UNIVERSITI PUTRA MALAYSIA

ANTIDIABETIC EFFECTS OF Melicope lunu-ankenda (GAERTN.) T.G. HARTLEY ON OBESE STZ-INDUCED DIABETIC RATS USING NMR-BASED METABOLOMICS

MIZHER HEZAM BAROOR AL-ZUAIDY

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By

MIZHER HEZAM BAROOR AL-ZUAIDY

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

December 2016
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DEDICATION

This thesis is dedicated to my parents, family, brothers, sisters and friends.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia, in fulfilment of the requirement for the degree of Doctor of Philosophy

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MIZHER HEZAM BAROOR AL-ZUAIDY

December 2016

Chairman : Prof. Azizah Abdul Hamid, PhD
Faculty : Food Science and Technology

In the present study, antioxidant and antidiabetic activities of different *Melicope Lunu-ankenda* (ML) ethanolic extracts were evaluated using *in vitro* and *in vivo* models. Proton nuclear magnetic resonance (*^1^H NMR*) and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) were used to profile the bioactive metabolites in ML leaf extracts. Sixty percent ethanolic ML extract showed the highest inhibitory effect against α-glucosidase, DPPH scavenging activity and ferric reducing antioxidant power. Results based on cell line investigations showed that the leaf extract stimulated the glucose uptake by both 3T3-L1 and HepG2 cells. A discriminatory study on the metabolites responsible for the variation between different ethanolic ML extracts was successfully performed using *^1^H*-NMR-based metabolomics. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) scores revealed clear and distinct separations by PC1 and PC2 with an eigenvalue of 69.9%. The main bioactive compounds found responsible for the separation were isorhamnetin, skimmianine, scopoletin and melicarpinone. The antidiabetic effect was also carried out *in vivo* using rat models. The extract exerted its effect by decreasing the blood glucose level, insulin resistance, and increasing insulin sensitivity. The treatment of obese diabetic rats with ML extract also resulted in significant decrease in TG, TC, and LDL levels. However, HDL levels were significantly increased. The impact of treatment was also observed in terms of regulation of the renal injury markers and activities of liver enzymes.

In addition, NMR-based metabolomics and multivariate data analysis showed clear metabolic differences in the serum and urine samples of healthy, diabetic and treated diabetic Sprague-dawley rats. The metabolomics results demonstrated that the observed metabolic changes were linked with diabetes progression, and metabolic biomarkers were reflected by the perturbed metabolites, hence providing clear understanding regarding the underlying mechanism involved in generation and progression of diabetes. This study presented potent antidiabetic activity of ML and
describes its mechanism of action. The NMR based metabolomics approach is supportive for the additional understanding of diabetes-related mechanisms and enhances the metabolic pathways affected in the diabetic rats. These results of the present study may further contribute towards understanding of the underlying molecular mechanism of this medicinal remedy.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN ANTIDIABETIK Melicope lunu-ankenda DI DALAM TIKUS DIABETIK TERARUH STZ OBES MENGUNAKAN METABOLOMIK BERASASKAN NMR

Oleh

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Di samping itu, metabolomik berasaskan-NMR dan analisis data pelbagai pembolehubah menunjukkan perbezaan metabolik yang jelas di dalam sampel-sampel serum dan air kencing tikus Sprague-Dawley sihat, berdiabetes, dan berdiabetes yang dirawat. Keputusan metabolomik menunjukkan bahawa perubahan-perubahan metabolisme yang diperhatikan adalah berkaitan dengan perkembangan
diabetes, dan penanda-penanda biologi metabolisme digambarkan oleh metabolit-metabolit yang terganggu, justeru itu memberikan kefahaman yang jelas mengenai mekanisme asas yang terlibat dalam penjanaan dan perkembangan diabetes. Kajian ini telah membentangkan aktiviti antidiabetik ML yang kuat dan menerangkan mekanisme tindakannya. Pendekatan metabolomik berasaskan NMR menyokong pemahaman tambahan terhadap mekanisme-mekanisme yang berkaitan dengan diabetes dan meningkatkan laluan metabolik yang terlibat di dalam tikus berdiabetes. Hasil-hasil kajian ini mungkin boleh selanjutnya menyumbang ke arah pemahaman mengenai mekanisme di paras molekul bagi remedi mengubat ini.
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I will like to express my profound and heartfelt gratitude to my wife, daughters, relatives, friends and all other well-wishers for their interest and immense contributions in prayers, guidance and moral support towards my success.
I certify that a Thesis Examination Committee has met on 19 December 2016 to conduct the final examination of Mizher Hezam Baroor Al-Zaaidy on his thesis entitled "Antidiabetic Effects of Melicope lunu-ankenda (Gaertn.) T.G. Hartley on Obese STZ-Induced Diabetic Rats 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.

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A4  Obese diabetic rat treated with high dose of ML extract at week8 of treatment

A5  Obese diabetic rat treated with low dose of ML extract at week8 of treatment
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>$^1$H NMR</td>
<td>Proton Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>$d$</td>
<td>Doublet</td>
</tr>
<tr>
<td>DPPH</td>
<td>Diphenyl picrylhydrazyl</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GAE</td>
<td>Gallic Acid Equivalent</td>
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<tr>
<td>gCOSY</td>
<td>Gradient Correlation Spectroscopy</td>
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<tr>
<td>gHMBC</td>
<td>Gradient Heteronuclear Multiple Bond Correlation</td>
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<tr>
<td>gHSQC</td>
<td>Gradient Heteronuclear Single-Quantum Coherence</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Inhibition Concentration at 50 percent</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography–Mass Spectrometry</td>
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<tr>
<td>m</td>
<td>Multiplet</td>
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<tr>
<td>m/z</td>
<td>Mass per Charge</td>
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<tr>
<td>MHz</td>
<td>Mega Hertz</td>
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<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MVDA</td>
<td>multivariate data analysis</td>
</tr>
<tr>
<td>°C</td>
<td>Degree in Celsius</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal Partial Least Squares–Discriminant Analysis</td>
</tr>
<tr>
<td>PC</td>
<td>Principal Component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
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<tr>
<td>PLS</td>
<td>Partial Least Squares</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>Partial Least Squares–Discriminant Analysis</td>
</tr>
<tr>
<td>ppm</td>
<td>Part Per Million</td>
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<tr>
<td>QTOF</td>
<td>Quadrupole–Time of Flight mass spectrometer</td>
</tr>
</tbody>
</table>
ROS  Reactive Oxygen Species
$s$  Singlet
SIMCA  Soft Independent Modelling of Class Analogy
TPC  Total Phenolic Contents
UV  Ultraviolet
UV/VIS  Ultraviolet/visible
VIP  variable importance in the projection
$\delta$  Chemical Shift in ppm
$\mu g$  Microgram
$\mu L$  Microliter
$^{13}C$  Carbon-13
CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes is a metabolic disorder usually characterized by hyperglycemia due to defects in insulin action, insulin secretion or both. Most of diabetes cases fall into two main etiopathogenetic categories. Type 1 diabetes mellitus (T1DM) results due to absolute deficiency of insulin secretion. The other category is type 2 diabetes mellitus (T2DM) caused by combination of resistance to insulin action and an insufficient compensatory insulin secretory response. Type 2 diabetes mellitus accounts for 90–95% of all diabetes cases (Inzucchi et al. 2010). Currently, around 387 million people are living with this disease all over the world, and the number is expected to increase up to 592 million by the year 2035 (Guariguata et al. 2014).

T2DM is a complex type of diabetes and may results in multiple complications including micro-vascular complications, retinopathy, nephropathy and neuropathy etc., (Fowler 2011).

Imbalance between antioxidant defences and reactive oxygen species (ROS) production causes oxidative stress, which can increase from rising generation and/or decreased elimination of ROS by antioxidant. Consequently, excess production of ROS and impairment of the antioxidant defence system leads to diabetes mellitus. In this regard, any substance that delays or inhibits or removes the oxidative stress is defined as antioxidant (Halliwell 2011). Some prospective studies support the assumption that the progress of type 2 diabetes may be reduced by the consumption of antioxidant-rich diets (Porter 2012).

The existing pharmacological treatment options based on sulfonylureas, thiazolidinedione, and metformin, do not improve adequately the underlying consequences of insulin resistance such as hyperglycemia, pancreatic β-cell damage and diabetic dyslipidaemia (Goldberg, Holman, and Drucker 2008). Although currently a number of effective Western medications are available for the treatment of T2DM, management of T2DM at lower cost with fewer side effects still remain a big challenge. These Western medicines although effective, but can have severe side effects including weight gain, increased risk of cardiovascular events and bone loss (Fowler 2011). These side effects could become more severe due to the continuous use of these synthetic drugs. Comparatively, herbal medications can be considered as good alternative with fewer side effects and low cost. Herbal medications can treat diabetes by number of curatives such as stimulation of insulin secretion, enhancement of insulin sensitivity, and/or reduction of carbohydrate absorption (Li et al. 2004, Prabhakar and Doble 2011). Unlike Western medicine, herbal medicines may contain numerous active ingredients targeting multiple mechanisms and therefore herbal medicine can be considered as potential candidate for the treatment
Recent studies established positive link between the plants and the decrease of chronic diseases, like diabetes mellitus (Bansal et al. 2012, Lee et al. 2014). Around 800 medicinal plants have been evaluated for their anti-diabetic potential for treatment or/and prevention of T2DM. (Prabhakar and Doble 2011). The natural product research is growing positively over the years and with improved “omics” technologies provides a platform to link traditional medicine and molecular pharmacology (Solanky et al. 2003, Wang, Lamers, et al. 2005, Yuliana et al. 2011). Out of all available “omics” technology, metabolomics is the latest which is recognized to be highly beneficial for the qualitative and quantitative characterization of all metabolites present in a cell, tissue, or/and organism under specific conditions (Colquhoun 2007). This emerging research field combines analytical chemistry, biochemistry and chemometrics and is highly emphatic for the analysis of thousands of small metabolites in any biological system. Mass spectrometry (MS) hyphenated with other analytical tools such as gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR) spectroscopy and/or capillary electrophoresis (CE) are being used (Roessner and Beckles 2009). Comparatively for the monitoring of many endogenous “low-molecular-weight” metabolites LC/MS, NMR, and GC/M are the leading analytical plate forms (Bjerrum et al. 2009).

Use of animal model is highly imperative for the better understanding of metabolic disorders. Simulation of diabetic conditions based on animal model has been carried out by numerous researchers worldwide to explore the underlying molecular aspects and complications of this disease (Beckonert et al. 2007a, Tian et al. 2013).

Recently, metabolomics has been successfully applied by the researchers for the diagnosis and evaluation of the therapeutic effects linked with diabetes mellitus (DM) and its complications (Wu et al. 2014, Liu, Wang, et al. 2015). Numerous studies have been conducted previously with NMR-based metabolomics for the effective characterization of metabolites in serum, urine, or kidney tissue samples using diabetic rat models (Zhao et al. 2010, Tian et al. 2013, Emwas et al. 2015). So, metabolomics exhibit potential to identify overall alteration in metabolite levels during the treatments of diabetic cases.

Malaysia with its extensive flora presents an untapped capacity for the natural product research. *Melicope lunu-ankenda* (ML) is one of the *Melicope* species belonging to family Rutaceae and found all around the world especially in tropical
Asia and Australia. In Malaysia twenty-four Melicope species have been identified (Kassim et al. 2013). Its leaves are popular as salad and condiment for food flavoring. The leaves and flower are also traditionally being consumed to manage hypertension, menstrual disorder and fever etc. (Ramli et al. 2004, Tan, Yin, and Chan 2012). Presence of secondary metabolites including; alkaloids, flavonoids, acetophenones and coumarins in several Melicope species further ascertain its emphatic health benefits (Fauvel et al. 1981, Parsons et al. 1994). However, there are no reported on antidiabetic effects of this plant in high fat diet (HFD) rats and induced into diabetic condition with a low-dose of streptozotocin (STZ).

1.2 Problem Statement

Present study is therefore aimed at evaluating the Melicope lunu-ankenda extract for its functionalities i.e., antioxidant and antidiabetic potential both in vitro and in vivo. It is also identify the main bioactive compounds (in plant) and detects the biomarkers (animal bio-fluids) relating to antidiabetic effect of standardized ML extract on obese diabetic rats and subsequently to establish its efficacy as potent natural antidiabetic agent that may be used as functional foods or as natural alternative or in combinations to the conventional drugs for the treatment of diabetes mellitus.

1.3 Hypothesis

This study hypothesizes that animal model of HFD induced diabetes may be linked with many perturbations in serum biochemistry and metabolic pathways. The leaf extract of ML may show therapeutic efficacy in vivo study. It is expected that a $^1$H NMR metabolomics method combined with suitable multivariate data analysis may be a good method to study these perturbations and the therapeutic effect of ML leaf extract.

1.4 Objectives of the study

1. To evaluate antidiabetic and antioxidant property of ML leaf extract.
2. To profile the bioactive compounds in ML leaf extract.
3. To evaluate the anti-diabetic activity of ML extract in obese diabetic Sprague-Dawley.
4. To determine the metabolic perturbations of obese diabetic Sprague-Dawley and the therapeutic effects of ML leaf extract.
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