

**POTENTIAL PERSISTENT MYCOBACTERIUM  
INHIBITORS FROM PLANTS AND  
ACTINOMYCETES TARGETING ISOCITRATE  
LYASE AND MALATE SYNTHASE IN THE  
GLYOXYLATE SHUNT OF *MYCOBACTERIUM* sp.**



**KHOO YAU LIANG**

**UMS**  
UNIVERSITI MALAYSIA SABAH

**SCHOOL OF SCIENCE AND TECHNOLOGY  
UNIVERSITI MALAYSIA SABAH  
2013**

**POTENTIAL PERSISTENT MYCOBACTERIUM  
INHIBITORS FROM PLANTS AND  
ACTINOMYCETES TARGETING ISOCITRATE  
LYASE AND MALATE SYNTHASE IN THE  
GLYOXYLATE SHUNT OF *MYCOBACTERIUM* sp.**

**KHOO YAU LIANG**



**UMS**  
UNIVERSITI MALAYSIA SABAH

**THESIS SUBMITTED IN FULFIMENT FOR THE  
DEGREE OF MASTER OF SCIENCE**

**SCHOOL OF SCIENCE AND TECHNOLOGY  
UNIVERSITI MALAYSIA SABAH  
2013**

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : \_\_\_\_\_

IJAZAH : \_\_\_\_\_

SAYA : \_\_\_\_\_ SESI PENGAJIAN : \_\_\_\_\_  
(HURUF BESAR)

Mengaku membenarkan tesis \*(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

TIDAK TERHAD

Disahkan oleh:

\_\_\_\_\_  
(TANDATANGAN PENULIS)

\_\_\_\_\_  
(TANDATANGAN PUSTAKAWAN)

Alamat Tetap: \_\_\_\_\_

\_\_\_\_\_  
(NAMA PENYELIA)

TARIKH: \_\_\_\_\_

TARIKH: \_\_\_\_\_

Catatan:

\*Potong yang tidak berkenaan.

\*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.

\*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

## DECLARATION

All the materials in this dissertation are original except for the quotations, excerpts, summaries and references, which have been duly acknowledged.

26 April 2013

---

Khoo Yau Liang  
PS20078269



UMS  
UNIVERSITI MALAYSIA SABAH

## CERTIFICATION

NAME : **KHOO YAU LIANG**

MATRIX NO. : **PS20078269**

TITLE : **POTENTIAL PERSISTENT MYCOBACTERIUM INHIBITORS FROM PLANTS AND ACTINOMYCETES TARGETING ISOCITRATE LYASE AND MALATE SYNTHASE IN THE GLYOXYLATE SHUNT OF *MYCOBACTERIUM* sp.**

DEGREE : **MASTER OF SCIENCE (NATURAL PRODUCTS DISCOVERY)**

VIVA DATE : **26 APRIL 2013**

 **DECLARED BY**

**1. SUPERVISOR**  
Assoc. Prof. Dr. How Siew Eng

**2. CO-SUPERVISOR**  
Assoc. Prof. Dr. Lee Ping Chin

Signature

**UMS**  
UNIVERSITI MALAYSIA SABAH

---

---

## ACKNOWLEDGEMENT

First of all, I would like to express my sincere thanks to School of Science and Technology, University Malaysia Sabah which provided a very good opportunity for me to complete my master project.

Secondly, I must thank to my supervisor, Assoc. Prof. Dr. How Siew Eng for her patient and supervision in the project. Next, I would like to express my gratitude to my co-supervisor, Assoc. Prof. Dr. Lee Ping Chin for her assistance, advice and knowledge on microbiology and thesis writing.

I am grateful to my teamwork partners, Ch'ng Ai Ying, Wong Siak Chung, and Ng Seong Wooi for their assistances and supports. I would like to thank my seniors and friends for their helps and friendship. I am grateful to other fellows from different laboratories and universities for their helps and supports during my master project.

Deepest appreciation to my NSF scholarship sponsor (MOSTI) for this accomplishment. At last, I would like to dedicate this dissertation to my family for their loves, understanding and supports.



UMMS  
UNIVERSITI MALAYSIA SABAH

## ABSTRACT

Multi- or extensive TB drug resistance, co-infection of HIV/TB and the burdensome persistent infection have placed tuberculosis (TB) as a global emergence that causes 2 million deaths annually. During persistency, *Mycobacterium tuberculosis* utilizes the glyoxylate pathway to survive, thus making pathway enzymes such as isocitrate lyase (ICL) and malate synthase (MLS) valuable drug targets to improve persistent-TB control. The main objective of this study was to identify potential persistent inhibitor(s) targeting the specific enzyme (MLS) in the acetate growth of *Mycobacterium* sp. A total of 117 extracts prepared from the 44 local plants and 60 soil actinomycetes were screened against MLS using the non-pathogenic form of mycobacteria (*M. smegmatis* mc<sup>2</sup>155, H8000) in agar diffusion assay. The potential crude extracts were further analyzed using modified Resazurin Microtiter Assay (REMA), MLS enzymatic assay and Tetrazolium Microplate Assay (TEMA). Among the extracts tested, *Hopea pentanarvia* (plant) and H7763 (actinomycete) gave the most potent growth inhibition activity on *M. smegmatis* in REMA. The H7763 extract produced most promising MLS growth inhibitory effect and the *Hopea pentanarvia* showed potential anti-mycobacterium activity against the pathogenic strain, *M. tuberculosis* H37Rv. Following this, both potential extracts were selected for bioassay-guided fractionation, and yielded a number of bioactive compounds which were characterized by spectroscopic methods [UV, IR, Mass Spectrometry (MS), 1D- and HMBC NMR]. *Hopea pentanarvia* yielded a known resveratrol derivative which was finally proposed as *cis*-Upunaphenol K. H7763 gave a known nucleoside compound named guanine 7-*N*-oxide. In addition to these structural studies, a minimum inhibition concentration (MIC) agar diffusion assay was performed using the nucleoside and resveratrol derivative against *M. smegmatis* with 3-nitropropionate (a known ICL prototypic inhibitor) as positive control. This nucleoside ( $8.1 \pm 2.3 \mu\text{g}/\text{disc}$ ) gave the lowest MIC value compared to the resveratrol derivative ( $70.0 \pm 14.1 \mu\text{g}/\text{disc}$ ) and the known inhibitor ( $37.5 \pm 3.5 \mu\text{g}/\text{disc}$ ). The nucleoside may require further research on toxicity before use in the development of antitubercular drug against *M. tuberculosis*.

## ABSTRAK

### **POTENSI PADA TUMBUHAN DAN AKTINOMISET SEBAGAI PERENCAT TERPENDAM SASARAN ISOSITRAT LIASE DAN MALAT SINTASE DALAM KITARAN GLIOKSILAT BAGI MYCOBACTERIUM sp.**

Rintangan pada ubatan anti-tuberkulosis, jangkitan bersama HIV dan TB terpendam menjadi penyebab kepada 2 juta kematian dalam setiap tahun. Semasa fasa pendam, kitaran glioksilat adalah salah satu keperluan untuk Mycobacterium tuberculosis supaya hidup berterusan, menjadikan enzim-enzim kitaran tersebut seperti isositrat liase (ICL) dan malat sintase (MLS) sasaran utama bagi meningkatkan kawalan TB terpendam. Objektif utama kajian ini adalah untuk mengenalpasti perencat terpendam yang menasaskan enzim (MLS) dalam asetat pertumbuhan Mycobacterium sp. Sejumlah 117 ekstrak daripada 44 spesies tumbuhan tempatan dan 60 sampel aktinomiset tanah telah disaringkan terhadap MLS dengan penggunaan mycobacteria tak patogenik (*M. smegmatis* mc<sup>2</sup>155, H8000) pada penyaringan agar. Kemudian, ekstrak kasar yang berpotensi diteruskan dengan analisis Resazurin Microtiter Assay (REMA) yang diubahsuai, MLS Enzymatic Assay dan Tetrazolium Microplate Assay (TEMA). Di antaranya, *Hopea pentanarvia* (tumbuhan) dan H7763 (aktinomiset) paling merencat terhadap *M. smegmatis* pada REMA. Ekstrak H7763 adalah ekstrak yang paling potensi untuk merencat pertumbuhan MLS dan *Hopea pentanarvia* pula berpotensi menghasilkan aktiviti anti-mycobacterium terhadap mycobacterium jenis patogenik, *M. tuberculosis* H37Rv. Oleh itu, kedua-dua ekstrak berpotensi ini dipilih untuk penulenan berasaskan bioasai, dan menghasilkan beberapa sebatian bioaktif melalui kaedah spektroskopi [UV, IR, Mass Spectrometry (MS), 1D- dan HMBC NMR]. *Hopea Pentanarvia* menghasilkan satu terbitan resveratrol yang dikenali sebagai cis-Upunaphenol K. H7763 pula menghasilkan satu sebatian bioaktif nucleoside iaitu guanine 7-N-oxide. Selain daripada penentuan struktur, penyaringan agar untuk menentukan aktiviti perencatan minimum (MIC) telah dijalankan terhadap nucleoside dan terbitan resveratrol tersebut dan dibandingkan dengan 3-nitropropionate (suatu prototaip perencat ICL). Nucleoside tersebut memberi nilai MIC ( $8.1 \pm 2.3 \mu\text{g}/\text{disc}$ ) terendah jika dibandingkan dengan terbitan resveratrol ( $70.0 \pm 14.1 \mu\text{g}/\text{disc}$ ) dan perencat ICL tersebut ( $37.5 \pm 3.5 \mu\text{g}/\text{disc}$ ). Kajian kadar toksik perlu dijalankan ke atas nucleoside tersebut sebelum diaplikasikan secara mendalam dalam perkembangan ubat antitubercular terhadap *M. tuberculosis*.



## TABLE OF CONTENTS

	Page
<b>TITLE</b>	i
<b>DECLARATION</b>	ii
<b>VERIFICATION</b>	iii
<b>ACKNOWLEDGEMENT</b>	iv
<b>ABSTRACT</b>	v
<i>ABSTRAK</i>	vi
<b>LIST OF CONTENTS</b>	vii
<b>LIST OF TABLES</b>	ix
<b>LIST OF FIGURES</b>	x
<b>LIST OF ABBREVIATIONS</b>	xii
<b>LIST OF SYMBOLS</b>	xiii
<b>LIST OF APPENDIX</b>	xiv
<b>CHAPTER 1: INTRODUCTION</b>	
1.1 Background	1
1.2 Objectives	4
1.3 Scope of study	4
<b>CHAPTER 2: LITERATURE REVIEW</b>	
2.1 The global burden of Tuberculosis (TB)	5
2.2 Physical nature of <i>Mycobacterium tuberculosis</i> ( <i>M. tuberculosis</i> )	6
2.3 Persistent infection of TB	7
2.4 Conventional antimycobacterial drugs	8
2.5 Role of natural products in drug development	9
2.6 Model of persistence and the mechanism of persistency	13
2.7 <i>In-vitro</i> assays for evaluation of anti-TB activity	18
2.8 Summary	22
<b>CHAPTER 3: MATERIALS AND METHODS</b>	
3.1 Plant and actinomycete extracts	23
3.2 Preparation of extracts	26

3.2.1	Plant extracts	26
3.2.2	Actinomycete extracts	26
3.3	Determination of anti-mycobacterium activity	27
3.3.1	Agar diffusion assay	27
3.3.2	Micro broth dilution assay	28
	a. TEMA	29
	b. REMA	30
3.4	Enzymatic inhibitory assay	31
3.4.1	Preparation of MLS enzyme	31
3.4.2	Determination of protein concentration using Bradford assay	32
3.4.3	Malate synthase (MLS) enzymatic assay	33
3.5	Isolation of anti-mycobacterium inhibitor(s)	34
3.5.1	Solvent-solvent partitioning of <i>H. pentanarvia</i> and H7763	34
	a. <i>H. pentanarvia</i>	34
	b. H7763	34
3.5.2	Procedures for isolation of bioactive inhibitor(s) from <i>H. pentanarvia</i> and H7763	35
	a. <i>H. pentanarvia</i>	35
	b. H7763	36
3.5.3	RP-HPLC purification of the bioactive fraction(s) from <i>H. pentanarvia</i> and H7763	38
3.6	Structure elucidation of isolated bioactive inhibitor(s)	39
3.6.1	Ultraviolet-visible (UV-vis) spectrophotometry	39
3.6.2	Infra-red (IR) spectrometry	39
3.6.3	Nuclear magnetic resonance (NMR) spectra	39
3.6.4	Liquid chromatography-Mass spectrometry (LCMS)	39
3.7	Chemical characterization	40
3.7.1	HP01 ( <i>cis</i> -Upunaphenol K)	40
3.7.2	H-2 [Guanine 7- <i>N</i> -oxide]	42
3.8	Summary of the study	43
<b>CHAPTER 4: RESULTS AND DISCUSSION</b>		
4.1.1	Selection of most active crude extract(s) for isolation of anti-mycobacterium inhibitor(s) targeting malate synthase (MLS)	44
4.1.2	Malate synthase (MLS) enzymatic assay on the most promising extract(s)	53
4.1.3	Selection of <i>H. pentanarvia</i> and H7763 for isolation of anti-mycobacterium and anti-persistent inhibitor(s) targeting malate synthase (MLS)	54
4.2	Isolation of anti-mycobacterium compound(s) targeting MLS	55
4.2.1	Plant, <i>Hopea pentanarvia</i>	55
4.2.2	Actinomycete, H7763	63
4.3	Chemical characterization of the isolated MLS and non-MLS inhibitors	75
<b>CHAPTER 5: CONCLUSION</b>		80
<b>REFERENCES</b>		83

## LIST OF TABLES

	Page
Table 2.1 Mechanism of action, resistance and half-line of the anti-TB drugs isolated from actinomycetes	11
Table 2.2 Principles, advantages and weakness of the <i>in-vitro</i> assays	20
Table 3.1 Part used, type of extract and some information of plant materials	23
Table 3.2 Type of extract and some information of actinomycete strains	25
Table 3.3 <sup>1</sup> H, <sup>13</sup> C-NMR ( $\delta$ scale) and HMBC spectral data for HP01	40
Table 3.4 <sup>1</sup> H, <sup>13</sup> C-NMR ( $\delta$ scale) and HMBC spectral data of H-2	42
Table 4.1 Anti-mycobacterium activity of various crude plant extracts against <i>M. smegmatis</i> mc <sup>2</sup> 155 (H8000)	45
Table 4.2 Anti-mycobacterium activity of various crude actinomycete extracts against <i>M. smegmatis</i> mc <sup>2</sup> 155 (H8000)	48
Table 4.3 The final MIC ( $\mu$ g/mL) of the active extracts against <i>M. smegmatis</i> mc <sup>2</sup> 155 (H8000) (REMA) and <i>M. tuberculosis</i> (H37Rv) (TEMA)	52
Table 4.4 Enzyme activity and percent of inhibition for comparative control and the active crude extracts against MLS encoded <i>E. coli</i>	54
Table 4.5 Anti-mycobacterium activity of the fractions and MIC ( $\mu$ g/disc) of the pure compound against the growth of <i>M. smegmatis</i> mc <sup>2</sup> 155 (H8000) using agar diffusion assay	57
Table 4.6 Anti-mycobacterium activity of the fractions and MIC ( $\mu$ g/disc) of the pure compound against the growth of <i>M. smegmatis</i> mc <sup>2</sup> 155 (H8000) using agar diffusion assay	65

## LIST OF FIGURES

	Page
Figure 2.1	The transmission electron micrograph of <i>M. tuberculosis</i> 6
Figure 2.2	Temporal scales in TB infection 8
Figure 2.3	Enzymic reactions of the glyoxylate shunt 15
Figure 2.4	The related reactions in glyoxylate shunt 15
Figure 3.1	Schematic diagram of a 96-wells microplate assay 28
Figure 3.2	Sephadex LH-20 column chromatography of methanolic fraction of <i>H. pentanarvia</i> 35
Figure 3.3	Silica gel 60 column chromatography of freeze-dried aqueous fraction 37
Figure 3.4	Sephadex LH-20 column chromatography of purer fraction(s) after silica gel chromatography 37
Figure 3.5	Flowchart of bioassay screening of potential anti-mycobacterium inhibitor(s) from plants and actinomycete extracts against MLS 43
Figure 4.1	Anti-mycobacterium activity of H7763 aqueous crude extract on carbon sources, a) Glucose and b) Acetate 55
Figure 4.2	HPLC chromatogram of Sephadex fractions, a) S05, b) S06, c) S07, d) S08, e) S09, f) S10 and g) S11 at 220 nm 58
Figure 4.3	Anti-mycobacterium activity of methanolic and hexanoic partitions (from solvent-solvent partitioning) on carbon sources, a) Glucose and b) Acetate 59
Figure 4.4	Anti-mycobacterium activity of HPLC fractions, HP01 and HP02 on carbon sources, a) Glucose and b) Acetate 60
Figure 4.5	HPLC chromatogram of a) Sephadex fraction-S11, b) HP01 and c) HP02 at 220 nm 61
Figure 4.6	Schematic representation of isolation of non-MLS inhibitor from methanolic extract of <i>H. pentanarvia</i> 62
Figure 4.7	Solvent-solvent partition from potential aqueous crude extract of H7763 63

Figure 4.8	Anti-mycobacterium activity of buthanol and aqueous partitions on carbon sources, a) Glucose and b) Acetate	66
Figure 4.9	TLC of aqueous extract under detection of Iodine vapor, I <sub>2</sub>	67
Figure 4.10	Anti-mycobacterium activity of silica gel fraction, F2 on carbon sources, a) Glucose and b) Acetate	68
Figure 4.11	Anti-mycobacterium activity of silica gel fraction, 2F1 on carbon sources, a) Glucose and b) Acetate	69
Figure 4.12	Anti-mycobacterium activity of sephadex fraction, S04 on carbon sources, a) Glucose and b) Acetate	70
Figure 4.13	HPLC Chromatogram of Sephadex fraction, a) S03, b) S04 at 210 nm	71
Figure 4.14	Anti-mycobacterium activity of HPLC fraction, H-2 on carbon sources, a) Glucose and b) Acetate	72
Figure 4.15	HPLC Chromatogram of a) Sephadex fraction, S04, b) H-1 and c) H-2 at 210 nm	73
Figure 4.16	Schematic representation of isolation of MLS inhibitor from aqueous partition of H7763	74
Figure 4.17	HMBC connectivities in <b>21</b>	76
Figure 4.18	Chemical structure of Halophilol B	77
Figure 4.19	a) Guanine 7- <i>N</i> -oxide ( <b>22</b> ) and b) HMBC connectivities in Guanine 7- <i>N</i> -oxide	79
Figure 4.20	Tautomeric structure of guanine 7- <i>N</i> -oxide	79
Figure 5.1	<i>Hopea Pentanarvia</i> in Herbarium of Forestry Research Center (FRC), Sandakan, Sabah, Malaysia	82

## LIST OF ABBREVIATIONS

<b>Acetyl-CoA</b>	Acetyl-coenzyme A
<b>AIDS</b>	Acquired Immunodeficiency Syndrome
<b>D<sub>2</sub>O</b>	Deuterium oxide
<b>DNA</b>	Deoxyribonucleic acid
<i>glcB</i>	Gene of malate synthase
<b>HMBC</b>	Heteronuclear Multiple Bond Correlation
<b>HRESI-MS</b>	High resolution electron spray ionization- Mass spectrometry
<b>i.d.</b>	Internal diameter
<i>icl</i>	Gene of isocitrate lyase
<b>IFN-<math>\gamma</math><sup>-/-</sup></b>	Interferon-gamma
<b>MeOD</b>	Deuterated Methanol
<b>NMR</b>	Nuclear Magnetic resonance
<b>ppm</b>	Part per million
<b>RP-HPLC</b>	Reverse phase-High pressure liquid chromatography
<b>rpm</b>	revolution per minute
<b>TB</b>	Tuberculosis
<b>TLC</b>	Thin layer chromatography
<b>WHO</b>	World Health Organization

## LIST OF SYMBOLS

<b>%</b>	Percentage
<b>μL</b>	microliter
<b>μm</b>	micrometer
<b>μg/mL</b>	Microgram per milliliter
<b>cm</b>	Centimeter
<b>cm<sup>-1</sup></b>	Reciprocal centrimeters
<b>g/L</b>	Gram per liter
<b>L</b>	Liter
<b>m/z</b>	Mass per charge
<b>mAU</b>	milliAbsorbance
<b>mg/mL</b>	milligram per milliliter
<b>mL</b>	milliliter
<b>mL/min</b>	milliliter per minutes
<b>mm</b>	millimeter
<b>mM</b>	millimole
<b>nm</b>	nanometer
<b>°C</b>	Degree of Celsius
<b>v/v</b>	volume per volume
<b>w/v</b>	Weight per volume



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF APPENDIX

	Page
Appendix A FT-IR and UV Spectra of HP01	96
Appendix B TOF-ESI-Mass Spectrum of HP01	97
Appendix C $^1\text{H}$ NMR Spectrum of HP01	98
Appendix D $^{13}\text{C}$ APT-NMR Spectrum of HP01	101
Appendix E HMBC-NMR Spectrum of HP01	105
Appendix F FT-IR and UV Spectra of H-2	115
Appendix G TOF-ESI-Mass Spectrum of H-2	116
Appendix H $^1\text{H}$ NMR Spectrum of H-2	117
Appendix I $^{13}\text{C}$ APT-NMR of H-2	120
Appendix J HMBC-NMR Spectrum of H-2	123
Appendix K HMBC-NMR Spectrum of P2	131
Appendix L Calculation of MLS protein concentration from <i>E. coli</i> with Bradford assay	140



# CHAPTER 1

## INTRODUCTION

*Labisia pumila* or commonly known as Kacip Fatimah in Malaysia is a herbaceous plant widely used in folk medicine for facilitating childbirth and post-partum recovery (Bodeker, 2009). The phytochemical constituents of this herb have been well documented with phenolics and flavonoids being the main compounds (Norhanisah *et al.*, 2013). Several scientific studies reported that *L. pumila* possesses biological activities such as antioxidant (Norhaiza *et al.*, 2009; Karimi *et al.*, 2011), anti-carcinogenic (Pihie *et al.*, 2011), anti-microbial (Karimi *et al.*, 2011), antifungal and anti-inflammatory activities (Karimi *et al.*, 2013).

Considering the interesting pharmacological values that *L. pumila* has to offer, raw materials of this herb is highly demanded for commercial production. However, the propagation and growth rate of wild *L. pumila* is rather slow and time consuming (Mohd. Noh *et al.*, 2002; Jaafar *et al.*, 2009). Hence, a propagation system that can supply *L. pumila* continuously must be established to accommodate the demand of bioactive compounds synthesised by this herb.

Plant cell culture is an ideal biotechnological approach for secondary metabolites production as it produce continuous and reliable source of plant-based pharmaceutical products (Rao & Ravishankar, 2002; Yue *et al.*, 2016). Research to date has successfully produces high yielding cultures from various medicinal plants in either undifferentiated or differentiated cultures (Yue *et al.*, 2016). Undifferentiated cell suspension cultures lack stability and uniformity (Habibi *et al.*, 2017) which resulted in lower production of high value natural products (Yue *et al.*, 2016). In contrast, organ culture, especially adventitious root culture is more

favourable due to its fast growth and stable production of secondary metabolites (Murthy *et al.*, 2008; Habibi *et al.*, 2017).

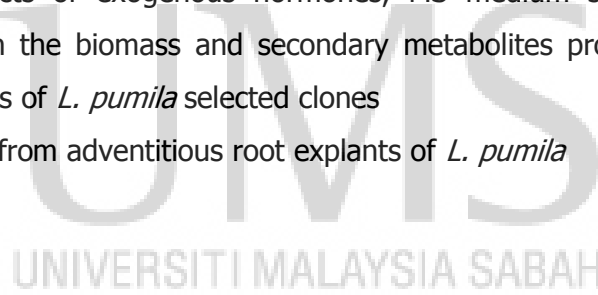
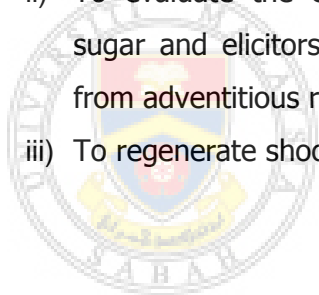
Establishment of organ cultures that produce large amounts of biomass with increased accumulation of secondary metabolites is possible through specific strategies (Murthy *et al.*, 2014a). These includes the selection of high-yielding clones, optimisation of medium composition such as type of basal medium, carbon source and plant growth regulators; and physical factors such as temperature, medium pH, agitation and aeration. Other approaches such as elicitation, precursor feeding, permeabilisation and immobilisation could also assist with the accumulation of metabolites (Abouzid, 2014; Malik *et al.*, 2014; Murthy *et al.*, 2014a; Ali *et al.*, 2016; Yue *et al.*, 2016; Andrews & Robert, 2017).

Through optimisation of *in vitro* culture conditions of adventitious root culture, high product concentration and efficacy can be achieved from the continuous source of secondary metabolites of root cultures (Murthy & Praveen, 2012). This study will highlight some of the strategies undertaken to increase *L. pumila* adventitious root metabolites yield including selection of clones, optimisation of plant growth regulators, MS medium strength and carbon source; and also elicitation. Initiation of organ cultures began with selecting parent plants that showed higher contents of the desired secondary product for organ induction (Murthy *et al.*, 2014a). The selection of a specific organ for the induction of *in vitro* adventitious roots is essential as the accumulation of metabolites varies in different organs of the same species. Following selection of high performing organ lines, another key consideration is to establish optimum media and culture composition (Ochoa-Villarreal *et al.*, 2016). Typical modifications to the adventitious root culture medium include the addition of phytohormones (Wu *et al.*, 2006; Baque *et al.*, 2010a; Fazal *et al.*, 2014), modification of the salt strength (Baque *et al.*, 2010b; Li *et al.*, 2015; Deepthi & Satheeshkumar, 2017) and sugar concentration (Baque *et al.*, 2012; Yin *et al.*, 2013; Li *et al.*, 2015). In addition, metabolite production in organ cultures can be stimulated *in vitro* by adding elicitors into the culture medium as metabolites are produced by plants in response to the imposed stresses (Naik & Al-Khayri, 2016; Andrews & Robert, 2017).

Apart from producing secondary metabolites, adventitious root can also serve as a reliable micropropagation method in tissue culture especially when numerous small shoots arise rapidly from each explant, hence leading to high rate of propagation. Previous studies on shoot regeneration of *L. pumila* only focused on leaf and stem explants (Hartinie, 2007; Ling *et al.*, 2013; Ozayanna, 2015; Syafiqah *et al.*, 2016). No attempt was done to explore the potential of adventitious roots explants of *L. pumila* for shoot regeneration purpose.

Therefore, the present study has focused on the aforementioned strategies to produce bioactives from adventitious root cultures of *L. pumila* with antioxidative properties. In addition, the potential of adventitious root explants of *L. pumila* for producing new shoots will also be investigated. The objectives of the study are;

- i) To select superior *in vitro* source materials from each variety of *L. pumila* (var. *alata*, var. *pumila* and var. *lanceolata*) for high antioxidative properties
- ii) To evaluate the effects of exogenous hormones, MS medium strength, sugar and elicitors on the biomass and secondary metabolites production from adventitious roots of *L. pumila* selected clones
- iii) To regenerate shoots from adventitious root explants of *L. pumila*



## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 *Labisia pumila* (Bl.) Fern. Vill

##### 2.1.1 Origin, distribution and taxonomy of *Labisia pumila*

*Labisia pumila* (Bl.) Fern. Vill is herbaceous plant which grows wildly in the rain forest of Malaysia, Indochina, Thailand and Papua New Guinea (Sunarno, 2005). The distribution of *L. pumila* is shown in Figure 2.1. In Malaysia, this herb is usually known as Kacip Fatimah. Other local names of *L. pumila* include Selusoh Fatimah, Kacit Fatimah, Tadah Matahari and Mata Pelanduk Rimba (Sunarno, 2005; Jamal, 2006).



**Figure 2.1 : Distribution of *L. pumila***

Source : Global Biodiversity Information Facility (GBIF) Secretariat (2016)

According to Sunarno (2005), there are eight varieties of *L. pumila* namely var. *alata*, var. *discoplacenta*, var. *gladiata*, var. *lanceolata*, var. *pumila*, var. *malintangensis*, var. *neriifolia* and var. *sessilifolia*. Among these eight varieties, only var. *alata*, var. *pumila* and var. *lanceolata* are well-known in Malaysia (Stone, 1990). These three varieties can be distinguished from each other via their petiole and leaf physical appearances (Sunarno, 2005).

The taxonomy of *L. pumila* is shown in Table 2.1. *Marantodes pumilum* (Blume) Kuntze is a heterotypic synonym of *L. pumila* that has been accepted by The Plant List (2013). This name was originally found in Post and Kuntze (1903) as accepted taxon in the genus *Marantodes* (family Primulaceae). Myrsinaceae and Primulaceae are two best known families in Ericales. The taxon limits of Myrsinaceae and Primulaceae have been substantially changed, therefore the limits of Primulaceae was extended based on numerous synapomorphies within the group (Mabberly, 2008; Bremer *et al.*, 2009).

**Table 2.1: Taxonomy of *L. pumila***

<b>Taxonomy</b>	
<b>Domain</b>	Eukaryota
<b>Kingdom</b>	Plantae
<b>Phylum</b>	Magnoliophyta
<b>Class</b>	Magnoliopsida
<b>Order</b>	Ericales
<b>Family</b>	Myrsinaceae
<b>Genus</b>	Labisia
<b>Species</b>	<i>Labisia pumila</i>

Source: Global Biodiversity Information Facility (GBIF) Secretariat (2016)

### 2.1.2 Morphological description

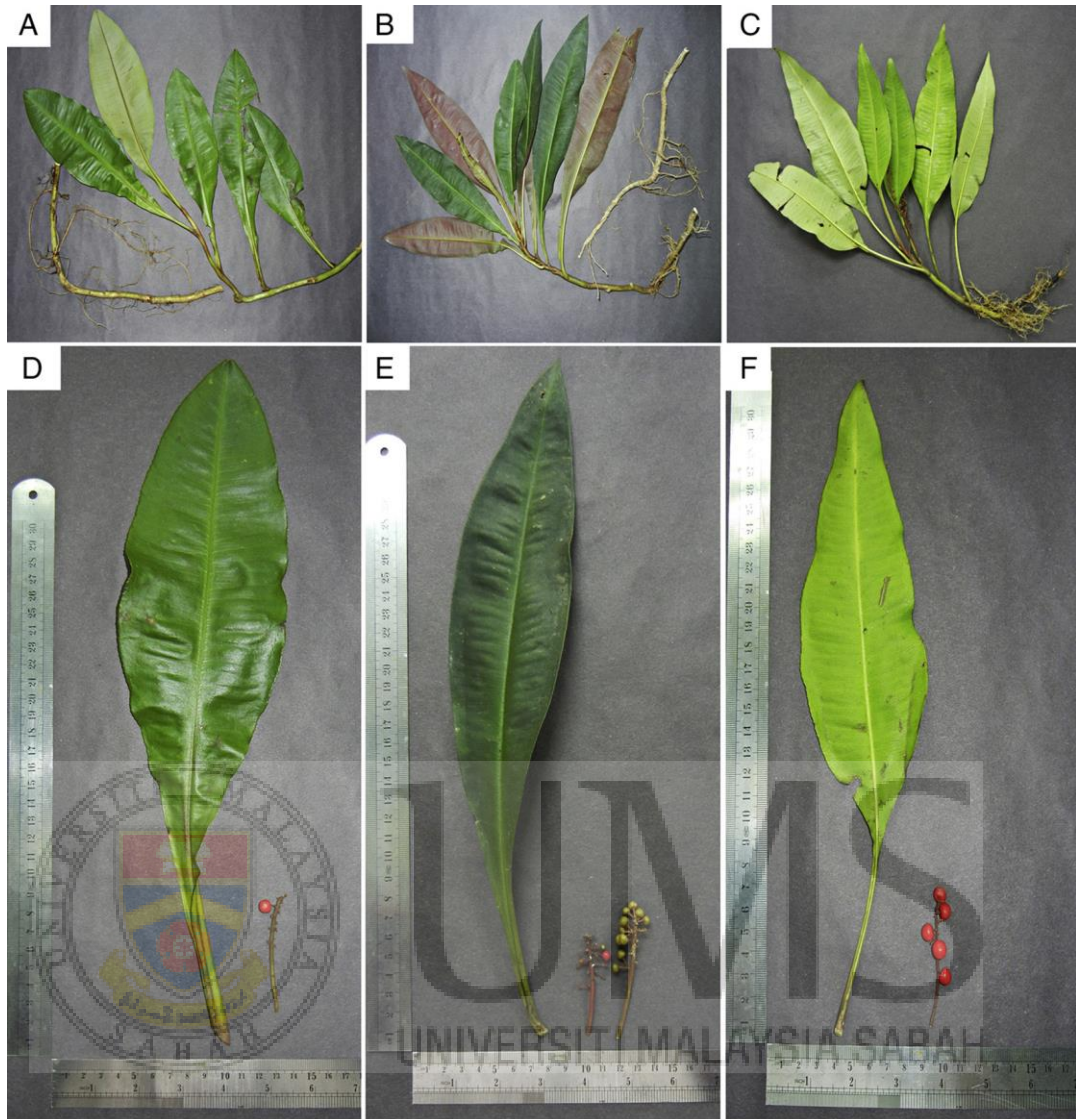
Wild *L. pumila* usually grows in habitat with humus-rich soils, sandy loam and sometimes in deep clay soil or granite soils. This plant is able to grow until 60 cm in height and carries four to twelve leaves per plant. Its leaf size is approximately around 5 to 35 cm long and 2 to 8 cm wide. In addition, *L. pumila* also produced flower and fruits. Their whites to pinkish flowers are quite small which grow in spike like panicle or small clusters. Meanwhile, the size of the fruit is about 0.5 cm in diameter which changes colour from green to red or purple when ripen (Stone, 1988; Zhari *et al.*, 1999; Sunarno, 2005). The comparison of morphological characteristics and the habitat of the three varieties of *L. pumila* are shown in Table 2.2. Figure 2.2 shows the three varieties of *L. pumila* which were grown in the field.

**Table 2.2: Morphological characteristics and habitat of *L. pumila***

Variety	var. <i>alata</i>	var. <i>pumila</i>	var. <i>lanceolata</i>
Petiole shape	Broad winged	Slightly winged	Terete
Length of petiole	5-12 cm	4-15 cm	6-21 cm
Length of anther	0.8 mm	1.2 mm	0.8 mm
Habitat	Lowland primary forests, shady secondary forest	Shady rain forests, edge of swampy forests	Shady primary forests, secondary and mossy forests

Source: Sunarno (2005)

Aladdin *et al.* (2016) conducted a comparative study of var. *alata*, var. *pumila* and var. *lanceolata* using microscopic technique to identify the anatomical characteristics presents in the leaf and stem parts of the plant. Based on the anatomical investigation; anisocytic stomata, scale and capitate glandular trichomes were present in all three varieties of *L. pumila*. From the study, Aladdin *et al.* (2016) concluded that the identification of anatomical features in terms type of stomata and trichomes, outline structure of stem and leaf margin, petiole and midrib, organisation of vascular system, areolar venation, pattern of anticlinal walls, the distribution of secretory canals and cell inclusion can be used to differentiate each variety of *L. pumila*.



**Figure 2.2: Three varieties of *L. pumila* (ex vitro conditions) (a) *L. pumila* var. *alata*, (b) *L. pumila* var. *pumila*, (c) *L. pumila* var. *lanceolata* and the macroscopic characteristics of leaf and fruit (d) *L. pumila* var. *alata*, (e) *L. pumila* var. *pumila*, (f) *L. pumila* var. *lanceolata***

Source : Aladdin *et al.* (2016)

### **2.1.3 Tissue culture of *Labisia pumila***

In the natural habitat, *L. pumila* propagates from its seeds (Mohd. Noh *et al.*, 2002). Zahari (2008) reported that *L. pumila* also can be propagated using its leaf, petiole and stem. Propagation of *L. pumila* var. *alata* high yielding clones using leaf cuttings had been conducted by Syafiqah *et al.* (2014). As *L. pumila* propagates in a slower rate in the wild (Mohd. Noh *et al.*, 2002), attempts have been made to cultivate this herb by using tissue culture techniques for the purpose of micropropagation and regeneration of healthy clones.

To date, there are only a few published studies on tissue culture of *L. pumila*. These *in vitro* studies include seeds germination and seedling development of *L. pumila* (Hartinie & Jualang, 2007), shoot regeneration (Hartinie, 2007; Ling *et al.*, 2013; Ozayanna, 2015; Shafiqah *et al.*, 2015), callus induction (Hartinie, 2007; Ling *et al.*, 2013; Ozayanna, 2015) and adventitious root induction (Hassan & Hussein, 2013; Ling *et al.*, 2013) on semi-solid medium. A recent study by Syafiqah *et al.* (2016) reported that the production of superior clone of *L. pumila* var. *alata* through tissue culture method is more feasible than using leaf cuttings for the production of future planting stocks of the herb.

### **2.1.4 Medicinal properties of *Labisia pumila***

Traditionally, *L. pumila* is consumed in the form of water decoction from its leaf, root or the whole plant. Between the three varieties of this herb, *L. pumila* var. *alata* is more commonly used in the Malay traditional medicine (Jamal, 2006). Indigenous Malay women drinks the water decoction in order to ease their childbirth as well as a post-partum medicine (Burkill, 1935). Other traditional usages of *L. pumila* are for treating flatulence, dysentery, dysmenorrhea and gonorrhoea, "sickness in the bones" (Burkill, 1935) and haemorrhoids (Rahman, 1998).