UNIVERSITI PUTRA MALAYSIA

NMR-BASED METABOLOMICS FOR QUALITY CONTROL OF DUKUNG ANAK (Phyllanthus niruri Linn.) AND IDENTIFICATION OF BIOMARKERS FOR ITS DIABETES-RELATED ACTIVITIES

AHMED MEDIANI

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By

AHMED MEDIANI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

August 2016

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DEDICATION

This thesis is dedicated to my parents, family, and friends.

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

NMR-BASED METABOLOMICS FOR QUALITY CONTROL OF DUKUNG ANAK (*Phyllanthus niruri* Linn.) AND IDENTIFICATION OF BIOMARKERS FOR ITS DIABETES-RELATED ACTIVITIES

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Chairman : Associate Professor Faridah Abas, PhD
Faculty : Food Science and Technology

Phyllanthus is a plant genus that has been used in traditional medicine due to its phytomedicinal metabolites content. However, there is a lack of consistency and efficacy of plant of this genus as well as not enough scientific reports to support its traditional uses specifically in treating diabetes. The main aim of this study was to investigate the antidiabetic properties of *Phyllanthus in vitro* and *in vivo* using metabolomics approach. The first part of the present study determined the variation between two *Phyllanthus* species (*P. niruri* and *P. urinaria*) at different growth stages (8, 10 and 12 weeks) using proton nuclear magnetic resonance (^{1}H NMR) combined with multivariate data analysis (MVDA). The results revealed that the Principal Component Analysis (PCA) and Partial Least Square (PLS) showed ideal differentiation between these species, suggesting the contributed metabolites, which were responsible for the discrimination. *Phyllanthus niruri* extracted with 80% ethanol possessed high antioxidant and α-glucosidase inhibitory activities compared to *P. urinaria* and 10 weeks was the valuable growth age that provides *P. niruri* extract with the highest content or/and number of bioactive metabolites. Thus, *P. niruri* harvested at 10 weeks was selected in the second part of the study to optimize the appropriate drying method and solvent for extracting these bioactive components. Three extracting solvents (methanol, ethanol and water) with different ratios were assessed for their effects on biological activities of *P. niruri* subjected to three drying treatments (air, oven and freeze). The freeze-dried *P. niruri* samples that were extracted with 80% ethanol exhibited higher biological activity values compared to the other extracts. The 80% ethanol extract had the quantities of epicatechin (172.0 µg/g), ellagic acid (46.67 µg/g), chlorogenic acid (44.33 µg/g), quercetin (1.73 µg/g), catechin (1.63 µg/g), phyllanthin (46.0 µg/g), and hypophyllanthin (23.17 µg/g) analyze using high performance liquid chromatography (HPLC). In the third part of this study, the correlation was also determined among the phytochemical constituents and the bioactivities using PLS regression. The identified phenolics and hypophyllanthin were strongly correlated with the antioxidant and α-glucosidase inhibitory activities, suggesting their great contributions to these activities. The optimized *P. niruri* extract was further

evaluated for *in vivo* antidiabetic properties at two concentrations (250 and 500 mg/kg body weight) and compared with metformin. The effect of *P. niruri* extract on the biochemical parameters of obese–diabetic rats was also investigated. The development of the obese diabetic model was done by feeding the high fat diet to Sprague-Dawley rats and inducing diabetic condition with a low dose of streptozotocin (STZ). The *in vivo* results indicated that *P. niruri* extracts of 500 mg/kg bw displayed the management of metabolites disorders of obese diabetic rats toward the normal state. The phytochemicals of *P. niruri* extracts might improve the metabolic disorders caused by diabetes. The extract at a concentration of 500 mg/kg bw also exhibited a noticeable effect in declining the plasma glucose level and improving lipid profile in obese diabetic rats as compared to 250 mg extract/kg bw. Both *P. niruri* doses showed reduction in the plasma cholesterol and low-density lipoprotein (LDL) levels and an increase in high-density lipoprotein (HDL). The effect of various metabolites in controlling the diabetes syndromes was discussed. The present study suggested that *P. niruri* can be a prominent and constructive medicinal plant. In conclusion, ^{1}H NMR-based metabolomics approach was successfully used to optimize the postharvest parameters, provide insights into the efficacy of *P. niruri* as a remedy for diabetes, and set the preliminary step towards developing this herb into high claim products.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENDEKATAN METABOLOMIK DALAM MENYIASAT SEBATIAN ANTIDIABETIK BAGI EKSTRAK DUKUNG ANAK (*Phyllanthus niruri* Linn.) DAN PENGENALPASTIAN PENANDA BIO DI DALAM TIKUS SPRAGUE-DAWLEY TERARUH DIABETES

Oleh

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Ogos 2016

Pengerusi : Profesor Madya Faridah Abas, PhD
Fakulti : Sains dan Teknologi Makanan

Phyllanthus merupakan genus tumbuhan yang telah digunakan dalam perubatan tradisional disebabkan oleh kandungan metabolit fitoperubatannya. Walau bagaimanapun, terdapat kekurangan konsistensi dan keberkesanan tumbuhan daripada genus ini dan juga laporan saintifik tidak cukup untuk menyokong kegunaan tradisional khusus dalam merawat diabetes.Tujuan kajian ini adalah untuk menyiasat sifat-sifat antidiabetik *Phyllanthus* secara *in vitro* dan *in vivo* menggunakan pendekatan metabolomik. Bahagian pertama kajian ini menentukan variasi antara dua spesies *Phyllanthus (P. niruri* dan *P. urinaria*) pada peringkat pertumbuhan yang berbeza (8, 10 dan 12 minggu) dengan menggunakan proton resonans magnetik nuklear (^{1}H NMR) digabungkan dengan analisis data multivariat (MVDA). Keputusan daripada Analisis Komponen Utama (PCA) dan Separa Dua Terkecil (PLS) menunjukkan perbezaan yang ideal antara spesies ini, mencadangkan metabolit yang menyumbang terhadap diskriminasi. Ekstrak *P. niruri* memiliki bioaktiviti yang lebih tinggi berbanding *P. urinaria* dan sepuluh minggu merupakan umur bermanfaat yang menyediakan ekstrak *P. niruri* dengan kandungan tertinggi atau/dan beberapa metabolit bioaktif. Oleh itu, *P. niruri* yang dituai pada 10 minggu telah dipilih untuk mengenal pasti kaedah pengeringan dan pelarut yang sesuai untuk mengekstrak komponen bioaktif. Tiga pelarut pengekstrakan (metanol, etanol dan air) dengan nisbah yang berbeza telah dinilai untuk kesannya terhadap aktiviti-aktiviti biologi *P.niruri* tertakluk kepada tiga rawatan pengeringan (udara, ketuhar dan sejuk beku). Sampel pengeringan sejuk beku *P. niruri* yang diekstrak dengan 80% etanol mempamerkan nilai aktiviti biologi yang lebih tinggi berbanding dengan ekstrak lain. Ekstrak bagi 80% etanol mempunyai kuantiti epikatekin (172.0 μg/g), asid elargik (46.67 μg/g), asid klorogenik (44.33 μg/g), kuersetin (1.73 μg/g), katekin (1.63 μg/g), filantin (46.0 μg/g), dan hipofilantin (23.17 μg/g) dianalisis menggunakan kromatografi cecair berprestasi tinggi (HPLC). Untuk bahagian ketiga kajian, korelasi antara juzuk fitokimia dan bioaktiviti ditentukan menggunakan regresi PLS. Metabolit-metabolit yang telah dikenalpasti mempunyai kaitan yang kukuh dengan aktiviti antioksidan dan perencatan α-glukosidase, menunjukkan sumbangan besar mereka kepada aktiviti-aktiviti ini. Ekstrak *P. niruri* yang telah

dioptimumkan seterusnya dinilai bagi pengaruh antidiabetik secara *in vivo* pada dua kepekatan (250 dan 500 mg/kg bw) dan dibandingkan dengan metformin. Kesan ekstrak *P. niruri* kepada parameter biokimia tikus gemuk-diabetes juga disiasat. Pembentukan model gemuk diabetes dilakukan dengan memberi diet lemak yang tinggi sebagai makanan kepada tikus Sprague-Dawley dan mengaruh kencing manis dengan dos yang rendah streptozotocin (STZ). Keputusan *in vivo* menunjukkan ekstrak *P. niruri* sebanyak 500 mg/kg bw memaparkan pengurusan metabolit yang terganggu bagi tikus gemuk-diabetes ke arah kondisi normal. Fitokimia ekstrak *P. niruri* mungkin membawa kepada peningkatan tahap insulin, sekali gus merangsang β-sel pankreas sedia ada dalam tikus kencing manis. Ekstrak pada kepekatan 500 mg/kg bw juga mempamerkan kesan yang ketara dalam penurunan paras glukosa plasma dan meningkatkan profil lipid dalam tikus gemuk diabetes berbanding dengan 250 ekstrak mg/kg bw. Ekstrak *P. niruri* pada kedua-dua kepekatan menunjukkan pengurangan dalam paras kolesterol plasma dan lipoprotein berketumpatan rendah (LDL) dan peningkatan dalam lipoprotein berketumpatan tinggi (HDL). Kesan sinergi pelbagai metabolit dalam mengawal sindrom diabetes telah dibincangkan. Kajian ini mencadangkan *P. niruri* boleh menjadi tumbuhan ubatan yang penting dan berharga. Kesimpulannya, pendekatan metabolomik berasaskan ^{1}H NMR telah berjaya digunakan untuk mengoptimumkan parameter lepas tuai, memberi maklumat keberkesanan *P. niruri* sebagai ubat untuk kencing manis, dan menetapkan langkah awal ke arah membangunkan herba ini sebagai produk bertuntutan tinggi.

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I certify that a Thesis Examination Committee has met on 29 August 2016 to conduct the final examination of Ahmed Mediani on his thesis entitled "NMR-Based Metabolomics for Quality Control of Dukung Anak (*Phyllanthus niruri* Linn.) and Identification of Biomarkers for its Diabetes-Related Activities" 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.

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

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LIST OF ABBREVIATIONS

^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
AA	Antioxidant Activity
AD	Air Drying
OD	Oven Drying
FD	Freeze Drying
CC	Column Chromatography
d	Doublet
DAD	Diode Array Detector
dd	Doublet of doublet
dH_2O	Deionized water
DPPH	Diphenylpicrylhdrazyl
ESI	Electrospray Ionization
g	Gram
GAE	Gallic Acid Equivalent
HMBC	Heteronuclear Multiple Bond Correlation
HCA	Hierarchical Cluster Analysis Hierarchical Cluster Analysis
HPLC	High Performance Liquid Chromatography
HREISMS	High Resolution Electron Impact Mass Spectroscopy
Hz	Hertz
IC_{50}	Inhibition Concentration at 50 percent
L	Litre
LC-MS	Liquid Chromatography–Mass Spectrometry
m	Multiplet
m/z	Mass to charge
MeOH	Methanol
EtOH	Ethanol
Aq	Water
MHz	MegaHertz
mL	Milliliter
MS	Mass Spectrometry
MVDA	multivariate data analysis
^{o}C	Degree in Celsius

PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares–Discriminant Analysis
ppm	Part Per Million
RMSEE	Root Mean Square Error of Estimation
RMSEP	Root Mean Square Error of Prediction
ROS	Reactive Oxygen Species
RPLC	Reversed Phase Liquid Chromatography
s	Singlet
SIMCA	Soft Independent Modeling of Class Analogy
TPC	Total Phenolic Contents
UV	Ultraviolet
UV/VIS	Ultravoilet/visible
VIP	variable Importance in the Projection
δ	Chemical Shift in ppm
µg	Microgram
µL	Microliter
^{13}C	Carbon-13
LDL	Low-density Lipoprotein
HDL	High-density Lipoprotein
STZ	Streptozotocin
DM	Diabetes Mellitus
WHO	World Health Organization
PNPG	p-nitrophenyl-α-D-glucopyranose
ANOVA	Analysis of Variance
CID	Collision-induced Dissociation
NaOD	Sodium Deuterium Oxide
D_2O	Deuterium Oxide
CD_3OD	Methanol-d_4
KH_2PO_4	Potassium Dihydrogen Phosphate
CMC	Carboxymethyl Cellulose
CH_3OH-d_4	Deuterated Methanol-*d4*
SD	Sprague-Dawley

HFD	High Fat Diet
ND	Normal Diet
2D	Two-Dimensional
HMDB	Human Metabolome Database
TG	Total Triglycerides

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is the dysregulation in metabolic function of an organism characterized by the disorder of the catabolic reaction of organic nutrients. This disorder is caused by a disturbance in the excretion and/or action of insulin, which is associated with chronic hyperglycemia. The DM is a chronic disease in which the pancreas does not produce enough insulin (Type 1) or the body is unable to use the insulin effectively (Type 2). Worldwide, 9% of adults aged 18 years and above were living with diabetes in 2014. In addition, DM caused 1.5 million deaths in 2013, with 80% of them occurring in low- and middle-income countries (WHO, 2014). One of the practical treatments of diabetes is the reduction of postprandial hyperglycemia by the retardation of glucose catabolism via the inhibition of hydrolysis enzymes (α-glucosidase and α-amylase). This inhibition reaction leads to the prolongation of the sugar digestion time and a decrease in the proportion of glucose absorption. These inhibitors can be provided by natural products.

Plant extracts as natural products that contain several phytochemicals are attracting more consideration for their potential usage in curing and preventing DM. Numerous natural biomolecules and plant extracts have been examined for their antidiabetic activities both *in vitro* and *in vivo*, and they showed very promising impacts, which indicates that phytochemical intake might be a key approach for diabetes prevention. Recent studies have indicated that plant substances possess different functions that aid to find the prominent candidate for innovative antidiabetic usage (Kordalewska & Markuszewski, 2015; Otunola & Afolayan, 2015; Patel et al., 2012).

The *Phyllanthus* genus (Euphorbiaceae) has significant medicinal properties used in the treatment of different diseases (Markom et al., 2007; Moreira et al., 2013). *Phyllanthus* genus is originally from India, and it spreads in tropical regions, including Malaysia (Ali et al., 2006). In Malaysia, it is locally known as ‘dukung anak’. Specifically, the genus *Phyllanthus* has been used historically for the treatment of intestinal parasites, problems of the liver, kidney and bladder and the treatment of diseases such as infectious diseases (Calixto et al., 1998). This genus contains several species, and the most popular ones are *Phyllanthus niruri* and *Phyllanthus urinaria* (Khatoon et al., 2006; Kumaran & Karunakaran, 2007). *Phyllanthus niruri* has been utilized as a traditional medicine to cure kidney problems, diarrhea, fever, diabetes and colic (Kumaran & Karunakaran, 2007). *Phyllanthus niruri* also has the ability to reduce lipids peroxidation and exhibit an analgesic effect (Amin et al., 2012; Kumaran & Karunakaran, 2007). In China, this plant is used as a traditional folk medicine and is known as ‘pearls under the leaves’ (Murugaiyah and Chan, 2009). It has also been acclaimed for its effective remedy to treat diabetic sufferers in Nigeria and India (Okoli et al., 2010; Okoli et al., 2011). However, *P. urinaria* has several uses in traditional medicine including liver

protection, fever reduction, laxative, cure for inflammation, suppression of hyperactivity of the liver, improve eyesight, and urine flow as well as detoxification of poisons from the body (Amin et al., 2012; Kumaran & Karunakaran, 2007). Currently, *P. urinaria* is also widely used as an anti-inflammatory and antioxidant agent as well as a smooth muscle relaxant (Catapan et al., 2000; Kumaran & Karunakaran, 2007). Various bioactive and phytochemical compounds are found in *P. urinaria*, including flavonoids, tannins, lignans and other benzenoid compounds (Chang et al., 2003; Khatoon et al., 2006; Kumaran & Karunakaran, 2007). In addition, corilagin, gallic acid, caffeoylquinic acid, geraniin, and rutin have been isolated from *P. urinaria* (Khatoon et al., 2006; Kumar et al., 2015).

Metabolites can have significant variation at several growth stages and may be negatively affected by some processing methods, such as drying, in terms of their bioactivities and chemical structures. Thermal drying conditions were stated to possess wide negative influences on the functionality of plant materials due to a significant reduction of its metabolites (Lim & Murtijaya, 2007). A large variability was also marked in the biological activity of plants and their metabolite constituents as a result of extraction with different solvents (Siddhuraju & Becker, 2003). Therefore, it would be valuable to accomplish the conservation, efficiency and consistency values of plants and their derivative products against the negative effects and changes of these aspects using the appropriate approach.

Metabolomics is related to the metabolome, which represents the small molecules of the cells of organisms. It is a competent approach to study and evaluate the changes in the small molecule composition of the biological system under various circumstances. Furthermore, metabolomics is a global profiling tool in the detection of metabolites and metabolic pathways (Kaddurah-Daouk et al., 2008). Its central value is dramatically increased in biological regulation, including the control and management of diseases. Metabolomics sheds light on medicine with the help of technological advances, which bring it to the point that it can grow rapidly in the biological and biomedical areas. New insights and ideas regarding metabolites and their mechanisms of action provide the platform to be further used for studying many diseases. Proton nuclear magnetic resonance (^{1}H NMR) is emerging as a leading analytical tool in metabolomics studies due to the large number and groups of identified compounds. Thus, ^{1}H NMR-based metabolomics can be used as an approach to assess the variation in phytochemicals content and bioactivity of plant material and changes in biofluids (Cevallos-Cevallos et al., 2009; Deja et al., 2013).

Animal models play an important role in understanding metabolic disorders. Many studies have used animal models in simulating diabetic conditions to understand the molecular events associated with this disease and its complications (Bakar et al., 2015; Beckonert et al., 2007). In addition, the metabolic changes of body fluids associated with treatment with several herbal drugs can also be evaluated. Recently, herbal drugs, herbal drug preparations and herbal medicinal products are extensively practiced in most countries. There is thus a need for an appropriate approach for rapid screening, identification and preclinical testing. Metabolomics can provide the accuracy, reproducibility, selectivity and specificity that are needed by applying

different tools as well as coupling these tools (Bilia et al., 2002). Its mission is also to detect, identify, and correlate the metabolic variations in the biological system, regardless of the individual cell. It can still directly reflect the functionality of the cells. Therefore, it is preferred in many cases over genomic and proteomic methods for predicting the efficacy of drugs and extracts in the control and management of diseases.

1.2 Scope and objectives

The current study was initiated to solve several issues that can serve as platforms in obtaining valuable extracts for the treatment of DM and the improvement of its metabolic disturbances. The main aim of this study was to evaluate the antidiabetic activity of *Phyllanthus* extracts by ^{1}H NMR based metabolomics. To accomplish this goal, numerous specific objectives were proposed. The first part of the study aims to compare the antioxidant and α-glucosidase inhibitory activities of two *Phyllanthus* species and their phytochemicals content at different growth stages (Chapter 3). The most active species at the selected growth age was chosen to evaluate the effect of different solvents and drying methods on antioxidant and α-glucosidase inhibitory activities and quantifying the metabolites by high performance liquid chromatography (HPLC) (Chapter 4). Additionally profiling of the chemical constituents of the most desirable and potent extract and correlate the chemical profile with biological activities was further evaluated on the active species (Chapter 5). Then, the animal model of normal- and obese-diabetic rats was optimized and the metformin effect was tested (Chapter 6). Finally, the overall bio-markers (in animal bio-fluids) relating to the effect of *P. niruri* in streptozotocin induced diabetic rats were evaluated (Chapter 7).

The five specific objectives of the study are presented below:

1. To determine the antioxidant and α-glucosidase inhibitory activities of *Phyllanthus niruri* and *Phyllanthus urinaria* and their phytochemicals content at different growth ages (8, 10 and 12 weeks) using ^{1}H NMR-based metabolomics.
2. To profile the chemical constituents of the most desirable and potent extract and quantify the metabolites by HPLC and correlate the chemical profile with biological activities.
3. To determine the effect of different solvents and drying methods on antioxidant and α-glucosidase inhibitory activities of *P. niruri* and its phytochemical constituents using ^{1}H NMR-based metabolomics.
4. To evaluate the metabolic disorders associated with Type 1 and Type 2 DM on the urine composition of obese- and normal-diabetic rats as well as the effect of metformin treatment.
5. To identify the metabolic disorders in obese diabetic rats and evaluate the functionality and benefits of *P. niruri* extract on ameliorating the complications associated using a ^{1}H NMR-based metabolomics approach.

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