



UNIVERSITI PUTRA MALAYSIA

***METABOLOMICS STUDY ON THE EFFECTS OF *Orthosiphon stamineus*
Benth. ON STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL***

AMALINA BINTI AHMAD AZAM

IB 2015 1



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By

AMALINA BINTI AHMAD AZAM

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

June 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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AMALINA BINTI AHMAD AZAM

June 2015

Chairman: Intan Safinar Ismail, PhD
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The main purpose of this research is to study the anti-diabetic effects of *Orthosiphon stamineus* Benth. Extract (OSE) in different solvent polarity (ethanol, ethanol: water, methanol, methanol: water and water) in streptozotocin (STZ)-induced diabetic rats by using metabolomics approach. The rat model of diabetic-induced by streptozotocin (STZ: 60 mg Kg⁻¹) from which urine and serum used as the media to detect pathological changes during the development and curing of diabetes mellitus. The second objective is to develop a validated model between the chemical profile of extracts and anti-diabetic activity of induced STZ-diabetic rats that treated orally with OSEs within 14 days and Glibenclamide used as reference drug. Proton Nuclear Magnetic Resonance (¹H NMR) spectra of the extracts, urine and serum were obtained and analyzed by multivariate data analysis (MVDA). Partial least square discriminant analysis (PLS-DA) of ¹H NMR spectra disclosed metabolic pattern differences among groups where the related metabolic changes in urine and serum identified. OSE of water showed significantly (P < 0.05) reduction in urine glucose by 31.4% through PLS-DA of ¹H NMR with changes nearing to the control group or similar to glibenclamide that reduced by 48.6%. The novelty of metabolomics approaches is their ability to give a broad overview of metabolites from complex biological samples. Interestingly, the spectroscopic data of urine STZ-diabetic rats revealed that glucose, allantoin, creatine, creatinine, and glutamate were near to normal group ranges after treatment. Furthermore, to propose the metabolism pathway affected by the OS treatment in diabetic rats, MetScape 3.0 software was used. Consequently, several metabolic pathways like carbohydrate, lipid, and amino acid metabolism have been affected during the treatment period. This study suggests that OSE of water may be beneficial in treating the hyperglycemic condition as it acts in a better way compared to glibenclamide drug. The significantly higher intensity of purine metabolites (allantoin) detected in OSE water indicates an increased in glomerular filtration rate that prove the ability in helping kidney function.

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

KAJIAN METABOLOMIK MENGENAI KESAN *Orthosiphon stamineus* Benth. PADA TIKUS ARUHAN STREPTOZOTOCIN-DIABETIS MODEL

Oleh

AMALINA BINTI AHMAD AZAM

Jun 2015

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Tujuan utama kajian ini adalah untuk menjelaskan kesan ekstrak *Orthosiphon stamineus* Benth. ekstrak (OSE) di dalam pelarut yang berbeza polariti (etanol, etanol:air, metanol, metanol:air dan air) pada tikus aruhan streptozotocin (STZ)-diabetis telah dijalankan menggunakan pendekatan metabolomik. Model tikus diabetis yang di rangsang oleh streptozotocin (STZ: 60 mg Kg⁻¹) yang mana air kencing dan serum darah telah digunakan sebagai medium bagi mengenalpasti perubahan patologi semasa perkembangan dan rawatan diabetis melitus. Objektif kedua adalah untuk membina model validasi antara profil kimia ekstrak dan aktiviti anti-diabetis tikus diabetis yang di rangsang oleh STZ, yang telah diberi makan OSE selama 14 hari dan glibenclamide telah digunakan sebagai ubatan rujukan kawalan. Proton Nuklear Magnetik Resonan (¹H NMR) diperolehi dari air kencing dan serum darah telah digunakan untuk analisa menggunakan analisis multivariat (MVDA). Analisa separa persegi diskriminan (PLS-DA) dari ¹H NMR spektra membuktikan corak perubahan metabolik di dalam air kencing dan serum darah antara kumpulan-kumpulan dikenalpasti. OSE air menunjukkan pengurangan signifikan ($P < 0.05$) pada glukosa dengan 31.4% melalui PLS-DA dari ¹H NMR dengan perubahan yang menghampiri kumpulan kawalan atau rujukan kawalan iaitu glibenclamide yang berjaya mengurangkan glukosa sebanyak 48.6%. Pendekatan metabolomik adalah novel kerana kebolehannya untuk memberi gambaran keseluruhan metabolit yang terlibat dalam sampel biologi yang kompleks. Lebih menarik, data spektroskopi dari air kencing tikus STZ-diabetis menunjukkan julat glukosa, allantoin, kreatin, kreatinin dan glutamat menghampiri julat kumpulan kawalan selepas rawatan. Untuk mengenalpasti laluan metabolik yang terjejas oleh rawatan OS, perisian Metscape 3.0 telah digunakan. Keputusan menunjukkan beberapa laluan metabolik seperti karbohidrat, lemak, dan metabolomik asid amino telah terjejas semasa tempoh rawatan. Kajian ini mencadangkan yang OSE air mungkin bermanfaat dalam merawat hiperglisemik oleh kerana ia bertindak lebih baik apabila dibandingkan dengan ubat glibenclamide. Peningkatan signifikan metabolik purin (allantoin) yang dikesan di dalam OSE air menunjukkan peningkatan pada kadar penapisan glomerular yang membuktikan kebolehannya dalam membantu fungsi buah pinggang.

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I certify that a Thesis Examination Committee has met on 15 June 2015 to conduct the final examination of Amalina binti Ahmad Azam on her thesis entitled *Orthosiphon stamineus* Benth. on Streptozotocin-Induced Diabetic 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 Master of Science.

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LIST OF ABBREVIATIONS

/	Chemical shift in ppm
APCI-MS	Atmospheric Pressure Chemical Ionization Mass Spectroscopy
<i>br</i>	Broad
¹³ C	Carbon-13
COSY	Correlation Spectroscopy
<i>d</i>	Doublet
<i>dd</i>	Doublet of doublets
<i>ddd</i>	Doublet of doublets of doublets
<i>dt</i>	Doublet of triplets
eV	Electron volt
FT-IR	Fourier Transform Infra-Red
¹ H	Proton
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single-Quantum Coherence
EIMS	Electron Impact Mass Spectrum
<i>J</i>	Coupling Constant in Hz
<i>m</i>	Multiplet
MHz	Megahertz
<i>s</i>	Singlet
<i>t</i>	Triplet
OSE	<i>Orthosiphon stamineus</i> extract
STZ	Streptozocin
CMC	Carboxymethylcellulose
Trimethylsilanepropionic Acid Sodium Salt	Trimethylsilanepropionic Acid Sodium Salt
ACUC	Animal Care and Use Committee
TCA	Tricarboxylic acid
OGTT	Oral Glucose Tolerance Test
CPMG	Carr-Purcell-Meiboom-Gill

CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM) is serious and increasing health burden, treatable but incurable lifelong disease. According to International Diabetes Federation (IDF) through *Diabetes Atlas* (2011), around 366 million people worldwide had diabetes, and it is projected to increase by years to 552 million in 2030. An alarming of 3.6 million adults was estimated to be affected by diabetes in Malaysia alone (The Star, 2014). Based on the definition by WHO (2011), diabetes is a condition primarily defined by the level of glycaemia which raise the risk of microvascular (retinopathy, nephropathy and neuropathy) and macrovascular complications (heart attack, stroke and peripheral vascular disease). In addition, DM is also a chronic metabolites disorder of multiple etiologies characterized by insulin deficiency that leads to the elevation in glycaemia (Gupta *et al.*, 2005), which in turn causes changes primarily in carbohydrate metabolism and secondarily of lipids such as cholesterol, triglyceride and lipoprotein in plasma (Fontes, 2002; Negri, 2005; Al-Hiari *et al.*, 2011).

Despite the vast understanding of its epidemiology, yet there are no effective therapies to cure diabetes (Maiti *et al.*, 2004) and a definite solution for its prevention and causes is still not forthcoming (Zhang *et al.*, 2008). Biguanides and sulfonylureas are drugs used alone or together with insulin to treat this disease. Unfortunately, these medications can cause side effects (Hwang *et al.*, 2005), which are also not suitable for use during pregnancy (Pari and Uma, 1999). Also, failure of this hypoglycemic synthetic drug in significantly altered diabetic complications (Rang and Dale, 1991) lead to demand for alternative therapy which is safer and efficient as antidiabetic agents. In recent years, there is a broad revolution in using natural products for pharmacological applications, wherein numerous published investigations done on laboratory animals showing various plant extracts actually reduced glycaemia with fewer side effects and lower costs than the usual antidiabetic agents drugs (Pushparaj *et al.*, 2000; Gupta *et al.*, 2005; Sohn *et al.*, 2010; Petchi *et al.*, 2014).

The first medical text reported on diabetes mellitus is over 2000 years ago (Vladmir and John, 2005). Since then, diabetes was treated orally by traditional remedies with indications of 800 plants used (Eidi *et al.*, 2005). However, only a few plants have undergone comprehensive scientific investigations. Even though traditional medicines have considerable benefits in illness recovery since early stages but the effectiveness and stability are in doubt since the little in-depth study was conducted. This is due to the herbs require standardization of their content for diabetic therapeutic activity consistency of the products. Therefore, maximal doses of toxicology and effective treatment are crucial to be disclosed (David *et al.*, 2001). WHO also recommended research to focus on this area. *Orthosiphon stamineus* is one of medicinal plants which gained popularity due to its extensive therapeutic uses.

Orthosiphon stamineus Benth (Lamiaceae) is a traditional medicinal plant in Southeast Asia countries such as Indonesia and Malaysia. Its use is recommended for the treatments of many diseases, especially those affecting the urinary tract, diabetes mellitus, hypertension, rheumatism, tonsillitis and menstrual disorder (Awale *et al.*, 2003). In Malaysia, the tea prepared from the leaves is believed to treat kidney disease, bladder inflammation, gout, and diabetes. Moreover, the major chemicals of *Orthosiphon stamineus* (OS), for example, polymethoxylated flavonoids and caffeic acid derivatives were shown to exert diuretic and uricosuric actions in rats (Olah *et al.*, 2003).

Various studies on the use of OS in treating DM were conducted both *in vivo* and *in vitro* assays. Dose of 200mg, 500mg and 1000 mg Kg⁻¹ of rat body weight (BW) of OS aqueous extract was reported effective for alleviating hyperglycemia and improving lipid profile in diabetic rats after 14 days of treatment (Sriplang *et al.*, 2007). According to the study conducted by Abdullah (2009), the acute toxicity test LD₅₀ of OS standardized extract was estimated to be >5000 mg Kg⁻¹ of body weight (BW) in Sprague-Dawley (SD) rats that proved that OS is safe to be consumed. These studies have utilized streptozotocin (STZ)-induced rats *in vivo* model. STZ (*N*-nitro derivative of glucosamine) is an antibiotic and cytotoxic chemical which is particularly toxic to the pancreas leading to the degeneration of Langerhans islets beta cells which are responsible for insulin production in mammals (Hayashi *et al.*, 2006; Ikebukuro *et al.*, 2002; Weiss, 1982).

Metabolomics is a tool to identify all metabolites in biological fluids such as urine and blood (Listudies *et al.*, 2007). It relates biological endpoints to multiple altered metabolites in which provide the biological information of health in a complex system (Viant *et al.*, 2003; Chen *et al.*, 2006). Metabolomics combines data rich of advanced analytical tools such as nuclear magnetic resonance (NMR)-spectroscopy or mass spectrometry or both with multivariate statistical analysis (MVA). However, ¹H NMR is preferable in this study because it is robust, rapid, has high reproducibility, and is capable in detecting broad range of metabolites changes in both normal and abnormal biochemical pathways. This provides great potential for revealing the unknown mechanism of pathophysiology of drug effects (Cho *et al.*, 2015). In metabolomics study, NMR spectral pattern and intensity are recorded, and then statistically compared. Their features were identified for the relevant spectra that discriminate sample classes. Identification technique usually involved unsupervised clustering (Principal Component Analysis or PCA) or supervised classification (Partial Least Squares Discriminant Analysis or PLS-DA) (David, 2008).

Although, there have been several studies on the antidiabetic activity of OS, none of them thoroughly described the alteration of blood glucose regulation or other DM biomarkers in both urine and serum profiles using metabolomics approach. Recently, metabolomics study has rapidly progressed and has provided mechanistic insights into biochemical activity. Hence, the validity of the model on anti-diabetic alteration using metabolomics approach will furnish better understanding of chemical profile on the extracts, their anti-diabetic properties and affected pathways. Taking all of

these into account, in this present study the streptozotocin-induced diabetic rats were treated with different OS extracts in 14 days to address all of the mentioned issues with the main aims to:

1. identify the different polarity of OS extracts that have the highest anti-diabetic effect observed through urine and serum profiles in diabetic rats after 14 days of oral administration
2. develop a validated model of NMR chemical profile of extracts and their anti-diabetic activity using Multivariate Data Analysis for the prediction of the bioactivity of new OS extracts
3. propose the metabolism pathway affected by the OS treatment in diabetic rats



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