UNIVERSITI PUTRA MALAYSIA

CHEMICAL AND BIOLOGICAL EVALUATIONS OF PEGAGA (Centella asiatica (L.) Urb., Hydrocotyle bonariensis Comm. ex Lam. and Hydrocotyle sibthorpioides Lam.) EXTRACTS AND THEIR EFFECTS ON AN OBESE DIABETIC RAT MODEL USING 1H NMR METABOLOMICS

MAULIDIANI

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By

MAULIDIANI

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfillment of the
Requirement for the Degree of Doctor of Philosophy**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

CHEMICAL AND BIOLOGICAL EVALUATIONS OF PEGAGA (*Centella asiatica* (L.) Urb., *Hydrocotyle bonariensis* Comm. ex Lam. and *Hydrocotyle sibthorpioides* Lam.) EXTRACTS AND THEIR EFFECTS ON AN OBESE DIABETIC RAT MODEL USING ^{1}H NMR METABOLOMICS

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July 2014

Chairman : Professor Khozirah Shaari, PhD

Institute : Bioscience

Pegaga is one of the important medicinal herbs in Malay culture. The herb comprises of several known varieties which raises the inevitable possibility of large variations with respect to their metabolites and bioactivities. Therefore, to ensure safety and efficacy of products derived from the herb, proper quality control and standardization (e.g. authentication *via* the use of marker compounds and consistent bioactivities) are necessary. Thus, the metabolites of three species of *Pegaga*, *viz Centella asiatica*, *Hydrocotyle bonariensis* and *H. sibthorpioides*, were analyzed using ^{1}H NMR-based metabolomics approach. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) resolved the three species into separate clusters, wherein, saponins such as asiaticoside and madecassoside, along with chlorogenic acids were the metabolites contributing most to the separation. Identification of these components was supported by LCMS/MS data and by comparison with literature data. In addition, the metabolite contents of *Pegaga* grown under different light conditions were also investigated. The extracts of *C. asiatica* grown under full exposure to sunlight exhibited stronger radical scavenging activity and contained higher amounts of triterpenes (asiaticoside and madecassoside), flavonoids, and chlorogenic acids as compared to plants grown under 50% shade.

The three Pegaga species were further investigated for their DPPH free radical scavenging activity. The combined bioactivity and ^{1}H-NMR data of the plant samples were subjected to multivariate data analysis to find possible correlations. The use of PLS, a linear method in modelling, was compared to that of artificial neural network (ANN), a non-linear method. The result showed that both were good models for the prediction of IC_{50} values of the extracts. However, on the overall, the PLS model is comparatively more appropriate for the prediction of free radical scavenging activity of *Pegaga* varieties. Better prediction was achieved for extracts at higher concentration in both models. The loading scatter plot for the PLS analysis revealed that caffeoylquinic acid derivatives, kaempferol and quercetin were responsible for the bioactivity. Further statistical analysis using ANOVA showed that

C. asiatica variety 3 (extract E) gave the most potent radical scavenging activity among the extracts. This metabolomic technique could be beneficial for the standardization of *C. asiatica* extract that possess radical scavenging activity.

As an extension to our research interests, this study also reported the application of ^{1}H-NMR-based metabolomics in the detection of physiological changes in the urine and serum samples of obese-diabetic (obdb) Sprague-dawley rats treated with standardized *C.asiatica* and *H. bonariensis* extracts. This model mimics the symptoms in Type 2 diabetes. After one month of treatment with 300 mg/kg body weight of *C. asiatica* and *H. bonariensis* extracts, results showed that the glucose level was decreased by 19.3% after the obdb rats were treated with *C. asiatica* (300 mg/kg), while treatment with *H. bonariensis* extracts did not show a similar decrease in glucose levels. Detailed analysis of the altered metabolites using mean peak height of ^{1}H-NMR signals revealed that the administration of *C. asiatica* extract to the obdb animal model improved glucose metabolism, creatine metabolism, lipid metabolism, tricarboxylic acid cycle, amino acid metabolism, trimethylamine metabolism, and purine metabolism.

^{1}H NMR-based metabolomics has been successfully applied in the chemical and biological evaluations of Pegaga extracts. The results of this metabolomics study will be useful in establishing standards for phytomedicinal preparations.

Keywords: *C. asiatica*, *H. bonariensis*, *H. sibthorpioides*, ^{1}H NMR metabolomics, LCMS, PCA, PLS, SUS-plot, radical scavenging activity, *in vivo* anti-diabetes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KIMIA DAN PENILAIAN BIOLOGI DARI EKSTRAK PEGAGA (*Centella asiatica* (L.) Urb., *Hydrocotyle bonariensis* Comm. ex Lam. dan *Hydrocotyle sibthorpioides* Lam.) DAN KESANNYA KEATAS MODEL TIKUS TERARUH DIABETES GEMUK MENGGUNAKAN ^{1}H NMR METABOLOMIK

Oleh

MAULIDIANI

Julai 2014

Pengerusi : Prof. Khozirah Shaari, PhD

Institut : Biosains

Pegaga merupakan salah satu herba perubatan penting dalam budaya Melayu. Herba ini terdiri daripada beberapa jenis yang dikenal pasti dan kemungkinan menghasilkan variasi yang besar berkenaan dengan metabolit dan bioaktiviti mereka. Oleh itu, untuk memastikan keselamatan dan keberkesanan produk yang berasal dari herba ini, kawalan kualiti yang tepat dan standardisasi (misalnya pengesahan melalui penggunaan sebatian penanda dan ketekalan bioaktiviti) adalah diperlukan. Untuk mencapai matlamat ini, metabolit daripada tiga spesies Apiaceae, juga dikenali sebagai Pegaga, dianalisis menggunakan ^{1}H NMR spektroskopi dan data multivariat analisis. Analisis komponen utama (PCA) dan analisis kelompok hierarki (HCA) berjaya mendiskriminasi spesies, *Centella asiatica, Hydrocotyle bonariensis dan Hydrocotyle sibthorpioides* kepada tiga kluster. Saponin, asiatikosida dan madekassosida, bersama-sama dengan asid klorogenik adalah metabolit yang menyumbang kepada pemisahan. Pengenalpastian komponen ini disokong oleh analisis LCMS/MS ekstrak Pegaga dan perbandingan dengan data kajian terdahulu. Tambahan pula, kesan keadaan pertumbuhan pencahayaan untuk kandungan metabolit juga telah disiasat. *C. asiatica* yang berkembang dalam pendedahan cahaya penuh menunjukkan aktiviti mengaut radikal lebih kuat, dan mengandungi lebih banyak sebatian triterpena (asiatikosida dan madekassosida), flavonoid, dan asid klorogenik berbanding dengan tumbuhan yang ditanam di tempat 50% teduh.

Tiga jenis *C. asiatica* dan dua taksa terdekat (*H. bonariensis* dan *H. sibthorpioides*) telah dikaji untuk aktiviti DPPH radikal bebas. Bersama-sama dengan data ^{1}H-NMR, gabungan data spektrum dan aktiviti ini selanjutnya dianalisis menggunakan data analisis multivariat. Prestasi model PLS (linear) telah dibandingkan dengan rangkaian neural tiruan (ANN) (tidak linear) untuk menentukan teknik multivariat yang boleh menyediakan model yang lebih baik. Hasilnya menunjukkan bahawa kedua-dua model mempunyai prestasi baik untuk ramalan nilai IC_{50} ekstrak, bagaimanapun model PLS secara keseluruhan adalah agak lebih baik bagi meramalkan aktiviti radikal bebas jenis Pegaga. Ramalan yang lebih baik dicapai untuk ekstrak pada kepekatan yang lebih tinggi pada kedua-dua model. Perincian plot muatan analisis PLS mendedahkan bahawa terbitan asid kaffeolkuinik, kaempferol, kuersetin bertanggungjawab untuk aktiviti radikal bebas. Analisis statistik ANOVA menunjukkan bahawa *C. asiatica* variati ke 3 (ekstrak E) memberikan aktiviti antioksidan yang paling kuat di kalangan ekstrak. Di samping itu, teknik metabolomik memberi manfaat untuk penyeragaman ekstrak *C. asiatica* yang mempunyai aktiviti antioksidan.

Demi melanjutkan penyelidikan ini ke atas aspek biologi daripada *Centella asiatica*, kajian metabolomik berasaskan ^{1}H-NMR dalam pengesanan perubahan fisiologi dalam urin dan serum tikus teraruh diabetes gemuk yang dirawat dengan ekstrak piawai *C. asiatica* telah dilakukan. Model ini meniru gejala dalam penyakit diabetis jenis 2 pada manusia. Selepas dirawat dengan 300 mg/kg ekstrak *C. asiatica* dan *H. bonariensis* selama sebulan, hasil menunjukkan bahawa tahap glukosa telah menurun sebanyak 19.33% untuk tikus diabetis gemuk yang telah dirawat dengan *C. asiatica* (300 mg/kg), manakala rawatan ekstrak *H. bonariensis* tidak memberikan hasil yang positif. Skor plot PCA menunjukkan bahawa ekstrak *C. asiatica* mempunyai potensi yang baik untuk merawat diabetis jenis 2. Analisis terperinci metabolit dengan menggunakan ketinggian puncak ^{1}H-NMR mendedahkan bahawa pentadbiran ekstrak *C. asiatica* baik dalam membaikpulih metabolisma glukosa, kreatina, lipid, kitaran asid trikarboksilik, asid amino, trimetillamina, dan purin pada diabetis jenis 2.

Metabolomik berasaskan ^{1}H NMR berjaya bagi penilaian kimia dan biologi daripada Pegaga (*C. asiatica, H. bonariensis* dan *H.sibthorpioides*) ekstrak. Keputusan tumbuhan ekstrak metabolomik akan berguna untuk mewujudkan standard untuk persiapan phytomedicinal.

Keywords: *C. asiatica*, *H. bonariensis*, *H. sibthorpioides*, ^{1}H NMR metabolomik, LCMS, PCA, PLS, SUS-plot, aktiviti radikal bebas, *in vivo* anti-diabetes.

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I certify that a Thesis Examination Committee has met on 23 July 2014 to conduct the final examination of Maulidiani on her thesis entitled “Chemical and Biological Evaluations of Pegaga (*Centella asiatica* (L.) Urb., *Hydrocotyle bonariensis* Comm. ex Lam. and *Hydrocotyle sibthorpioides* Lam.) Extracts and their Effects on an Obese Diabetic Rat Model using ^{1}H NMR Metabolomics” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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Date: 23 October 2014

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

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

δ	Chemical shift in ppm
°C	Degree in Celsius
ANN	Artificial Neural Network
br	Broad
CA	*Centella asiatica*
CMC	Carboxymethyl celullose
d	Doublet
dd	Doublet of doublets
DMA	Dimethylamine
DMG	Dimethylglycine
ESIMS	Electron Soft Ionization Mass Spectrum
EtOAc	Ethyl acetate
eV	Electron volt
^{1}H	Proton
HB	*Hydrocotyle bonariensis*
HDL	High-density lipoprotein cholesterol
HFD	High fat diet
HS	*Hydrocotyle sibthorpioides*
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC50	Inhibition concentration at 50 percent
LC-DAD	Liquid Chromatography- Diode Array Detector
LDL	Low-density lipoprotein cholesterol
m	Multiplet
Nd	Normal Diet
Obdb	Obese diabetic
PCA	Principal Component Analysis
PLS	Partial Least Square
STZ	Streptozotocin
t	Triplet
TCA	Tricarboxylic acid
TMA	Trimethylamine
TMS	Tetramethylsilane
TSP	3-Trimethylsilyl-propionic acid

Biodata of the Student

Maulidiani was born on 7th February 1980 in Bogor, West Java, Indonesia. She received her primary education at Sekolah Dasar Negeri Pengadilan 3 Bogor and secondary education at Sekolah Menengah Pertama Negeri 1 (SMPN 1) Bogor. Her high school education was obtained in three year of study at SMPN 1 Bogor and another three year of study at Sekolah Menengah Umum Negeri 1 (SMUN 1) Bogor. In 1998, she was offered to pursue her degree in Institut Pertanian Bogor (IPB), majoring in Agroindustrial Technology. After she received her degree in 2002, she continued her Master (M.S.) at Institute of Bioscience, Universiti Putra Malaysia in the field of drug discovery study (2002-2004). As her interests on natural products research continue to increase, in 2008, she decided to register for her PhD degree, also under the field of drug discovery.

LIST OF PUBLICATIONS

Journals

1. Maulidiani, H., Khatib, A., Shaari, K., Abas, F., Shitan, M., Kneer, R., Neto, V., Lajis, N.H., 2012. Discrimination of three pegaga (*Centella*) varieties and determination of growth-lighting effects on metabolites content based on the chemometry of ^{1}H nuclear magnetic resonance spectroscopy. *Journal of Agricultural and Food Chemistry* 60, 410-417.

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CHAPTER 1

INTRODUCTION

Plant-based traditional (herbal) medicine is still a popular alternative for primary health care and treatment of various diseases (Cordell and Colvard, 2012). The World Health Organization (WHO) estimated that as much as 80% of the world population still relies on the use of various forms of traditional medicine for its primary healthcare (Cragg et al., 1999; Narins, 2000). Due to this and in addition to rising costs as well as emergence of new diseases, there is a strong demand for and a necessity to accelerate research in phytomedicine. The world market for phytopharmaceuticals has charted a steady growth over the past decade. The estimated turnover in 1995 alone was reported to be US$12.4 billion. Today, the herbal industry has a turnover of about US$ 62 billion, with still a strong growth potential (Patwardhan et al., 2005). The World Bank Reports stated that trade in medicinal plants, botanical drug products and raw materials are growing at an annual growth rate between 5% and 15% (WHO, 2002).

In recent years, there has been a change in paradigm with respect to research approaches in understanding the effects of a complex mixture such as that of phytomedicines. Such complexity is certain to have a big influence on interactions between genes, proteins and ligands in the total manisfestation of a disease or illness. Thus, the determination of efficacy of phytopreparations and their mode of action are major challenges for an evidence-based phytotherapy. Even though the assessment of the efficacy of phytopreparations by placebo controlled trials, together with bioavailability and pharmacokinetics holds the key for a rational and fully accepted phytotherapy. The application of “metabolomics” technology unfolds the possibility to investigate phytopreparations without prominent active principle(s) for their complex mechanisms of action. Metabolomics can help to rationalize the therapeutic superiority of many plant extracts over single isolated constituents. Provided that phytopreparation-specific signatures in the form of gene/protein expression profiles can be developed, this technology will be useful for the chemical and pharmacological standardization and evidence of the toxicological potential of a plant extract. Since the mode of action of a drug combination can differ substantially from the mode of action of the same drugs applied individually, the thrust for the search of the single active principle may lose its importance.

Currently, conventional standardization and quality control (QC) of medicinal herbs relies on a technology that has been successfully applied for the QC of single ingredient medicines, i.e *via* HPLC quantification of the active ingredient. Even though a perfect tool for single ingredient medicines, HPLC quantification of active or marker compounds does not seem to be fair to the complex mixtures present in herbs and potentially ignores synergistic activities of multiple active compounds on multiple mechanisms of action for which more and more evidences are being found (Stermitz et al., 2000).

NMR-based metabonomics in combination with multivariate data analysis, especially Principal Component Analysis (PCA), has been recognized as a powerful tool to classify samples according to their total chemical composition. The low molecular weight metabolite contents of cells, organs or organisms can contain a significant amount of information on their physiological status. High field ^{1}H-NMR is the fingerprinting method of choice for two obvious and crucial advantages: every organic molecule contains hydrogen atoms and hydrogen is the only atom where the isotope with the highest natural abundance has the NMR responsive ½ spin. Due to this maximum sensitivity, high quality spectra can be generally obtained in less than 5 minutes. NMR-based metabolomics has been successfully applied in many research areas such as medical (Brindle et al., 2002), physiological (Gavaghan et al., 2000; Wang et al., 2005), and toxicological problems (Holmes et al., 2000). Metabolomics is also useful for the applications in the QC of plant-based product, for example in characterizing the metabolite patterns of apple juices (Belton et al., 1998), *Strychnos* species (Frederich et al., 2004) and tobacco (Choi et al., 2004).

'*Ulam*' refers to the name of vegetables and herbs that are eaten raw, blanched or boiled, forming a part of the local cuisine of the Malay community in Malaysia. It is recommended as a healthy diet since the vegetables and herbs provide essential vitamins and fibers. According to Husain et al. (2004) there are at least 109 'ulam' in Malaysia, and one of the most popular is Pegaga. Pegaga in Malaysia refers to some plants from the two genera *Centella* and *Hydrocotyle*, both belonging to the family Apiaceae. Characteristically, these species are creepers, commonly found in various South East Asian countries. Although, the two genera can be taxonomically distinguished from each other based on the differences in the leaf form and features (Downie et al., 1996), the differences are still rather difficult to recognize by the general consumers. Hence, these species are generally vernacularly referred to as Pegaga. It is a common assumption that these pegaga plants have the same chemical and biological properties. To the best of our knowledge there has been no proof of this and thus we were interested to investigate the differences in their metabolite profiles and biological properties. Three Pegaga plants of the more common species (*C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*) were therefore selected for investigation. Also included were several varieties of the species. Among the selected species, the phytochemical information on *Centella asiatica* is quite well established. Some characteristic phytochemical constituents have been identified (e.g. asiaticoside, madecasoside, acid, and madecassic acid) and their pharmacology (e.g. antioxidant and immunomodulatory properties) have been assessed. However, not much is known about *Hydrocotyle*. Thus, a comprehensive metabolite analysis of these plants will allow us to establish the phytochemical relationship between them. Further, a metabolomics approach in the assessment of their biological or pharmacological properties will indicate to us the more preferred species or variety for their phytomedicinal use.

There has been a long association between oxidative stress, inflammation and diabetes. Therapeutic approaches aimed at ameliorating stress and inflammation may be promising targets for the development of new treatments for diabetes (Montane et al., 2014). *C. asiatica* which has been reported previously to have antioxidant (Zainol et al. 2003, Hamid et al. 2002) and anti-oxidative stress (Hussin et al. 2007) properties, may be a good candidate in treating diabetes as well. Several studies have reported anti-diabetic property of *C. asiatica* (Chauhan et al., 2010) and asiatic acid, its characteristic chemical constituent or marker compound (Liu et al., 2010, Ramachandran and Saravanan, 2013a). However, the molecular mechanism of this biological effect remains unclear. Thus, utilizing ^{1}H-NMR-based metabonomics, we are also interested to understand more about the metabolic changes in an obese diabetic (obdb) animal model, brought about upon the oral consumption of *C. asiatica* extract.

Thus, the major aim of the study was to develop phytochemical quality control methods that can be used to guarantee the biological activity of medicinal plants, and to use this platform for further in depth studies of the pharmacology that might give evidence for its traditional use. These are translated into four separate objectives as follows:

1. To establish the chemical profiles of selected Pegaga species (*C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*) *via* metabolite profiling using NMR and LC-MS/MS^n spectroscopy.
2. To predict the antioxidant activity of Pegaga extracts based on ^{1}H NMR spectroscopy using PLS and ANN model.
3. To determine the metabolic changes in the biofluids of obese and obese-diabetic animal models using ^{1}H-NMR-based metabolomics approach.
4. To evaluate the *in vivo* anti-diabetic property of selected Pegaga extracts (*C. asiatica* and *H. bonariensis*) using ^{1}H NMR-based metabolomics approach.

Outlines of the thesis:

The thesis consists of nine chapters. Chapter 1 gives the introductory and Chapter 2 gives a review on the available literature on Pegaga. General materials and methods used throughout the investigation follow in Chapter 3. The results of the study are broken down into five chapters. Chapter 4 describes the discriminative analysis of the Pegaga species based on their metabolite profiles obtained using NMR spectroscopy while the LC-MS/MS^n profiles are described in Chapter 5. Chapter 6 dealt with the prediction of antioxidant activity of Pegaga extracts based on ^{1}H NMR-based metabolomics. Comparison between PLS and ANN models and their validation are also reported. Chapter 7 focuses on the analysis of the biochemical changes observed in obese and obese diabetic animal models and the proposed biochemical pathways. Chapter 8 discusses the examination of metabolic changes in the biofluids (urine and serum) of rats treated with Pegaga extracts based on ^{1}H NMR metabolomics.

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