UNIVERSITI PUTRA MALAYSIA

METABOLITE CHARACTERIZATION OF Neptunia oleracea Lour. AND ITS CORRELATION WITH ANTIOXIDANT AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES USING NMR-BASED METABOLOMICS APPROACH

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By

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October 2017

Chairman : Associate Professor Faridah Abas, PhD
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Diabetes mellitus is a metabolic disease characterized by high blood glucose levels. Due to the unpleasant side effects of currently available synthetic drugs, traditional medicinal plants have drawn the attention of researchers who are seeking natural compounds for the control of diabetes. This present study focused on the profiling and characterization of metabolites in local medicinal plants with potential antioxidant and α-glucosidase inhibitory activities. The first part of the study assessed the total phenolic contents (TPC), DPPH free radical scavenging and α-glucosidase inhibitory activities of 5 local medicinal plants. Of these plants, *Neptunia oleracea* exhibited the highest TPC and most potent DPPH free radical scavenging and α-glucosidase inhibitory activities. Subsequently, the second part of the study examined the influence of different drying methods on metabolite variations among *N. oleracea* leaves and stems using an NMR-based metabolomics approach. The results showed that freeze drying was the most suitable drying method for preserving the metabolites in *N. oleracea*; whereas the *N. oleracea* leaves possessed higher TPC and better bioactivities than its stems. Multivariate data analysis (MVDA) revealed that the identified phenolics, including catechin, caffeic and gallic acids and derivatives of apigenin, quercetin, kaempferol and myricetin were responsible for the discrimination and potent bioactivities of freeze-dried *N. oleracea* leaves. Then, the third part of the study focused on an investigation of the relationship between these phenolics and the tested bioactivities. The effects of different extraction methods and ethanol ratios were also investigated. Both partial least square (PLS) and random forests (RF) analysis identified caffeic acid and derivatives of apigenin, quercetin and kaempferol as the most effective DPPH scavengers and α-glucosidase inhibitors of *N. oleracea*. To extract these phenolics, sonication with absolute ethanol was the most suitable extraction condition. Consequently, in the next part of the study, the freeze-dried *N. oleracea* leaf extracts obtained via sonication with absolute ethanol were fractionated using solid phase extraction (SPE) to obtain hexane (HF), chloroform (CF), ethyl acetate (EF) and methanol (MF) fractions. Of

these fractions, EF and MF displayed the most significant bioactivities, and were then subjected to UPLC-MS/MS analysis, which resulted in the identification of 37 metabolites, with mostly phenolics. Through various chromatographic techniques, 5 phenolics including quercetin-3-O-β-D-xylopyranoside (**28**), quercetin-3-O-α-L-arabinopyranoside (**30**), quercetin-3-O-α-L-rhamnoside (**32**), methylgallate (**11**) and rutin (**22**) were isolated from these fractions. Individual testing of the bioactivities of these phenolics showed that methylgallate (**11**) exhibited the most potent DPPH scavenging and α-glucosidase inhibitory activities, with the IC_{50} values of 17.25 and 50.76 µM, respectively. In summary, this study suggested that *N. oleracea* is a prominent source of phenolics which can be potential antioxidant and α-glucosidase inhibitors for the management of diabetes. This study is the first report on the antidiabetic potentiality of *N. oleracea*, and the first application of metabolomics approach for extensive study of the phytoconstituents and their correlation with antioxidant and α-glucosidase inhibitory activities of this plant. The results of this study lay the foundation for future research regarding the antidiabetic effect of *N. oleracea*.

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

PENCIRIAN METABOLIT *Neptunia oleracea* Lour. DENGAN KORELASI KEPADA AKTIVITI ANTIOKSIDAN DAN PERENCATAN ALFA–GLUKOSIDASE MENGGUNAKAN PENDEKATAN METABOLOMIK BERASASKAN NMR

Oleh

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Kencing manis merupakan satu penyakit metabolik yang dicirikan oleh kandungan gula yang tinggi dalam darah. Disebabkan oleh kesan sampingan yang tidak menyenangkan daripada ubat sintetik yang sedia ada, ubatan tradisional berasaskan tumbuhan telah menarik perhatian penyelidik dalam pencarian sebatian semulajadi untuk mengawal penyakit kencing manis. Kajian ini memberi tumpuan kepada profil dan pencirian metabolit dalam tumbuhan ubatan tempatan yang berpotensi untuk aktiviti antioksidan dan perencatan α-glukosidase. Bahagian pertama kajian ini adalah menilai jumlah kandungan fenolik (TPC), aktiviti pemerangkap radikal bebas DPPH dan perencatan α-glokosidase bagi 5 tumbuhan ubatan tempatan. Dalam kalangan tumbuhan ini, *Neptunia oleracea* mempamerkan TPC yang paling tinggi dan aktiviti pemerangkapan radikal bebas DPPH dan perencatan α-glokosidase yang paling kuat. Seterusnya, bahagian kedua kajian ini adalah mengkaji kesan kaedah pengeringan yang berbeza terhadap variasi metabolit antara daun dan batang *N. oleracea* dengan menggunakan metabolomik berasaskan NMR. Keputusan menunjukkan pengeringan sejuk beku adalah kaedah pengeringan yang paling sesuai untuk mengekalkan metabolit dalam *N. oleracea*; manakala daun *N. oleracea* memiliki TPC yang lebih tinggi dan bioaktiviti lebih baik daripada batang. Analisis data multivariat (MVDA) membuktikan bahawa fenolik yang dikenalpasti, antaranya katekin, asid kafeik dan galik, dan terbitan apigenin, kuersetin, kaempferol dan miricetin bertanggungjawab terhadap diskriminasi dan bioaktiviti yang tinggi pada daun *N. oleracea* yang dikering dengan pengeringan sejuk beku. Selanjutnya, bahagian ketiga kajian ini adalah memberi tumpuan kepada kajian hubungan antara fenolik dan bioaktiviti yang diuji. Pengaruh kaedah pengekstrakan dan nisbah etanol juga dikaji. Analisa separa persegi (PLS) dan hutan rawak (RF) telah mengenalpasti asid kafeik dan terbitan apigenin, kuersetin dan kaempferol sebagai pemerangkap DPPH dan perencat α-glukosidase yang paling mempengaruhi dalam *N. oleracea*. Bagi pengekstrakan fenolik ini, sonication dengan etanol mutlak merupakan kaedah

pengekstrakan yang paling sesuai. Kesannya, ekstrak daun *N. oleracea* yang dihasilkan dari pengeringan sejuk beku dan sonication dengan etanol mutlak telah difraksionasi menggunakan pengekstrakan fasa pepejal (SPE) untuk mendapatkan fraksi heksans (HF), klorofom (CF), etil asetat (EF) dan metanol (MF). Di antara kesemua fraksi, EF dan MF mempamerkan bioaktiviti yang paling ketara, dan kemudian dijadikan subjek untuk analisis UPLC-MS/MS, yang membawa kepada pengenalpastian 37 metabolit, dengan kebanyakannya fenolik. Melalui pelbagai teknik kromatografi, 5 fenolik termasuk kuersetin-3-O-β-D-xilopiranosida (**28**), kuersetin-3-O-α-L-arabinopiranosida (**30**), kuersetin-3-O-α-L-rhamnosida (**32**), metilgallate (**11**) dan rutin (**22**) telah diasingkan dari kedua-dua fraksi ini. Kajian individu pada fenolik ini dalam bioaktiviti menunjukkan bahawa metilgallate mempamerkan aktiviti pemerangkapan radikal bebas DPPH dan perencatan α-glokosidase yang paling kuat dengan nilai IC_{50} masing-masing sebanyak 17.25 dan 50.76 µM. Kesimpulannya, kajian ini mencadangkan *N. oleracea* adalah sumber utama fenolik yang boleh berpotensi sebagai antioksidan dan perencat α-glokosidase bagi menangani masalah kencing manis. Kajian ini merupakan laporan pertama mengenai potensi antidiabetik *N. oleracea*, serta aplikasi pertama pendekatan metabolomik untuk analisis secara meluas kandungan fitokimia *N. oleracea*, dan korelasi dengan aktiviti pemerangkapan radikal bebas DPPH dan perencatan α-glokosidase tumbuhan ini. Hasil kajian ini meletakkan asas untuk penyelidikan masa depan mengenai aktiviti antidiabetik *N. oleracea*.

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I certify that a Thesis Examination Committee has met on 25 October 2017 to conduct the final examination of Lee Soo Yee on her thesis entitled "Metabolite Characterization of *Neptunia oleracea* Lour. and its Correlation with Antioxidant and Alpha-Glucosidase Inhibitory Activities using NMR-Based Metabolomics Approach" 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 fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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I hereby confirm that:

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This is to confirm that:

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AD	Air drying
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
API	Atmospheric ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CF	Chloroform fraction
CID	Collision-induced dissociation
d	Doublet
DAD	Diode array detector
dd	Doublet of doublet
DPPH	2,2-diphenyl-1-picrylhydrazyl free radicals
EF	Ethyl acetate fraction
ESI	Electrospray ionization
FD	Freeze drying
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
gCOSY	Gradient homonuclear correlation spectroscopy
gHMBCAD	Adiabatic gradient heteronuclear multiple bond coherence
gHSQCAD	Adiabatic gradient heteronuclear single quantum correlation
HF	Hexane fraction

HPLC	High performance liquid chromatography
HRAM	High resolution accurate mass
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
m	Multiplet
m/z	Mass-to-charge ratio
MF	Methanol fraction
MS	Mass spectrometry
MVDA	Multivariate data analysis
NMR	Nuclear magnetic resonance
OD	Oven drying
ORAC	Oxygen radical absorption capacity
PCA	Principle component analysis
PLS	Partial least squares
PLS-DA	Partial least squares–discriminant analysis
PNP	*p*-nitrophenol
PNPG	*p*-nitrophenyl-α-D-glucopyranose
ppm	Part per million
QTOF	quadrupole-time of flight
RF	Random forests
RNS	Reactive nitrogen species
s	Singlet
SPE	Solid phase extraction
t	Triplet

TIC	Total ion chromatogram
TLC	Thin layer chromatography
TMS	Tetramethyl silane
TOF	Time of flight
TPC	Total phenolic content
TPU	Agricultural Park
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry analysis
UPM	Universiti Putra Malaysia
UV	Ultraviolet
WHO	World Health Organization
δ	Chemical shift in ppm
1D	One-dimensional
^{1}H	Proton
2D	Two-dimensional
^{13}C	Carbon-13

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus is a metabolic complicated disease in which the patient experiences a high level of blood glucose. Over the past few decades, the occurrence of diabetes mellitus have increased drastically. Globally, the number of diabetic cases in adult population has increased from 108 million to 422 million from 1980 to 2014 (World Health Organization, 2016). Among the diabetic patient, majority are suffering from type 2 diabetes. In Malaysia, 20.8% of its adults are affected by type 2 diabetes (Hussein et al., 2015). Diabetes ultimately results in complications, such as cardiovascular disease, retinopathy, nephropathy and impaired wound healing. As a result, it is claimed to be a major factor contributing to blindness, kidney failure, heart attacks, stroke and amputation of lower limb.

Currently, there are many synthetic drugs available for the treatment of diabetes, such as acarbose, voglibose and miglitol. However, they meet few of the needs of diabetic patients and cause many undesirable side effects (Hung et al., 2012). On the other hand, accumulated evidence suggests that antioxidants and α-glucosidase inhibitors from natural sources may exert antidiabetic effects (Hung et al., 2012; Yao et al., 2010; Zhang et al., 2013). Hence, these natural compounds have drawn the attention of researchers worldwide. Phenolic compounds are one of the most important groups of secondary natural compounds. They have been reported to exhibit various biological effects, including antioxidant and carbohydrate hydrolyzing enzyme inhibition activities (Pukalskienė et al., 2015; Yao et al., 2010). Traditional medicinal plant is one of the most studied source of these compounds.

The tropical rainforest in Malaysia harbors precious medicinal plants. These plants has been traditionally used by the folks to enhance their health and as remedy to treat several ailments, including diabetes. The examples of some well-known Malaysian medicinal plant used traditionally for controlling diabetes are *Andrographis paniculata* (hempedu bumi), *Cosmos caudatus* (ulam raja), *Orthosiphon stamineus* (misai kucing), *Centella asiatica* (pegaga), *Ficus deltoidea* (mas cotek), *Piper sarmentosum* (sireh) and *Phyllanthus niruri* (dukung anak). Many studies had been performed to scientifically prove the antidiabetic efficacy of these plants and to identify the active principles (Mustaffa et al., 2011; Sekar et al., 2014). This shows that the traditional medicinal plants in Malaysia offers a great opportunity for seeking the antidiabetic components.

Neptunia oleracea is a floating aquatic plant widely distributed over tropical regions of the world. It is consumed as a vegetable, especially in Southeast Asia, and as a traditional medicinal plant. The leaf is traditionally used as antipyretic and antidote (Daduang et al.,

2011). The roots are used to treat necrosis of the nose and hard palate and as a remedy to cure fever whereas the juice from stem is squeezed into the ear to cure earache (Deb et al., 2013). Several biological activities had been reported for this plant, including antioxidant (Thalang et al., 2001), antiulcer (Bhoomannavar et al., 2011a), analgesic and anti-inflammatory (Paul et al., 2012), α-glucosidase inhibitory (Wongsa et al., 2012) as well as hepatoprotective (Bhoomannavar et al., 2011b) activities. Though the presence of flavonoids, carbohydrates, tannins and triterpenes had been reported in this plant (Vijayashree et al., 2006), the extensive metabolite profiling and correlation between metabolites with the claimed bioactivities have not yet been reported.

Metabolite composition can have significant variation at the different parts of the plant and may be altered by the post-harvest processing, such as drying and extraction. Generally, drying is applied to remove moisture from plants to retard the enzymatic degradation and hence extend their shelf life for storage. Air-, oven- and freeze drying are some of the common drying methods applied in medicinal plants research. There are various study reported regarding the effect of different drying methods on the plant metabolites (Borchani et al., 2011; Chan et al., 2009). Furthermore, extraction is a crucial step in the assessment of metabolites present in plant materials as extraction helps to release the valuable metabolites from the complex plant matrix. There is no single extraction procedure to simultaneously extract all classes of metabolites with great efficiency due to the complexity of the metabolites in their physical as well as chemical properties. Hence, depending on the plant samples, the effect of different drying as well as the extraction conditions should be evaluated and optimized.

Metabolomics is a competent approach for evaluating the metabolite variation in organism under certain conditions. The variation in the metabolite composition arise from different processing parameters can hence be measured by metabolomics approach, using advanced analytical methods coupled with multivariate statistical analysis. Nuclear magnetic resonance (NMR) spectroscopy is one of the most utilized analytical tools for applications in plant metabolomics. It provides information about the metabolites in plant. The metabolites identification is made possible based on the chemical shift and coupling constants provided by NMR spectroscopy (Kim et al., 2006). Multivariate data analysis (MVDA) such as principle component analysis (PCA) and partial least squares (PLS) are useful to manage the huge dataset generated from NMR analyses and to highlight the important phytochemical markers contributing to the studied bioactivities of the samples (Kim et al., 2010b). Recently, a machine learning method, random forests (RF) has drawn increased popularity in the application as MVDA tool in omics research as well.

Often at times, the metabolite profile of crude extracts are complicated due to the numerous amount of metabolites that could be present. Hence, fractionation of the crude extract by different polarity may facilitate the analysis and make identification of the important metabolites more effective (Grkovic et al., 2014). Fractionation can also enrich the important metabolites in certain fractions and these fractions can be subjected to different chromatographic techniques to isolate the metabolites. The isolated metabolites can be further characterized based on their physical properties and spectral

data obtained from advance spectroscopic method such as mass spectrometry (MS) and NMR to confirm their structures and identities. In addition, the isolated metabolites can be individually tested for the studied bioactivities to validate their contributions towards the bioactivities.

1.2 Problem statements

Negative effects arise from available synthetic drugs such as acarbose, voglibose and miglitol has drawn the attention of researchers to seek for antioxidants and α-glucosidase inhibitors from natural sources. The medicinal plants in Malaysia is a potential natural resources for this purpose. However, there are still a large number of these plants which have none or limited study regarding their antidiabetic potency. Little is known regarding the metabolite profile of *N. oleracea* and its relationship between the metabolites and antidiabetic-related bioactivities. There is also no information has been reported on the effects of different post-harvest processing on the metabolites in this plant. Besides, the metabolite profile of crude extract is often complicated owing to the large amount of metabolites present. This makes the analysis and identification of the important metabolites less effective.

1.3 Objectives

To address the aforementioned problems, the present study was designed and conducted, with the goal to seek for the phytochemical components to be used for the management of diabetes and its complications. To achieve these goals, several specific objectives were proposed. First part of the work aimed to screen the antioxidant and α-glucosidase inhibitory activities of 5 local traditional medicinal plants (section 4.1). The most active plant was chosen to evaluate the effect of different drying methods on the antioxidant and α-glucosidase inhibitory activities using ^{1}H NMR-based metabolomics approach (section 4.2). Following this, two regression models applied in MVDA were compared for evaluating relationship between phenolics and the bioactivities studied (section 4.3). The effect of different extraction conditions on the phenolics were also assessed (section 4.3). Subsequently, the plant extract obtained from the most suitable drying and extraction conditions were fractionated and profiling of the chemical constituents in the bioactive fractions were performed (section 4.4). Lastly, attempt was made to isolate the phenolics from the bioactive fractions, followed by individually assessment for their bioactivities and quantitative determination (section 4.5).

The five specific objectives are listed below:

1. To screen the leaves and stems of 5 selected traditional medicinal plants (*Mitragyna speciosa, Clinacanthus nutans, Strobilanthes crispus, Neptunia oleracea* and *Mentha asiatica*) for antioxidant and α-glucosidase inhibitory activities.

(From screening results, *N. oleracea* was identified as having the highest activity and was thus selected for further study.)

2. To evaluate the effect of drying methods on the phytochemical constituents of different parts of *N. oleracea* and the correlation with antioxidant and α-glucosidase inhibitory activities using ^{1}H NMR-based metabolomics.

3. To compare partial least squares (PLS) and random forests (RF) for evaluating relationship between phenolics and bioactivities of *N. oleracea* and to evaluate the effects of different extraction methods and ethanol ratios on the phenolics in *N. oleracea*.

4. To differentiate the fractions of *N. oleracea* leaves extract and correlate with antioxidant and α-glucosidase inhibitory activities using ^{1}H NMR-based metabolomics, as well as to profile the bioactive fractions using UPLC–MS/MS.

5. To isolate and characterize the phenolics in the bioactive fractions of *N. oleracea* and to quantitate their contents in their respective fractions using HPLC.

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