UNIVERSITI PUTRA MALAYSIA

ANTIOXIDANT, α-GLUCOSIDASE AND NITRIC OXIDE INHIBITORY ACTIVITIES OF Phyllanthus acidus L. LEAVES IN CORRELATION WITH THEIR METABOLITES USING ^{1}H-NMR- AND LCMS-BASED METABOLOMICS

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By

SITI ZULAIKHA BINTI ABD GHAFAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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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 Master of Science

ANTIOXIDANT, α-GLUCOSIDASE AND NITRIC OXIDE INHIBITORY ACTIVITIES OF *Phyllanthus acidus* L. LEAVES IN CORRELATION WITH THEIR METABOLITES USING ^{1}H-NMR- AND LCMS-BASED METABOLOMICS

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April 2019

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

Phyllanthus acidus L. also known as star gooseberry, is a well-known medicinal plant used as a traditional remedy. Different parts of this plant have been used to manage several disorders associated with oxidative stress diseases, including inflammation. Solvent polarity has a significant influence on the extraction of metabolites and need to be optimized. In this study, the total phenolic (TPC), antioxidant (DPPH and nitric oxide scavenging), α-glucosidase and nitric oxide (NO) inhibitory activities from two parts (leaf and fruit) of *P. acidus* extracted with various ethanol ratios (0, 50 and 100%) were evaluated. Furthermore, proton nuclear magnetic resonance (^{1}H-NMR)- and liquid chromatography mass spectrometry (LCMS)-based metabolomics approaches were applied to identify the metabolites in the active parts of *P. acidus* extracted with various ethanol ratios. To support the identification of compounds, correlation analysis was performed with the ^{1}H-NMR- and LCMS data. The results showed that leaf extraction with 50% ethanol gave the most active extract with the lowest IC_{50} value for α-glucosidase with 1.53 µg/mL. It also showed moderate NO scavenging and inhibitory activities (IC_{50} = 158.17 and 180.06 µg/mL, respectively) as well as the highest TPC with 6.92 mg GAE/g dried sample. The 50% ethanol extract from the fruit had the highest TPC, DPPH free radical scavenging, NO scavenging, and α-glucosidase and NO inhibitory activities with values of 3.20 mg GAE/g dried sample, 48.41%, 49.39%, 2.44 µg/mL and 43.30%, respectively. Both metabolomics approaches showed different analytical selectivities and sensitivities. In total, 27 metabolites were tentatively identified based on the ^{1}H-NMR characteristics including phenolics, flavonoids, sugars, amino acids and organic acids compound groups. Using LCMS, 39 metabolites were detected and identified, which include derivatives of quercetin, kaempferol, epicatechin, coumaric, and

cinnamic acids. Based on multivariate analysis, partial component analysis (PCA) of both the ^{1}H-NMR- and LCMS databases revealed clear separation between the *P. acidus* extracts. The partial least square analysis (PLS) biplot based on the ^{1}H-NMR data showed that the metabolites that contributed to α-glucosidase and nitric oxide (NO) inhibitory activities were kaempferol, quercetin, myricetin, phyllanthusol A, phyllanthusol B, chlorogenic, cinnamic and ellagic acids, while according to the PLS biplot for the LCMS data, the metabolites that correlated to bioactivities were the derivatives of kaempferol, quercetin, catechin, and coumaric, caffeic, quinic, citric, ellagic and malic acids. Additionally, the ^{1}H-NMR and LCMS correlations allowed the tentative identification of caffeoyl glucarate, *p*-coumaroyl glucarate, mucic acid, epigallocatechin, catechin-3'-methyl ether, kaempferol-3-glucoside-7-rhamnoside, epicatechin, phyllanthusiin E, quercetin-3-*O*-rhamnoside, kaempferol-3-rhamnoside-4'-xyloside and peonidin-3-glucoside as metabolite signals with highly positive correlation. In conclusion, the 50% ethanol extract from the *P. acidus* leaves showed antioxidant activity, a high TPC, and substantial inhibition of α-glucosidase and NO, which strengthened its traditional claim. The present study shows that the combination of ^{1}H-NMR- and LCMS-based metabolomics would be the best strategy for improving the discovery and identification of metabolites from *P. acidus* extracts and established the extract that possesses the best antioxidant, anti-diabetic, and anti-inflammatory properties.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANTIOKSIDAN, AKTIVITI PERENCATAN α-GLUKOSIDASE DAN NITRIK OKSIDA DAUN *Phyllanthus acidus* L. DALAM PERKAITAN DENGAN METABOLIT MENGGUNAKAN METABOLOMIK BERASASKAN ^{1}H-NMR DAN LCMS

Oleh

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Pengerusi : Profesor Madya Faridah Abas, PhD
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Phyllanthus acidus L. juga dikenali sebagai cermai, merupakan tumbuhan ubatan yang terkenal dalam kegunaan ramuan tradisional. Bahagian yang berbeza pada tumbuhan ini telah digunakan untuk menangani beberapa permasalahan yang berkaitan dengan penyakit tekanan oksidatif, termasuk radangan. Polariti pelarut mempunyai pengaruh penting terhadap pengekstrakan metabolit dan perlu dioptimumkan. Dalam kajian ini, jumlah kandungan fenolik (TPC), antioksidan (pemerangkapan DPPH dan nitrik oksida), aktiviti perencatan α-glukosidase dan nitrik oksida (NO) dari dua bahagian (daun dan buah) *P. acidus* yang diekstrak dengan pelbagai nisbah etanol (0, 50 dan 100%) telah dikaji. Tambahan pula, metabolomik berasaskan resonans magnetik nuklear proton (^{1}H-NMR) dan kromatografi cecair spektroskopi jisim (LCMS) digunakan untuk mengenalpasti kandungan metabolit dalam bahagian aktif *P. acidus* yang diekstrak dengan pelbagai nisbah etanol. Untuk menyokong pengenalpastian sebatian, analisis korelasi dilakukan pada data ^{1}H-NMR dan LCMS. Keputusan menunjukkan bahawa pengekstrakan daun dengan etanol 50% memberikan ekstrak paling aktif dengan nilai IC_{50} paling rendah untuk α-glukosidase pada nilai 1.53 µg/mL. Ia juga menunjukkan aktiviti yang sederhana bagi pemerangkapan dan perencatan NO (IC_{50} = 158.17 dan 180.06 µg/mL, masing-masing) serta TPC tertinggi dengan nilai 6.92 mg sampel kering GAE/g. Ekstrak buah pada etanol 50% mempunyai TPC, pemerangkapan radikal bebas DPPH, pemerangkapan NO, dan aktivit perencatan α-glukosidase dan NO yang tertinggi dengan nilai 3.20 mg sample kering GAE/g, 48.41%, 49.39%, 2.44 µg/mL dan 43.30%, masing-masing. Kedua-dua kaedah metabolomik menunjukkan perbezaan analisis dalam pemilihan dan sensitiviti. Secara keseluruhannya, 27 metabolit telah dikenal pasti berdasarkan ciri-ciri ^{1}H-NMR termasuk fenolik, flavonoid, gula,

asid amino dan kumpulan sebatian asid organik. Melalui LCMS, 39 metabolit dikesan dan dikenalpasti, termasuk derivatif kuersetin, kaempferol, epikatekin, koumarik, dan asid sinamik. Berdasarkan analisis multivariat, analisis komponen utama (PCA) dari kedua-dua data ^{1}H-NMR dan LCMS menunjukkan pemisahan yang jelas di antara ekstrak-ekstrak *P. acidus*. Berdasarkan biplot analisis kuasa dua terkecil separa (PLS) pada data ^{1}H-NMR menunjukkan metabolit yang menyumbang kepada aktiviti perencatan α-glucosidase dan NO adalah kaempferol, kuersetin, myricetin, phyllanthusol A, phyllanthusol B, asid klorogenik, sinamik dan elagik, manakala menurut PLS biplot untuk data LCMS, metabolit yang dikaitkan dengan bioaktiviti adalah derivatif kaempferol, kuersetin, katekin, dan asid koumarik, kafeik, kuinik, sitrik, elagik dan malik. Selain itu, korelasi ^{1}H-NMR dan LCMS, membenarkan pengenalpastian tentatif pada glucarate kaffeoyl, *p*-coumaroyl glucarate, asid mucic, epigallokatekin, katekin-3'-metil eter, kaempferol-3-glukosida-7-rhamnosida, epikatekin, phyllanthusiin E, kuersetin-3-*O*-ramnosida, kaempferol-3-ramnosida-4'-xyloside dan peonidin-3-glukosida sebagai isyarat metabolit dengan korelasi yang sangat positif. Sebagai kesimpulan, ekstrak etanol 50% pada daun *P. acidus* menunjukkan aktiviti antioksidan, TPC yang tinggi, dan perencatan kuat dalam α-glukosidase dan NO, dimana mengukuhkan dakwaan traditionalnya. Kajian ini menunjukkan bahawa gabungan metabolomik berasaskan ^{1}H-NMR dan LCMS boleh menjadi strategi terbaik untuk meningkatkan penemuan dan pengenalan metabolit dari ekstrak *P. acidus* dan menghasilkan ekstrak yang mempunyai sifat antioksida, anti-diabetes, dan anti-radang.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. 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;
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

^{1}H-NMR	Proton nuclear magnetic resonance
2D	Two-Dimensional
ABTS	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
AChE	Acetylcholinestrase
BChE	Butyrylcholinestrase
CD_3OD	Deuterated methanol
CO_2	Carbon dioxide
D	Doublet
Dd	Doublet of doublet
dH_2O	Deionized water
DMSO	Dimethylsulfoxide
DPPH	2,2-Diphenyl-1-Dipicrylhydrazyl
ESI	Electrospray ionization
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
HMDB	Human metabolome database
Hz	Hertz
IC_{50}	Inhibition concentration at 50 percent
IFN-γ	Interferon-γ
J	Coupling constant
LCMS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharides

M	Multiplet
m/z	Mass to charge
MS	Mass spectrometry
MVDA	Multivariate data analysis
NO	Nitric oxide
NOS	Nitric oxide synthase
P. acidus	*Phyllanthus acidus*
PCA	Principal component analysis
PLS	Partial least squares
PNPG	ρ-nitrophenyl-α-D-glucopyranosidase
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
S	Singlet
SIMCA	Soft independent modeling of class analogy
TIC	Total ion chromatogram
TPC	Total phenolic content
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
UV/Vis	Ultraviolet–visible
VIP	Variable importance in the projection

CHAPTER 1

INTRODUCTION

1.1 Background

The importance of medicinal plants is being highlighted as a source of natural antioxidant and functional foods. In a review of plants, most of the plant extracts have a potential source of bioactive compounds as an alternative way to replace synthetic antioxidant (Norziah et al., 2015). Different parts of plant differ markedly in the quantity and types of phenolic antioxidants and their derivatives (Wang et al., 2009). Phenolic compounds in plants also possess strong antioxidant and may prevent the cells from oxidative damage caused by free radicals (Liu et al., 2008). These free radicals play a major role in some pathogenesis of serious diseases, such as neurodegenerative disorders, liver cirrhosis, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). Besides, the natural compounds that can scavenge the free radicals, have a great potential in curing or preventing these diseases (Ara et al., 2003). They showed potent antioxidant activities due to the existence of noble bioactive compounds.

Phyllanthus species have become the main focus in most researches due to its therapeutic potential properties that can help in curing many oxidation related diseases. They were traditionally used for treating cough, jaundice, gonorrhoea, dysentery, diabetes, skin ulcers, headache, stomach-ache, eye wash, sore throat, and dressing of wounds (Eldeen et al., 2011). The isolated compounds and extracts of *Phyllanthus* species revealed several pharmacological activities, such as anti-viral, anti-malarial, anti-bacterial, anti-plasmodial, anti-microbial, anti-cancer, anti-diabetic, hypolipidemic, antioxidant, hepatoprotective, and nephroprotective (Chopade & Sayyad, 2014). Among the available and endemic genus of *Phyllanthus* found in Malaysia, India and South America is *Phyllanthus acidus* L.

P. acidus L. has several common names, including star gooseberry, “Otaheiti” gooseberry or “Mayom” (Thailand) and in Malaysia, it is known locally as “Chermai”, kemangul or chermala (Leeya et al., 2010). The fruits are usually pickled or processed into juice, jam or jelly (Quijano et al., 2007). It has long been used in numerous medical treatments. Traditionally, *P. acidus* is used in the management of several disorders associated with pain, inflammation and oxidative stress, including rheumatism, bronchitis, asthma, respiratory disorder, hepatic disease, diabetes and gonorrhoea (Devi & Paul, 2011; Banik et al., 2010). In addition, it is identified as one of the anti-diabetes plants of Southern Assam that used in treatment and management of diabetes (Banik et al., 2010). In Thai, this plant is known in folk medicine, where every part of it can be used to treat several diseases. The leaves are mostly used for the treatment of

hypertension, liver disease and itchy skin (Chongsa et al., 2015; Jain et al., 2011; Jain & Singhai, 2011). The fruits are used as a liver tonic and blood purifier, as well as for the management of several pathological conditions, such as bronchitis, constipation, vomiting and diabetes (Jain et al., 2011; Jain & Singhai, 2011; Banik et al., 2010).

Besides, the different parts of plants have an immense source of various bioactive compounds with different molecular structure and function, where the compounds are primarily derived from the secondary metabolism of plants as a function to protect the plants against predation such as microorganisms, insects and herbivorous (Jeyaseelan et al., 2012). Different parts of plants might have different levels of chemical compounds with varied bioactivities (Ismail et al., 2012; Abu et al., 2009). The review of Chakraborty et al. (2012) stated the different parts of *P. acidus* exhibited various bioactivities. In facts, the chemical composition and antioxidant of every plant were depending on different parts of plant extracts (Marzoug et al., 2011).

Prior to testing the bioactivities of plant materials, the drying and extraction process should be conducted. The drying and extraction processes of the fruits and leaves prior to any analysis are crucial as they may affect the bioactivity and benefit of the plant. Thus, the selection of the valuable drying method and the suitable solvent polarity are important. There are several drying methods can be carried out such as ambient air-drying, oven-drying, freeze-drying and trap-drying (Harbourne et al., 2009). However, oven-drying with low temperature was usually preferred due to its easy handling and low cost (Kamiloglu et al., 2016; Soysal & Öztekin, 2001). It is one of the proper methods to preserve phytochemical contents with appropriate temperature and time of drying (Mediani et al., 2013). This method is one of the most widely used in term of crops for further analysis (Binte et al., 2014; Eldeen et al., 2011). Furthermore, previous researches have established that the different polarity solvent extraction might influence the solubility of chemical constituents and other antioxidant compounds in the plant (Mediani et al., 2015; Abdul-Hamid et al., 2015). Therefore, there is a need for optimization of these processing parameters using the appropriate tools in order to save the quality and efficiency of the plant.

Metabolomics as an important field of “omics”, has been defined to be the noble approach for detecting all metabolites in an organism with high accuracy in terms of quality and quantity (Kusano et al., 2007; Verpoorte et al., 2007). In order to achieve this, a variety of analytical techniques can be used in metabolomics applications. The most commonly used techniques are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), such as gas chromatography-mass spectrometry (GC-MS) and/or liquid chromatography mass spectrometry (LCMS) (Nagana & Raftery, 2015; Kusano et al., 2007). The NMR and MS have ability to analyse hundreds of metabolites in a single measurement (Nagana & Raftery, 2015).

The most analytical tool that has been used in metabolomics study is proton NMR (^{1}H-NMR), due to its benefit in detecting and quantifying a wide range of metabolites with a single measurement, simple preparation, potent robustness, and high-throughput analysis (Nagana & Raftery, 2015; Maulidiani et al., 2014). However, the limitation of NMR spectroscopy is its less sensitivity compared to MS (Maulidiani et al., 2013; Kim et al., 2011). MS is more advantageous in targeted metabolomics and has shown increased sensitivity and selectivity through combination with chromatographic separation (Bedair & Sumner, 2008). In recent years, LCMS has grown in popularity for metabolomics studies and has been successful application in the identification and quantification of metabolites due to its high throughput, soft ionization and good coverage of metabolites (Zhou et al., 2012).

LCMS metabolomics is one of the abundant choices, which have the ability to separate small molecules that differ in physical and chemical properties of size, polarity and charges through positive or negative ionization mode (Lu et al., 2008). However, LCMS limitations includes the need to select a chromatographic column for a good metabolite separation, the ionization capacity of the analytes and limited molecular elucidation, such as the resolution of isomers (Moco et al., 2008). The combination of high-throughput NMR and LCMS analyses can provide information about the chemical composition and offer a more comprehensive metabolic profiling as well as useful insights regarding the identification of chemical biomarkers (Farag et al., 2012; Moco et al., 2008).

The huge dataset was obtained from the analytical techniques, the need for proper data processing and advanced statistical analysis is crucial. Therefore, the multivariate data analysis (MVDA) is one of the statistical approaches that can handle the huge dataset of the metabolomics analysis (Kim & Verpoorte, 2010). Principal component analysis (PCA) and partial least squares (PLS) are the most widely used tests in MVDA for the cluster recognition and correlation analyses (Kim & Verpoorte, 2010; Fumagalli et al., 2009).

1.2 Problem statements

Most of *Phyllanthus* genus are well studied. However, *P. acidus* has been claimed to possess several medicinal properties and limited studies were carried out, especially on its phytochemical profile and biological activity. To provide better clarification and scientific prove, *P. acidus* has been selected in this study. Besides, in traditional claims about *Phyllanthus* species, several vegetative parts are used. However, the selection of the valuable part that has the potent medicinal value is unrevealed. The leaves and fruits of *P. acidus* are compared to find the better site of bioactive compounds responsible for the bioactivities tested.

The different factors, including the processing parameters, might affect the biological samples. The solvent extraction is one of processing parameter used for the preparation of plant extracts, but the efficiency of the extraction depends on the polarity of the analyte in the sample matrix and the polarity of solvents. The metabolomics can be applied to resolve these issues. Both NMR- and LCMS-metabolomics approaches are complementarily used to optimize the processing conditions and analyse their variations. All these parameters ought to be optimised in order to maximize its valuable medicinal benefits and ensuring its quality.

1.3 Significance of study

The increasing public demand for natural medicines has resulted in increased commercial activity and production of these medicines. This has led to a growing concern in ensuring the standardization of medicinal plants and herbal drugs preparation. *Phyllanthus* genus has significant medicinal properties used in the treatment of several diseases. One of the available and edible *Phyllanthus* genus in Malaysia is *P. acidus* that every part of the plant has several uses in traditional medicine. This study evaluated the antioxidant, anti-diabetic by α-glucosidase inhibition and anti-inflammatory by nitric oxide inhibition of *P. acidus* extracts. The results can be used in further supporting and evidencing the traditional claims of the plant.

Besides, this study has been analysed the efficiency of extraction by applied in metabolomics approaches. The metabolomics can be conducted in the profiling and identifying small metabolites as many as possible in the extracts. Therefore, this study is a fast and efficient analytical used to find preferable method processing. Thus, this can be provided the guidance in the development of medicinal preparation, nutraceuticals, and functional food as the alternatives way for food consumption toward all generation.

1.4 Objectives

In this study, *P. acidus* leaves and fruits extracts were assessed for antioxidant, total phenolic content, α-glucosidase and nitric oxide inhibitory activities to find credence of traditional claims of *P. acidus* for oxidative stress related disorders. The identification of the *P. acidus* metabolites that contribute significantly to bioactivities was also performed using NMR- and LCMS-based metabolomics approaches. Therefore, the objectives of this study are:

1. To evaluate the antioxidant, total phenolic content, α-glucosidase and nitric oxide inhibitory activities of different parts (leaf and fruit) of *P. acidus* extracted with different ethanol ratios.

2. To identify phytochemical constituents of *P. acidus* extracts and correlate them with biological activities using ^{1}H-NMR-based metabolomics.
3. To correlate the biological activities of *P. acidus* extracts with the identified phytochemical constituents using LCMS-based metabolomics.
4. To validate the phytochemical that contribute to biological activities by using ^{1}H-NMR and LCMS correlation analyses.

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