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ROOT CULTURES AND ELICITATION OF SECONDARY METABOLITES FROM Labisia pumila Benth & Hook. f. var. alata

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By

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ROOT CULTURES AND ELICITATION OF SECONDARY METABOLITES FROM *Labisia pumila* Benth & Hook. f. var. alata

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Labisia pumila var alata (Kacip Fatimah) is currently one of the eighteen main herbs under the EPP 1 of Agriculture cluster of the Economic Transformation Programme, Malaysia. Due to the slow and non-uniform growth of this plant, destructive harvesting and the lack of plant material to sustain the demand of the bio-pharmaceutical industry, an alternative approach is crucially needed to ensure sustainable supply of the herbal raw material. The development of organ cultures such as hairy roots and adventitious roots is a suitable approach to curb the problem of sustaining raw material for the production of pharmaceuticals. Furthermore, the secondary metabolites produced in *L. pumila* has not been well studied. In this study, hairy roots of *L. pumila* were successfully established from leaf with petiole explants using three strains of *Agrobacterium rhizogenes* (A4, NCPPB2629 and NCPPB1855) which produced 16.67%, 70% and 40% transformation efficiencies, respectively. Strain A4 showed a rapid growth rate compared to strain NCPPB2629 and NCPPB1855 with a doubling time of 14 days. Subsequently, liquid cultures were established using strain A4. The presence of *rol C* gene (strain A4) and *rol B* (strain NCPPB2629 and NCPPB1855) genes were determined using PCR analysis. Adventitious roots were also successfully initiated using a combination of auxins indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA), both supplemented in 2.69 mM concentration in GM1 medium. A total of 100% frequency of adventitious root induction with 9.93 ± 1.18 mean number of roots formed per explant were produced after 14 days of culture. Tandem mass spectrometry platforms [time of flight (TOF) based and quadruple ion-trap based (QTRAP)] were used to detect and compare secondary metabolites produced in different tissues of *L. pumila* using Principle Component Analysis (PCA). The time-of-flight based tandem mass spectrometer managed to tentatively identify three compounds (FG1, FG2 and AP1) which were produced abundantly in the aqueous extracts of tissue cultured leaves. The production of triterpene saponin, TSrA was also noted in the aqueous extracts of the natural stem (51.33 mg/g), natural roots (132.48 mg/g), tissue cultured leaves (135.65 mg/g), tissue cultured roots (250.75 mg/g) and hairy roots (361.26 mg/g). Methanol extracts of elicited hairy roots in shake flasks using Methyl jasmonate elicitor using different two stage elicitation procedures produced higher concentrations of TSrA (11.8-fold increase in GM3-PM1 media) and AP1 (2.8-fold increase in GM3-PM3 media). Another triterpene saponin, TSrB was also obtained from methanol extracts of hairy roots elicited in GM3-PM2 medium (0.52 mg/g). This compound has never been reported found in any other tissues of *L. pumila*. Production of hairy root biomass was upscaled further in laboratory scale bioreactors. In terms of biomass production, stirred tank bioreactor with elephant ear impeller, bubbled flask and balloon type bubble bioreactor (BTBB A) produced 6.94-fold, 4.0-fold and 8.42-fold of increase in biomass respectively. The production of hairy root biomass was proven to be effective in BTBB A. Hence, further optimization were made to enhance the production of secondary metabolites. A total of 13 compounds (TSrA, TSrB, TScA, TScB, TScD, TScH, TSmE, TSmH, TSrp, TSdxp, AP1, AP2 and FG1) were detected from various tissues of *L. pumila*. Among these compounds, TSrB, TScA, TScB, TScD, TScH, TSrp and TSdxp productions were enhanced in the BTBB D bioreactor. This bioreactor produced a maximum biomass accumulation of 15.04 fold. The findings in this study concluded that the supply of herbal raw material can be enhanced through the usage of organ cultures, elicitation and upscaling in bioreactor platforms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

KULTUR AKAR DAN ELISITASI METABOLIT SEKUNDER DARI *Labisia pumila* Benth & Hook. f. var. alata

Oleh

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Labisia pumila var alata (Kacip Fatimah) merupakan salah satu daripada lapan belas herba utama di bawah EPP1 Kluster Pertanian di bawah Program Transformasi Ekonomi, Malaysia. Ekoran daripada itu, kelambatan dan ketidakseragaman tumbesaran tumbuhan ini, penuaian yang berleluasa dan kekurangan bahan mentah untuk memenuhi keperluan pasaran dalam industri farmaseutikal, pendekatan yang alternatif amatlah diperlukan bagi memastikan kesinambungan bekalan bahan mentah herba. Penghasilan kultur organ seperti akar rerambut dan akar adventisius adalah sesuai untuk menyelesaikan untuk memastikan bekalan bahan mentah yang berterusan untuk pengeluaran ubat farmasiutikal. Tambahan pula, penghasilan metabolit sekunder di dalam *L. pumila* masih belum dikaji dengan teliti. Dalam kajian ini, akar rerambut *L. pumila* telah berjaya dihasilkan daripada eksplan daun dengan petiole hasil daripada penggunaan tiga strain *Agrobacterium rhizogenes* (A4, NCPPB2629, NCPPB1855) di mana 16.67%, 70% dan 40% kadar transformasi diperolehi. Di antara tiga strain *A. rhizogenes* yang digunakan, akar rerambut yang dihasilkan daripada strain A4 mempunyai kadar pertumbuhan yang lebih baik berbanding dengan strain NCPPB2629 dan NCPPB1855. Biomas akar rerambut digandakan dua kali ganda dalam masa 14 hari. Justeru itu, kultur akar rerambut di dalam media cecair dihasilkan daripada akar rerambut yang dihasilkan daripada strain A4. Kehadiran gen *rol C* (strain A4) dan gen *rol B* (strain NCPPB2629 dan NCPPB1855) ditentukan oleh analisis PCR. Akar adventisius juga telah berjaya dihasilkan menggunakan kombinasi auksin indole-3-butyric acid (IBA) dan α-naphthalene acetic acid (NAA) di mana kepekatan 2.69 mM untuk setiap auksin dibekalkan bersama-sama dengan medium GM1. Frekuensi pengaruhan akar adventisius adalah 100% manakala purata bilangan akar yang dihasilkan pada setiap eksplan adalah 9.93 ± 1.18, 14 hari selepas kultur. Platform Tandem Mass Spectrometry [time-of-flight (TOF) dan quadruple ion trap (QTRAP)] digunakan untuk mengesan dan membuat perbandingan metabolit sekunder dengan analisis konponen principal (Principle Component Analysis) (PCA). Analisis yang menggunakan

time-of flight berjaya membuat pengenalan tentatif kepada tiga jenis sebatian iaitu FG1, FG2 dan AP1 di mana tiga jenis sebatian ini dihasilkan dengan kuantiti yang banyak di dalam ekstrak akuas daun dari kultur tisu. Triterpene saponin, TSrA dihasilkan di dalam ekstrak akuas batang semulajadi (51.33 mg/g), diikuti oleh akar semulajadi (132.48 mg/g), daun kultur tisu (135.65 mg/g), akar kultur tisu (250.75 mg/g) dan akar rerambut (361.26 mg/g). Ekstrak methanol untuk akar rerambut yang dielisitasi di dalam kelalang menggunakan elisitor methyl jasmonate serta menggunakan prosedur elisitasi yang berlainan berjaya menghasilkan nilai kepekatan yang lebih tinggi untuk TSrA (11.8 kali ganda peningkatan di dalam media GM3-PM1) dan Fatimahol (2.8 kali ganda kepekatan di dalam media GM3-PM3). Satu lagi triterpene saponin , TSrB dijumpai di dalam ekstrak methanol yang dielisitasi di dalam media GM3-PM2 (0.52 mg/g). Sebatian ini masih belum pernah dijumpai di dalam tisu-tisu *L. pumila* yang lain. Penghasilan biomas akar rerambut diskalakan dengan lebih besar di dalam bioreaktor skala makmal. Penghasilan biomas akar rerambut di dalam bioreaktor tangki kacau dengan pendesak telinga gajah, bioreaktor kelalang gelombang dan bioreaktor belon kolum gelombang (BTBB A) adalah masing-masing 6.94 kali ganda, 4.0 kali ganda dan 8.42 kali ganda. Penghasilan biomas akar rerambut adalah efektif di dalam BTBB A. Oleh itu, optimasi dibuat untuk meningkatkan penghasilan metabolit sekunder di dalam bioreaktor ini. Sebanyak 13 sebatian semulajadi (TSrA, TSrB, TScA, TScB, TScD, TScH, TSmE, TSmH, TSrp, TSdxp, AP1, AP2 dan FG1) dikesan dari pelbagai tisu *L. pumila* . Di antara sebatian-sebatian ini, penghasilan TSrB, TScA, TScB, TScD, TScH, TSrp dan TSdxp ditingkatkan di dalam bioreaktor BTBB D. Bioreaktor ini menghasilkan biomas maksimum sebanyak 15.04 kali ganda. Kesimpulannya, hasil daripada kajian ini boleh menambahbaik bekalan bahan mentah herba melalui kultur organ, elisitasi dan penskalaan besar dalam platform bioreaktor.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AP	Alkylated phenolic compounds *(compound names are kept confidential at this stage due to patent filing in progress)*
aux	Auxin biosynthesis gene
BAP	6-Benzylaminopurine
BTBB	Balloon Type Bubble Bioreactor
EPP	Entry Point Project
ETP	Economic Transformation Programme
FG	Flavonoid glycoside compounds *(compound names are kept confidential at this stage due to patent filing in progress)*
GM	Growth Medium *(Media formulations and names are kept confidential at this stage due to patent filing in progress)*
IBA	Indole-3-butyric acid
LC	Liquid Chromatography
MJ	Methyl Jasmonate
MS	Murashige & Skoog
NAA	α-naphthalene acetic acid
NCPPB	National Collection of Plant Pathogenic Bacteria
NKEA	National Key Economic Areas
PCA	Principle Component Analysis
PM	Production Medium (*Media formulations and names are kept confidential at this stage due to patent filing in progress*)
QTRAP	Quadruple-ion-trap
rol	Root loci
TOF	Time-of-flight

TS	Triterpene saponin compounds (*compound names are kept confidential at this stage due to patent filing in progress*)
vir	Virulence gene

CHAPTER 1

INTRODUCTION

1.1 Background Information

Traditional medicinal plants from the tropical green forest of Malaysia have gain popularity due to the presence of bioactive compounds that are much needed by local biopharmaceutical industry. In fact, the herbal industry in the world is expected to reach an income of USD115 billion by the year 2020 (Global Industry Analyst Inc, 2015).

Labisia pumila Benth & Hook. f. var. alata. (Myrsinaceae), locally known as Kacip Fatimah can be found in the shady part of lowland primary forest and humus-rich secondary forest in Malaysia. Scientists and several publications has shown that the plant may have phytoestrogenic properties, traditionally that have been used as a post-partum medicine, to facilitate child birth, regulate menstrual, treat dysentery, flatulence and dsymenorrhoea (Burkhill 1966, Rozihawati et al., 2003). According to Sunarno (2005), seven varieties of *L. pumila* can be found in Malaysia and Indonesia region namely *L. pumila* var *alata, L. pumila* var *pumila*, and *L. pumila* var lanceolata. *L. pumila* var discoplacenta, *L. pumila* var gladiata, *L. pumila* var nerrifolia, *L. pumila* var malintangensis and *L. pumila* var sessilifolia. Currently, Kacip Fatimah (*Labisia* pumila) is listed as one of the five main herbs in the Entry Point Project (EPP1) under the 12 agriculture National Key Economic Areas (NKEA) under Malaysia's Economic Transformation Programme (ETP). The other four herbs are Tongkat Ali, Misai Kucing, Hempedu Bumi and Dukung Anak. In 2014, the number of medicinal plants under the EPP1 NKEA under ETP programmed have been increased to 18 plants which included the addition of Pegaga, Mengkudu, Roselle, Mas Cotek, Belalai Gajah, Halia, Peria Katak, Gelenggang, Lempoyang, Sambung Nyawa, Sireh/Kadok, Senduduk and Merunggai (Borang NRGS—A1(R), Herbal Development Office, Ministry of Agriculture & Agro-Based Industries Malaysia).The focus of Agriculture NKEA is to transform small scale herbal production sectors into large scale biobusiness sectors.

Under the Entry Point Project 1 (EPP1), the production of the five herbs including *Labisia pumila* are ensured in order to provide sufficient supply of raw plant material for the purpose of research and development and clinical trials. High value commercialization of herbal based products will take place subsequently. This EPP1 has the aim of producing an income of RM2,213.90 mil by the year 2020 and to project a number of 1822 jobs in Malaysia (Economic Transformation Programme, 2010).

The production of ginseng raw material by field cultivation and bioreactor grown adventitious root were compared previously by Murthy et al (2014). At a slightly higher cost of production of USD47/kg in the bioreactor compared to USD35/kg for field cultivation, the production of plant material was enhanced 57.36-fold with significant reduction of harvesting time from 5 to 7 years in the field compared to a one year in the bioreactor platform.

1.2 Justification

Under the EPP1 project for commercialization of high value herbal products in the Economic Transformation Programme, the cultivation of *Labisia pumila* was produced by cultivation in the field. Currently, the cultivation practices were highly affected by the weather, geographical factors, slow and non-uniform growth. *L. pumila* originated from seeds took 16 weeks to germinate and grow before they could be transferred to growth medium (Ahmad Fauzi, 2013). According to Aminah and Farah Fazwa (2012), due to the slow growth of the plant, the optimum harvesting age for *L. pumila* is 7 to 9 months old which was considered long and not suitable for large scale planting. Furthermore, *L. pumila* whole plants are commonly used in traditional herbal preparations. This leads to the lack of plant material to sustain the demand of the biopharmaceutical industry. Moreover, the secondary metabolites produced from the field-grown plants are present in minute quantities (less than 1%). The differences may be due to the morphological differentiation of field grown plants and tissue cultured plants. Furthermore, hairy root cultures has the ability to synthesize higher amounts of secondary metabolites (Niżyński et al., 2014, Kolewe et al., 2008 and Sauerwin et al., 1992).

Therefore, an alternative approach to planting should be considered to obtain sustainable production for raw plant material of *Labisia pumila* with relatively high content of bioactive secondary metabolites. Plant tissue culture is a clonal propagation method in controlled environment which can provide sustainable supply of *L. pumila* plant material. The utilization of *in vitro* organ cultures such as adventitious root and hairy root cultures can be more beneficial in the long term as these differentiated cultures are genetically stable and can be grown in controlled environment in a bioreactor. Production of specific and desired natural compounds in *L. pumila* can be manipulated in organ cultures to enhance quantity or amount of bioactive content in the herbal plant material grown under standardized conditions. This will indirectly accelerate the product to market time for *L. pumila* herbal products in Malaysia.

Organ cultures developed in a close environment such as bioreactors are needed to ensure sustainable supply of raw materials by the pharmaceutical industry. The physical and biochemical environment can be controlled and manipulated for production of specific compounds of interest.

However, the cultivation of hairy roots in bioreactors needs special considerations as hairy root cultures are sensitive to high shear stress and has tendency to form clumps which hinders the efficient delivery of oxygen to the roots. Different physical and biochemical parameters need to be assessed in order to determine suitable physical and biochemical conditions to cultivate *L. pumila* hairy roots in bioreactor. The biomass of hairy roots and highly specific secondary metabolites can be manipulated under clean and controlled conditions of a bioreactor platform.

Various strategies can be applied such as elicitation, precursor feeding, permealization, immobilization, selective absorption, biotransformation and nutrient replenishment to enhance the production of secondary metabolites (Murthy et al., 2014). In this way, continuous production of organ culture biomass and secondary metabolites can be performed at the same time to ensure continuing supply of plant material and phytochemical products to the bio-pharmaceutical industry.

The availability of highly sophisticated and sensitive instruments for the discovery of secondary metabolites in recent years such as Tandem Mass Spectrometry with various detectors such as triple quadruple, quadruple ion trap and quadruple time-of-flight facilitated the discovery and detection of new secondary metabolites in medicinal plants. The process of identification and quantification of secondary metabolites become relatively easier, more accurate and faster.

Since *L. pumila* is an important local herb, and has a high demand in the bio-pharmaceutical industry, a sustainable production of high quality herbal raw material with relatively high amount of bioactive compounds is needed. In this study, sustainable organ cultures (hairy roots and adventitious roots) of *L. pumila* were initiated from leaf with petiole explants. Secondary metabolites' production of various tissues of *L. pumila* were investigated using high end instrumentation. Following that, scaling up of biomass and secondary metabolites production will be performed at lab scale bioreactor platforms.

1.3 Objectives

Therefore, the objectives of this study were

1) to establish hairy root and adventitious root cultures of *Labisia pumila* var alata,
2) to compare the production of secondary metabolites in various tissues of *Labisia pumila* var alata, and
3) to assess the secondary metabolites produced in the hairy root cultures of *Labisia pumila* var alata using bioreactors.

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