic fibrosis, pneumonia and neutropenia in immunocompromised patients (Chatzinikolaou et al., 2000). Infections caused by *P. aeruginosa* generally disseminate to other patients through contact with medical equipment and nursing staff (Yayan, Ghebremedhin & Rasche, 2015). *P. aeruginosa* is capable of forming biofilms and this has become a growing global concern (Tran et al., 2014). The biofilms which develop during the course of an infection could result in increased morbidity and mortality in patients who are seriously ill. In addition, the bacteria in these biofilms are resistant to various antibiotics including carbapenems, penicillin G, ceftazidime and quinolones (Tam et al., 2010). Furthermore, characteristics including production of periplasmic beta-lactamases, efflux system and restricted outer-membrane permeability also contribute to the antibiotic resistance which intrinsically a feature in these bacteria (Hancock & Speert, 2000).

2.1.2 Gram-positive Bacteria

2.1.2.1 *Enterococcus faecalis*

E. faecalis is non-motile, Gram-positive bacterium that is commonly found in the human intestine (Gajan et al., 2013). Among all of the species of enterococci, it is the third most common pathogen that is isolated from healthcare institutions and has become a serious public health issue (Hidron et al., 2008; Brooks, Carroll, Butel, Morse & Mietzner, 2013). *E. faecalis* usually infects wounds, blood and urinary tract of patients and can be transmitted from one patient to another through hospital personnel or contaminated medical equipment (Brooks et al., 2013). In addition, the advent of antibiotic treatment has led to the development of antibiotic resistance in *E. faecalis* since the 1960s (Miller, Munita, & Arias, 2014). The mechanism of horizontal gene transfer was first discovered in 1970s and showed that bacteria could acquire the antibiotic-resistant gene both intra-specifically as well as inter-specifically (Clewell & Franke, 1974). Strains of *E. faecalis* can be resistant to antibiotics including cephalosporins, monobactams and vancomycin (Brooks et al., 2013). They can also be resistant to the synergistic effect of aminoglycosides and some other antibiotics due to the expression of aminoglycoside-modifying enzymes (Brooks et al.,