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

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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^2155, 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 ± 2.3 µg/disc) gave the lowest MIC value compared to the resveratrol derivative (70.0 ± 14.1 µg/disc) and the known inhibitor (37.5 ± 3.5 µg/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 tuberkulosis *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 mensasarkan enzim (MLS) dalam asetat pertumbuhan* Mycobacterium *sp. Sejumlah 117 ekstrak daripada 44 spesis tumbuhan tempatan dan 60 sampel aktinomiset tanah telah disaringkan terhadap MLS dengan penggunaan mycobacteria tak patogenik* (M. smegmatis *mc²155*, *H8000*) *pada penyaringan agar*. *Kemudian, ekstrak kasar yang berpotensi diteruskan dengan analisis Resazurin Microtiter Assay (REMA) yang diubahusuai, 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 struktrur, 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 ± 2.3 µg/disc) terendah jika dibandingkan dengan terbitan resveratrol (70.0 ± 14.1 µg/disc) dan perencat ICL tersebut (37.5 ± 3.5 µg/disc). Kajian kadar toksik perlu dijalankan ke atas nucleoside tersebut sebelum diaplikasikan secara mendalam dalam perkembangan ubat antitubercular terhadap* M. tuberkulosis*.*

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LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl-coenzyme A
AIDS	Acquired Immunodeficiency Syndrome
D_2O	Deuterium oxide
DNA	Deoxyribonucleic acid
glcB	Gene of malate synthase
HMBC	Heteronuclear Multiple Bond Correlation
HRESI-MS	High resolution electron spray ionization- Mass spectrometry
i.d.	Internal diameter
icl	Gene of isocitrate lyase
IFN-$\gamma^{-/-}$	Interferon-gamma
MeOD	Deuterated Methanol
NMR	Nuclear Magnetic resonance
ppm	Part per million
RP-HPLC	Reverse phase-High pressure liquid chromatography
rpm	revolution per minute
TB	Tuberculosis
TLC	Thin layer chromatography
WHO	World Health Organization

LIST OF SYMBOLS

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

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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*

Taxonomy	
Domain	Eukaryota
Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Ericales
Family	Myrsinaceae
Genus	Labisia
Species	*Labisia pumila*

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. *alata***	**var. *pumila***	**var. *lanceolata***
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*.

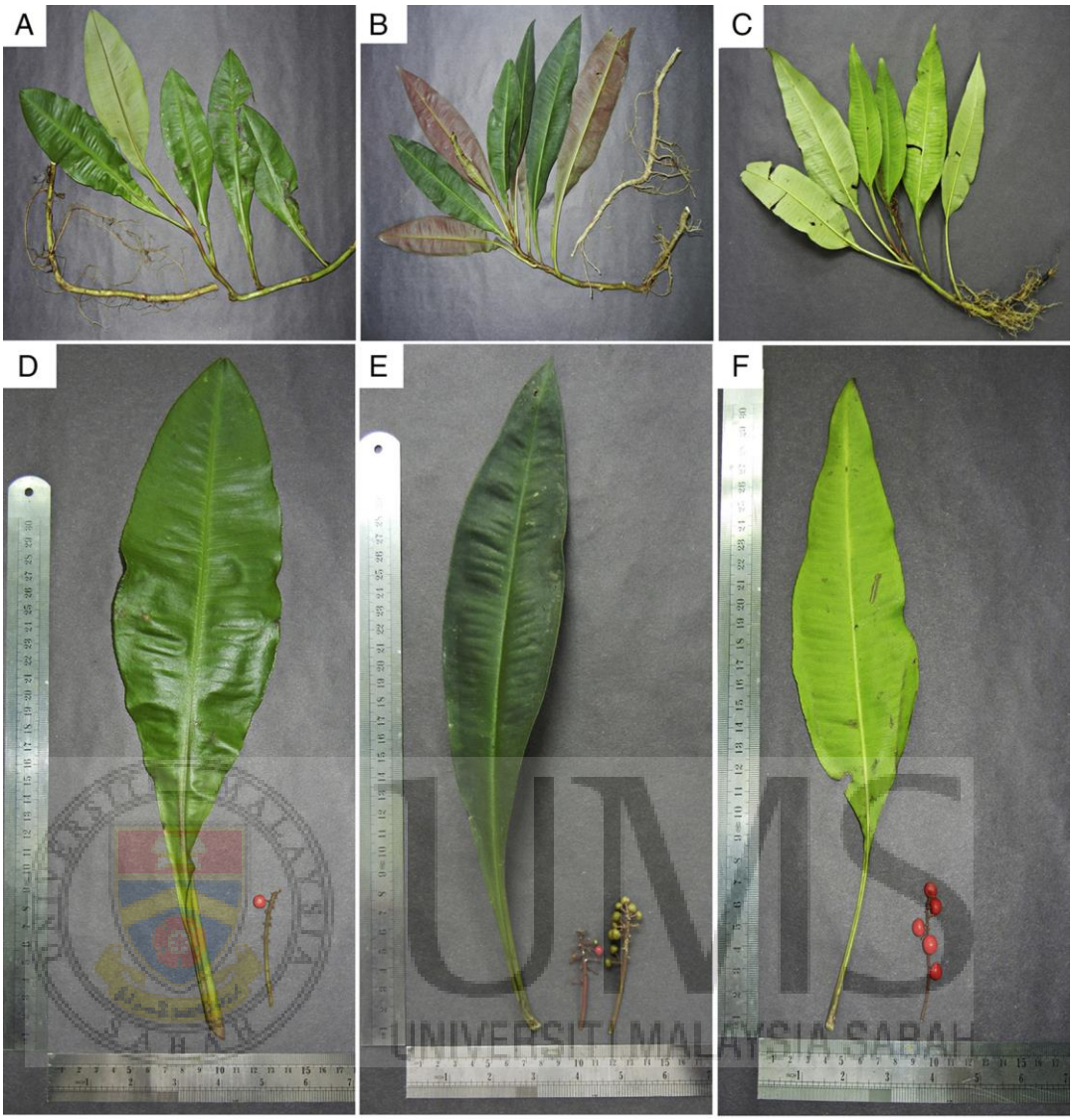

Figure 2.2: Three varieties of *L. pumila* (*ex vitro* conditions) (a) *L. pumila* var. *alata*, (b) *L. pumila* var. *pumila*, (c) *L. pumila* var. *lanceolata* and the macroscopic characteristics of leaf and fruit (d) *L. pumila* var. *alata*, (e) *L. pumila* var. *pumila*, (f) *L. pumila* var. *lanceolata*

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).