UNIVERSITI PUTRA MALAYSIA

CORRELATION BETWEEN METABOLITE PROFILE AND PHYTOCHEMICAL CHARACTERISTICS OF Ipomoea aquatica Forssk. WITH ITS ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY ACTIVITIES USING NMR-BASED METABOLOMICS

UMAR LAWAL

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By

UMAR LAWAL

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

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DEDICATION

This thesis is dedicated to my parents and family

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

CORRELATION BETWEEN METABOLITE PROFILE AND PHYTOCHEMICAL CHARACTERISTICS OF *Ipomoea aquatica* Forssk. WITH ITS ANTIOXIDANT AND α-GLUCOSIDASE INHIBITORY ACTIVITIES USING NMR-BASED METABOLOMICS

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UMAR LAWAL

March 2016

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

Ipomoea aquatica Forssk. (morning glory) is a green leafy vegetable that is rich in minerals, proteins, vitamins, amino acids and secondary metabolites. The aims of the study were to discriminate *Ipomoea* extracts by ^{1}H NMR spectroscopy in combination with chemometrics method and to determine their antioxidant and α-glucosidase inhibitory activities. In the first part of the study, different types of *I. aquatica* cultivar such as the upland type has narrow leaves (K-11), the low-land and aquatic type, have broader, arrow-shaped leaves, (K-25) and bamboo shaped leaves (K-88), extracted using water and methanol at various concentrations were investigate for their effects on the total phenolics, antioxidant and α-glucosidase inhibitory activities. This study indicates that 70% methanol was the most efficient solvent for the extraction of phenolic compounds from *I. aquatica* cultivars. Thus, for the second part of the study ^{1}H NMR combined with multivariate data analysis was applied for the metabolic profiling of three cultivars of *I. aquatica* extracted with 70% methanol. The orthogonal partial least squares discriminant analysis (OPLS-DA) indicated a clear separation among cultivars. The relative levels of various compounds such as amino acids, organic acids, sugars and phenolic compounds were specific to each cultivar. Among the three cultivars, the K-11 was the most active for the antioxidant and α-glucosidase inhibitory activities and was selected for the third part of the study. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was combined with multivariate data analyses to distinguish the K-11 cultivar at different developmental stages. A principal component analysis (PCA) of *I. aquatica* provides clusters based on the different developmental stages by combining principal components PC1 and PC2 with a total variance of 65.1%. The initial stages (weeks 3 and 4) showed comparatively low contents of phenolic and organic acids, such as citric and maleic acid; the latter stages (weeks 5 and 6) exhibited higher glucose and phenolic compound contents. The sugar, phenolic compound, fatty and formic acid contents increased based on the developmental stages of the *I. aquatica*. The latent structures were projected using a partial least squares (PLS) model to predict the biological activity of the *Ipomoea*

extracts based on their ^{1}H NMR spectra. The results showed that six-week-old plants were the most active, showing an accumulation of epicatechin, protocatechuic acid, rutin and maleic acid. In addition, three compounds namely 3,5-di-*O*-caffeoylquinic acid (**83**) and 4,5-di-*O*-caffeoylquinic acid (**84**) and quercetin-3-*O*-β-glucoside (**85**) were isolated from six-week-old of the K-11 cultivar. The structures of these compounds were elucidated using various spectroscopic techniques including UV, ESIMS, 1D- and 2D-NMR. Compounds (**83**) and (**84**) exhibited good antioxidant activity with IC_{50} values of 4.85±0.06 µg/mL and 6.65±0.12 µg/mL, respectively, and compound (**85**) exhibited the lowest activity with IC_{50} values of 7.66±0.19 µg/mL compared to standard BHT; 6.25±0.15 µg/mL. A similar trend was observed for α-glucosidase inhibitory activity. In conclusion, this study may serve as a starting point for further research on phytochemicals from *I. aquatica* and can aid in the development of medicinal preparation, nutraceutical and functional food.

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

PROFIL METABOLIT DAN PENCIRIAN FITOKIMIA *Ipomoea aquatica* Forssk. DENGAN KORELASI AKTIVITI ANTIOKSIDAN DAN PERENCATAN ALFA-GLUKOSIDASE MENGGUNAKAN METABOLOMIK BERASASKAN NMR

Oleh

UMAR LAWAL

Mac 2016

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

Ipomoea aquatica Forssk. (kangkong) adalah sayuran berdaun hijau yang kaya dengan mineral, protein, vitamin, asid amino dan metabolit sekunder. Tujuan kajian ini adalah untuk membezakan ekstrak *Ipomoea* menggunakan ^{1}H NMR spektroskopi kombinasi dengan kaedah chemometrics dan untuk menentukan aktiviti antioksidan dan perencatan α-glukosidase. Dalam bahagian pertama kajian ini, pelbagai jenis kultivar *I. aquatica* seperti jenis tanah tinggi yang mempunyai daun yang tirus (K-11), tanah rendah dan jenis akuatik, yang mempunyai daun yang lebih lebar, berbentuk anak panah (K-25) dan daun berbentuk daun buluh (K-88), diekstrak menggunakan air dan metanol pada pelbagai kepekatan telah disiasat untuk kesannya atas jumlah fenolik, aktiviti antioksidan dan perencatan α-glukosidase. Kajian ini menunjukkan bahawa 70% metanol adalah pelarut yang paling berkesan untuk pengekstrakan sebatian fenolik dari kultivar *I. aquatica*. Oleh itu, untuk bahagian kedua kajian ^{1}H NMR yang digabungkan dengan analisis data multivariat telah digunakan untuk profil metabolik tiga kultivar *I. aquatica* yang diekstrak dengan 70% metanol. Analisis diskriminan-kuasa dua terkecil separa ortogonal (OPLS-DA) menunjukkan pemisahan yang jelas antara kultivar. Kepekatan relatif pelbagai sebatian, seperti asid amino, asid organik, gula dan sebatian fenolik adalah khusus kepada setiap kultivar. Antara tiga kultivar, K-11 adalah yang paling aktif untuk aktiviti antioksidan dan perencatan α-glukosidase dan telah dipilih untuk bahagian ketiga dalam kajian ini. Proton resonans magnetik nuklear (^{1}H NMR) spektroskopi telah digabungkan dengan analisis data multivariat untuk membezakan sampel di peringkat perkembangan yang berbeza. Analisis komponen utama (PCA) dari *I. aquatica* menunjukkan kelompok berdasarkan tahap perkembangan yang berbeza dengan jumlah keseluruhan varian adalah 65.1% daripada komponen utama PC1 dan PC2. Peringkat awal (minggu 3 dan 4), menunjukkan kandungan sebatian fenolik dan asid organik, seperti asid sitrik dan malik yang agak rendah; peringkat akhir (minggu 5 dan 6) mempamerkan kandungan glukosa dan sebatian fenolik yang lebih tinggi. Kandungan gula, sebatian fenolik, lemak dan asid formik meningkat berdasarkan peringkat perkembangan *I. aquatica*. Struktur terpendam diunjurkan menggunakan model kuasa dua terkecil separa (PLS) untuk meramalkan aktiviti biologi ekstrak *Ipomoea* daripada spektrum ^{1}H NMR. Hasil kajian

menunjukkan bahawa *I. aquatica* yang berusia enam minggu adalah yang paling aktif, menunjukkan pengumpulan epikatekin, asid protocatechuic, rutin dan asid malik. Di samping itu, tiga sebatian tulen termasuk asid 3,5-di-*O*-caffeoylquinic (**83**) dan asid 4,5-di-*O*-caffeoylquinic (**84**) dan kuersetin-3-*O*-β-glukosida (**85**) telah diasingkan daripada daripada kultivar K-11 yang berusia enam minggu. Struktur sebatian ini telah dijelaskan dengan menggunakan pelbagai teknik spektroskopi termasuk UV, ESIMS, 1D- and 2D-NMR. Sebatian (**83**) dan (**84**) menunjukkan aktiviti antioksidan yang baik dengan nilai-nilai IC_{50} masing-masing ialah 4.85±0.06 μg/mL dan 6.65±0.12 μg/mL, dan sebatian (**85**) menunjukkan aktiviti yang paling rendah dengan nilai IC_{50} sebanyak 7.66±0.19 μg/mL berbanding BHT; 6.25±0.15 μg/mL. Pemerhatian yang sama dapat dilihat untuk aktiviti perencatan α-glukosidase. Kesimpulannya, kajian ini dapat bermanfaat sebagai titik permulaan untuk penyelidikan lanjut mengenai fitokimia dari *I. aquatica* dan boleh membantu dalam pembangunan penyediaan perubatan, nutraseutikal dan makanan berfungsi.

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I certify that a Thesis Examination Committee has met on 22 March 2016 to conduct the final examination of Umar Lawal on his thesis entitled "Correlation between Metabolite Profile and Phytochemical Characteristics of *Ipomoea aquatica* Forssk. with its Antioxidant and α-Glucosidase Inhibitory Activities using NMR-Based 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:

Nazamid bin Saar, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Azizah binti Abdul Hamid, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Irwandi Jaswir, PhD
Professor
International Islamic University Malaysia
Malaysia
(External Examiner)

T. Parimelazhagan, PhD
Professor
Bharathiar University
India
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 25 May 2016

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:

Faridah Abas, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairperson)

Intan Safinar binti Ismail, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Alfi Khatib, PhD
Associate Professor
Faculty of Pharmacy
International Islamic University Malaysia
(Member)

_____
BUJANG BIN KIM HUAT, PhD
Dean and Professor
School of Graduate Studies
Universiti Putra Malaysia

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This is to confirm that:

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Signature: 
Name of Chairman of Supervisory Committee: Associate Professor Dr. Faridah Abas

Signature: 
Name of Member of Supervisory Committee: Associate Professor Dr. Intan Safinar binti Ismail

Signature: 
Name of Member of Supervisory Committee: Associate Professor Dr. Alfi Khatib

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LIST OF ABBREVIATIONS

^{1}NMR	Proton Nuclear Magnetic Resonance Spectroscopy
AA	Antioxidant activity
APCI	Atmospheric Pressure Chemical Ionization
CC	Column Chromatography
$CHCl_3$	Chloroform
d	Doublet
DAD	Diode Array Detector
dd	Doublet of doublet
ddd	Doublet of double of doublet
ddH_2O	Distilled deionized water
DEPT	Distortionless Enhancement by Polarization Transfer
DPPH	Diphenylpicrylhdrazyl
ESI	Electrospray Ionization
FTIR	Fourier Transmission Infrared spectroscopy
g	Gram
GAE	Gallic Acid Equivalent
gCOSY	Gradient Correlation Spectroscopy
gHMBC	Gradient Heteronuclear Multiple Bond Correlation
gHSQC	Gradient Heteronuclear Single-Quantum Coherence
HCA	Hierarchical Cluster Analysis
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IC_{50}	Inhibition Concentration at 50 percent
IR	Infra-red
L	Litre
LC-MS	Liquid Chromatography–Mass Spectrometry
m	Multiplet
m/z	Mass per Charge
MeOH	Methanol
MHz	MegaHertz
mL	Milliliter

MS	Mass Spectrometry
MVDA	multivariate data analysis
ºC	Degree in Celsius
OPLS-DA	Orthogonal Partial Least Squares–Discriminant Analysis
PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares–Discriminant Analysis
ppm	Part Per Million
QTOF	Quadrupole–Time of Flight mass spectrometer
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
TLC	Thin Layer Chromatography
TPC	Total Phenolic Contents
UV	Ultraviolet
UV/VIS	Ultravoilet/visible
VIP	variable importance in the projection
	Chemical Shift in ppm
µg	Microgram
µL	Microliter
^{13}C	Carbon-13

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Diabetes represents one of the most significant global health problems because they are associated with a large economic burden. The global burden of diabetes is growing rapidly worldwide and it is estimated that the total number of patients with diabetes mellitus may increase from 382 million in 2013 to 592 million by 2035 (Guariguata et al., 2014). Type 2 diabetes mellitus is the most prevalence and it is continuously increasing worldwide. It accounts for about 90% of the total population with diabetes.

In Malaysia, a survey conducted by the Malaysian National Health Morbidity Survey III in 2006 involving 34,539 respondents age ≥ 18 years old covering all states of Malaysia, showed that the overall prevalence of diabetes mellitus was 11.6% among which Indians had the highest prevalence of 19.9%, followed by Malays 11.9% and Chinese 11.4% (Letchuman et al., 2010). A more recent study reported in 2013 showed that the prevalence rate of diabetes mellitus in Malaysia has increased significantly from 11.6% (in 2006) to 22.9%, with Indians (37.9%) having most prevalent then followed by the Malays (23.8%), hence showing an increase of almost two fold in the disease among Malaysians aged ≥30 years over the last two decades (Wan et al., 2013). The global financial burden of diabetes is on the increase; it was estimated to account for 12% of health expenditures in 2010, or at least $376 billion and the figure is expected to hit $490 billion in 2030 (Zhang et al., 2010). In Malaysia, studies were done in some localities that measure the economic burden of diabetes. One of the studies observed that the care cost for outpatients per diabetic patient per year varies with health clinic with specialist been more expensive at RM1127 (RM906.088)[USD363.13(291.95)] compared to health clinic without specialist with RM802.15 (RM626.266) [USD258.46 (201.79)]. For inpatient care, the author measured the provider cost of type 2 diabetics care admitted to medical wards was RM2,161 (RM1,322)[USD696.31(425.97)] per patient per admission (Ibrahim et al., 2010).

Among the different ways of managing type 2 diabetes mellitus, inhibition of α-glucosidase is regarded as one of the effective measures for regulating the disease. Mammalian α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is the key enzyme that catalyzes the digestive process of carbohydrates. Thus, α-glucosidase inhibitors can retard the liberation of D-glucose of disaccharides and oligosaccharides from carbohydrates and delay glucose absorption resulting in reducing postprandial plasma glucose levels and inhibiting postprandial hyperglycemia (Zhang et al., 2013). Common synthetic α-glucosidase inhibitors used in the treatment of patients with type 2 diabetes includes voglibose, acarbose and miglitol. They have been used as oral antidiabetic drugs since the early 1990s; nevertheless they also cause various side-effects like liver diseases, abdominal discomfort, flatulence and diarrhea. Because of

the side effects that are associated with these drugs safer natural α-glucosidase inhibitors are desired and many potential compounds have been reported from plants (Benalla et al., 2010; Ieyama et al., 2011; Rengasamy et al., 2013). Polyphenols are the major phytochemicals with antioxidant properties present in vegetables and fruits, which partly contribute to their beneficial effect on the prevention of CVD. The study of Yao et al. (2009) showed positive correlation between α-glucosidase inhibition activity and total phenolic contents. Thus, phenolic phytochemicals can potentially provide a natural source of α-glucosidase inhibitors.

Water spinach (*Ipomoea aquatica*, Forssk.) is a green leafy vegetable with many natural and health benefits. Different cultivars of *I. aquatica* are consumed as food in Hong kong, Taiwan, Sri Lanka, Malaysia, China (Ismail & Fun, 2003). The leaves are considered important because they provide adequate amounts of carotene, crude fiber, vitamin A and C, folic acids, mineral salts and iron. They also contain a reasonable amount of proteins that are equivalent to soy beans, legumes or whole egg (Aletor et al., 2002). The leaf extract of the plant showed hypoglycemic effect (Malalavidhane et al., 2001).

Metabolomics is a modern approach used to access environmental influence on living systems, it enables both the quantitative and qualitative analysis of all metabolites found in an organism. From the researches done over the years NMR has proven to be an adequate and suitable method to carry out such analyses; one of the most important reason is that it allows simultaneous detection of diverse groups of secondary metabolites besides abundant primary metabolites (Kim et al., 2010). In order to correlate *I. aquatica* chemical composition with the possible effects of maturation/development stages, NMR based metabolomics approach combined with MVDA was applied. Moreover, this approach was also used to differentiate the effect of the development stages on antioxidant activity (AA) and α-glucosidase inhibition activities of *I. aquatica*. More detailed research on metabolomic profiling of *I. aquatica* will definitely enhance the knowledge and appreciation for its use as vegetables and possible selection for cultivars with added nutritional effects. Following this, it was hypothesized ^{1}H NMR metabolomics approach can be useful in differentiating *I. aquatica* cultivars and correlate their metabolites with α-glucosidase inhibitors activity. In addition, the chemical composition of *I. aquatica* can be affected by different developmental stages.

Due to the aforementioned problems, the present study was designed and conducted in order to address some of the important issues that will aid in the selection of the best developmental stage and cultivars. The main goal of the present study was to discriminate *I. aquatica* extracts by ^{1}H NMR spectroscopy in combination with chemometrics tools and determine their biological activities. To achieve this goal, different specific objectives were defined. The first part of the study aimed to screen the *I. aquatica* extracted using different solvents for antioxidant and α-glucosidase inhibitory activities (Chapter 3). The most efficient solvent for extracting phenolic compounds will be chosen for further studies in characterization *I. aquatica* cultivars and correlating their biological activity using ^{1}H NMR based metabolomics (Chapter 4). These would be followed by study of the most active cultivar for biological activity

at different developmental stages and metabolites characterization using ^{1}H NMR based metabolomics (Chapter 5). To further elucidate and identify the chemical constituents in the active cultivar at the best developmental stage by using different chromatographic techniques (Chapter 6).

1.2 Study Objectives

The specific objectives of the study are:

1. To evaluate the antioxidant and α-glucosidase activities of different methanolic extracts of *Ipomoea aquatica.*
2. To discriminate *I. aquatica* cultivars and correlate with the biological activity using ^{1}H NMR based metabolomics.
3. To evaluate the effects of harvesting time on the metabolites of *I. aquatica* using ^{1}H NMR based metabolomics.
4. To isolate and identify the chemical constituents in active extract using various chromatographic techniques.

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