

FRACTIONATION AND CHARACTERIZATION OF POLYPHENOLS
RICH EXTRACT FROM *LABISIA PUMILA* LEAVES

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Engineering (Bioprocess)

Faculty of Chemical Engineering
Universiti Teknologi Malaysia

MARCH 2015

Dedicated to my beloved family, my husband, Mohd Nazrul, and my daughter,
Safiyyah Nur Humaira

ACKNOWLEDGEMENTS

Alhamdulillah. Thanks to the ALLAH S.W.T, for having made everything smooth along the journey to complete my research. Thanks to people who directly involved to this research work. To people who are giving advised, ideas, encouragement and help, your kindness is highly appreciated.

I would like to express my sincere gratitude to my supervisor, Prof. Dr Mohamad Roji Sarmidi and my co-supervisor, Dr Chua Lee Suan, for their helpful, guidance, support and encouragement until throughout this research. Thank you very much for giving me this opportunity to conduct this interesting research.

Thank to Institute of Bioproduct Development (IBD), the staffs under research and development (R&D) division, for moral support and advised. IBD is providing good research environment, human resources and facilities for this project. Thanks to all my colleagues; Metabolite Profiling Laboratory, Herb and Phytochemical Laboratory for your kindness helps to complete the research tasks. To my research group at IBD, your kindness and inspiration is much appreciated. Also special thank to Faculty of Chemical Engineering (FKK) for good facility and human resources

Last but not least, to both parents; Abdul Latiff Md Isa, mother Jaimah Md Jai, my husband Mohd Nazrul Mohd Noor and my beloved daughter Safiyyah Nur Humaira for passion and understanding until study completed. Without them, it is possible on me in completing this project.

ABSTRACT

Herbal plant extracts rich in polyphenols are known to exhibit high antioxidant activity. In this study, the correlation between antioxidant and polyphenols content of *L. pumila* (Kacip Fatimah) leaves was investigated. The polyphenol rich fractions were prepared by ultrasonic assisted extraction (UAE) and followed by solid phase extraction (SPE). The UAE extraction was carried out by using methanol, acetonitrile, water and methanol-water mixture as the solvents. The *L. pumila* crude extracts were subsequently fractionated using SPE using mixtures with different methanol-water ratio. The antioxidant and total polyphenol content of the crude extracts and fractions were evaluated using spectrophotometric assays. The crude extract, extracted using 60% methanol-water mixture was found to give the highest antioxidant activity of $IC_{50} = 59.30 \pm 0.12$ ppm with the content of flavonoid (285.94 $\mu\text{g}/\text{mg}$ dry weight of rutin equivalent, RE) and phenolic (213.5 $\mu\text{g}/\text{mg}$ dry weight of gallic acid equivalent, GAE) content. Whereas the *L. pumila* fraction, fractionated using 40% methanol-water was found to exhibit the highest antioxidant activity ($IC_{50} = 1109.46 \pm 4.45$ ppm), phenolic (266.64 ± 0.12 dry weight of gallic acid equivalent, GAE) and flavonoid (27.66 ± 0.07 $\mu\text{g}/\text{mg}$ dry weight of rutin equivalent, RE). High correlation ($r = 0.60-0.96$) was found between the antioxidant activity, phenolic and flavonoid content based on spectrophotometric results. Based on liquid chromatography mass spectrometry (LC-MS/MS) analysis, twenty three polyphenols constituents consisted of flavonoids and phenolic acid were identified from the fraction. Among of these constituents, epigallocatechin, myricetin and rutin were identified as the key polyphenols in the fraction. The validation of these compounds was carried out with the linearity from 0.9840 to 0.9983, limit of detection (LOD) from 0.03 $\mu\text{g}/\text{ml}$ to 0.11 $\mu\text{g}/\text{ml}$, limit of quantification (LOQ) from 0.11 $\mu\text{g}/\text{ml}$ to 0.35 $\mu\text{g}/\text{ml}$ and recoveries from 75% to 88 %. The findings showed that epigallocatechin, myricetin and rutin can be used for the quality control of this herb.

ABSTRAK

Ekstrak tumbuhan herba kaya dengan polifenol diketahui menunjukkan aktiviti antioksidan yang tinggi. Dalam kajian ini, korelasi antara kandungan antioksidan dan polifenol dari daun *L. pumila* (Kacip Fatimah) telah dikaji. Pecahan yang kaya dengan polifenol telah disediakan melalui kaedah pengekstrakan dibantu oleh ultrasonik (UAE) diikuti dengan pengekstrakan fasa pepejal (SPE). Pengekstrakan UAE dijalankan menggunakan metanol, acetonitril, air dan campuran 60% metanol-air sebagai sistem pelarut ekstrak mentah. Ekstrak mentah *L. pumila* seterusnya dipecahkan menggunakan SPE dalam campuran metanol-air yang berbeza (0-100%). Sifat antioksidan dan kandungan polifenol dinilai menggunakan analisa spektrofotometri. Ekstrak mentah, diekstrak menggunakan campuran 60% metanol-air didapati memberikan aktiviti antioksidan yang tertinggi ($IC_{50} = 59.30 \pm 0.12$ ppm) dengan kandungan flavonoid ($285.94 \mu\text{g}/\text{mg}$ berat kering bersamaan rutin, RE/mg) dan fenolik ($213.58 \mu\text{g}/\text{mg}$ berat kering bersamaan asid gallic). Manakala, pecahan *L. pumila*, dipecahkan menggunakan 40% metanol-air didapati mempamerkan aktiviti antioksidan tertinggi ($IC_{50} = 1109.46 \pm 4.45$ ppm), kandungan fenolik ($266.64 \pm 0.12 \mu\text{g}/\text{mg}$ berat kering bersamaan asid gallic) dan flavonoid ($27.66 \pm 0.07 \mu\text{g}/\text{mg}$ berat kering bersamaan rutin, RE/mg). Kolerasi yang tinggi ($r = 0.60-0.96$) didapati antara aktiviti antioksidan, kandungan fenolik dan flavonoid berdasarkan keputusan spektrofotometri. Berdasarkan keputusan analisis kromatografi cecair gandingan spektrometri jisim (LC-MS/MS), dua puluh tiga sebatian polifenol yang terdiri daripada flavonoid dan asid fenolik telah dikenal pasti dari pecahan ini. Epigallocatechin, myricetin dan rutin dikenalpasti sebagai polifenol utama dalam pecahan ini. Sebatian tersebut telah divalidasikan dengan kelinearan 0.9840-0.9983, had pengesanan (LOD) dari $0.03 \mu\text{g}/\text{ml}$ kepada $0.11 \mu\text{g}/\text{ml}$, had pengesanan kuantiti (LOQ) dari $0.11 \mu\text{g}/\text{ml}$ kepada $0.35 \mu\text{g}/\text{ml}$ dan pemulihan dari 70% kepada 88%. Keputusan menunjukkan sebatian epigallocatechin, myricetin dan rutin adalah sesuai digunakan untuk kawalan kualiti herba ini.

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LIST OF ABBREVIATIONS

LC-MS/MS	-	Liquid Chromatography Mass Spectrometry
SPE	-	Solid Phase Extraction
MeOH	-	Methanol
DPPH	-	1,1-diphenyl-2-picrylhydrazyl
TPC	-	Total phenolic content
TFC	-	Total flavonoid content
IC ₅₀	-	Concentration to inhibit 50% of scavenging activity
GAE	-	Gallic acid equivalent
RE	-	Rutin equivalent
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-visible
UAE	-	Ultrasonic assisted extraction
MAE	-	Microwave assisted extraction
LC	-	Liquid chromatography
HPLC	-	High performance liquid chromatography
PDA	-	Photodiode detector array
GC-MS	-	Gas chromatography mass spectrometry
NMR	-	Nuclear magnetic resonance
IR	-	Infrared radiation
EPI	-	Enhanced product ion
MS	-	Mass spectrum
MRM	-	Multiple reaction ion monitoring
SFE	-	Supercritical fluid extraction
[M-H] ⁻	-	Negative ionisation
[M-H] ⁺	-	Positive ionisation

LOD	-	Limit of detection
LOQ	-	Limit of quantification
FDA	-	Food Drug Administration
EURACHEM	-	European Analytical Chemistry
PCA	-	Principle component analysis

LIST OF SYMBOLS

mg	-	Miligram
g	-	Gram
cm	-	Centimeter
L	-	Litre
ml	-	Mililiter
ppm	-	Concentration unit (part per million, $\mu\text{g/ml}$)
mMol	-	Milimol
a.m.u	-	Atomic mass unit
m/z	-	Molecular weight
Da	-	Dalton
psi	-	Pounds per square inch
δ	-	Dielectric constant
$^{\circ}\text{C}$	-	Degree celcius
<	-	Less than
\leq	-	Equal and less than
>	-	Greater than
%	-	Percentage
r	-	Correlation index
r^2	-	linear regression

CHAPTER 1

INTRODUCTION

1.1 Research Background

Malaysia is becoming become a the key herbal medicine producer for global and local demands due to its diverse biodiversity and strong knowledge on herbal technology. It was estimated that the Malaysian herbal market increase expand by 15% per year by 2020. The expanded production of herbal based food and health products could rake in lucrative return by contributing to the gross national income (GNI) by 2020.

There are around 2,000 plant species located in the tropics, which had been reported to have medical values (Jaganath and Ng, 2000). The Malaysian government has drafted a transformation program to elevate herbal industry under the National Key Economic Areas (NKEAs). This program provides opportunity to the private sector to increase their income based on high value agri-based products and services. Eleven plant species were recognized and specified to be the Malaysian top herbal plants in the first Entry Point Project (EPP1) under the National key economic area (NKEA) program. The herbs includes the *Kacip Fatimah*, *Tongkat Ali*, *Misai Kucing*, *Dukung Anak*, *Hempedu Bumi*, *Mas Cotek*, *Mengkudu*, *Belalai Gajah*,

Roselle, Ginger and *Temulawak*. Through this program, more incentives and research funds were offered to develop high value herbal products.

Biological active substances from plant were increasing significantly for commercial usages. Natural products derived from many plants such as resin and saponin were widely used in industrial oils, steroids and alkaloids in pharmaceutical industry, as well as vanillin and caffeine for beverages industry (Balandrin *et al.*, 1985). For example, two famous Malaysia herbal plants, *Tongkat Ali* and *Kacip Fatimah* contain chemical substances which acted similarly to the human hormones and reproductivity (Ang *et al.*, 2002; Ezumi *et al.*, 2007). Moreover, numerous medicinal benefits from various Malaysian herbal plant has been discovered nowadays (Karimi and Jaafar, 2011; Nik Hussain *et al.*, 2013; Wasman *et al.*, 2011; Mustafa *et al.*, 2010; Dolečková *et al.*, 2012). These findings have increased the demand for Malaysian herbal plants for future development of new products and elevating the economy, especially for agricultural industry.

One of the most important biological active plant-derived chemical substance was polyphenolics, type of antioxidant compound distributed widely in herbal plants. The polyphenolics including phenolic acids, flavonoids, tannin, lignans, quinones and coumarins were known to give health benefits to the human life—such as an antioxidants, anti-aging activity, antimutagenic and antiangiogenesis, antibacterial and antifungal effects (Huang *et al.*, 2010; Wahle *et al.*, 2010).

Numerous researches regarding the pharmacological effects of *Labisia pumila* (*L. pumila*) or Kacip Fatimah had been done in recent years. However, prior to 2010, the phytochemical component in this plant extract was not discovered. Preliminary phytochemical study with corresponding polyphenols component was initiated in this research since 2010 to fulfill the correlation between biological effect and polyphenols. In the earlier publication, *L. pumila* fraction contained polyphenolic compounds such as flavonoid and phenolic acid (Chua *et al.*, 2010^a, Chua *et al.*, 2010^b, Chua *et al.*, 2011). Beside, the anti-aging property has increased the demand

of this plant for usage in cosmeceutical industry (Choi *et al.*, 2010, Chua *et al.*, 2012, Abdullah *et al.*, 2013).

1.2 Problem statement

The relationship between phytochemical components in *L. pumila* var. *alata* (leaves) and its bioactivities are not well established. The biological effects such as of anti-oxidant, anti-cancer and anti-aging should be related to specific phytochemical components in the plant extract. Thus, the role of phytochemical such as polyphenol components should be elucidated. However, due to the complexity of the plant, fractionation process should be carried out to further isolate the polyphenol components from *L. pumila* var. *alata* (leaves).

1.3 Hypothesis

The central hypothesis of this study was *L. pumila* var. *alata* (leaves) fraction could exhibit high antioxidant property due to the presence of polyphenol components.

1.4 Objective of the study

The objectives of this study was to establish the correlation between polyphenols and their scavenging activity as antioxidant from *L. pumila* var. *alata* leaves.

1.5 Scopes of the study

The scopes of the study were highlighted as follows:

1. To fractionate the crude extract of *L.pumila* var. *alata* using a reversed phase C18 chromatographic method.
2. To determine the polyphenols content for the crude extract and its fractions by using total phenolic and flavonoid content.
3. To determine the antioxidant activity of the crude extract and its fractions expressed as free radical scavenging activity based on spectrophotometric assay.
4. To validate the presence of key polyphenols from the fraction by using LC-MS/MS based on statistical approach and fragmentation pattern.

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