UNIVERSITI PUTRA MALAYSIA

CHEMICAL PROFILE AND ANTI-DIABETIC ACTIVITY OF Ipomoea aquatica Forssk. EXTRACT ELUCIDATED BY NMR-BASED METABOLOMICS

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IB 2016 15

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By

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

September 2016

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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September 2016

Chairman : Assoc. Prof. Faridah Abas, PhD
Institute : Bioscience

Diabetes mellitus (DM) is one of non-communicable disease (NCDs) that is characterized by high glucose content in blood or hyperglycemia, which can lead to long term complications and even death in the case of no proper treatment performed. Therefore, effective control of blood sugar has been known to be one the main issue in dealing with DM and its associated complications. In this study, the influence of various ethanol ratios (0, 20, 50, 80, and 100%) as an extraction solvent and different drying methods including air drying (AD), sun drying (SD) and oven drying (OD) on phytochemical constituents of *I. aquatica* were investigated using a proton nuclear magnetic resonance (^{1}H NMR) based metabolomics approach. The highest α-glucosidase inhibitory activity was observed for absolute ethanol extract from the OD method with an IC_{50} value of 204.0 ± 59.0 µg/mL and TPC value of 22.0 ± 0.7 µg GAE/mg extract. Correlation between the α-glucosidase inhibitory activity and the metabolite were analyzed using a partial least square (PLS) analysis. The metabolites that might be responsible for the activity were quercetin derivatives, chlorogenic acid derivatives, sucrose and fructose.

Along with our *in vitro* study, the ^{1}H NMR based metabolomics also been applied to the *in vivo* model (Sprague-Dawley rats). The *in vivo* model was first evaluated for understanding the metabolic link between the obesity (OB), lean diabetic (ND+STZ) and obese diabetic (OB+STZ). In this model, the OB+STZ rats mimics the symptom in the type 2 diabetes (T2DM), whereas the lean diabetic rats (ND+STZ) mimics type 1 diabetes (T1DM). The results of multivariate data analysis (MVDA) managed to highlight several similarities and dissimilarities in metabolites level in OB, ND+STZ and OB+STZ. This finding indicates both of the diabetic group (ND+STZ and OB+STZ) and OB rats shared some similar features especially in metabolic traits (2-oxoglutarate, succinate, tryptophan (TRP) and dimethylamine (DMA)), where it manage to highlights the importance of tricarboxylic acid cycle (TCA) and tryptophan (TRP) metabolism in diabetes progression. On the other hand, the differences between

ND+STZ and OB+STZ can be seen in the synthesis of ketone bodies and branched chain amino acid (BCAA).

Additionally, the effectiveness of the *I. aquatica* (IA) extracts as a hypoglycemic agent was also tested *in vivo* using obese Sprague-Dawley streptozotocin (STZ) -induced rats (OB+STZ). The rats were treated for 1 month, and the pathophysiological changes in serum and urine of these treated rats (OB+STZ+IA) and non-treated obese-diabetic rats (OB+STZ) were compared. The serum was assessed for biochemical parameter while the urine was evaluated using ^{1}H NMR. The result from serum showed there was no significant difference ($p > 0.05$) between the serum glucose of OB+STZ+IA (20.32 ± 8.79 mmol/L) and OB+STZ (24.60 ± 1.67 mmol/L) due to huge variation between the individuals. Interestingly, we found that there was clear discrimination between the urine spectra of OB+STZ+IA and OB+STZ by using ^{1}H-NMR metabolomic approach. The differences between the biochemical results from serum as compared to urine are probably due to the sensitivity of the instruments and the nature of the sample. Analysis of altered metabolites reveals that administration of *I. aquatica* extracts affects TCA cycle, creatine and creatinine metabolism, amino acids metabolism and nicotine and nicotinamide metabolism. This study highlights the basis for future investigations of *I. aquatica* as a source of food that has the potential for nutraceutical enhancement and as an ingredient in medicinal preparation.

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

PROFIL KIMIA DAN AKTIVITI ANTI-DIABETIK EKSTRAK *Ipomoea aquatica* Forssk. DIJELASKAN OLEH METABOLOMIK BERASASKAN NMR

By

AZLIANA BINTI ABU BAKAR SAJAK

September 2016

Pengerusi : Prof. Madya Faridah Abas, PhD
Institut : Biosains

Kencing manis (DM) merupakan salah satu penyakit tidak berjangkit (NCD) yang dicirikan oleh kandungan gula yang tinggi dalam darah atau hiperglisemia, di mana ia boleh membawa kepada komplikasi jangka panjang dan kematian jika tidak dirawat dengan sebaiknya. Justeru, keberkesanan mengawal gula dalam darah telah dikenalpasti sebagai salah satu isu utama di dalam menangani DM dan kompilaksi yang berkaitan dengannya. Di dalam kajian ini, pengaruh nisbah etanol (0, 20, 50, 80, and 100%) sebagai pelarut pengekstrakan dan teknik pengeringan yang berbeza termasuk pengeringan udara (AD), pengeringan matahari (SD) and pengeringan ketuhar (OD) ke atas sebatian fitokimia *Ipomoea aquatica* dikaji menggunakan pendekatan metabolomik berasaskan proton resonans magnet nukleus (^{1}H NMR). Perencatan aktiviti α-glukosidase yang tertinggi didapati pada ekstrak 100% etanol daripada pengeringan OD dengan nilai IC_{50} 204.0 ± 59.0 µg/mL dan jumlah kandungan fenolik (TPC), 22.0 ± 0.7 µg GAE/mg ekstrak. Korelasi di antara perencatan aktiviti α-glukosidase dan metabolit telah dianalisa menggunakan analisis kuasa dua terkecil separa (PLS). Metabolit yang mungkin mempengaruhi aktiviti tersebut adalah terbitan kuasertin, terbitan asid klorogenik, sukrosa dan fruktosa.

Di samping kajian *in vitro*, pendekatan metabolomik berasaskan ^{1}H-NMR juga dijalankan ke atas model *in vivo* menggunakan tikus *Sprague Dawley*. Tujuan utama model *in vivo* ini adalah untuk memahami hubungan diantara obesiti (OB), bukan-obes diabetes (ND+STZ) dan obes diabetes (OB+STZ) dalam mengenal pasti jenis diabetes di alami oleh tikus streptozotocin (STZ) teraruh. Dalam model ini, tikus OB+STZ mengalami gejala atau simptom yang meyamai diabetes jenis kedua (T2DM), manakala tikus dari ND+STZ menyerupai diabetes jenis pertama (T1DM). Keputusan daripada analisis multivariat (MVDA) berjaya menemukan beberapa persamaan dan perbezaan pada kandungan metabolit tikus OB, ND+STZ dan OB+STZ. Penemuan ini menandakan kedua-dua kumpulan diabetes (ND+STZ dan OB+STZ) dan tikus OB berkongsi ciri-ciri khususnya di dalam trait metabolit (2-oksoglutarat, suksinat, triptofan (TRP) dan dimetilamina (DMA)), di mana ia berjaya menunjukkan

kepentingan dalam kitaran trikarbosilik asid (TCA) dan metabolisma TRP di dalam patogenesis diabetes. Di samping itu, perbezaan di antara ND+STZ dan OB+STZ boleh didapati pada jasad keton dan sintesis asid amino bercabang (BCAA).

Di samping itu, keberkesanan ekstrak *I. aquatica* (IA) sebagai agen hipoglisemik juga di kaji melalui kajian *in vivo* menggunakan tikus OB+STZ. Tikus-tikus ini telah dirawat selama 1 bulan, dan perubahan patofisiologi di dalam serum dan urin (air kencing) tikus yang dirawat (OB+STZ+IA) dan tikus yang tidak dirawat (OB+STZ) dibandingkan. Serum dianalisis untuk parameter biokimia, manakala urin dinilai menggunakan ^{1}H-NMR. Keputusan serum menunjukan tiada perubahan signifikan di antara serum glukosa tikus daripada kumpulan OB+STZ+IA (20.32 ± 9.49 mmol/L) dan OB+STZ (24.60 ± 1.67 mmol/L) kerana terdapat variasi yang besar di antara tikus-tikus di dalam kumpulan yang sama. Walaubagaimanapun, terdapat perbezaan di antara OB+STZ dan OB+STZ+IA melalui spektrum urin menggunakan metabolomik berasaskan ^{1}H-NMR. Perbezaan keputusan biokimia di antara serum dan urin mungkin disebabkan sensitivi instrumen dan sifat semulajadi sampel. Analisis menunjukan *I. aquatica* berjaya merubah metabolisma seperti kitaran TCA, kreatin dan kreatinine, asid amino dan nikotin dan nikotinamida. Kajian ini berjaya menekankan potensi *I. aquatica* sebagai sumber makanan nutraseutikal yang berpotensi dan juga sebagai bahan dalam pemyediaan ubat-ubatan.

ACKNOWLEDGEMENTS

Alhamdulillah, all praise to Allah S.W.T who have given me the strength and health to finish this thesis. First and foremost I owe my deepest gratitude to my supervisor, Assoc. Prof. Dr. Faridah Abas, who has supported me throughout my thesis without failed, her patience and knowledge whilst allowing me the room to work in my own way. I simply could not wish for a better or friendlier supervisor. I also want to give my warm appreciation to my committee members; Prof. Amin Ismail and Assoc. Prof. Dr. Alfi Khathib for their helpful suggestion, insights and comments.

This might take a while but I need to address them all. All of these people have been dear to me, as I have been through my ups and down throughout my study years here with them. So, please bear with me; to my friends, Amalina Azam and Norzaini – thank you for helping me out during my study years. Without their help, I do not think I would be able to finish my lab work. Only Allah S.W.T can repay all their good deeds to me.

To my research teammate Ahmed Mediani - your valuable insights and suggestion really helps me a lot. To my other lab mates; Ilya, Shazwani, Nur Ashikin, Raghu, Nur Athifah, Siti Nazirah, Khoo, Soo Yee, Tahani, Dr. Leong, Umar and Mohammed – of all us have been thorough a lot together, I am blessed having you guys as my lab mates. To our lab mood maker "twins", Khaleeda and Kenny – thank you for making my days with your antics.

Since I work in the various laboratories in Natural Product Laboratory for many years in running my experiments – special gratitude should be given to the man behind the Nuclear Magnetic Resonance (NMR), Mr. Salehuddin - a fine and dedicated science officer who always available to be contacted even though its weekend. The smooth running of the NMR is much more a testament to his efforts than my own.

To Mr. Azizul Isha, our research officer in Phytochemistry lab – I spend most my study days here, I reckon my table is still there in the lab! To Assoc. Dr. Intan Safinar, our hardworking head of Natural Product Laboratory - thank you for giving me the permission to use the lab during the weekend. Not to forget – Dr. Diani who helped to tutor me during the animal study and how to analyze metabolomics data from the biofluids. To others that I might forget to mention or to those people that I encounter during my study years here – thank you for your prayers, wishes and help. May Allah S.W.T blessed you and your family always. Amin.

Lastly, special mention to both of my parents, Mr. Abu Bakar Sajak, Mrs. Zaliha – I am sorry that I seldom going back to the hometown due to commitment that I have here and I am also sorry for taking a longer time to finish this thesis. Their prayers and support that led me to finish this thesis. I hope both of you will always under Allah S.W.T care and safety. To my sisters and brother – thank you for your prayers and love. Be healthy always. Amin.

I certify that a Thesis Examination Committee has met on 8 September 2016 to conduct the final examination of Azliana binti Abu Bakar Sajak on her thesis entitled "Chemical Profile and Anti-Diabetic Activity of *Ipomoea aquatica* Forssk. Extract Elucidated by 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of Supervisory Committee are as follows:

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- the research conducted and the writing of this thesis was under our supervision;
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^{1}H	Proton
3-HAA	3-hyroxyanthranilic
3-HB	3-hydroxybutyrate
ACUC	Animal Care and Use Committee
AD	Air dry
ATP	Adenosine triphosphate
BCAA	Branched chain amino acid
br	Broad
CMC	Carboxymethyl cellulose
CPMG	Carr-Purcell-Meiboom-Gill
d	Doublet
dd	Doublet of doublet
DM	Diabetes mellitus
DMA	Dimethylamine
DMG	Dimethylglycine
EGP	Endogenous glucose production
ER	Endoplasmic reticulum
GAD_{65}	Glutanic acid decarboxylase
HCA	Hierarchical cluster analysis
Hfd	High fat diet
HLA	Human leukocyte antigen
HMBC	Heteronuclear multiple bond correlation
IA	*Ipomoea aquatica*
IAA	Insulin autoantibodies
ICA	Islet cell autoantibodies
IDDM	Insulin dependent diabetes mellitus
J	Coupling constant in Hz
KK	Kuo kondo mouse
KYN	Kynurenine
LADA	Latent autoimmune diabetes
LDH	Lactate dehydrogenase
m	Multiplet
MHz	Megahertz
MNA	1-Methylnicotinamide
MOH	Ministry of Health Malaysia
mRNA	Messenger ribonucleic acid
MSG	Monosodium glutamate
NAD	Nicotinamide
Nd	Normal diet
ND	Normal/Lean
ND+STZ	Lean diabetic
ND+STZ+MET	Lean diabetic treated with metformin
NDDM	Insulin dependent diabetes mellitus
NMR	Nuclear magnetic resonance
NZO	New Zealand obese mouse
OB	Obese
OB+STZ	Obese diabetic
OB+STZ+IA	Obese diabetic treated with *I. aquatica* extract
OB+STZ+MET	Obese diabetic treated with metformin

OD	Oven dry
OGTT	Oral glucose tolerance test
OPLS-DA	Orthagonal partial least square
PC	Pyruvate complex
PCA	Principle component analysis
PDC	Pyruvate dehydrogenase complex
PLS	Partial least square
PLS-DA	Partial least square discriminant analysis
QA	Quinolinic acid
RBC	Red blood cell
s	Singlet
SD	Sun dry
STZ	Streptozotocin
t	Triplet
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
TCA	Tricarboxylic acid
TPC	Total phenolic content
TRP	Tryptophan
TSP	Trimethylsilyl propionic acid sodium salt
VIP	Variable importance in the projection
WHO	World Health Organization
δ	Chemical shift in ppm

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is one of the major degenerative diseases that haunt major populations in this world today (Van den Driessche et al., 2009). It is usually associated with other diseases such as hypertension, atherosclerosis, and microcirculatory disorders (Milicevic et al., 2008; Moore et al., 2009). It can also cause long-term complications such as retinopathy, nephropathy, neuropathy and angiopathy (Nicolucci et al., 1996). In Malaysia, the prevalence of adults with DM is nearly 1.5 million, and this disease increased by 3.3% over the last few decades (Azimah et al., 2009).

DM can essentially be divided into two main groups, Type 1 and Type 2, based on the requirement for insulin. Type 1 is insulin-dependent diabetes mellitus, while Type 2 is non-insulin dependent. Approximately 90% of DM cases worldwide are Type 2 diabetes (Alberti & Zimmet, 1998), characterized by a combination of defects in insulin secretion and insulin sensitivity. Type 2 DM is usually linked with another disease such as obesity (Grundy, 2004; Parton et al., 2007; Pradhan, 2007) and cardiovascular (Desouza et al., 2003; Wright & Frier, 2008) disease, associated with glucose metabolism deregulations. All these factors make Type 2 DM a complex disease that is difficult to understand (Leahy, 2005; McIntyre & Walker, 2002).

One of the ways to manage DM is by controlling the glycemic/glucose level in the blood. Previous studies have confirmed that the effective control of the blood glucose level in DM can significantly reduce the risk of developing diabetes complications (Ohkubo et al., 1995; UKPDS, 1998). The search for effective and safer anti-hyperglycemic drugs is increasing as the current insulin therapy and oral hypoglycemic agents contain side effects that are harmful to health (Holman & Turner, 1991). To overcome this matter, alternative medicine sources such as plants are used in treating DM. From the literature, there are more than 800 plant species traditionally used as anti-hyperglycemic agents (Harbilas et al., 2009). These include fruits and vegetables that are known to contain high levels of vitamins and phytochemicals that are beneficial to humans (Franceschi et al., 1998).

Fruits and vegetables such as *Momordica charantia* (bitter melon), *Coriandrum sativum* (coriander), *Brassica juncea* (leaf mustard) and *Ipomoea batatas* (sweet potato) have been tested for their hypoglycemic activities using animal models, and all showed promising results (Day et al., 1990; Khan et al., 1995; Kusano & Abe, 2000; Swanston et al., 1990). Previously, an infusion and diet containing *Coriandrum sativum* was shown to reduce hyperglycemia during the development of streptozotocin (STZ) - induced diabetes in mice (Swanston et al., 1990). *Ipomoea batatas* L. (sweet potato) produced a significant reduction in the glucose levels of Zucker fatty rats after the third week of treatment, and this result is comparable to a standard anti-diabetic drug,

troglitazone (Kusano & Abe, 2000). However, the search to find more plant extracts that can be scientifically proven to contain hypoglycemic activities is still ongoing. Demands for the use of these plants are increasing due to their low cost, easy availability and low side effects (Harbilas et al., 2009).

In the present study, *Ipomoea aquatica* or locally known as "kangkung" has been chosen as the potential plant to treat obese rats with induced diabetes. *I. aquatica* is a green leafy vegetable, a semi-aquatic plant that belongs to the Convolvulaceae family. It is easily grown and has been cultivated throughout Southeast Asia and consumed as a vegetable (Meira et al., 2012). Chen and Chen (1992) reported that among the vegetables, *I. aquatica* is one that is rich in carotenoids and chlorophylls. Based on a previous study conducted by Hamid et al. (2011), there is a significant difference ($p < 0.001$) in the hypoglycemic activity of *I. aquatica* extracts at 200 mg/kg compared to the control of Swiss albino mice. Malalavidhane et al. (2001) also reported that the hypoglycemic activity of *I. aquatica* is on par with the standard anti-diabetic drug, tobultamide. However, the mechanism of this plant in diabetes is yet unknown.

An animal model was used in this study to mimic human DM. Streptozotocin (STZ) is a broad spectrum antibiotic and a powerful alkylating agent that was injected into the rats, resulting in the destruction of insulin-producing β-cells (Bolzán & Bianchi, 2002). A previous study (Nieman et al., 2006) found that a diabetic state changes metabolites such as the methyl group, choline, and homocysteine compared to the control. Due to the complexity of the disease, Zhang et al. (2008) suggested that a metabolomics approach should be used in conducting diabetes research. Metabolomics studies can be defined as an attempt to measure all the metabolites that are present in a cell, tissue or organism due to genetic modification or physiological stimulus (Bino et al., 2004; Nicholson et al., 2005; Nicholson et al., 1999; Oliver, 2006; Oliver et al., 1998).

Metabolomics studies use biofluids or cell or tissue extracts to collect metabolic data. These metabolic data are important for the identification of sensitive and specific traits of metabolic disorders, and this information is crucial, especially for early-stage detection and evaluation of therapy in treating DM (Sebedio et al., 2009). In addition, biofluids from animal and human studies are usually relatively easy to obtain high in volume and produce a consistent result (Sebedio et al., 2009). In general, the most common technologies that have been used in metabolomics studies are NMR, LC-MS, GC-MS and HPLC. Compared to the others, NMR-based metabolomics is preferred due to its robustness, reproducibility and non-destructive nature (Nicholson et al., 2002).

1.2 Problem statements

The effect of the extraction solvent on plant phytochemicals may offer much information on the extraction efficiency to maximize the benefit of plants as medicine or food ingredients. However, little information has been reported on the effects of solvent ratios on *I. aquatica* bioactive compounds, and although extensive studies have been performed on this plant as an anti-diabetic agent (Hamid et al., 2011;

Malalavidhane et al., 2001; Malalavidhane et al., 2000), little is known regarding chemical analyses that can describe the metabolic alterations resulting from phytochemical intervention in diabetic-induced animal models.

1.3 Objectives

Therefore, the present study was conducted to evaluate the anti-diabetic activity of *I. aquatica* extract by using metabolomics approach. This approach will allow better understanding in terms of the mechanism of action and also help to identify chemical and biomarkers associated with anti-diabetic and *I. aquatica* treatment. Hence, the objectives of this study were:

1. To evaluate the effect of drying methods and solvent ratio on phytochemicals constituents and α-glucosidase inhibitory activity of *I. aquatica* using ^{1}H NMR based metabolomics.
2. To identify metabolite variation in lean and obese streptozotocin (STZ)-induced diabetic rats.
3. To determine the bio-markers relating to the anti-diabetic effect of *I. aquatica* extracts in streptozotocin (STZ)-induced diabetic rats via ^{1}H NMR-based metabolomics approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes mellitus

2.1.1 Overview

Diabetes mellitus (DM) is one of the non-communicable diseases that usually marked by higher glucose content in blood (Sebedio et al., 2009). Looking back into history, the disease has been cited since Greeks and Romans time. The word *mellitus* which meant sweet in Latin was derived when the Romans noticed that urine of certain individuals were sweeter as compared to the others; while the words *diabetes* originated from the Greeks when they noticed that individual with sweeter urine often drank regularly and urinate frequently like a siphon (Rubin, 2012).

All of the symptoms that have been mentioned above are among of the symptoms that usually associated with DM which is, known as polyuria (production of a large amount of urine), polydipsia (abnormal of great thirst), and glycosuria (excretion of glucose in the urine) (WHO, 2013). Poorly treated DM can cause long term complication such as nephropathy (kidney disease by diabetes), glaucoma (eye disease causes damage to optic nerve), neuropathy (problem with peripheral nerves), and in the worst case, DM can be fatal (WHO, 2002).

In Malaysia, it is reported that more than 2.6 million adults having this disease in 2011 and this number is expected to increase each year (MOH, 2013). This matter is quite alarming because it becomes one of the economic burdens. About 80% of the registered patients seek treatments at the local government hospitals which cost Ministry of Health of Malaysia about RM 836 million in 2009; where it compromised about 2.2% of 16% nation's expenditures on health sector (Yoon et al., 2006; Zhang et al., 2010). Also, it affects the productivity and the quality of life of the patients as they need to deal with the complications in their daily life.

One of the ways to treat DM is by controlling the glucose level in the blood (Ohkubo et al., 1995). Carbohydrate is one of the main sources of energy in the body that can be one of the factors contributing to higher glucose level (hyperglycaemia) because carbohydrate will break down into smaller molecules as glucose (Leu & Zonszein, 2010). For a healthy individual, a hormone called insulin will be secreted and acted as a 'key' that allows the muscle and liver cells to absorb the glucose for the production of energy or adenosine triphosphate (ATP) (Wallymahmed, 2006).

Besides lowering blood glucose, insulin is important to store glucose in the form of glycogen that would be used when the energy is insufficient. Meanwhile, insulin also

acts antagonistically against protein breakdown when there is enough of glucose (Brass et al., 2010). In the case of individual with DM, lack of insulin or insulin deficiency causes the blood glucose level to rise especially after eating (post prandial glucose) and maintain constant after a while (Rubin, 2012).

2.1.2 Endocrine pancreas

Insulin is the most important hormone in the body and being produced by the beta cells (β-cells) in islets of Langerhans (endocrine glands) that located in the pancreas (Rubin, 2012). Besides producing insulin, pancreas is also important in producing digestive enzyme via exocrine glands such as lipase, amylase, and protease in the small intestine. Generally, islets of Langerhans comprises of 4 types of cells which are, β-cells, alpha cells (α-cells), delta cells (δ-cells), and pancreatic polypeptide (PP) cells (Brass et al., 2010; Rubin, 2012). The majority of islets of Langerhans are constituted with β-cells where they are concentrated in the anterior head, body, and the tail of the pancreas (Brass et al., 2010). Whereas, the posterior portion of the head (derived from the primordial ventral bud of the pancreas) consists mostly of PP cells. As been mentioned earlier, β-cells are insulin-secreting cells while α-cells are the cells that secrete glucagon, a hormone that raises the blood glucose when the blood glucose level gets too low. Other cells such as δ-cells are cells that produce somatostatin, a hormone that function by blocking another hormone and PP cells produce pancreatic polypeptide (Brass et al., 2010; Rubin, 2012).

2.1.3 Insulin and its function

Insulin and glucagon are functioning antagonistically with each other in glucose and nutrient homeostasis. Insulin is built from 51 amino acids that make it one of the smallest proteins (Wallymahmed, 2006). Structurally, insulin consists of two polypeptide chains (A and B) and they are linked by disulfide bonds. In addition, another disulfide bonds can be found within the A chain itself (Brass et al., 2010).

In brief, an inactive single sequence precursor called preproinsulin is first translated from insulin mRNA to rough endoplasmic reticulum (ER) of β-cells. It is immediately converted to proinsulin by removing of its signal peptide (Figure 2.1). Conversion of proinsulin to insulin is done by the action of two prohormone-converting enzymes (PC1/3 and PC2) that resulted in an insulin molecule and amino acid residue, C-peptide (Brass et al., 2010).

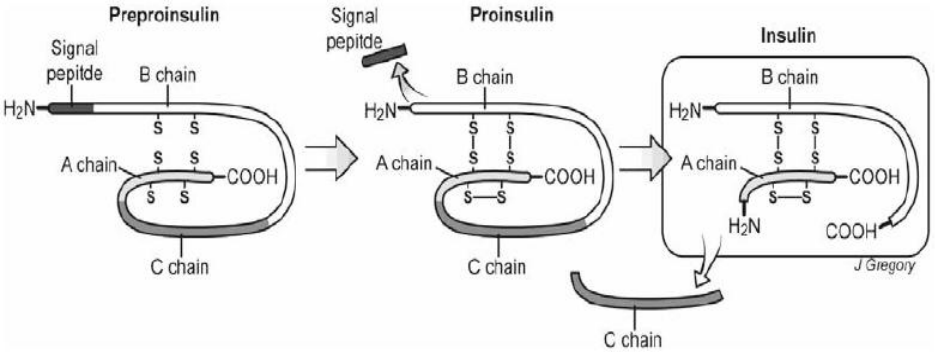


Figure 2.1: Sequence in insulin synthesis; from preproinsulin to insulin (Brass et al., 2010).

Stimulation of insulin can be initiated by the ingestion of meal or by intravenously administered glucose (Brass et al., 2010). Ingestion of meal can increase the plasma glucose that resulting in an increase in plasma insulin up to three to fourth fold within 30-60 minutes. In contrast, the decrease in plasma glucose below 50 mg/dl can result in plasma insulin reduction up to 80-90% (Brass et al., 2010; Wallymahmed, 2006). Other factors such as acute increases in amino acids or free fatty acids (FFA) have also been reported to increase insulin secretion (Brass et al., 2010).

Glucose homeostasis is maintained by insulin through direct and indirect actions (Rubin, 2012). It will bind to its receptor in the kidney, muscle, liver, and adipose tissue which will activate its signaling pathways that involved cascades of protein kinase and regulatory proteins (e.g. IRS-1 and IRS-2). Eventually, this will cause (1) suppression of glucose releases from liver and kidney; (2) increase of glucose uptakes by the muscle and adipose tissues due to translocation of glucose transporters (e.g. GLUT4); and (3) increase of FFA clearance in the circulation due to FFA inhibition. Interestingly, even though insulin does not directly involve in glucose transport in the liver, inhibition of glucose-6-phosphatase and phosphorylase (glycogenolysis enzymes) during the stimulation of glycogen synthase insulin promotes glycogen accumulation (Brass et al., 2010; Rubin, 2012).

2.1.4 Classification of diabetes

Diabetes can be classified into four categories as shown in Figure 2.2. Among the four categories, type 1 (T1DM) and type 2 (T2DM) are the most frequent groups that occur among the diabetes patients in which 90% of the cases involved T2DM (WHO, 2002). Previously, T1DM was known as insulin dependent diabetes mellitus (IDDM) and T2DM was known as non-independent diabetes mellitus (NIDDM) before they were revised by National Diabetes Data Group (NDDG) and World Health Organization (WHO) in 1979 (Leu & Zonszein, 2010). Meanwhile the third and fourth groups are diabetes that associated with or caused by certain specific conditions or syndrome, and gestational diabetes (GDM) that occurs during pregnancy. In this chapter, the

discussion would be focused more on the common groups that suffered by the diabetes patients, which are T1DM and T2DM.

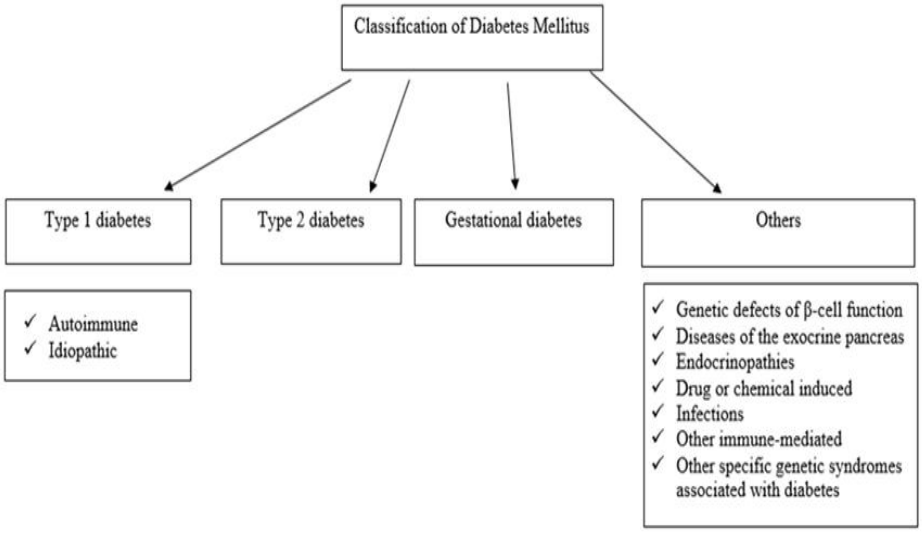


Figure 2.2: Four types of categories in diabetes.

2.1.4.1 Type 1 diabetes (T1DM)

This form of diabetes compromises about 5-10% out of all cases. T1DM is used to be called as *juvenile diabetes* (aside to IDDM) because it occurs most frequently in children (Rubin, 2012). However, due to the increased cases in adults, the term was revised and renamed as T1DM (Leu & Zonszein, 2010; Rubin, 2012). T1DM is often associated with complete insulin deficiency resulting from a progressive cellular-mediated autoimmune destruction of β-cells (Rubin, 2012). This β-cell failure can lead to ketoacidosis which is often mark as the first manifestation of the disease (Gillespie, 2006; Leu & Zonszein, 2010). In addition, there are also cases where severe hyperglycemia or ketoacidosis occurs when the patients are exposed to stress condition or severe infections (Leu & Zonszein, 2010).

Since patients with T1DM are severely insulin deficient and are depending on insulin treatment, the management of glucose levels is crucial. For T1DM patients, exogenous insulin is provided as a replacement for endogenous insulin hormone (Leu & Zonszein, 2010). Early diagnosis of T1DM on the new-onset patient can be made by detecting biomarkers of β-cell destruction such as islet cell autoantibodies (ICAs), insulin autoantibodies (IAAs), glutamic acid decarboxylase autoantibodies (GAD_{65}), and autoantibodies to the tyrosine phosphatase IA-2 and IA-2α (Kaufman et al., 1992; Lan et al., 1996; Lu et al., 1996; Myers et al., 1995).

Furthermore, T1DM has a close connection with human leukocyte antigen (HLA) with the linkage to DQA and B alleles; where it is influenced by DRB genes and link with other immune disorder (Mosaad et al., 2012; Noble et al., 2010). This type of immune-mediated diabetes can onset at any age and the slow rate of β-cell destruction is making it difficult to distinguish from T2DM. The only way that can differentiate it from T2DM is the positive result in pancreatic autoantibody test. This type of diabetes is known as Latent Autoimmune Diabetes in Adults (LADA) which is common in the Caucasian population (Leu & Zonszein, 2010; Rubin, 2012).

The other uncommon type of T1DM is idiopathic diabetes. It is less comprehended as compared to LADA with various degrees of β-cell dysfunctions. Lack of evidence for β-cell autoimmunity and not HLA associated is a form of diabetes that is inherited. The disease is often demonstrated by severe insulinopenia and/or ketoacidosis. Since the β-cell functions are always recovered in idiopathic diabetes, the insulin treatment is only necessary during the initial phase and may not be needed after the recovery phase (Leu & Zonszein, 2010).

2.1.4.2 Therapy for T1DM

Since T1DM is associated with complete insulin deficiency, replacement of endogenous insulin with exogenous insulin is essential to balance out the glucose homeostasis. However, there are several issues arise when using exogenous insulin such as: (1) insulin delivered to the whole body is through peripheral circulation instead of portal circulation; (2) imbalance in between exogenous insulin with secretions of another hormone such as glucagon, amylin, incretins, and other hormones to balance blood sugar level; and (3) difficulties in monitoring the blood glucose level after injection that can lead to hypoglycemia (Cohan & Peters, 2010; Leu & Zonszein, 2010).

All of these issues have led to the development of newer technology for insulin analogues that are easier to use and with fewer side effects such as hypoglycemia. A wide variety of insulin delivery devices such as pens and pumps are also available on the market for patients to choose (Leu & Zonszein, 2010; Wallymahmed, 2006). Furthermore, monitoring blood glucose becomes an ease as monitoring devices such as glucose meters make it possible to monitor the glucose level at home without requiring the patients to visit the hospital regularly.

Until today, there are several types of insulin that are available for the patients including traditional insulin and insulin analogues. This insulin can be divided into 4 types (rapid, short, intermediate, and long acting) based on their time course of actions (onset, peak, and duration) as shown in Table 2.1. The rationale of having different time courses in insulin is because the volume of insulin and the site of injection (i.e. intramuscular, subcutaneous, and intradermal) may influence the rate of absorption in the body (Cohan & Peters, 2010; Wallymahmed, 2006). In addition, internal and external factors such as exercise, skin temperature, and hydration can affect the blood flows that will affect the rate of absorption (Cohan & Peters, 2010). With variability

types of insulin, T1DM patient is able to use the most suitable insulin and injecting insulin at the sites that most effective for them.

Table 2.1: Time course of action of insulin

Type	Example	Onset (min)	Peak (h)	Duration (h)
Rapid acting	Lispro Aspart Glulisine	5-15	0.5-2.0	3.0-5.0
Short acting	Regular	0.50-1.0	2.0-4.0	6.0-8.0
Intermediate acting	Neutral Protamine Hagedron (NPH)	2.0-4.0 h	4.0-10.0	10.0-16.0
Long acting	Glargine Detemir	2-4 3-8	None None	~24 6-23*

*Duration depends on dose was given (Cohan & Peters, 2010)

2.1.4.3 Type 2 diabetes (T2DM)

This form of diabetes accounts 90% of cases from all individuals with diabetes (WHO, 2002). T2DM is considered as heterogeneous condition as it is often associated with other metabolic diseases such as obesity and cardiovascular risk factors namely hypertension. T2DM patients often suffer abnormalities in insulin resistance and β-cell dysfunction. Insulin resistance occurs when the insulin secretion is initially high in the early stage of the disease and become less sensitive towards glucose level as the disease progressing which will lead to hyperglycemia (Fonseca & John, 2010).

There are many factors that can lead to the development of T2DM. Genes and environmental factors such as unhealthy lifestyles have been reported to be the causes. Most individuals with T2DM are born with genes of insulin resistance. Individual with a family history of T2DM are 40% at risk getting T2DM as compared to individual with non-family history. Previous studies have also conducted in twins that showed genetic makeup accounts for 60-90% of the susceptibility to T2DM where the identical twins (monozygotic twins) rate is 70-90% as compared to fraternal twins (dizygotic twins) which is only 15-25% (Leu & Zonszein, 2010; Rubin, 2012).

Intricate plays between environmental and genetic factors can also affect insulin action and/or insulin secretion (Leu & Zonszein, 2010). Increased numbers of individuals with T2DM in developing countries have been remarkably high due to people opt for processed food such as fast food (Zhang et al., 2010). Moreover, physical inactivity and obesity may increase the chances of getting diabetes in genetically predisposed individual (Ramachandran et al., 2012).

2.1.4.4 Therapy for T2DM

Glycemic control has been shown to prevent and delay the short and long term complications of diabetes (UKPDS, 1998). One of the ways to monitor blood glucose in the body is by monitoring hemoglobin A1c (HbA1c). Hemoglobin is predominant protein in red blood cells (RBC) that carries oxygen and gives RBC its red colour. About 90% of this hemoglobin is hemoglobin A and its components are A1c where glucose is bound (Rubin, 2012). Current treatment guidelines from both American Diabetes Association and the European Association for the Study of Diabetes suggested that individual with diabetes needs to lower their HbA1c to < 7% and to get it near to normal range as possible (normal HbA1c range: < 5.7%) (Herzlinger & Abrahamson, 2010).

The development of DM drugs has begun since the 1940s when a group of scientists accidently discovered that certain sulphonamide antibiotics can cause the side effect of hypoglycaemia. This discovery has led to the development of synthetic hypoglycaemic drugs from sulfonylurea hypoglycemic agents. The first drugs that have been approved for use in 1967 in the US are tolbutamide, followed by the second generation of drugs such as glyburide and glicazide (Marles & Farnworth, 1995). Besides treating DM, the patients that are using these drugs need to bear the consequences of side effects such as severe hypoglycaemia, hyperactivity, and even worst it can also lead to death. The current methods of treatment for all types of diabetes mellitus have failed to achieve the normoglycemia and the prevention of diabetic complications (Marles & Farnworth, 1995). Therefore, to overcome this matter, alternative sources of medicine such as plants have been used in treating DM. From literature, there are more than 800-1200 species of plant traditionally or experimentally been used as anti-hyperglycemic agent (Marles & Farnworth, 1995; Thorne, 1981). Out of 295 traditional plants that traditionally claimed to have anti-hyperglycemic activity, 81% of them were tested and gave positive results. However, the search is still continuous to find more plants' extracts which are, scientifically proven contain hypoglycemic activities. Demands for the use of these plants are increasing due to their low price, ease of availability, and fewer side effects.

2.2 Plant derived drugs

Plants play a vital role in our life. First of all, they provide us oxygen to breathe. They are our main sources for food, medicine, wood, and many more (Cowan, 1999). With higher phytochemical constituents, plants have been used to treat diseases since early centuries. Before the arrival of synthetic drugs, 80% of medicines were obtained from

roots, barks, and leaves (McChesney et al., 2006). Historically, the isolation of morphine from opium by Seruturner in the early 19th century and the discovery of penicillin from *Penicillium notatum* mold have opened the new era in plant derived drugs as medicines (Gilani & Atta-ur-Rahman, 2005; Drews, 2000). Today, commercial drugs such as aspirin, atropine, quinine, morphine, vincristine, and vinblastine are from plant derived drugs (Gilani and Atta-ur-Rahman, 2005). Nevertheless, the number of newly approved drugs remained constant throughout these years even though there are about ~250,000 species of plant in the world (Verpoorte, 1998). From 2008 till 2013 only a total of 25 natural product and naturally derived drugs were approved for the consumers, where 5 of them were classified as natural products, 10 as natural products derived and the remaining 10 as semi synthetics natural products (Murad, 2014).

2.3.1 Genus *Ipomoea*

Genus *Ipomoea* belongs to Convolvulaceae family, comprising about 500-600 species (Austin and Huáman, 1996). The genus is distributed throughout the tropical and subtropical regions of the world although some species have reached temperate zones (Cao et al., 2005). They are dominated by twining or climbing woody or herbaceous plants that have heart-shaped leaves and funnel-shaped flowers (Austin, 1997). Plant under *Ipomoea* genus is commonly consumed as vegetable and also being used for other purposes such as medicinal, ritual, and agricultural (Pereda and Bah, 2003). The traditional uses of this genus are summarized below in Table 2.2. The knowledge of ethnopharmacology of this genus is important as guidance in selecting the potential plants to be evaluated in chemical studies. The constituents of the *Ipomoea* genus have been studied since 1950. Scientific studies have shown that several *Ipomoea* species possess antimicrobial, analgesic, spasmogenic, hypotensive, psychomimetic, and anticancer activities. The most common bioactive compounds that have been isolated from this genus are ergoline alkaloids, indolizidine alkaloids, nortropane alkaloids phenolics compounds, coumarins, norisoprenoids, diterpene, isocoumarins and benzenoids, flavonoids and antocianosides, glycolipids, lignin, and triterpenes (Miera et al., 2012).

Table 2.2: Traditional uses of genus *Ipomoea*

Species	Traditional uses
I. aquatica	• Anti-diabetes (Jayaweera, 1982; Malalavidhane et al., 2001). • Antidote for Scorpion venom (Uawonggul et al., 2006). • Hypertension (Perry, 1980). • Emetic, diuretic, purgative (the seed of *I. aquatica*), leucoderma, leprosy and antipyretic (Ghani, 1989; Mamun, et al., 2003).
I. asarofiloa	• Anti-pruritic (itching) (Silva, 2002).
I. batatas	• Anti-anaemic, hypertension and diabetes (Ludvik et al., 2004). • Treat tumours of the mouth and throat. Aphrodisiac, astringent, bactericide, demulcent, fungicide, laxative and tonic (leaf decoction). Treatment of asthma, bug bites, burns, and whitlows using sweet potato (Duke & Wain, 1981).
I. cairica	• Anti-inflammatory and treat rheumatism (Ferreira et al., 2006)
I. campanulata	• Antivenom for snake poison (Singh et al., 2003)
I. carnea	• Treatments for Acquired Immunodeficiency Syndrome (AIDS) (Woradulayapinij et al., 2005) • Anti-hypertension (Lamidi et al., 2000).
I. digtata	• Emaciation in children, tonic, alterative, aphrodisiac, demulcent, constipitation, lactogogue, and cholagogue (Singh et al., 2004).
I. indica	• Purgative agent and heal broken bones (Abbott & Shimazu, 1985)
I. leptophylla	• Anti-anxiety - roots (Gilmore, 1977). • Stomach distress (the roots) and tonic (Barnes, 2003).
I. muricata	• Treat skin ailments (Ysrael, 2003).
I. muricoides	• Mosquito repellent and anti-venom for scorpion (Léon at al., 2005).
I. nil	• Anti-cancer (Ko et al., 2004).

I. orizabensis	• Purgative agent (Pereda, 1995). • Anti-helmintic and to treat abdominal fever, dysentery, epilepsy, hydrocephaly, meningitis and tumours (Martinez, 1990).
I. pes -caparae	• Anti-inflammatory and algesic processes (Souza et al., 2000). • Treat digestive disorders, colic, internal and external pain, dysentery, inflammation, fatigue, strain arthritis and rheumatism. Treatment for diuretic disorder and constipation (Pereda et al., 2005; Lorenzi & Abreau, 2002; Martinez, 1989).
I. purga	• Purgative agent (Pereda & Bah, 2003).
I. purpurea	• Diuretic, anti-hemorrhagic, purgative and to treat syphilis (Camargo, 1998).
I. stans	• Treatment for epileptic seizures (root), nephritis ophthalmic diseases and paralysis, as antispasmodic and sedative agent (Diaz, 1976). • Purgative agent (Pareda & Bah, 2003).
I. stolonifera	• Diuretic and to relieve pain after childbirth, stomach problems, inflammations, wounds, tumefactions and furunculosis (Paula et al., 2003).

2.3.1.1 *Ipomoea aquatica* Forssk.

Figure 2.3: Broad leaves of *Ipomoea aquatica.*

Water spinach (*Ipomoea aquatica*) or locally known as kangkung (Figure 2.3) is a green leafy vegetable that is native to Asia, and the south-western Pacific Islands. Morphologically, *I. aquatica* can be distinguished by its soft, hollow and long stems (2-3 metres) and trumpet like shaped flowers that usually white in colour with the mauve centre. It can grow easily and have been cultivated widely in Southeast Asia as food (Austin, 2007).

Besides being consumed, this green leafy vegetable has been used as traditional medicine since at least A.D. 300 ago, and perhaps since 200 B.C. (Austin, 2007). Traditionally, *I. aquatica* has been used in treating nose bleed and high blood pressure (Perry, 1980), diabetes in Sri Lanka and China (Jayaweera, 1982; Malalavidhane et al., 2001), antidote for scorpion venom (Uawonggul et al., 2006), leucoderma, leprosy and fever (Ghani, 1989; Mamun et al., 2003) and many more.

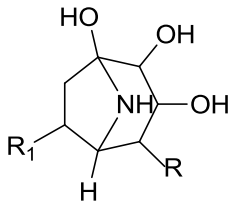
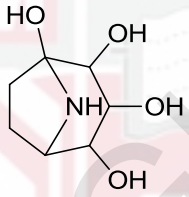
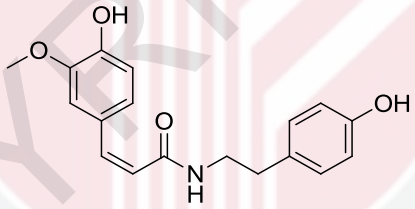
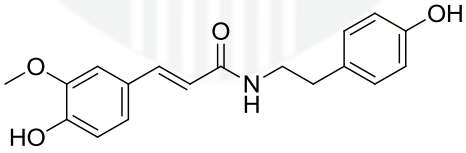
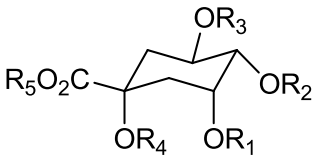
It is been reported that *I. aquatica* is rich with carotenoids and chlorophylls (Chen and Chen, 1992). The leaves contain adequate essential amino acids and thus it has potential for utilization as a food supplement (Prasad et al., 2008). Phytochemical studies of *I. aquatica* have revealed the presence of several phytochemical constituents such as carotenes (β-carotene, cryptoxanthin, lutein, lutein epoxide, violaxanthin and neoxanthin) (Tee et al., 1991), flavonoids (myricetin, quercetin, luteolin and apigenin) (Daniel, 1989) and some alkaloids (Tofern et al., 1999) (Table 2.3).

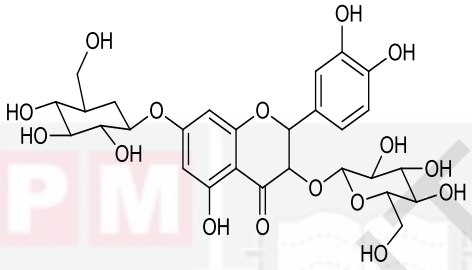
However, there are only a few studies have been conducted in this plant. This includes the inhibition of prostaglandin synthesis (Tseng et al., 1992), anti-venom (Uawonggul

et al., 2006), eye diseases, constipation (Jain et al., 2007), antioxidant and antimicrobial activity (Shekar et al., 2011) and hypoglycaemic effects (Malalavidhane, 2001).

An aqueous extract of *I. aquatica* has shown as effective as the oral hypoglycaemic drug tolbutamide in reducing blood sugar levels in rats (Malalavidhane et al., 2001; Malalavidhane et al., 2000). While isochlorogenic acids *a*, *b* and *c* that isolated from this species showed the promising result in inhibiting disaccharide-degrading enzyme activity. All of this information highlighted the potential of this plant as a food additive and as an alternative remedy in treatment and prevention of disease such as diabetes and obesity (Okudaira et al., 2005).

Table 2.3: Bioactive compounds from *Ipomoea aquatica* (Meira et al., 2012).

Substance	Structure	Activities
Notropane alkaloids		
calystegine B1 (1) calystegine B2 (2) calystegine C1 (3)	 1 R=H; R1=OH 2 R=OH; R1=H 3 R=R1=OH	Potent inhibitory activity toward rat lysomal β-glucosidase
calystegine B3		Moderate inhibitory activity toward rat lysomal β-glucosidase
Phenolic compounds		
N-cis-feruloyl tyramine		Inhibition of prostaglandin synthesis
N-trans-feruoloyl tyramine		Inhibition of prostaglandin synthesis
3,5-di-*O*-caffeoyl-quinic acid (isochlorogenic acid a) (4) 3,4-di-*O*-caffeoyl-quinic acid	 4 R_1=R_3=caffeoyl; R_2=R_4=R_5=H	Hypoglycemic, antimutagenic, antioxidant and inhibition of HIV replication.

(isochlorogenic acid b) (5)	5 R_1=R_2=caffeoyl; R_3=R_4=R_5=H 6 R_2=R_3=caffeoyl; R_1=R_4=R_5=H	
4,5-di-*O*-caffeoyl-quinic acid (isochlorogenic acid c) (6)		
Norisoprenoids, diterpene, isocoumarin and benzenoids		
3α,7β-*O*-D-diglycopyranosyl-dihydro-quercetin		Antioxidant, cytotoxic (*in vitro*).

2.3 Animal models in diabetes research

Over the years, animal model has been used to study the pathophysiology of diabetes. The used of the animal model in diabetes study started in the 1880s when Minkowski suggested the removal of the pancreas (pancreatectomies) to von Meering, who was working on fat absorption in the intestine of a dog (Rees & Alcolado, 2005; Srinivasan & Ramarao, 2007). The pancreas removal had led the dog to develop symptoms such as polyuria and polydipsia, and was found to have diabetes mellitus. Since then, studies have been conducted on rabbits and dogs followed with a notable finding from Banting and Best (1920) where they managed to isolate and purify the insulin from a dog (Rees & Alcolado, 2005). The most straightforward ways of studying the effects of hyperglycemia are by removing the pancreas partially or wholly (Srinivasan & Ramarao, 2007).

Generally, the large animal model is very costly and hard to manage. Therefore, small animals such as rodents are preferable than large animals (Chatzigeorgiou et. al, 2009; King, 2012). In addition, rodents such as rats and mice are known to have a shorter lifespan (about 22 months) that makes the experiments is suitable even for toxicology tests (Chatzigeorgiou et al., 2009). However, there are claims saying that using rodents may not adequately reflect the human situation. Thus, some researchers used this justification as a reason to use larger animals such as cats, pigs, and primates in diabetes studies. Nonetheless, the discovery of diabetes study using rodent as an animal model can be a platform or give a preliminary view of the disease before using the larger animals or even humans as the subject (Srinivasan & Ramarao, 2007; Zhao et al., 2011).

Also, there are non-surgical methods such as using chemical agents to induce hyperglycemia by damaging the pancreas (Srinivasan & Ramarao, 2007). Chemicals such as alloxan (ALX) and streptozotocin (STZ) are widely used in the animal model of diabetes studies since they are not only inducing hyperglycemia but also, the animal induced by them are exhibited diabetes complications (Chatzigeorgiou et al., 2009; King, 2012). Besides surgical and chemical induced animal models, there are also inbred animal models. These animals have homogenous genetic makeup are valuable especially in studies related to genetic factor in diabetes disease (Srinivasan & Ramarao, 2007). Large number of inbred models have been developed over the years with different traits and characterizations that added more information in the diabetes research (Rees & Alcolado, 2005; Srinivasan & Ramarao, 2007). However, it is still difficult to choose the right model as diabetes progression involved a complex interaction of multiple genes and also between genetic and environmental factors. The uses of rodents in T2DM research is discussed briefly below since the majority of diabetes patients are T2DM.

2.3.1 Animal models of T2DM

Similar to human, animals are used in T2DM because they exhibited the same symptoms and characteristics as T2DM patients such as insulin resistance and impaired

insulin secretion. Animal models in T2DM can be divided into two main groups namely spontaneous and non-spontaneously induced models. Spontaneous induced model is a model that involved animals with single (monogenic) or multiple genetic (polygenic) mutations (Srinivasan & Ramarao, 2007).

For this type of model, the animal can be either an animal with hereditary of certain traits such as hyperglycemia or obesity (e.g. ob/ob, db/db mice); or by selectively from non-diabetic outbred animals by repeated breeding over several general generation (e.g. Goto-Kazaki (GK) rat, Tsumara Suzuki Obese Diabetes (TSOD) mouse) (Rees & Alcolado, 2005; Srinivasan & Ramarao, 2007). Monogenic mutation can occur in animal with dominant gene (e.g. Yellow obese or KK/A^y mouse) or recessive gene (diabetic or db/db mouse, Zucker fatty rat); whereas polygenic mutation can be originated from its polygenic origin (e.g. New Zealand obese (NZO) mouse, Kuo Kondo (KK) mouse) (Srinivasan & Ramarao, 2007).

Experimentally or non-spontaneous induced model is an animal model that consist of non-genetic animal strains that originally normal or healthy under normal conditions (Bolzán & Bianchi, 2002). The price of animals in this model is cheaper than spontaneous animal model and widely available to researchers worldwide. In addition, this model is highly sought through due to its resemblance of pathogenesis in T2DM patients (Rees & Alcolado, 2005; Srinivasan et al., 2005). T2DM in this model can be induced through surgically (e.g. partial pancreatectomy), chemically (e.g. STZ, ALX or monosodium glutamate (MSG)), dietary manipulation (e.g. high fat fed (Hfd) or high fructose fed diet), or by any combination from those three (Figure 2.4) (Islam & Wilson, 2012).

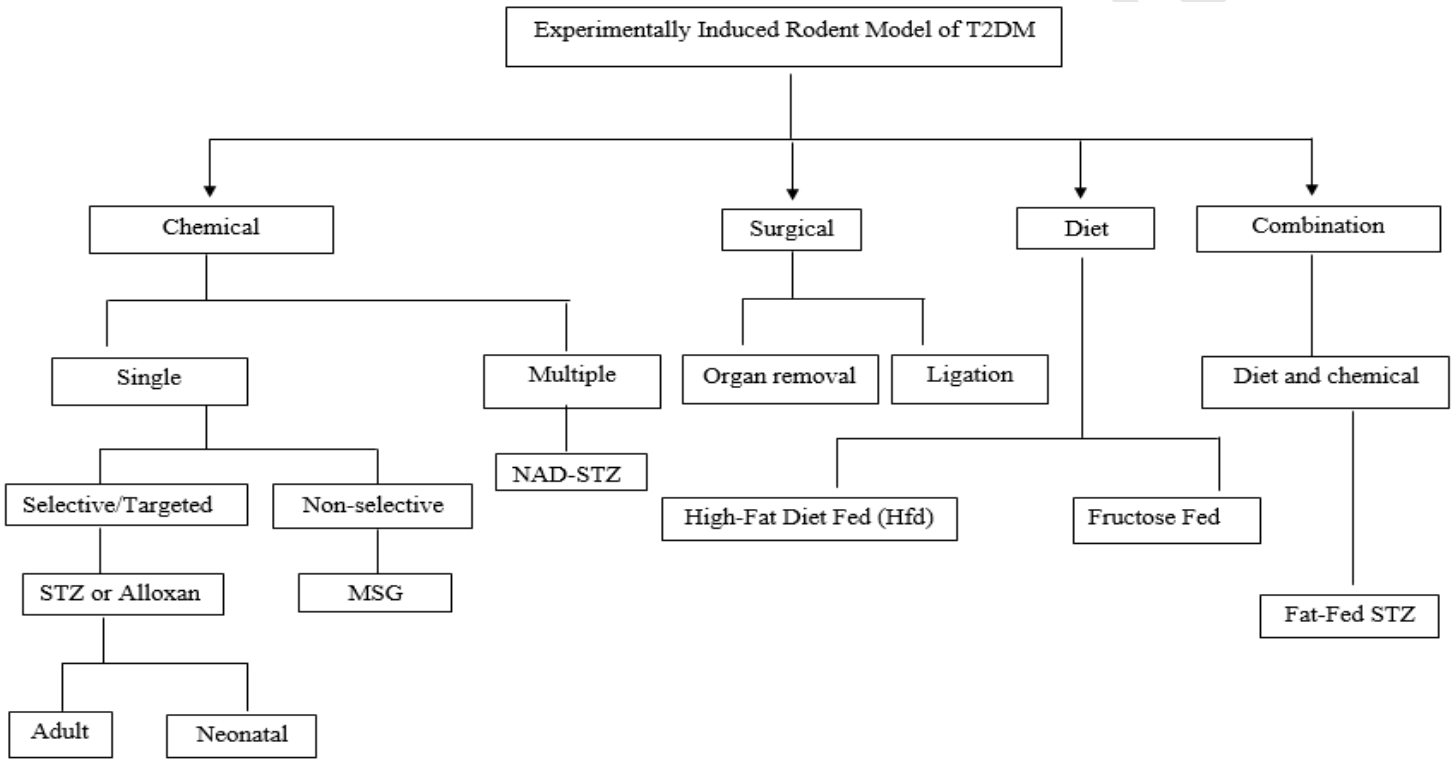


Figure 2.4: Summarize of the experimentally induced diabetic model in T2DM

2.3.1.1 Chemically induced model

2.3.1.2 Streptozotocin (STZ)–induced diabetic animal

Streptozotocin (STZ) is a broad spectrum antibiotic which is isolated from *Streptomyces achromogenes* and is nitrosourea derivatives. Diabetogenic action of STZ was first demonstrated in dogs and rats in 1963 by Rakieten and his associates (Rakieten et al., 1963). Similar to ALX, STZ functions by targeting the pancreatic β-cells through free radical involvement. The N-acetyl glucosamine analogue in STZ acts as a potent alkylating agent that disturbs the glucose transport, glucokinase activity, and breakdown multiple of DNA strands while deoxyglucose moiety facilitates transport across the cell membrane (Bolzán & Bianchi, 2002).

Development of hyperglycemia in STZ-induced animal is depending on several factors such as doses, age, and species (includes strain) (Srinivasan & Ramarao, 2007). STZ have a longer half-life as compared to ALX which is 15 minutes. The hyperglycemia sustained longer and incidents such as ketosis as well as mortality rate are lower in STZ induced animal (Jones et al., 1997). Both ALX and STZ models are widely used for pharmacological studies especially for the screening of the compounds or extracts for their capabilities as insulin mimetics, insulinotropic as well as other activities such as hypoglycemic (Islam & Wilson, 2012; Srinivasan & Ramarao, 2007).

2.4 Metabolomics

Down from genomics, transcriptomics and proteomics, metabolomics assesses end products of gene expression, and environmental influences along the pathway (Claudino et al., 2007). Metabolomic can be defined as an attempt to measure the complete metabolic response of living systems to any physical or environmental stimuli or alteration of genes (Oliver et al., 1998; Nicholson et al., 2005; Oliver, 2006; Bino et al., 2004). Metabolomics study is an interdisciplinary study that combines the knowledge of chemistry (e.g. analytical and organic chemistry, chemometrics), biology (e.g. genomics, transcriptomics), and statistics (informatics) that encompass almost all aspects of life sciences research (Chen et al., 2015).

The information that being collected from metabolomic studies can lead to better understanding of the global metabolic regulation of an organism and its symbiotic partners such as case plant phytochemical (Nicholson and Wilson, 2003). Metabolomics study has shown to have enormous potential when it can be applied to various kind of field such as subjects plant genotype discrimination (Taylor et al., 2002; Fiehn, 2002) toxicological mechanisms (Mally et al., 2007; Lindon et al., 2003), disease model (Nebert et al., 2006), and drug discovery (Aranibar et al., 2001; Fell, 2001; Nicholson et al., 2002).

2.4.1 The use of NMR in metabolomics studies

The most common used analytical tools in metabolomic are NMR, HPLC, LC-MS, and GC-MS. However, the NMR-based metabolomic approach is the most used due to the wide range and classes of metabolites identified, and the better understanding of metabolites interactions (Mediani et al., 2013). For instance, ^{1}H NMR of the biofluids spectra exhibits numerous metabolites signals, signifying the presence of many endogenous molecules such as amino acids, sugar, and lipids that involved in biochemical pathways. The huge numbers of metabolites are generated from NMR and can be useful in studying the metabolic changes. Nevertheless, the complication of the data obtained and their interpretation of the spectra involving the natural biological variation of a set of samples obligate the use of advanced statistical tools such as pattern recognition techniques (Shanaiah et al., 2008).

2.4.2 Multivariate data analysis

Prior to the analysis using multivariate data analysis (MVDA), the NMR spectra is preprocessed by aligning, phasing, and normalizing the data as well as binning them into small bins. The MVDA is useful for pattern recognition statistical tools in extracting information from the huge datasets of variables. Among these tools, principal component analysis (PCA), partial least squares (PLS), and hierarchical cluster analysis (HCA) are mostly used for assessing the metabolic changes and the biochemical disturbance (Diao et al., 2004; Zhao et al., 2010). The MVDA coupled with NMR approach is used to detect the possible single biomarkers or group of biomarkers in biofluids related to diseases, drug toxicity, or function of organs (Nicholson et al., 2002; Fernie et al., 2004).

2.4.3 Metabolomics in studying biological systems

Metabolomics studies in disease model are normally used in biofluids, cell, or tissue extracts to obtain information about the metabolic changes and associated complications. For the early detection of disease and therapy assessment, the identification of potential particularly early biomarkers in metabolites profiles of biofluids samples is important to control the disease and to find the treatment. The recent development in science field enables the simultaneous assessment of varied dataset of metabolites. This comprehensive analysis can develop the description of the general complications associated with disease conditions and finding the novel biomarkers as well as suggesting the biosynthetic pathways (Sébédio, 2009).

CHAPTER 3

EFFECT OF DIFFERENT DRYING TREATMENTS AND SOLVENT RATIOS ON PHYTOCHEMICAL CONSTITUENTS OF *Ipomoea aquatica* AND ITS CORRELATION WITH α-GLUCOSIDASE INHIBITORY ACTIVITY

3.1 Introduction

Ipomoea aquatica, which is locally known as kangkung, is a green leafy vegetable that belongs to the Convolvulaceae family, which comprises more than 1650 different species (Meira et al., 2012). Traditionally, *I. aquatica* has been used in treating hypertension, diabetes, fever, leucoderma and leprosy and can act as an emetic, diuretic, purgative, anthelmintic, and antipyretic agents (Chen & Chen, 1992; Malalavidhane et al., 2001; Prasad et al., 2005). Several pharmacological studies conducted on this plant have shown its potential use as anti-oxidant, anti-diabetic, anti-bacterial, anti-proliferative, anti-hyperlipidemic, anxiolytic and hepatoprotective agents (Hamid et al., 2011; Malalavidhane et al., 2000; Shekhar et al., 2011; Uawonggul et al., 2006).

In addition, it has also been reported to be rich in metabolites such as flavonoids, alkaloids, reducing sugar, amino acids, soluble carbohydrates, steroids, phenols, glycosides, β-carotene, saponins and tannins (Chen & Chen, 1992; Igwenyi et al., 2011; Miean & Mohamed, 2001). Phytochemical studies of this plant have isolated several compounds, such as quercetin, luteolin and isochlorogenic acid (Manvar & Desai, 2013; Okudaira et al., 2005; Prasad et al., 2005). Although numerous studies have been conducted on the bioactivity of *I. aquatica*, little is known regarding the correlation between its metabolites and their bioactivities.It is well known that recovery of bioactive metabolites from plant extracts depend largely on the efficiency of the extraction process and the composition of the metabolites in the plant cell matrix (Mediani et al., 2013).

Treatments such as heat, extraction solvents, solvent-to-sample ratio and time of the extraction have a profound effect on the extractable yield of bioactive metabolites. Drying is a process that eliminates excess moisture to avoid spoilage and to extend the shelf life of food (Mediani et al., 2012). Drying can involve heat, such as oven drying (OD), sun drying (SD), and microwave drying or not involve heat such as freeze drying (FD). Among these methods, air drying (AD) with or without sun exposure and OD are usually preferred due to their low cost and ease of handling (Kamiloglu & Capanoglu, 2015; Soysal & Öztekin, 2001). However, this process requires time and drying plants with high water content, such as leafy vegetables and herbs needs long time (Hossain et al., 2010). This can lead to deterioration in the quality of the samples, including changes in the taste and color and most importantly a loss of some bioactive metabolites (Slatnar et al., 2011). Furthermore, recovery of bioactive metabolites can be also affected by their uneven distribution in the cell matrix. To resolve this issues, ratios of different solvents with diverse polarities can also be used (Sultana et al., 2009). Nevertheless, it is still difficult to optimize the best solvent and drying method in

processing plant material. Therefore, it is important to investigate the effect of these two factors to determine the optimum conditions for extracting the plant having the most potential bioactivity with beneficial bioactive metabolites using an advanced approach.

Metabolomics is a competent approach for studying and evaluating changes of small molecule of biological system under various circumstances (Nicholson et al., 1999; Saito & Matsuda, 2010). In recent years, metabolomics has been widely used in several fields, such as agriculture, food chemistry, toxicology and medicine (Dunn, 2013; Wu et al., 2015). A lot of high throughput metabolomics methodologies are currently available, and among them, proton nuclear magnetic resonance (^{1}H NMR) is more preferred due to its robustness, reproducibility, and non-destructiveness (Nicholson et al., 2002; Nicholson et al., 1999). The ^{1}H-NMR is an emerging leading analytical tool in metabolomics studies due to a large number of identified compounds (Teng, 2013; Ward & Beale, 2006). The ^{1}H NMR coupled with multivariate analysis (MVDA) can be used for monitoring phytochemical changes in plants and can even correlate their metabolites with bioactivity (Mediani et al., 2013; Mediani et al., 2012).

Despite numerous studies that have already been conducted, there is still little information on phytochemical metabolites of *I. aquatica* and their variations based on different drying methods from sample extracted with various solvents types or/and at different ratios. Currently, polar solvents with various polarities are mostly used to extract phenolics and other bioactive compounds from plants. In addition, ethanol is not toxic solvent to be practically used in food industry. Therefore, the aims of this chapter were to determine the metabolic and bioactivity variation among the *I. aquatica* extracts that were dried with various methods and extracted with various ethanol ratios and to examine the correlation between the metabolites and bioactivities. The outcome of this study may benefit the future research on *I. aquatica* and may become an applied model for similar plant samples.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Deuterated methanol-*d4* (CD_3OD), non-deuterated KH_2PO_4, sodium deuterium oxide (NaOD), trimethylsilyl propionic acid-*d4* sodium salt (TSP), deuterium oxide (D_2O), and ethanol were obtained from Merck (Darmstadt, Germany). α-Glucosidase (EC 3.2.1.20) was purchased from Megazyme (Wicklow, Ireland) and Folin-Ciocalteu reagent, sodium carbonate, p-nitrophenyl-α-D-glucopyranoside, quercetin and glycine were supplied by Sigma Aldrich (St. Louis , USA).

3.2.2 Plant material

The *I. aquatica* plant was cultivated in an open field at the University Agricultural Park, Universiti Putra Malaysia in April 2012. A 14m x 2m plot was established and treated with organic fertilizer before leveling the soil and making holes at a distance of 10 cm. Then, 3-5 seeds were placed in each hole. Irrigation was performed twice a day using an automatic irrigation system. Weekly weeding was performed, as no herbicides and pesticides were used during the planting period. The plants were harvested four weeks after the germination date.

3.2.3 Sampling and extract preparation

Upon harvest, the plot was divided into six parts, and samples in each part were divided into three groups for the three different drying methods; air drying (AD), oven drying (OD)and sun drying (SD). Whole plants were collected and the roots were removed before being washed with tap water and cleaned with tissue paper. The AD samples were kept at room temperature (25 ºC) for one week. The SD samples were arranged on a drying rack and exposed to the sun for 10 h per day for 10 days. The OD samples were prepared by placing at a temperature of 40 ºC in a Memmert laboratory oven for 5 days. All samples were checked for complete dryness by taking their weights until constant before proceeds with further analysis. The samples were then ground to a fine powder using laboratory grinder. Then, 10 g of each sample was sonicated after mixing with 200 mL of different ethanol ratios (0, 20, 50, 80, and 100%). There was a total of 30 samples for each drying method, considering six replication of each group. Each extract was filtered through a Whatman filter paper no 1, dried using a rotary evaporator, and lyophilized to ensure no water remained in the crude extract. All of the extracts were kept at 4 ºC before further analysis.

3.2.4 *In vitro* assays

3.2.4.1 α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was carried out according to Deutschlander et al. (2009) with slight modification. The substrate solution (10 mM p-nitrophenyl-α-D-glucopyranoside) was prepared in 50 mM phosphate buffer (pH 6.5). Next, 10 µL of sample, 100 µL of 30 mM of phosphate buffer solution and 15 µL of α-glucosidase solution (3 U/mL) were pre-incubated in 96 well plates at 25 ºC for 5 min. After pre-incubation, 75 µL of the substrate was loaded into the well and the reaction mixtures incubated for another 15 min at 25°C. The reaction was stopped by adding 50 µL of 2 M glycine (pH 10). Absorbance readings were recorded at 405 nm. The percentage of inhibition (%) was calculated as follows:

% Inhibition = (Δ Ac – Δ Ae/ Δ Ac)

Where the Δ Ac is the difference in absorbance between the control (with enzyme) and the blank control (without enzyme). Δ Ae is the difference in absorbance between a sample (with enzyme) and the blank sample (without enzyme). The control was conducted in the same way as the experimental sample but with 5% DMSO. For the blank control and experimental samples, the enzyme solution and substrate were replaced by 30 mM phosphate buffer solution and glycine was replaced with distilled water. The percentage inhibition was plotted against the concentrations of each sample to determine the concentration required to inhibit 50% of the α-glucosidase enzyme.

3.2.4.2 Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu reagent following a modified method by Kähkönen et al. (1999). In this experiment, gallic acid was used as a standard. Briefly, 10 µL of each sample was transferred in triplicate to the 96-well microplate followed by 25 µL of the Folin-Ciocalteu reagent (50%, v/v), 25 µL of 20% (w/v) sodium carbonate solution and distilled water was added, attaining a final volume of 200 µL. After 1 h of incubation at room temperature, the absorbance was measured at 760 nm using spectrophotometer (SPECTRAmax PLUS). The same procedures were used for gallic acid as control compound with different concentration (15.6, 31.25, 62.5, 125, 250, and 500 µg/mL) and to plot the calibration curve. The total phenolic content was expressed as µg gallic acid equivalent (GAE) per mg extract (µg GAE/mg extract).

3.2.5 NMR measurement

For NMR measurement, a modified method by Mediani et al. (2012) was used for the preparation of NMR sample. In microcentrifuge tubes, 10 mg of each extract was weighed and dissolved in 0.375 mL of CD_3OD- solvent and 0.375 mL of KH_2PO_4 buffer in D_2O (pH 6.0), containing 0.1% TSP. The microcentrifuge tubes were then vortexed for 2-3 min and ultrasonicated for 30 min at room temperature to obtain a homogenous solution. This mixture was then centrifuged for 10 min at 13,000 rpm. Then, 0.6 mL of the supernatant was transferred to an NMR tube for ^{1}H-NMR analysis. The ^{1}H-NMR measurements were performed using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA), functioning at a frequency of 499.91 MHz and maintained at 26 °C. The NMR analysis was performed on all 90 samples. The acquisition time of each ^{1}H-NMR spectrum was 3.53 min, consisting of 64 scans with a width of 10 ppm. Additional support for identification was obtained using a two dimensional (2D) NMR-*J*-resolved.

3.2.6 Data processing and multivariate analysis

The ^{1}H-NMR and 2D NMR spectra were processed using Chenomx software (v. 5.1, Alberta, Canada) and ACD/NMR Processor Academic Edition software (v. 12.0, ACDLABS). Phasing and baseline corrections were performed on each spectrum with a consistent setting for all sample spectra, and TSP was set as the chemical shift index at

δ 0.00. The *I. aquatica* extracts spectral region (δ 0.50 to δ 10.00 ppm) was binned into a 0.04 ppm width that resulted in 231 integral regions per NMR spectrum. The regions containing water (δ 4.70-4.90) and methanol (δ 3.26-3.28) were excluded from the spectra in order to retain only the signals from the endogenous metabolites that were changed due to the drying process and solvent extraction. Multivariate data analysis was conducted on the normalized NMR dataset with the SIMCA-P software (v. 13.0, Umetrics, Umea, Sweden). Initially, principal component analysis (PCA) of the NMR spectral dataset was performed to visualize and discriminate the samples according to their metabolites, thus providing a general idea of the structure of the dataset. It was also used to observe possible outliers within the model. The partial least square (PLS) was then performed to identify the metabolites that significantly contribute to intergroup differentiation (Liu et al., 2014). Both PCA and PLS were conducted using Pareto scaling.

3.2.7 Statistical analysis

The results were presented as the mean ± standard deviation. The statistical significance of the difference was evaluated using one way ANOVA with Tukey's post hoc test $p < 0.05$ is considered significant. For the relative quantification purposes, only the metabolites in the best drying method and solvent were quantified based on the mean peak area of the ^{1}H NMR signals of interest after binning using Chenomx software (v. 5.1). The quality of the PLS model was expressed by the R^2X and Q^2 and the validity of the model tested using the permutation test and CV ANOVA. According to Eriksson et al. (2006), the R^2Y-intercept must be < 0.3-0.4, while the Q^2Y-intercept must be <0.05 and the p value of regression of CV ANOVA scores should be lower than 0.05 to be considered a valid model.

3.3 Results and discussion

3.3.1 Extraction yield of *I. aquatica* after different drying methods and solvent extractions

As indicated in our results, the yield percentage of the *I. aquatica* extracts ranged from 5 to 27 % depending on the extraction solvent and drying methods (Table 3.1). Both the drying method and the type of solvent extraction have significant effect ($p < 0.05$) on the extraction yield of *I. aquatica*. Although the highest yield can be found in the OD samples, there are no significant differences ($p > 0.05$) in its yield compared to the SD samples. Based on the drying method, the trend for the maximum yield is OD ≥ SD > AD. With regards to the effect of ethanol on the extraction yield, the highest yield was observed for water extracts, with the yield become decreasing as the ethanol ratio increases. This result indicates that a higher yield can be obtained as the polarity of the solvent increases. The result was in agreement with the work of Javadi et al. (2014) and Sultana et al. (2009), whom both found that aqueous solvents extract more than the absolute organic solvent.

Table 3.1: Percentage of extraction yield at different concentration of ethanol

Ethanol ratio (%)	% Yield		
	OD	SD	AD
100	6.37 ± 0.97^{dHI}	5.37 ± 0.02^{cI}	4.57 ± 0.04^{cI}
80	20.71 ± 0.55^{bcBCD}	19.62 ± 0.92^{bCDE}	10.84 ± 0.05^{bGH}
50	16.53 ± 2.26^{cDEF}	20.45 ± 0.92^{bBCD}	14.05 ± 0.22^{aFG}
20	25.10 ± 3.14^{abAB}	24.23 ± 1.00^{aABC}	10.23 ± 1.77^{bGH}
0	26.76 ± 1.32^{aA}	20.96 ± 1.91^{abBCD}	15.01 ± 0.40^{aEFG}

Different letter indicates there are significance differences ($p < 0.05$) using Tukey's test, where the lower case represent differences between solvent within the same drying method and upper case represent differences among the sample. Values are expressed as mean ± standard deviation (n=6). Whereas, AD: Air drying; SD: Sun drying, OD: Oven drying.

3.3.2 Effect of drying treatments and solvents on total phenolic content (TPC) and α-Glucosidase inhibitory assays

Table 3.2 shows the activity results of *I. aquatica* from the various drying methods and ethanol extractions. *I. aquatica* was tested in two *in vitro* assays; α-glucosidase inhibitory assay and total phenolic content (TPC) assay. The α-glucosidase inhibitory assay was used to screen the anti-diabetic potential of *I. aquatica*, while the TPC test was used to estimate the concentration of phenolic compounds in *I. aquatica*.

Table 3.2: TPC and α-glucosidase inhibitory activity of *I. aquatica* extracts

Ethanol ratio (%)	Drying Treatment	Total Phenolic Content (µg GAE/mg extract)	α-Glucosidase inhibitory assay	
			% α-Glucosidase inhibitory (500µg/ml)	IC_{50} (µg/ml)
100	OD	22.0 ± 0.7^{a}	84.0 ± 1.0^{a}	204.0 ± 59.0^{a}
	SD	12.0 ± 2.0^{de}	72.0 ± 1.0^{b}	373.0 ± 28.0^{b}
	AD	17.0 ± 0.7^{bc}	41.0 ± 0.9^{e}	nd
80	OD	14.0 ± 0.3^{cd}	41.0 ± 1.0^{e}	nd
	SD	13.0 ± 1.0^{de}	51.0 ± 0.1^{d}	518.0 ± 15.0^{c}
	AD	18.0 ± 0.6^{b}	20.0 ± 0.7^{g}	nd
50	OD	10.0 ± 0.7^{ef}	55.0 ± 0.8^{c}	468.0 ± 17.0^{d}
	SD	12.0 ± 3.0^{de}	51.0 ± 0.9^{d}	472.0 ± 16.0^{d}
	AD	14.0 ± 0.6^{cde}	22.0 ± 0.3^{f}	nd
20	OD	5.0 ± 0.2^{gh}	nd	nd
	SD	8.0 ± 0.1^{fg}	10.0 ± 0.8^{h}	nd
	AD	8.0 ± 0.2^{fg}	nd	nd
0	OD	2.0 ± 0.2^{j}	nd	nd
	SD	7.0 ± 0.1^{gh}	nd	nd
	AD	4.0 ± 0.5^{hi}	nd	nd
Quercetin				4.0 ± 0.1

Different letter in different column indicates there are significance differences ($p < 0.05$) using Tukey's test. Values are expressed as mean ± standard deviation (n=6). Whereas AD: Air drying; SD: Sun drying, OD: Oven drying and nd: not detected.

As can been seen in the summarized results in Table 3.2, the percentages of α-glucosidase inhibition for all samples is in the range of 10 to 84% for 500 µg/mL of extract. Overall, the α-glucosidase inhibition from the different drying methods and ethanol ratios are ranked in the following order: OD 100% ethanol > SD 100% ethanol > OD 50% ethanol > SD 80% ethanol ≥ SD 50% ethanol > OD 80% ethanol ≥ AD 100% ethanol > AD 20% ethanol > SD 20% water. Only a few extracts had an inhibitory effect of more than 50% and none of the AD extracts had α-glucosidase inhibitory activity of more than 50%. It was obvious that the polar solvents had the

most ability to extract most of the phytochemical constituents of *I. aquatica*. This might be due to most of the metabolites of *I. aquatica* are hydrophilic in the matrix, showing greater attraction to polar solvents.

Previously, Lin et al. (2012) found that there were insignificant differences in the α-glucosidase activity of *Rabdosia serra* leaf prepared by AD and SD methods. While Mediani et al. (2015) reported that AD extracts of *Phyllanthus niruri* exhibited higher α-glucosidase activity compared to OD extracts. Differences in our findings and previous reports are probably due to differences in plant composition (i.e metabolites, water content, and cell structure). We found that the best solvent for α-glucosidase inhibitory activity was absolute ethanol, as no activity was detected in the water extracts at 0.5 mg/mL. These findings were consistent with the previous study by Javadi et al. (2014) in which the absolute ethanol extracts were found to have the best α-glucosidase inhibitory activity.

The results of the TPC (Table 3.2) assay for AD were in the range of 18.0 ± 0.6 to 8.0 ± 0.2 µg GAE/mg. The results of the other drying method such as SD were from 12 ± 2 to 8.0 ± 0.2 µg GAE/mg and OD from 22.0 ± 0.7 to 5.0 ± 0.2 µg GAE/mg. These results were inconsistent with the observed α-glucosidase inhibitory activity, where the OD sample had the highest TPC value (22.0 ± 0.7 µg GAE/mg). The second best sample in the TPC assay was from the AD group followed by the SD samples. In general, higher TPC contents can be found in the higher ethanol content extracts (50 ~ 100%) rather than in the higher aqueous solvents. The difference in the TPC contents indicates that drying and solvent extraction play a major role in extracting phenolic compounds.

3.3.3 Identification of metabolites in *I. aquatica* by 1D and 2D NMR

The representative ^{1}H NMR spectra of *I. aquatica* is presented in Fig. 3.1. A total of 21 metabolites were identified, including amino acids (valine, alanine, proline, aspartate, *N*,*N*-dimethylglycine and tyrosine), organic acids (acetic acid, glutaric acid, succinic acid, citric acid, malic acid and formic acid), and sugars: (fructose, sucrose, β-glucose and α-glucose; phenolics: luteolin). The other metabolites include quercetin derivatives, chlorogenic acid derivatives, 1-*O*-ethyl-*β*-glucoside and choline), which are listed in Table 3.3. Identification of the metabolites was based on information from software (Chenomx Profiler), online database (HMDB: http://www.hmdb.ca/) (Wishart et al., 2009), and from the literature (aided by 2D NMR *J*-resolved (Figure 3.2)). Amino acids such as valine, alanine, proline, aspartate and *N*,*N*-dimethylglycine were mainly identified by their aliphatic region (δ 0.5-3.5). Additionally, organic acids, including acetic acid (δ 1.93), glutaric acid (δ 2.30), succinic acid (δ 2.45), citric acid (δ 2.54), ethylene glycoside, and 1-*O*-ethyl-β-glucoside were also successfully identified. In general, the signals from the carbohydrate region especially from δ 3.0 to 5.5, highly overlapped with other signals, making the identification of metabolites a complex process (Kim et al., 2010). However, we ascertained the identity of several metabolites, such as malic acid (δ 4.29), sugars; fructose (δ 4.01), sucrose (δ 4.18 and δ 5.41), β-glucose (δ 4.59), α-glucose (δ 5.19), the amino acid; proline (δ 3.32) and choline (δ 3.22). In the region from δ 5.5 to 8.5, metabolites such as quercetin derivatives (δ 6.89,

δ 7.46), chlorogenic acids derivatives (δ 3.86, δ 3.54, δ 6.28 and δ 7.66), luteolin (δ 6.51, δ 6.92 and δ 7.49), formic acid (δ 8.47) and tyrosine (δ 6.48) were present. Confirmation of quercetin derivatives and chlorogenic acid derivatives was performed by comparison of their ^{1}H-NMR spectral data with those in the literature.

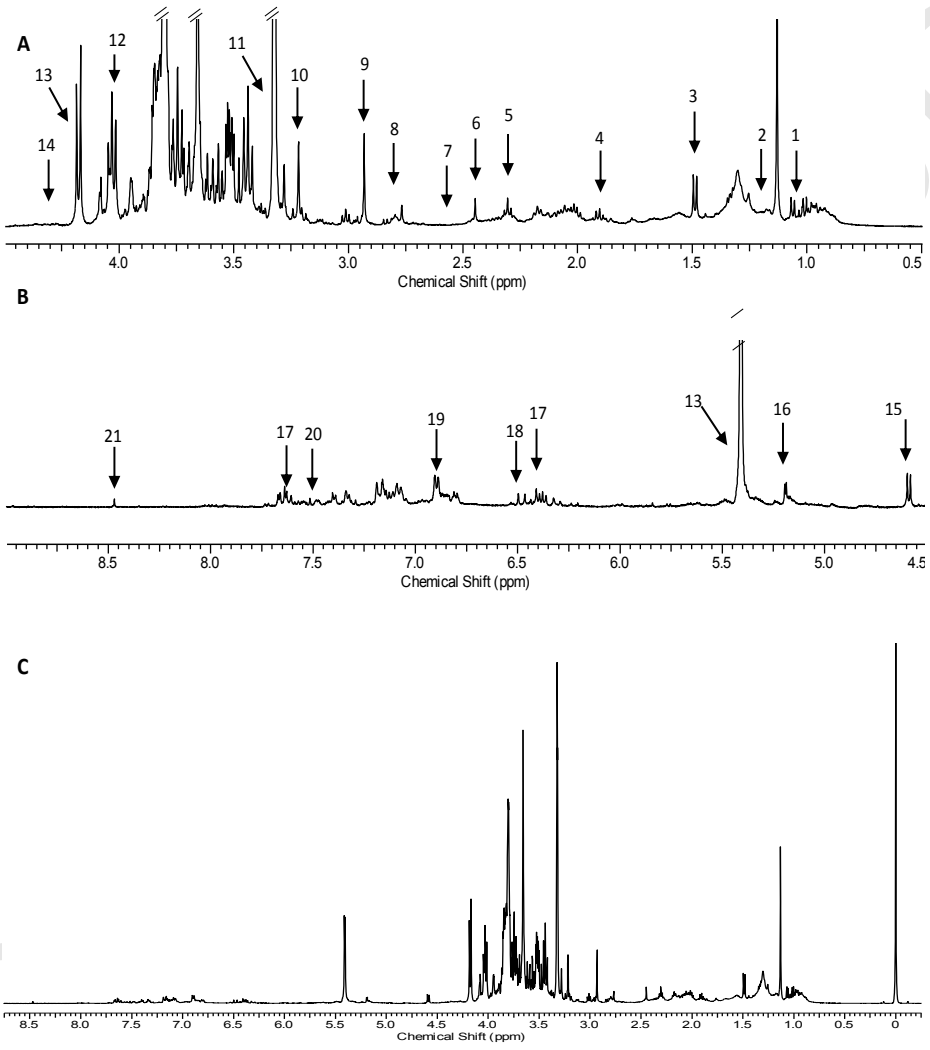


Figure 3.1: Representative ^{1}H NMR spectra of *I. aquatica*. (A) Spectra from 0.5 to 4.5 ppm (B) Spectra from 4.5 to 8.7 ppm (C) Full spectra from 0 to 8.6 ppm. Identified signals: 1, Valine; 2, 1-*O*-ethyl-β-glucoside; 3, Alanine; 4, Acetic acid; 5, Proline; 6,Glutaric acid; 7, Succinic acid; 8, Citric acid; 9, Aspartate; 10, *N*,*N*-Dimethylgycine; 11, Choline; 12, Fructose; 13, Sucrose; 14, Malic acid; 15, β-glucose; 16, α-glucose; 17, Chlorogenic acid derivatives;18, Tyrosine; 19, Luteolin; 20, Quercetin derivatives and 21, Formic acid.

Table 3.3: Assignment of ^{1}H NMR spectra peaks of *I. aquatica* (s: singlet, d: doublet, t: triplet, m: multiplet, and dd: doublet of doublet)

Metabolites	^{1}H (ppm) and multiplicity
Amino acids	
Valine (Kim et al., 2010)	1.06 d (7.0 Hz), 1.01 d (7.0 Hz)
Alanine (Kim et al., 2010)	1.49 d (7.5 Hz)
Proline (Kim et al., 2010)	2.04 m, 3.32 m
Aspartate (Yang et al., 2012)	2.82 dd (16.0 Hz, 8.0 Hz)
N,*N*- Dimethylglycine (Wishart et al., 2009)	2.93 s
Tyrosine (Fotakis et al., 2013)	6.48 d (15.5 Hz)
Organic acids	
Acetic acid (Yang et al., 2012)	1.93 s
Glutaric acid (Yang et al., 2012)	2.30 t (7.0 Hz)
Succinic acid (Kim et al, 2010)	2.45 s
Citric acid (Kim et al., 2010)	2.54 d (16.5 Hz)
Malic acid (Yang et al., 2012)	4.29 dd (9.0 Hz, 3.5 Hz)
Formic acid (Kim et al., 2010)	8.47 s
Sugars	
Fructose (Fotakis et al., 2013)	4.01 m
Sucrose (Mediani et al., 2012)	4.18 d (8.5 Hz), 5.41 d (4.5 Hz)
β-glucose (Mediani et al., 2012)	4.59 d (8.0 Hz)
α-glucose (Mediani et al., 2012)	5.19 d (3.5 Hz)
Phenolic compounds	
Quercetin derivatives (Mediani et al., 2012)	6.89 d (8.5 Hz), 7.46 dd (8.0 Hz, 4.0 Hz)
Chlorogenic acid derivatives (Maulidiani et al., 2012)	3.54 dd (10.0 Hz, 4.0 Hz),3.86 dd (11.5 Hz, 4.5 Hz), 6.28 d (16.0 Hz), 7.66 (16.0 Hz)
Luteolin (Gohari et al., 2010)	6.51 s, 6.92 d (8.0 Hz), 7.49 d (8.0 Hz)
Others	
1-*O*-ethyl-β-glucoside (Fotakis et al., 2013)	1.19 t (7.0 Hz)
Choline (Kim et al., 2010; Mediani et al., 2015)	3.22 s

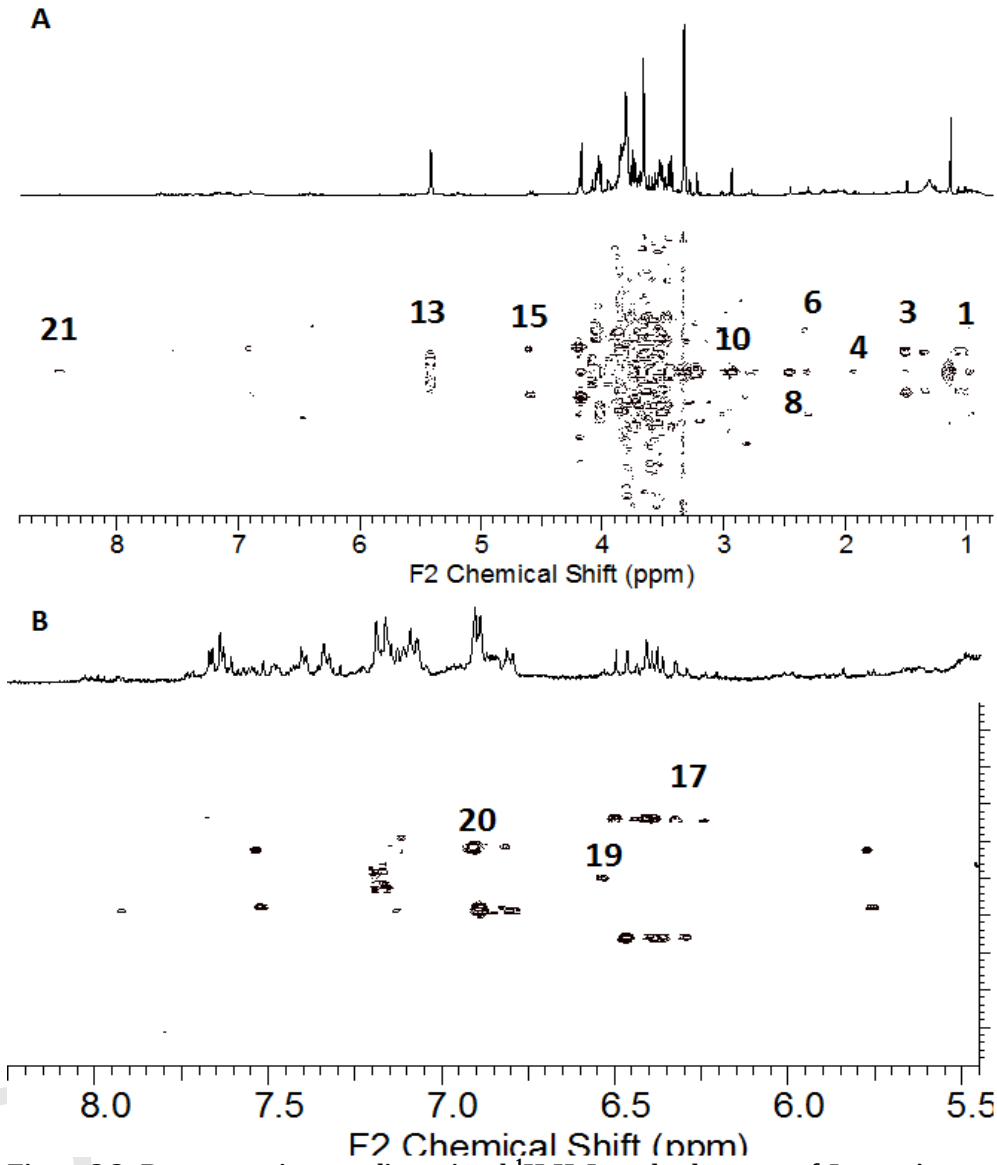


Figure 3.2: Representative two-dimensional ^{1}H-H *J*-resolved spectra of *I. aquatica* extract. (A) Spectral region from δ 0.70 – 8.80. (B) Spectral region from δ 5.45 – 8.25. The label represent signals; 1, Valine; 3, Alanine; 4, Acetic acid; 6, Glutaric acid; 8, Citric acid; 10, *N*,*N*-dimethylglycine; 13, Sucrose; 15, β-glucose; 17, Chlorogenic acid derivatives; 19, Luteolin; 20, Quercetin derivatives and 21, Formic acid.

3.3.4 Classification of *I. aquatica* extracts in different drying methods and ethanol concentration

In this study, variations in the extracts were achieved by performing PCA, a pattern recognition method from MVDA. The PCA model was used to provide an overview of all of the extracts that have been prepared by different drying processes and extract solvents (Figure 3.3). A model with 13 principal components (PCs) was achieved, with the first two components covering 54% of the variance. This model was considered a good model because the goodness of fit, $R^2X_{(cum)}$ and $Q^2_{(cum)} > 0.5$ and the differences between $R^2X_{(cum)}$ and $Q^2_{(cum)} < 0.3$, indicating that each of the subjects (i.e extract samples) equally and uniformly contributes to the observed group separation (Wheelock and Wheelock, 2013).

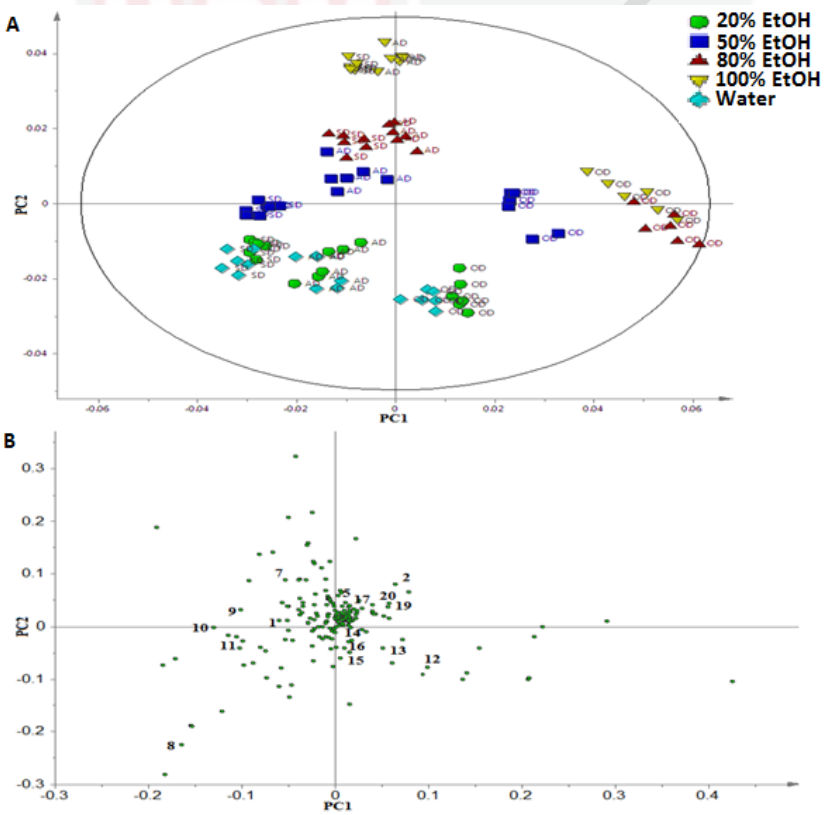


Figure 3.3: PCA derived score plot (A) and loading scatter plot (B) of *I. aquatica* extracts in different drying methods (OD: oven dry, SD: sun drying and AD: air drying) and solvent ratios (100% EtOH: 100% ethanol, 80% EtOH: 80% ethanol, 50% EtOH: 50% ethanol, 20%j EtOH: 20% ethanol and water). Different number on loading scatter represents: 1, Valine; 2, 1-*O*-ethyl-β-glucosidase; 5, Proline; 7, Succinic acid; 8, Citric acid; 9, Aspartate; 10, N,N-Dimethylglycine; 11, Choline; 12, Fructose; 13, Sucrose; 14, Malic acid; 15, β-glucose;16, α-glucose; 17, Chlorogenic acid derivatives ; 19, Luteolin; and 20, Quercetin.

From the score plot (Figure 3.3A), a clear cluster can be seen from PC1, separating the OD samples from the AD and SD samples. This cluster was due to variation in metabolites between the drying samples (Figure 3.3B). Metabolites such as 1-*O*-ethyl-β-glucoside, fructose, sucrose, tyrosine, malic acid, chlorogenic acid derivatives and quercetin derivatives were higher in the OD samples compared to the SD samples. In addition, metabolites such as citric acid, aspartate, *N*,*N*-dimethylglycine, choline, succinic acid, glutaric acid, proline and alanine were higher in the SD and AD samples. A distinguish separation can also be observed from the PC2 score plot (Figure 3.3A) between the SD and AD samples extracted with higher ethanol ratios (50, 80, 100%). The loading score (Figure 3.3B) shows that the higher ethanol extracts in the SD and AD groups contain higher level of phenolics (luteolin, chlorogenic acid derivatives and quercetin derivatives), amino acids (proline, alanine and valine) and organic acids (succinic acid and glutarate). The 20% ethanol and water extracts contain many sugars (α-glucose, β-glucose, sucrose and fructose) and organic acids such as citric acid and malic acid.

There were distinguishing features of the OD samples extracted with different ethanol ratio that can be seen in PC2 and they were all gathered at the positive side of the plot by PC1). However, due to the greater effect of the drying treatment, the metabolites contributing to the separation of different ethanol ratios extracts were very difficult to be assigned. Therefore, we performed another PCA specifically on the OD samples to see if there are any differences with the different ethanol ratios. A PCA model with three components in which the first two components explained 76.10% of the variation is shown in Figure 3.4. The effect of the ethanol solvent ratios can be clearly seen from the separation in PC1, where a higher ethanol ratio extract was located on the positive side as opposed to the extracts obtained with higher water contents (20% ethanol and water). Metabolites such as phenolics (luteolin, chlorogenic acid derivatives and quercetin derivatives), sucrose and 1-*O*-ethyl-β-glucoside were high on the positive side, while the negative side had more sugar groups (α-glucose and β-glucose), amino acids (proline, alanine and glutarate) and organic acids (citric acid and malic acid).

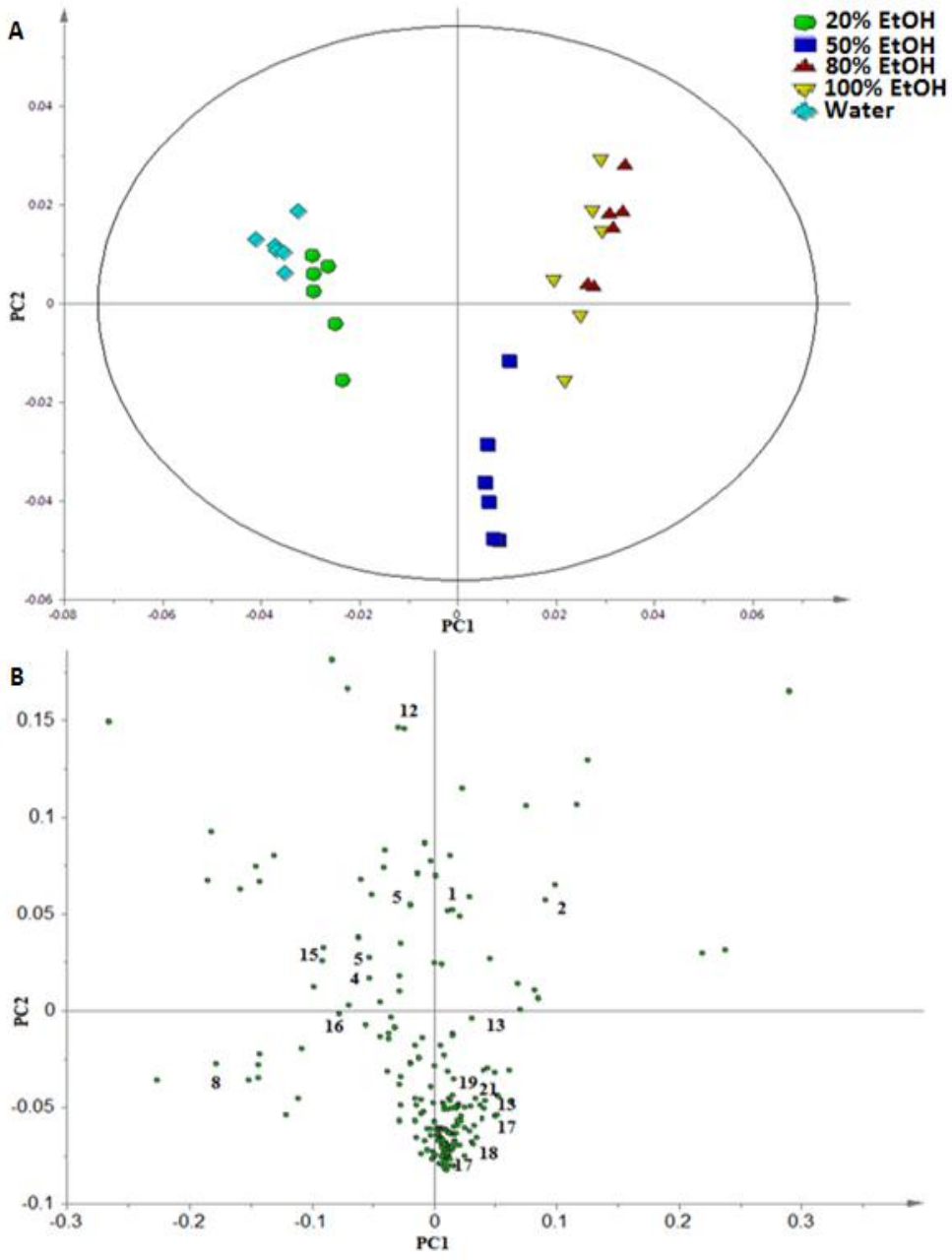


Figure 3.4: PCA derived score plot (A) and loading scatter plot (B) for oven dry. Different number on loading scatter represents: 1, Valine; 2, 1-*O*-ethyl-β-glucoside; 4, Acetic acid; 5, Proline; 6,Glutaric acid; 8, Citric acid; 12, Fructose; 13, Sucrose; 15, β-glucose; 16, α-glucose; 17, Chlorogenic acid derivatives; 18, Tyrosine; 19, Luteolin and 21, Formic acid.

A clear cluster between the different drying methods (Figure 3.3A) shows that drying treatments were the main factor that responsible for the metabolite compositions of the extracts. Various studies had reported on the changes in phytochemical constituent level in plant matrices when exposed to drying treatment (Hossain et al., 2010; Mediani et al., 2012). However these changes varied from plant to another as each plant has a different cell matrix composition and the presence of metabolites in the extracts were depending on their thermal stability (Hossain et al., 2010). Mediani et al. (2012) previously noted that there were differences in phenolic contents (flavonoids and flavonoid glycoside) in AD compared to OD in *Cosmos caudatus* samples. In that study, he found that the level of flavonoids and flavonoid glycoside was higher in the AD group. However, in the present study, the levels of phenolic compounds (quercetin and chlorogenic acid derivatives) were the highest in OD samples compared to AD and SD samples.

A lower concentration of phenolic compounds in AD and SD samples may be due to the residual degrading enzymes, polyphenol oxidase (PPO) and peroxidase (POD) in the AD and SD samples (Hossain et al., 2010; Mediani et al., 2012). Long exposure to atmospheric oxygen during AD and SD processing can also be a reason of affecting the samples bioactivity as they promote oxidation of the sample (Hossain et al., 2010). In addition, there have been several reports noted that UV radiation, especially from UV-B may affect plant components such as nucleic acid, amino acid, lipid, protein and even plant growth regulators (Zhang & Björn, 2009). However, the differences in metabolites concentration, especially between SD and AD in the absolute ethanol extract (Figure 3.6), can only be seen in valine, aspartate, *N*,*N*-dimethylglycine and fructose whereas no significant differences ($p > 0.05$) in chlorogenic acid derivative and quercetin derivative concentrations as compared to others. All of this indicates that the UV irradiation may not solely contribute to the differences, but the combination of solar radiation with an external factor such as environment factor such as air humidity may attribute to the differences.

Apparently, the OD samples have exceptionally high contents of fructose in the 80 and 100% ethanol extracts and sucrose in 50% ethanol extract compared to the other drying methods (Figure 3.3 and 3.4). This result was in agreement with the work of Mediani et al. (2012), who found that the OD samples have high sucrose content compared to FD and SD samples. The OD process may not only remove water and inactivate degradation enzymes, but it can also cause the plant to become brittle and fragile, resulting in cell wall rupture during the grounding steps of the sample preparation procedure (Hossain et al., 2010). The combination of these rupture cells with a suitable solvent in the extraction process may enhance the release of phenolic content and other metabolites, such as fructose and sucrose into the solvent, thus resulting in higher metabolites content in the OD samples.

With regards to solvent effects, a higher level of sugar groups (α-glucose and β-glucose) and organic acids (citric acid and malic acid) can be found in the low ethanol solvents in the OD, AD and SD samples. High phenolic contents were also observed in the high ethanol solvents for all drying methods. The differences in metabolite contents may be due to the abilities of the solvents to extract metabolites. The capability of the solvents to extract metabolites is largely dependent upon the solubility of the

metabolite and the interaction between the solvent and the plant cell matrix or the cell permeability (Sultana et al., 2009). High phenolic compounds can be extracted with ethanolic aqueous mixture (100, 80 and 50%) as they capable to extracts both non-polar and polar compound.

3.3.5 Correlation between *in vitro* assays and metabolites variation among the samples treated with three drying methods

The PLS is a supervised method in MVDA. Previously, PLS has been successfully used to correlate the bioactivity with phytochemical constituents (Mediani et al., 2012; Mediani et al., 2015). In PLS, the data are separated into two blocks, the X-block represents the predictor variables (NMR chemical shift of the metabolites) and the Y-block represents the response variables (bioactivity) (Wheelock and Wheelock, 2013). By using PLS, the relationship between bioactivities ($1/IC_{50}$ of α-glucosidase inhibitory and total phenolic content) and the metabolites in the ethanol extracts can be established.

Based on the *in vitro* assays, the best drying method (the OD samples different solvent ratios) and the best solvent, the 100% ethanol extracts (in different drying methods) were selected for further analyses for identification of metabolites that contributes to the bioactivity of the samples. Three clusters can be clearly seen from the bi-plot of 100% ethanol extracts in different drying method (Figure 3.5A). The OD samples were strongly correlated with the α-glucosidase inhibitory activity and TPC. Meanwhile, the SD and AD samples were projected at the negative side of the PC1. The metabolites that contribute to this separation were quercetin derivatives, chlorogenic acid derivatives, sucrose and fructose. All of these metabolites were located close to the OD samples, α-glucosidase inhibitory and TPC assays compared to AD and SD samples. The AD and SD were separated due to their high of organic acid and amino acid contents. This result was in agreement with our *in vitro* result where the AD and SD samples had lower bioactivity compared to the OD samples. As discussed earlier, the better result in the OD samples may be because of the shorter drying time and deactivation of residual degrading enzymes that can alter metabolites in *I. aquatica*.

In addition, bi-plot of different ethanol solvents of the OD samples (Figure 3.5B) showed two clear clusters in PC1. The higher ethanol ratio extracts (50-100%), are located in the positive region of the bi-plot, while the 20% ethanol and water extracts cluster together at the negative side of the bi-plot. The clustering of the extracts was due to metabolites such as sucrose, valine and chlorogenic acid derivatives. In line with previous *in vitro* assay result, this clustering suggests that these metabolites were the one, contributing to the bioactivity of the samples especially in the absolute ethanol extracts. Additionally, high amounts of organic acids (citric acid and acetic acid), *N*,*N*-dimethylglycine, α-glucose and β-glucose were found in the 20% ethanol and water extracts. As been previously observed, both extracts had the lowest *in vitro* activity (Table 3.2) and this might be an indication extracts with higher concentration of these metabolites might not be active in α-glucosidase inhibitory and TPC. Both of these PLS models showed excellent goodness-of-fit ($R^2Y_{(cum)} > 0.8$) and good predictive ability ($Q^2_{(cum)} > 0.8$). A permutation test with 100 permutation was also conducted to validate

the PLS models and all these models demonstrated good validity with the R^2Y-intercept < 0.3-0.4 and Q^2Y-intercept < 0.05. The CV ANOVA values for all of these models were also significant ($p < 0.05$).

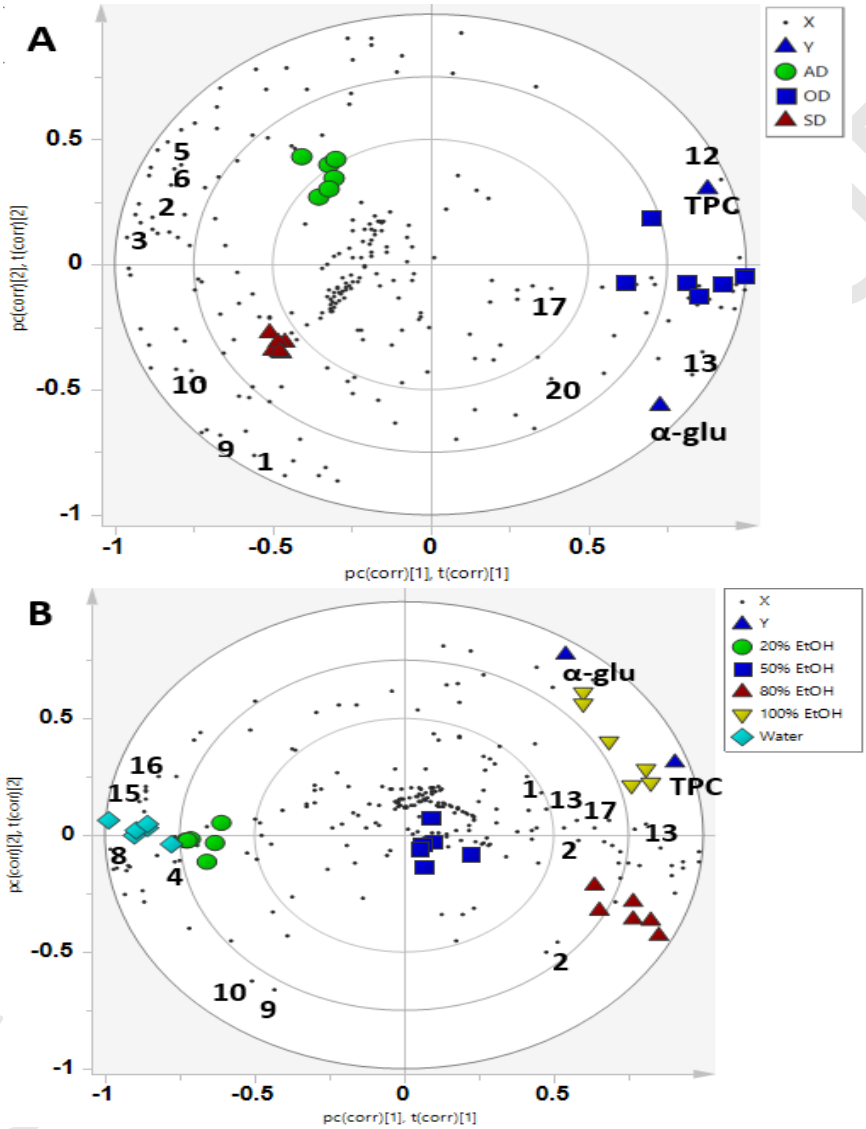


Figure 3.5: The biplot obtained from PLS describing the variation between the drying methods (A), solvent ratio (B) and the correlation between the metabolites and the bioactivities. Where; OD: oven dry, SD: sun dry, AD: air dry, 100% EtOH: 100% ethanol, 80% EtOH: 80% ethanol, 50% EtOH: 50% ethanol, 20% EtOH: 20% ethanol and water). Different number represents: 1, Valine; 2, 1-O-ethyl-β-glucoside; 3, Alanine; 4, Acetic acid; 5, Proline; 6, Glutaric acid; 8, Citric acid; 9, Aspartate; 10, *N*,*N*-dimethylglycine; 12, Fructose; 13, Sucrose; 15, β-glucose; 16, α-glucose; 17, Chlorogenic acid derivatives and 20, Quercetin derivatives.

3.3.6 Relative quantification of metabolites

Based on the PLS, we conducted relative quantification of the metabolites in the absolute ethanol extracts (from different drying methods) and the OD samples (in different solvents). We found, differences in the metabolite concentrations that affect discrimination among the samples and contribute to the activities. Representative of each drying methods OD, SD and AD in absolute ethanol (Figure 3.6A) and at different ethanol ratios in the OD samples (Figure 3.6B) shows all of the relative quantification of the metabolites that were previously identified in Figure 3.5.

As seen in Figure 3.6A, a high concentration of fructose and sucrose can be detected in the OD samples compared to the SD and AD samples. A lower concentration of metabolites such as 1-*O*-ethyl-β-glucoside, alanine, acetic acid, glutaric acid, aspartate and *N*,*N*-dimethylglycine was also observed. The OD samples had high content of chlorogenic acid derivative and quercetin derivative compared to SD and AD samples. Based on the solvent ratios in the OD samples (Figure 3.6B), there were significant differences ($p < 0.05$) in the metabolites of the absolute ethanol extracts including acetic acid, citric acid, aspartate and *N*,*N*-dimethylglycine compared to the 20% ethanol and water extracts. This result was in agreement with previous PLS results (Figure 3.5B), that suggested these metabolites might negatively contribute to the overall bioactivity of this sample.

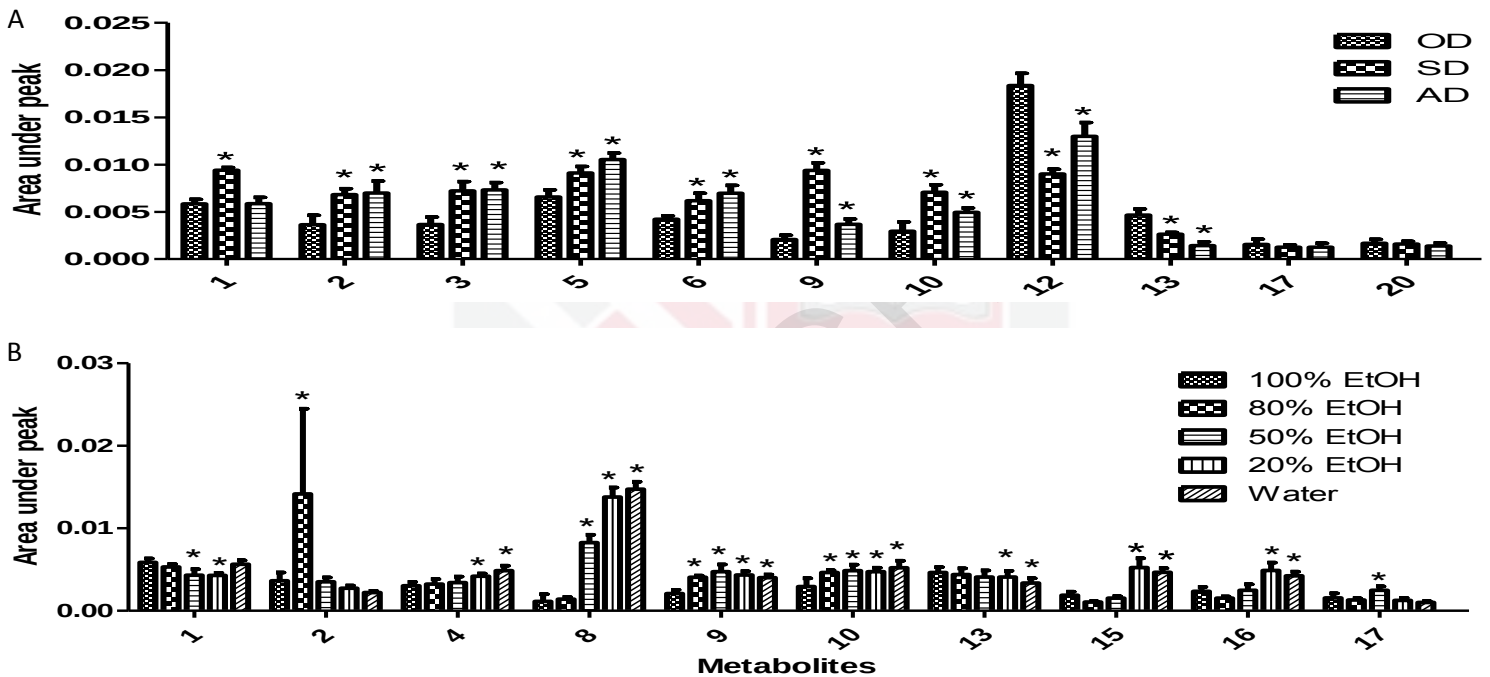


Figure 3.6: Relative quantification of metabolites in *I. aquatica* extracts: (A) Different drying method of absolute ethanol extracts and (B) Different ethanolic solvent ratio of OD samples. Different number represents: 1, Valine (δ 1.01); 2, 1-O-ethyl-β-glucoside (δ 1.19); 3, Alanine (δ 1.49); 4, Acetic acid (δ 1.93); 5, Proline (δ 2.04); 6,Glutaric acid (δ 2.30); 8, Citric acid (δ 2.54); 9, Aspartate (δ 2.82); 10, *N,N*-dimethylgycine (δ 2.93); 12, Fructose (δ 4.01); 13, Sucrose (δ 4.18); 15, β-glucose (δ 4.59); 16, α-glucose (δ 5.19); 17, Chlorogenic acid derivatives (δ 7.68) and 20, Quercetin derivatives (δ 7.48). (*) at A indicate significant differences ($p < 0.05$) as compared to OD, while (*) at B significant differences ($p<0.05$) as compared to absolute ethanol. Values are expressed as mean ± standard deviation (n=6).

3.4 Conclusion

^{1}H NMR metabolomics was successfully used to classify *I. aquatica* extracts from different solvent ratios and different drying processes. The best ethanol ratio for all drying methods was absolute ethanol. The overall best drying method was OD followed by SD. Among the 21 metabolites identified, chlorogenic acid derivatives, quercetin derivatives, fructose and sucrose were the metabolites that might contribute the most to the bioactivity of the samples. A bi-plot of PLS analysis shows a strong correlation between the metabolites of the OD samples with the *in vitro* assays (total phenolic content and α-glucosidase inhibition activity). This study suggests that both solvent and drying method play a significant role in the distribution and content of metabolites extracted from *I. aquatica*. Therefore in the next chapter, the optimized *I. aquatica* extract was evaluated for its anti-diabetic properties using an animal model.

CHAPTER 4

METABOLITE VARIATION IN LEAN AND OBESE STREPTOZOTOCIN (STZ)-INDUCED DIABETIC RATS VIA ^{1}H NMR-BASED METABOLOMICS APPROACH

4.1 Introduction

Diabetes Mellitus (DM) is generally characterized by a high glucose content in blood or hyperglycemia, which can cause long-term complications in the targeted cells (e.g., β-cells) and organs. Thus, it often causes other metabolic syndrome diseases such as cardiovascular (CVD), hypertension and microcirculatory disorder (Diao et al., 2014; Rubin, 2012). Generally, DM can be categorized into two main types based on the requirement for insulin, where Type 1 (T1DM) is insulin-dependent diabetes mellitus, and Type 2 (T2DM) is non-insulin-dependent DM (Sebedio et al., 2009). T1DM is an autoimmune disease, where the body itself (immune system) destroys the insulin-producing β-cells of the pancreas, whereas T2DM is more likely a combination of insulin insensitivity, inadequate function of the β-cell mass and an unhealthy lifestyle, such as obesity (Rubin, 2012).

For a better understanding of DM, many studies have been conducted on animal models (Chatzigeorgiou et al., 2009; Rees & Alcolado, 2005; Srinivasan & Ramarao, 2007). Both spontaneous or genetically modified models and non-spontaneous models (experimentally induced diabetes model) are currently available, in which non-spontaneous/experimentally-induced diabetes models are much preferred due to their lower cost, ease of maintenance and close relationship to human diabetes (Diao et al., 2014; Zhang et al., 2008). For the non-spontaneous diabetes model, the diabetic condition can be induced by diet/nutrition (e.g., high sucrose and high fat content diet), chemically (i.e., streptozotocin (STZ) or alloxan), surgically (by removing organs such as pancreas) or any combination of these methods (Srinivasan & Ramarao, 2007).

Streptozotocin (STZ) is a broad-spectrum antibiotic and a powerful alkylating agent that is known to induce diabetes by selectively destroying the pancreatic β-cells that produce insulin. This hormone regulates the glucose levels in the body (Diao et al., 2014; Zhang et al., 2008). Therefore, the STZ-induced diabetic rat model has been used widely in diabetes studies, where it provides additional information on the mechanisms and complications that are associated with the disease. A high dose of STZ (50 - 60 mg/kg) has been used for inducing the T1DM model, while a low dose of STZ (15 - 35 mg/kg) together with a modified diet, such as a high fat fed diet (Hfd), are used to mimic the obese-diabetic state in humans or T2DM (Srinivasan et al., 2005; Zhang et al., 2008). Although numerous diabetes studies have been conducted in the STZ-induced rat model, there is still a lack of information about the differences and similarities in the metabolites and pathways involved in the T1DM and T2DM rat models. Furthermore, there is still confusion about these models, especially whether the high-dose STZ-induced rat can represent the T1DM model (Skovsø, 2014). Hence, it is

important to investigate this matter as it can provide useful information, especially in the drug-targeted research field.

Recently, a combination of high resolution nuclear magnetic resonance (NMR) spectroscopy and multivariate analysis (MVDA) known as NMR-based metabolomics has been applied in diabetes studies (Diao et al., 2014; Zhang et al., 2008; Zhao et al., 2010). By using NMR-based metabolomics, any changes in metabolites will be detected, and this information is crucial as it can provide a view of how the biochemical pathways work (Diao et al., 2014; Zhang et al., 2008). Studies conducted by Zhang et al. (2008), Diao et al. (2014) and Zhao et al. (2010) managed to highlight several metabolites and pathways that are affected in diabetic rats. However, Zhao et al. (2010) noted that the strain might affect the separation as the control group (Wistar rats) is a different strain from the diabetic rats (GK rats). No previous study has focused on comparing between lean diabetic rats (ND+STZ) and obese diabetic rats (OB+STZ). Therefore, this chapter aimed to identify disturbed metabolic pathways in STZ-induced diabetic rats, ND+STZ and OB+STZ and to confirm which type of diabetes animal model these groups belong to by using the ^{1}H NMR-based metabolomics approach. In addition, in this chapter the similarities and dissimilarities in terms of metabolites among the groups and also the relationship between obesity and diabetes were also highlighted.

4.2 Materials and methods

4.2.1 Solvents and chemicals

Non-deuterated potassium phosphate monobasic (KH_2PO_4), sodium deuterium oxide (NaOD), trimethylsilyl propionic acid-d4 sodium salt (TSP), deuterium oxide (D2O), diethyl ether and a standard drug (metformin) were purchased from Merck (Darmstadt, Germany). Streptozotocin (STZ), carboxymethyl cellulose (CMC), and sodium azide were obtained from Sigma–Aldrich (St. Louis, MO, USA).

4.2.2 Experimental design for *in vivo* study

Approval for the animal study was obtained from the Institutional Animal Care and Use Committee (IACUC) of the Faculty Medicine and Health Sciences, Universiti Putra Malaysia (IACUC No. UPM/FPSK/PADS/BRUUH/00470, Appendix A). This approval was also used for the animal study in the Chapter 5. Forty five male Sprague-Dawley rats (five-week-old), weighing 100-150 g were used in the study. The rats were housed individually in plastic cages with stainless steel covers and put in acclimatization conditions for 1 week at room temperature (26-28 ºC) under a 12 h dark/12 h light cycle. During the acclimatization period, the rats were fed with normal rat chow and tap water ad libitum.

4.2.2.1 Obesity induction

Following acclimatization, the rats were divided randomly into 2 groups (Figure 4.5) in which the lean/control group (ND, *n* = 20) was fed with normal rat chow/normal diet (Nd) from Gold Coin, Malaysia and the other group, obese (OB, *n* = 25), with a modified high fat diet (Hfd). This Hfd contained 49% fat, 32% carbohydrate and 19% protein for the total energy, which consists of fat from ghee (milk fat) and corn oil (Jalil et al., 2008). The Nd contained 14% fat, 61% carbohydrate and 25% protein for the total energy. These diets were continuously given throughout this study, even after the induction of obesity (week 12). The final number of rats for ND was *n* = 10, and for OB, it was *n* = 9 after separating the rats for diabetes induction.

4.2.2.2 Diabetes induction

After the induction of obesity, ten out of twenty rats from the lean (ND) and fifteen out of twenty five of obese (OB) rats were then randomly chosen and injected with streptozotocin (STZ) intravenously. For this purpose, STZ was freshly prepared in citrate buffer (0.1 M, pH 4.5). The dosage of STZ for the lean diabetic group (ND+STZ) was 60 mg/kg, while for the obese diabetic group (OB+STZ), it was 25 mg/kg (Zhang et al., 2008; Srinivasan et al, 2010). Three days after the injection, the blood glucose was measured by tail prick using a glucometer (Accu-chek Performa, Roche, Mannheim, Germany). The final number of rats for both the ND+STZ and OB+STZ groups was *n* = 5 after separating the rats out for the metformin sensitivity test.

4.2.2.3 Sensitivity towards T2DM oral drugs

To test their sensitivity towards the T2DM oral anti-diabetic drug metformin, the rats from the lean diabetic group (ND+STZ, *n* = 10) and obese diabetic group (OB+STZ, *n* = 10) were once again randomly divided into other groups: treated lean diabetic (ND+STZ+MET, *n* = 5) and treated obese diabetic (OB+STZ+MET, *n* = 5). Metformin (150 mg/kg) suspended in 0.03% (w/v) carboxymethyl cellulose (CMC) was administered for 4 weeks in designated diabetic groups (Salemi et al., 2016; Akinola et al., 2012). The lean/control (ND), obese (OB) and non-treated diabetes group (ND+STZ and OB+STZ) were given 1 ml of 0.03% (w/v) CMC, as CMC was used as a vehicle in this study (Jalil et al., 2008; Adam et al., 2010).

4.2.2.4 Blood and urine collection

Urine was collected at weeks 12, 13 and 17 by placing the rats individually in plastic metabolic cages with urine bottles containing sodium azide (0.1%). The blood was collected into sterile tubes through retro orbital (week 12 and week 13) and cardiac puncture (week 17) after anesthetizing the rats with diethyl ether. Serum samples for the biochemical test were obtained by high-speed centrifugation for 6000 g for 10 min

at 4 ºC. All urine and serum samples were labeled carefully and kept at -80 °C until used. A summarized experimental diagram is shown in Figure 4.1.

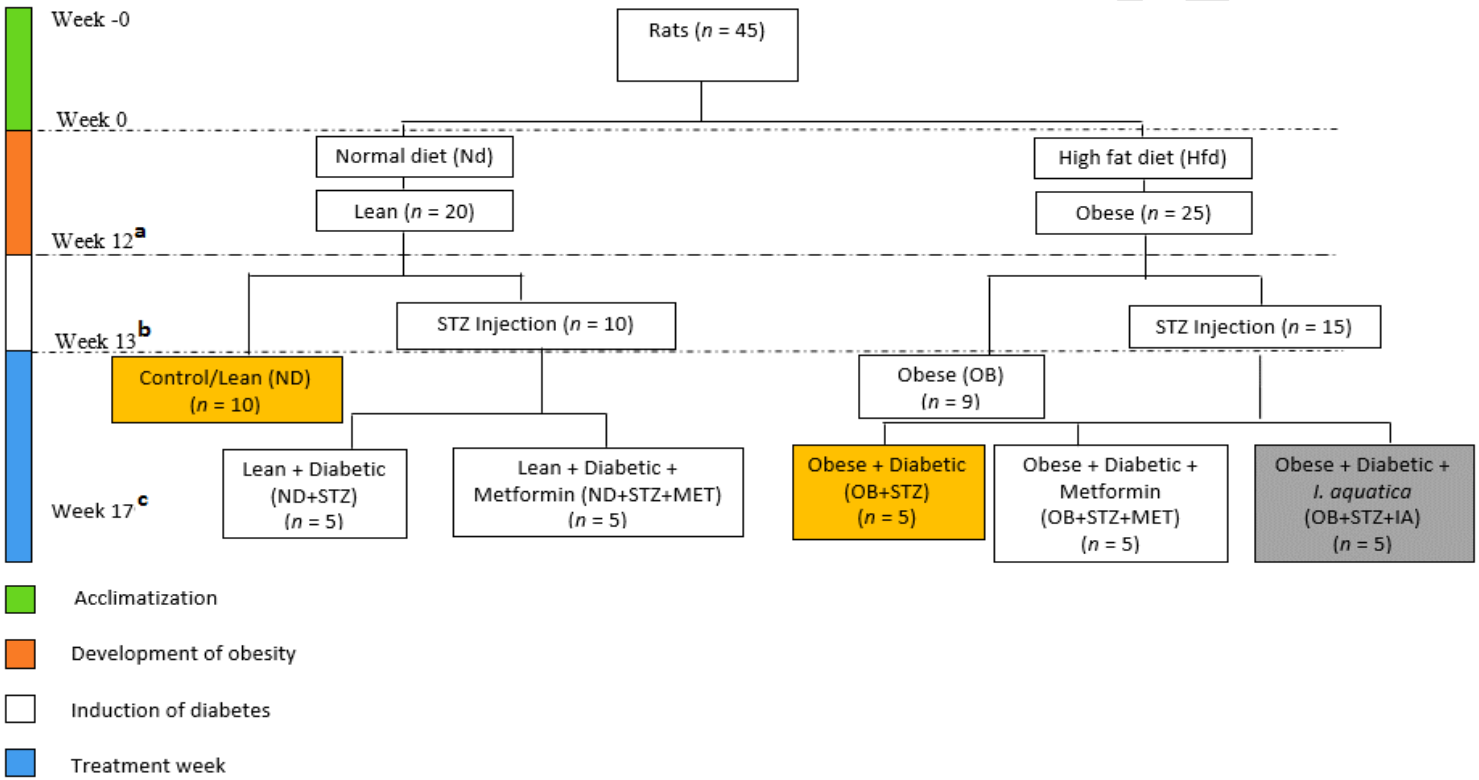


Figure 4.1: Overall schematic diagram for animal experimental design. Superscript letters a, b and c represent the time for biofluids collection. Where (a) and (b): Urine collection, glucose tail prick and blood collection and (c): Urine and blood collection. Boxes in white represent the groups used for optimization of type 1 and type 2 diabetes models (Chapter 4). While, boxes in yellow represent the control groups that are both used in Chapter 4 and 5. Lastly, the box in grey represent the group treated with *I. aquatica* extract (Chapter 5).

4.2.3 Biochemical analyses

For biochemical analyses, the serum glucose, total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured using an automatic biochemical analyzer (Hitachi 902, Roche, Germany). The values are expressed as the mean ± SD.

4.2.4 Biofluids preparation and NMR measurements

Frozen urine samples were thawed and centrifuged at 3000 rpm before the supernatant was used. In microcentrifuge tubes, 0.40 mL of supernatant was mixed with 0.20 mL of phosphate buffer solution (0.308 g of KH_2PO_4 in 25 mL of D_2O, pH 7.4, containing 0.1% TSP). All samples were vortexed for 1 min to obtain a homogenous mixture. The mixture (0.5 mL) was transferred to an NMR tube and subjected to ^1H NMR analysis. The ^1H NMR measurements were performed using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA) functioning at a frequency of 499.91 MHz and maintained at 26 °C. The NMR analysis was performed for all of the urine samples. For the urine samples, a standard water-suppressed one-dimensional NMR was obtained using the PRESAT sequence (64 scans, acquisition time 206 s). The identification of the metabolites was assisted by the information from the software and online databases (Chenomx Profiler and Human Metabolome Database (HMDB): http://www.hmdb.ca/) (Wishart et al., 2009) as well as from the literature data.

4.2.5 Data processing and multivariate analysis

The phasing and baseline corrections were conducted using the Chenomx software (v. 5.1, Alberta, Canada) with a consistent setting for all sample spectra. Phasing and baseline corrections were performed manually on each spectrum, and TSP was set as the reference peak at δ 0.00. Integration, which resulted in 231 integral regions per spectrum, was conducted on the urine sample spectral region from δ 0.50 to 10.0. The regions containing the water (δ 4.57-4.95) and urea (δ 5.55-5.95) were excluded from all the spectra. However, for the urine NMR spectra for weeks 13 and 17, another dataset were attained by excluding the glucose region (δ 3.15-3.24, δ 3.31-3.52, δ 3.63-3.9, δ 5.15-5.24), and each of these points were normalized again to the sum row to compensate for the variation in the total volumes.

The multivariate data analysis was conducted on the preprocessed NMR dataset with the SIMCA-P software (v. 13.0, Umetrics, Umea, Sweden). First, the principal component analysis (PCA) was performed to visualize and discriminate the groups according to their metabolites, thus giving the general idea within the dataset. The partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-DA) models of the NMR spectral data were also carried out for class discrimination and potential biomarker selection (Eriksson et al., 2006). Potential biomarkers in PLS-DA were determined based on the combination of loading scatter plot and variable importance in the projection (VIP) values using a VIP > 1 (An

et al., 2015), while in OPLS-DA, the determination of potential metabolites was made based on selection from the S-plot and loading column plot (Zhang et al., 2008). Validation of the PLS-DA model was described by the cross-validation parameter Q^2, indicating the predictability of the model, and R^2 for the total explained variation for the X matrix. In the OPLS-DA model, a CV-ANOVA value < 0.05 was required for the model to be considered valid (An et al., 2015; Song et al., 2013).

4.3 Results

4.3.1 Basal parameters (weight and biochemical)

Basal parameters, including weight and biochemical data are shown in Figure 4.2. Obese rats (OB) showed a significant increase in weight after 12 weeks of high fat feed (Hfd). About 20% of increment in weight was observed in OB rats compared to normal/lean rats (ND). After the STZ induction (week 13), the rat weight began to decrease as the weeks progressed, especially in the diabetes groups: obese diabetes (OB+STZ) and lean diabetes (ND+STZ). These result, is in line with previous study by Jalil et al. (2008), where they noted an increased in weight in rats after 12 weeks of Hfd (~10% weight increment) and decreased in weight after diabetes induction. Meanwhile for biochemical data, the diabetes group, especially the obese diabetes group (OB+STZ and OB+STZ+MET), showed increased levels of serum glucose, low density lipoprotein (LDL), total cholesterol (TC) and triglyceride (TG) after diabetes induction in week 13. All of these results suggested that STZ induction/progression affected the weight and metabolism (lipid and carbohydrate) of the diabetes group.

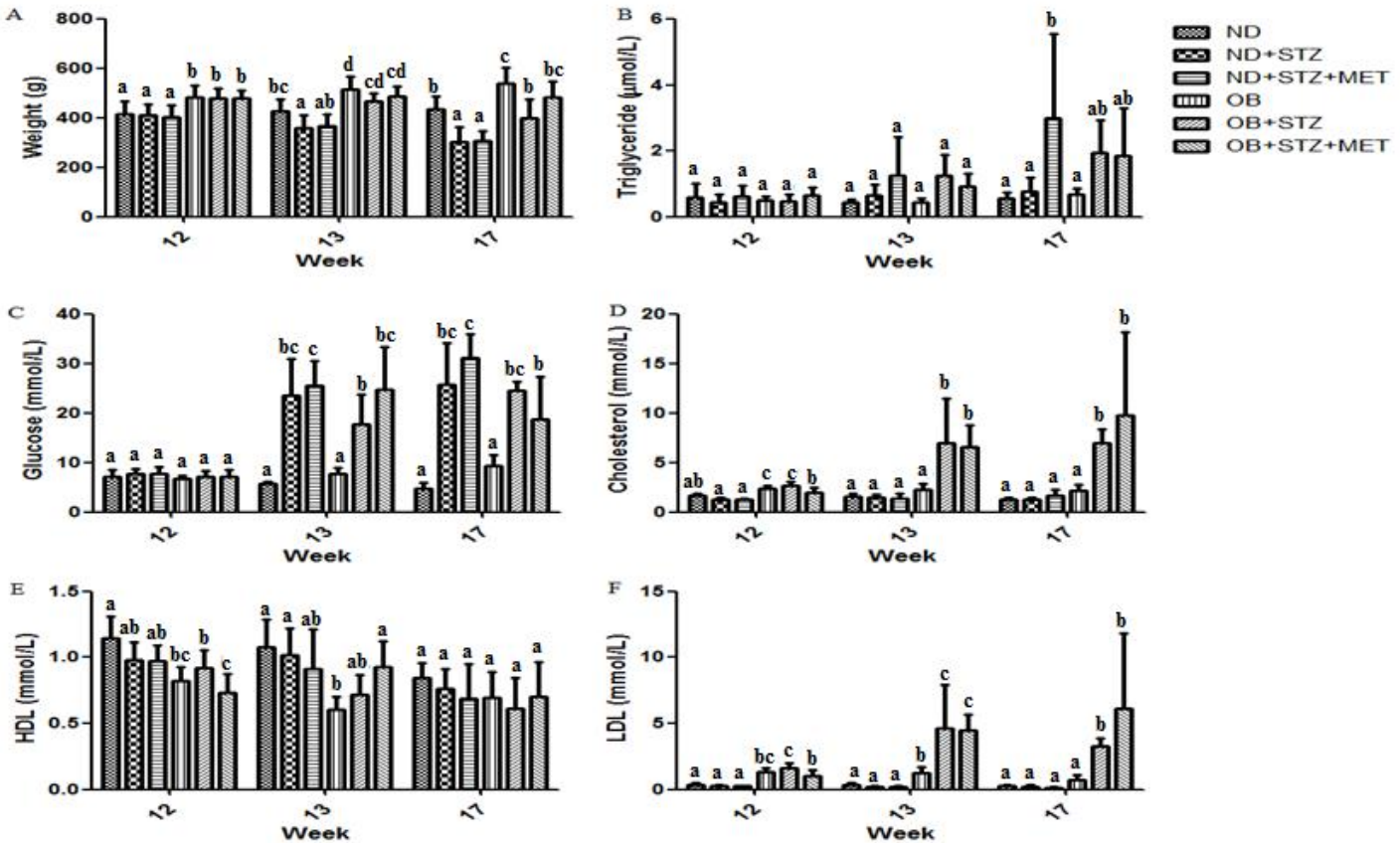


Figure 4.2: Basal parameter for lean (ND), obese (OB), lean diabetes (ND+STZ) and obese diabetes (OB+STZ) for week 12, 13 and 17. Where; A : Weight, B,C, D and F : Clinical biochemistry. Different letters indicate significant differences using one way ANOVA and Tukey post hoc test based on their respective weeks.

4.3.2 ^{1}H NMR spectral analysis of urine samples

Figure 4.3 shows a comparison of the general ^{1}H NMR spectra of urinary samples from normal (ND), obese (OB), lean diabetic (ND+STZ) and obese diabetic (OB+STZ) rats. The urinary spectra were assigned according to Chenomx NMR Suite 5.1 (Chenomx Inc., Edmonton, Canada), HMDB databases and published assignments. A total of 27 metabolites (excluding water and TSP) from various groups of endogenous metabolites ranging from amino acids such as leucine, taurine and lysine, organic acids such as formate, acetate and allantoin and tricarboxylic cycle (TCA) intermediates such as succinate, citrate and 2-oxoglutarate were identified (Table 4.1). Note that exceptional levels of glucose can be observed in the STZ-induced diabetic rats compared to the ND rat spectra (Figure 4.3).

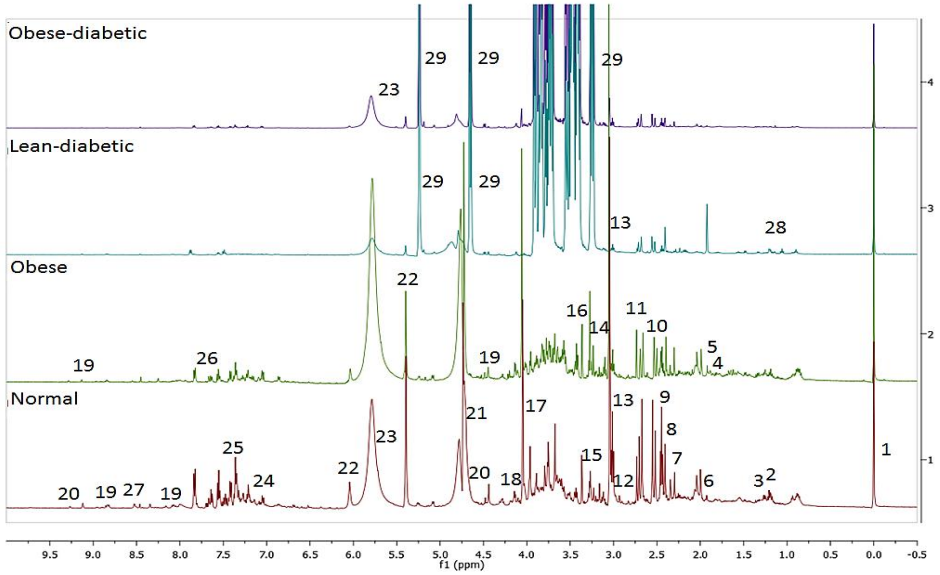


Figure 4.3: Representative of 500 MHz ^{1}H NMR spectra of urine samples for normal (ND), obese (OB), lean diabetic (ND+STZ) and obese diabetic (OB+STZ). Label: 1, TSP; 2, Isoleucine; 3, Lactate; 4, Leucine; 5; Leucine/Lysine; 6, Acetate; 7, Acetoacetate; 8, Succinate; 9, 2-oxoglutarate; 10, Citrate; 11, Dimethylamine; 12, *N*,*N*-Dimethylglycine; 13, Creatine; 14, Carnitine; 15, Taurine; 16, Methanol; 17, Creatinine; 18, Kynurenine; 19, Trigonelline; 20, 1-Methylnicotinamide; 21, Water; 22, Allantoin; 23, Urea; 24, Tryptophan; 25, *N*-phenylacetylglycine; 26, Hippurate, 27, Formate, 28, 3-Hydroxybutryrate (3-HB) and 29; Glucose.

Table 4.1: Assignment of ^{1}H NMR spectra peaks of rat urine spectra

Metabolites	δ ^{1}H (ppm) and multiplicity
Isoleucine	1.25(m), 1.46(m), 0.92(t)
Lactate	4.12(q), 1.33(d)
Leucine	1.76(m)
Lysine	1.86(m)
Acetate	1.90(s)
Acetoacetate	2.28(s)
Succinate	2.37(s)
2-oxoglutarate	2.44(t), 3.00(t)
Citrate	2.33(d), 2.50(d), 2.68(d)
Dimethylamine (DMA)	2.72(s)
N,*N*-Dimethylglycine (DMG)	2.91(s)
Creatine	3.04(s)
Carnitine	3.22(s)
Taurine	3.23(t), 3.41(t)
Methanol	3.35(s)
Creatinine	3.04(s), 4.04(s)
Kynurenine (KYN)	4.12(t) , 7.44(t)
Trigonelline	8.84(m), 9.12(s)
1-Methylnicotinamide (MNA)	4.47(s), 8.89(d), 8.95(d), 9.27(s)
Allantoin	5.38(s)
Urea	5.80(s)
Tryptophan (TRP)	7.20(t)
N-phenylacetyglycine	7.35(m), 3.74(s)
Hippurate	7.54(t), 7.63(t), 7.81(d)
Formate	8.44(s)
3-Hydroxybutyrate (3-HB)	1.12(d), 2.3(dd), 2.4(dd), 4.14(m)
Glucose	5.23(d), 4.64(d), 3.89(m), 3.53(dd), 3.48(t), 3.44(t), 3.40(dd), 3.23(dd)

4.3.3 Multivariate analysis of spectral data

4.3.3.1 Obesity development

Multivariate data analysis (MVDA) was conducted on the normalized NMR spectra dataset to identify the metabolic perturbations associated with diabetes. First, the unsupervised method PCA (Figure 4.4) was conducted on the 1D ^{1}H NMR urine spectra after the 12th week of Hfd feeding for a general overview of the sample (sample) distribution. A PCA with a goodness of fit $R^2X = 0.68$ and $Q^2_{(cum)} = 0.34$ was achieved. From the overall view, there was a trend that separates between the two groups.

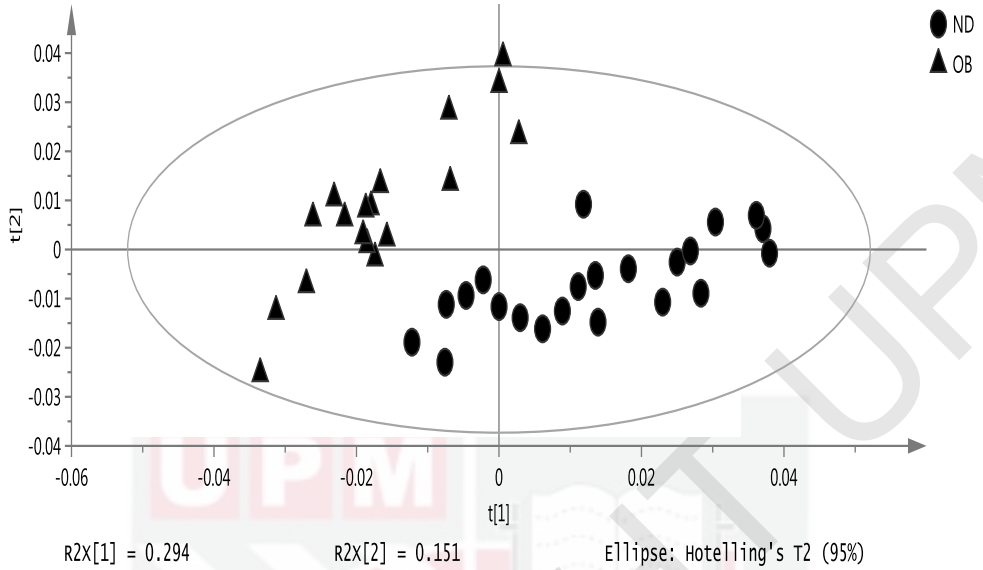


Figure 4.4: PCA scores of lean (ND) group against obese (OB) group at week 12.

For further and clearer separation, another MVDA method (OPLS-DA) was conducted. A distinctive discrimination can be seen between the OB group and the ND group from OPLS-DA on the 12th week (Figure 4.5A), with goodness of fit $R^2X_{(cum)}$ = 0.56, $R^2Y_{(cum)}$ = 0.98, $Q^2_{(cum)}$ = 0.94. This model was considered as a good model because the goodness of fit, $R^2Y_{(cum)}$ and $Q^2_{(cum)}$ were > 0.9, and the difference between $R^2Y_{(cum)}$ and $Q^2_{(cum)}$ was < 0.3, indicating that each of the subjects (i.e., urine samples) equally and uniformly contributes to the observed group separation (Wheelock & Wheelock, 2013). This result was in agreement with the previous body weight result from week 12, which proves that obesity induction is successful. The clear discrimination in OPLS-DA indicates that diet plays important roles in the development of obesity.

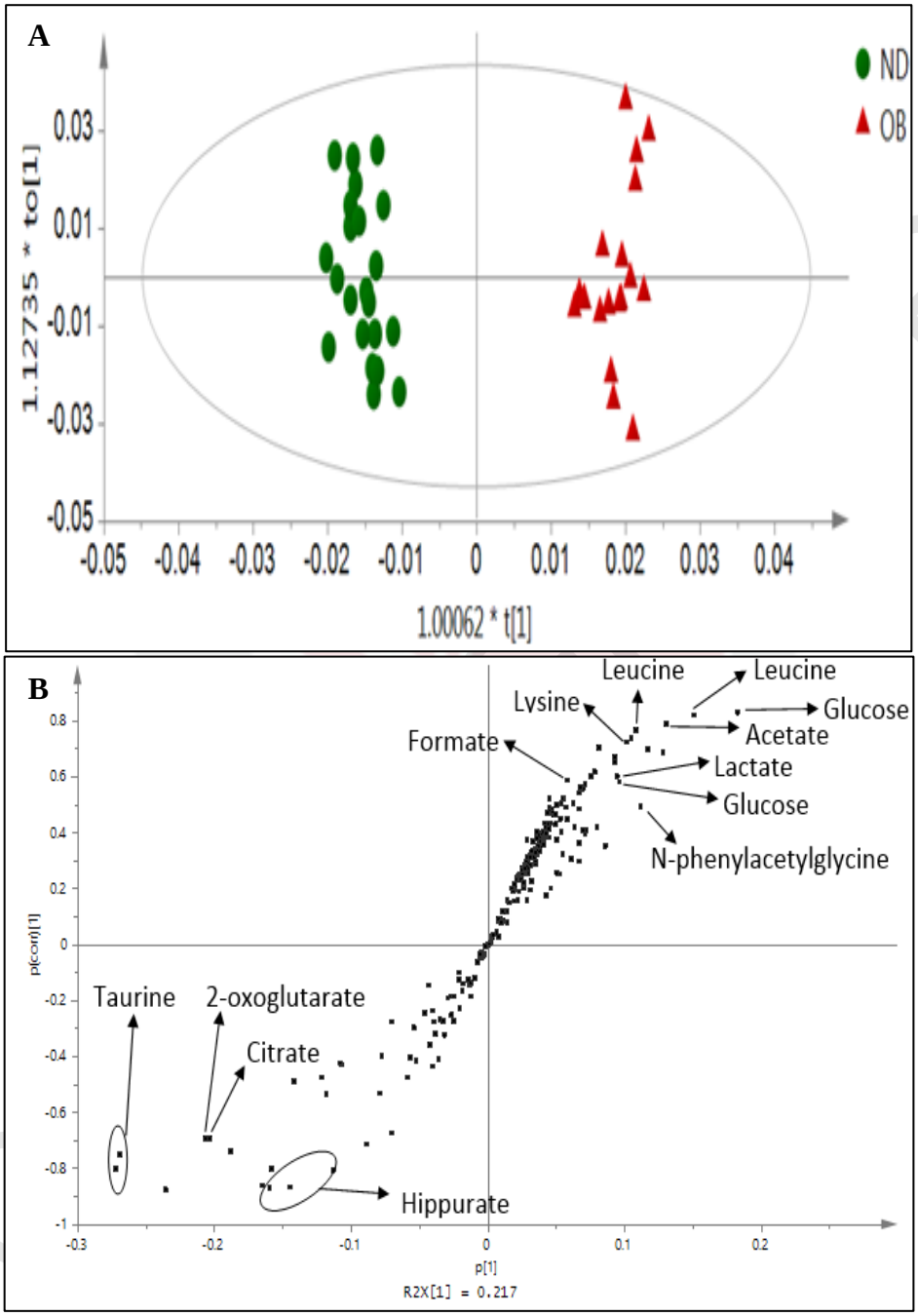


Figure 4.5: OPLS-DA score plot (A) between lean (ND) and obese (OB) rats. The S-plot (B) corresponding to OPLS-DA showed the putative biomarker that responsible for the separation.

4.3.3.2 Justification of model by sensitivity test towards metformin

To identify which type of diabetes model (T1DM or T2DM) obese diabetes and lean diabetes resemble, a sensitivity test towards the T2DM oral drug metformin was conducted on the diabetes group for 4 weeks (after the induction of diabetes at week 13). The OB+STZ and ND+STZ rats were divided into two groups where they were tested with metformin. The OB+STZ metformin-treated group was labeled as OB+STZ+MET, while the ND+STZ group treated with metformin was labeled as ND+STZ+MET. The PLS-DA was first performed on urine spectra data for week 17, where we achieved a good model with goodness of fit $R^2X_{(cum)} = 0.93$, $R^2Y_{(cum)} = 0.63$, $Q^2_{(cum)} = 0.54$ (Figure 4.6). As illustrated in Figure 4.6, clear separation from PC1 can be seen where the non-diabetic groups ND and OB were separated from the diabetic groups. This separation was due to the high concentration of glucose in the urine. However, there was a movement of individuals in the OB+STZ+MET group in which 2 out of 5 rats shifted from the negative to the positive side of PC1, where the non-diabetic group (OB) located.

The movement of individuals (rats) from OB+STZ+MET shows that metformin managed to lower the glucose level in the obese diabetic rats similarly to the non-diabetic OB but it did not manage to revert the condition in OB+STZ+MET back to ND. Metformin is a biguanide oral antihyperglycemic agent that used alone or in conjunction with another therapy to treat T2DM (Quaile et al., 2010). It functions by decreasing hepatic glucose production and improves insulin sensitivity by encouraging glucose uptake by the muscle (Inzucchi, 2002). Generally, the only therapy available for T1DM is insulin, either from insulin injection or insulin pump therapy (Wallymahmed, 2006). Therefore, this result suggests that OB+STZ+MET is sensitive towards the drugs compared to ND+STZ+MET, which also indicates that the obese diabetic model (OB+STZ) is more similar to T2DM.

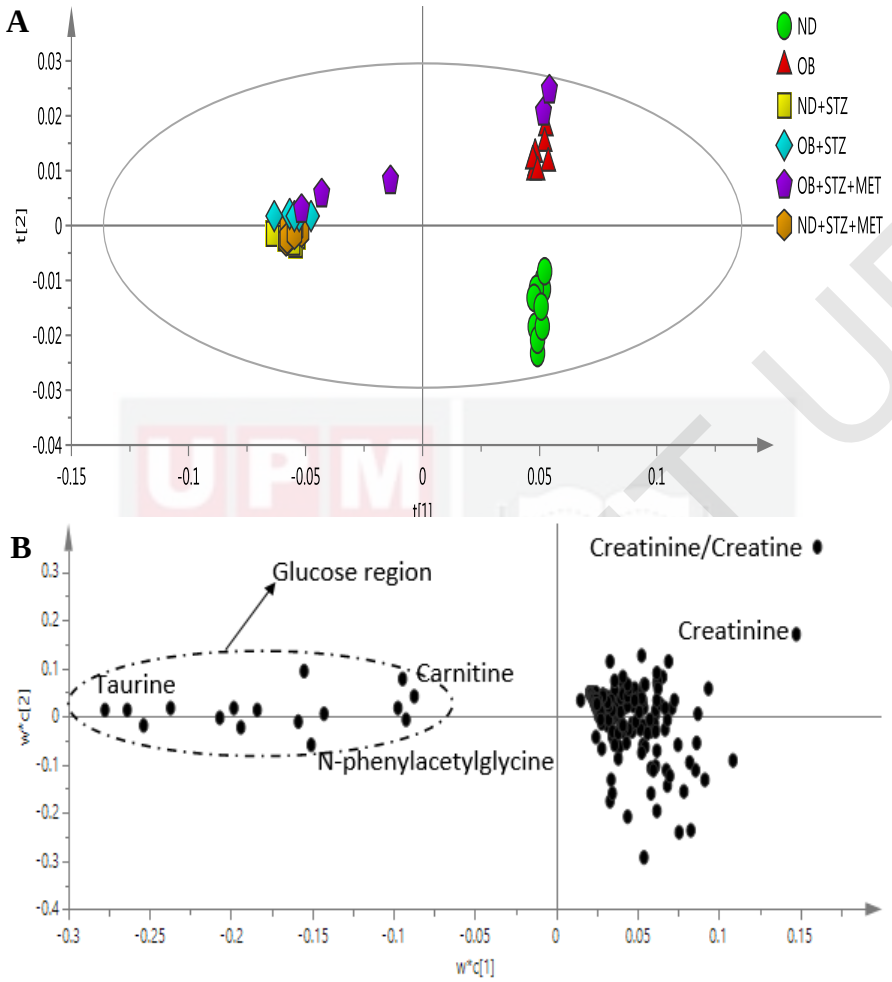


Figure 4.6: PLS-DA score (A) and its corresponding plot (B) of ^{1}H NMR urinary spectra obtained after sensitivity test/treated with oral T2DM drugs, metformin at week 17.

4.3.3.3 Metabolites variation in obesity, lean diabetes and obese diabetes after diabetes induction

To see the metabolic link in the ND, OB, OB+STZ and ND+STZ groups, the PLS-DA was performed on the urinary data after induction of diabetes at week 13. The PLS-DA (Figure 4.7), which had excellent goodness of fit ($R^2X_{(cum)}$ = 0.95, $R^2Y_{(cum)}$ = 0.89, $Q^2_{(cum)}$ = 0.83) and validity (R^2Y-intercept < 0.3-0.4 and Q^2Y-intercept < 0.05), shows a distinctive cluster formed, separating the non-diabetic group from the diabetic group in PC1. As expected, the separation between these groups was mainly due to the higher content of glucose and the other metabolites that overlapped with the glucose region such as taurine, carnitine, and *N*-phenylacetylglycine in the diabetic group (Figure 4.7).

However, it is difficult to identify the other metabolites in the STZ-induced diabetic group as the glucose signals (including other metabolites that overlap with the glucose region) suppress the other metabolite signals in the NMR spectrum. To improve the identification of new potential metabolites in the diabetic group, we decided to exclude the glucose region in the new NMR spectra dataset. To avoid biases or ambiguous results due to the influence of the glucose region, the metabolites that overlapped with the glucose region, such as taurine, carnitine and *N*-phenylacetylglycine were excluded from the selection of potential biomarkers.

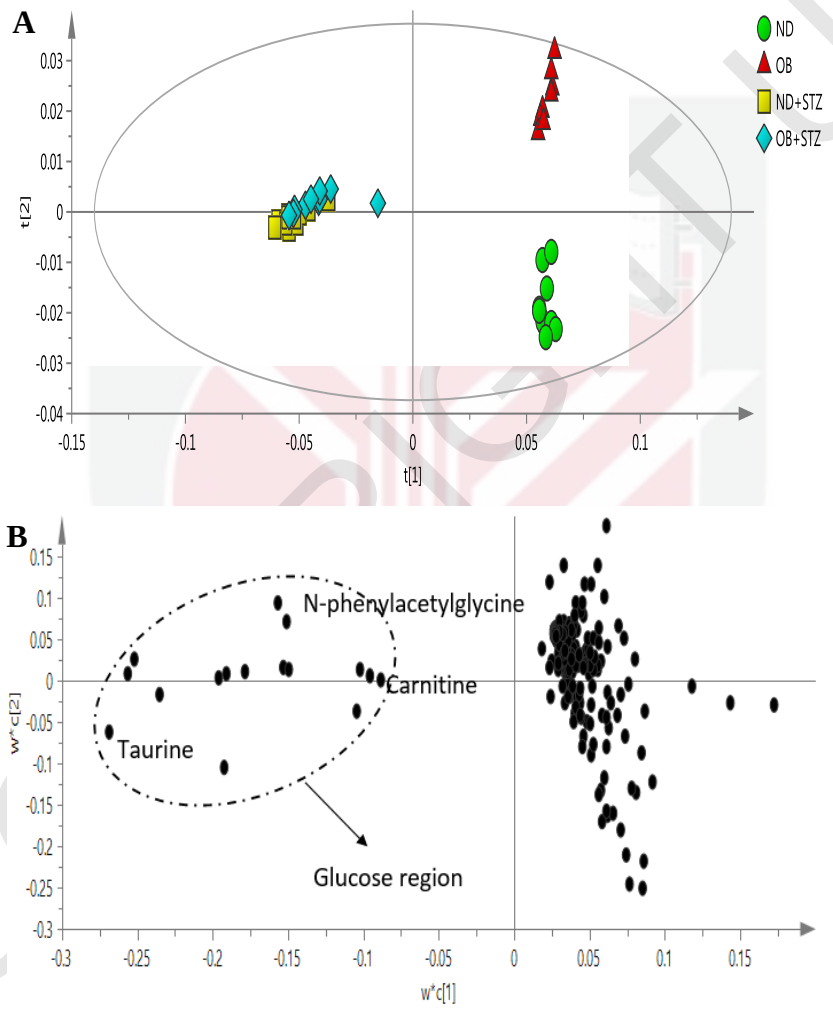


Figure 4.7: PLS-DA score (A) and its corresponding plot (B) before removal of glucose resonance obtained from normal (ND), obese (OB) and diabetic group (obese diabetic (OB+STZ) and lean diabetic (ND+STZ)) at week 13.

The PLS-DA was then performed on the new normalized dataset (Figure 4.8), where the goodness of fit was $R^2X_{(cum)}$ = 0.61, $R^2Y_{(cum)}$ = 0.71, $Q^2_{(cum)}$ = 0.58. This model is considered a good model, in terms of its goodness-of-fit ($R^2Y_{(cum)}$ > 0.7) and good predictive ability ($Q^2_{(cum)}$ > 0.5). A permutation test with 100 permutations was also conducted to validate the two component models, and the model demonstrated model validity with the R^2Y-intercept < 0.3-0.4 and Q^2Y-intercept < 0.05. The model exhibited a clear cluster that separated ND rats from OB, ND+STZ and OB+STZ. Interestingly, we also noted a trend in OB rats, where few of the OB rats overlapped with OB+STZ at the upper part of the positive side in PC1, and three out of ten rats from OB+STZ were nearer to ND+STZ rats (lower part of the positive side in PC1). As illustrated in the loading scatter plot (Figure 4.8B), endogenous metabolites from the TCA cycle such as 2-oxoglutarate, citrate, succinate and others, including hippurate, dimethylamine (DMA) and tryptophan (TRP), were higher in ND than in other groups. Meanwhile, metabolites such as creatinine were high in OB rats that located at the upper part of the negative side of PC1, and metabolites such as allantoin, leucine, lysine, lactate and acetate were high in the overlapped group of OB and OB+STZ. Lastly, the metabolites that affected the clustering in ND+STZ and some of the OB+STZ rats were trigonelline, 3-hydroxybutyrate (3-HB), kynurenine (KYN) and acetoacetate (AcAc).

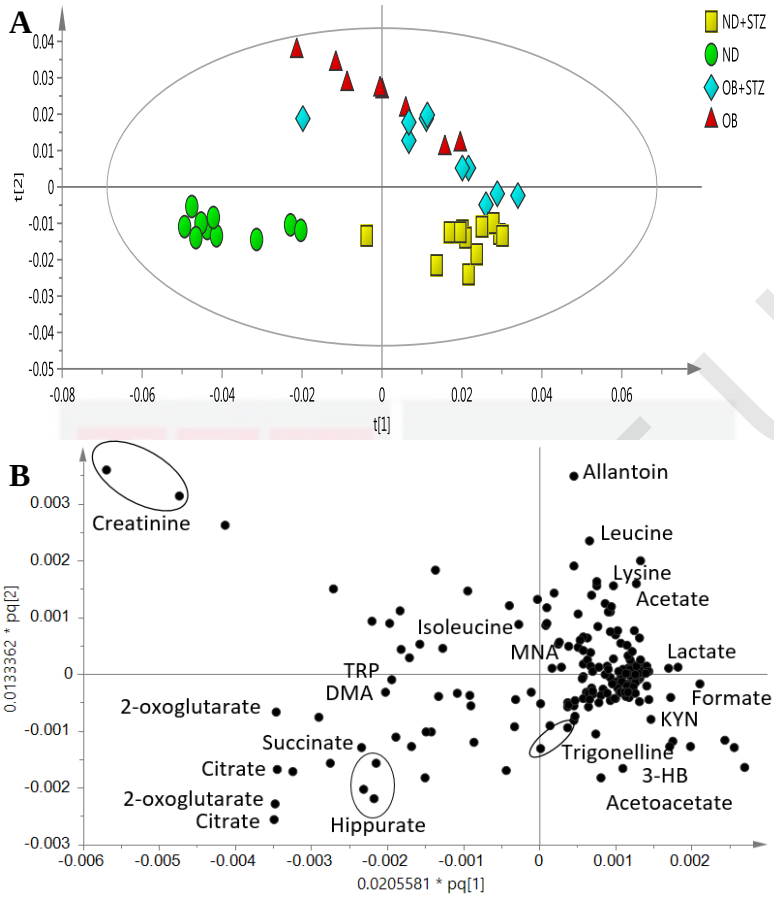


Figure 4.8: PLS-DA score (A) and its corresponding plot (B) after removal of glucose signals of ^{1}H NMR urinary spectra obtained from normal (ND), obese (OB) and diabetic group (obese diabetic (OB+STZ) and lean diabetic (ND+STZ)) at week 13.

To further validate the identified potential biomarkers, only the metabolites with VIP ≥ 1 were selected from PLS-DA models in Figures 4.7 and 4.8. From Table 4.2, only 15 metabolites were identified as potential biomarkers from ND, OB, ND+STZ and OB+STZ, and their relative concentrations were compared. Noted that several metabolites showed the same trend in OB and OB+STZ rats (leucine, allantoin and trigonelline) and also between the OB and diabetic groups, ND+STZ and OB+STZ (2-oxoglutarate, citrate, succinate, lysine, TRP, DMA and hippurate) when compared to the ND. In the comparison between ND+STZ and OB+STZ, metabolites such as allantoin, creatinine, leucine and lysine were significantly higher ($p < 0.05$) in OB+STZ rats compared to ND+STZ rats, while metabolites such as hippurate, 3-HB and trigonelline were higher in ND+STZ rats than OB+STZ. In addition, ND+STZ and OB+STZ also had no significant difference ($p > 0.05$) between them in the amounts of certain metabolites such as glucose, citrate, TRP and AcAc.

Table 4.2: Summary of the relative amount of urine metabolites level in normal (ND), obese (OB), lean diabetes (ND+STZ) and obese diabetes (OB+STZ) by ^{1}H NMR spectra (mean ± sd) after diabetes induction at week 13.

Metabolites	δ ^{1}H (ppm)	OB vs ND	OB+STZ vs ND	ND+STZ vs ND	ND+STZ vs OB+STZ	OB+STZ vs OB
Acetate	1.90	↑***	↑*	↑***	↓	↓
Leucine	1.25	↑***	↑***	↑	↓*	↓
Lysine	1.86	↑***	↑***	↑***	↓*	↓
Glucose	5.23	↑	↑***	↑***	↓	↑***
Citrate	2.50	↓***	↓***	↓***	↑*	↑
2-oxoglutarate	2.44	↓**	↓**	↓***	↑*	↓*
Hippurate	7.54	↓***	↓***	↓***	↑**	↓
Allantoin	5.38	↑**	↑**	↓***	↓*	↓*
Creatinine	4.04	↓	↓**	↓**	↓*	↓*
Trigonelline	9.12	↓**	↓**	↑**	↑**	↑
Tryptophan (TRP)	7.20	↓***	↓***	↓***	↓	↓
3-Hydroxybutyrate (3-HB)	1.12	↑*	↑*	↑*	↑*	↓
Dimethylamine (DMA)	2.72	↓*	↓***	↓***	↑**	↓
Succinate	2.37	↓**	↓**	↓*	↑*	↑
Acetoacetate	2.28	↓***	↓	↑	↑	↑

↑ denotes increase or upregulated metabolites, while ↓ denotes decrease or downregulated metabolites concentration compared to the respective group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ in rows indicated there was significant differences compared to respective group based on one way ANOVA and post hoc, Tukey test.

4.4 Discussion

After induction of diabetes by STZ, changes in metabolite levels were observed, especially for ND+STZ and OB+STZ. Both groups shared similarities in some of the metabolite changes, which indicate that both groups shared some similar features, especially in biochemical traits (e.g., glucose). In addition, the closeness of OB and OB+STZ rats in PLS-DA suggested the association or link of obesity with diabetes. This may denote that the dietary effect plays a significant role in diabetes progression (Khazrai et al., 2014). Figure 4.9 showed the summarized altered metabolites compared to the ND group and the possible pathways and aspects of metabolism involved using MetaboAnalyst 3.0 (Xia et al., 2015).

4.4.1 Metabolites involved in carbohydrate metabolism

In this study, a high accumulation of glucose in ND+STZ and OB+STZ was observed (Figure 4.2 and Figure 4.7). In addition, lower level of TCA intermediates, such as 2-oxoglutarate, citrate and succinate were found in the OB and diabetic groups (Figure 4.8 and Table 4.2). These findings were in agreement with the previous dietary effect studies (Dai et al., 2010; Jiang et al., 2013) and diabetes studies (Diao et al., 2014; Zhao et al., 2010), where they found elevated glucose levels and lower levels of 2-oxoglutarate and citrate in obese Goto-Kazaki and STZ-induced diabetic rats.

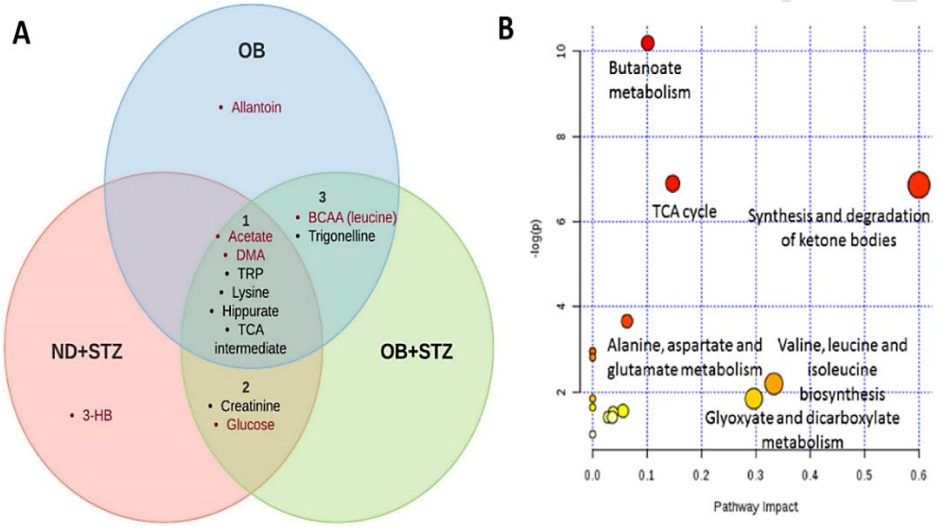


Figure 4.9: Summary of metabolite changes (A) in obese (OB), lean diabetic (ND+STZ) and obese diabetic (OB+STZ) groups as opposed to normal (ND). Where metabolites in red are significantly increased and black significantly decreased in the respective group. Numbers represents the region metabolites shared by the group; 1: OB, ND+STZ and OB+STZ; 2: ND+STZ and OB+STZ and 3: OB and OB+STZ. (B) Simplified schematic of some of the disturbed pathways as identified by MetaboAnalyst 3.0.

Glucose buildup in the urine can be contributed by the breakdown of non-carbohydrate substrates (e.g., pyruvate, lactate and glycerol) via gluconeogenesis, the degradation of glycogen via glycogenolysis or thru the breakdown of carbohydrates from the diet (Zhao et al., 2010). Moreover, glycosuria also indicates impairment in insulin function and sensitivity, as high glucose levels should trigger the release of insulin that is supposed to lower the glucose level, which did not happen in this case.

Higher lactate in the urine also indicates disturbance of pyruvate metabolism, where pyruvate is converted to lactate instead being converted to acetyl-CoA, resulting in lower TCA intermediates in the OB and diabetic groups. Theoretically, the conversion

of pyruvate to acetyl-CoA was accomplished by the pyruvate dehydrogenase complex (PDC) and pyruvate complex (PC) in the mitochondria during aerobic conditions (Srinivasan et al., 2010). While in an anaerobic condition, lactate dehydrogenase (LDH) catalyzed the reduction of pyruvate to lactate (Ainscow et al., 2000).

It is unclear how cellular respiration affects diabetes progression in the OB and diabetic group. However, it was previously found that the overexpression of the LDH enzyme, especially LDH-A, can lead to perturbation in β-cell mitochondria and insulin secretion (Ainscow et al., 2000). It was also reported that PDC deficiency can impair glucose-stimulated insulin secretion in a β-cell-specific PDH deficiency (β-PDHKO) mouse model (Srinivasan et al., 2010). Therefore, these results suggested that high anaerobic respiration occurs in OB and diabetic rats, which leads to the conversion of pyruvate to lactate. However, further studies are needed with regard to correlates of anaerobic respiration, enzymes and their effect on OB and diabetic rats.

4.4.2 Metabolites involved in lipid metabolism

Acetate is one of the intermediary metabolites involved in glucose metabolism (glycolysis and gluconeogenesis), fatty acid β-oxidation and glycerophospholipid metabolism (Wishart et al., 2009). Increases in the level of urine acetate in the OB and OB+STZ groups can be a sign of accumulation of phospholipids in the blood, which was also supported by a lipid profile test (LDL and TG) result of the serum biochemistry. A similar outcome has also been reported by Zhao et al. (2011) and Diao et al. (2014), where they found increased levels of lipid profiles after injection with STZ.

The accumulation of lipids in the body has been associated with reduced mitochondrial density and mitochondrial dysfunction in insulin-resistant individuals with T2DM (Befroy et al., 2007). This finding is also supported by the above results, where low levels of TCA intermediates were found in OB, ND+STZ and OB+STZ. As mitochondria are one of the sites where ATP production happens via the TCA cycle, the results show that the diabetic condition not only causes lipid accumulation but also leads to the deterioration of the mitochondrial density and function that later affects energy metabolism.

Ketone bodies (3-hydroxybutyrate (3-HB), acetoacetate (AcAc) and acetone) are the metabolic product of lipid metabolism in the liver mitochondria (Dedkova & Blatter, 2014). Higher 3-HB and AcAc production in ND+STZ as opposed to OB+STZ suggests that mitochondrial dysfunction and ketosis are more severe in ND+STZ than in OB+STZ. It is well known that ketosis usually occurs in T1DM rather than in T2DM (Diao et al., 2014; Zhang et al., 2008). The accumulation of 3-HB and AcAc is due to the β-oxidation of free fatty acids (FFAs) that leads to the production of acetyl-CoA, which is later convert to ketone bodies (Filla et al., 2014).

Therefore, this result suggested that the metabolites characteristic of the ND+STZ group are more closely related to T1DM. This suggestion is also in line with the previous result where OB+STZ was more sensitive towards metformin than was ND+STZ. In summary, the diabetic condition affected not only glucose and energy metabolism but also lipid metabolism. Limited energy availability due to the lack of absorption of glucose in the skeletal muscle and tissues has caused the breakdown of fats via gluconeogenesis and fatty acid β-oxidation, thus resulting in the accumulation of lipids (TC and TG) and free fatty acids (Diao et al., 2014).

4.4.3 Metabolites involved in amino acid metabolism

Amino acids play crucial roles in cellular function, including the building blocks of proteins, participating in cellular growth, repairing damaged cells and being involved in lipid synthesis (Wurtz et al., 2013). From the result, a high level of amino acids such as leucine and lysine can be noted from OB and OB+STZ ($p < 0.05$). Previous cohort studies conducted by Newgard et al. (2009) and Wurtz et al. (2013) have concluded that several aromatic and branched-chain amino acids (BCAAs), such as leucine and valine, could contribute to insulin resistance in obese and young adults. High levels of BCAAs can interfere with insulin signaling through the stimulation of mTOR-S6K1 (protein and enzyme complex) and the phosphorylation of insulin receptor substrate 1 protein (IRS-1) on its serine residues (Krebs et al., 2007; Tremblay et al., 2005). Therefore, BCAAs have been considered as biomarkers for predicting diabetes risk in an obese individual (Newgard et al., 2009).

In addition to BCAAs, changes in tryptophan (TRP) metabolism can also be noted in both diabetic and OB rats. Low levels of TRP in OB and both diabetic groups indicate that most of the TRP have been converted to kynurenine (KYN). In the body, 95% of the major metabolic pathways involved in TRP metabolism are KYN pathways. KYN then further goes through two different pathways, which are a kynurenine-kynurenine acid (KYN-KYNA) pathway and kynurenine-nicotinamide adenine dinucleotide (KYN-NAD) pathway, where NAD was the end product of the KYN-NAD pathway (Unluturk & Erbas, 2015). Numerous studies have reported that the accumulation of endogenous metabolites or kynurenines from KYN pathways can cause cell damage to the organism, which includes neurological disorders, psychiatric disturbances and systemic disorders such as hypertension, osteoporosis, lipid metabolism disorder and anemia (Stone & Darlington, 2007; Unluturk & Erbas, 2015). Among them, kynurenines such as 3-hydroxyanthranilic acids (3-HAA) and quinolinic acids (QA) have been known to induce apoptosis, and a high accumulation of L-kynurenine and QA has been linked to renal insufficiency (Pawlak et al., 2001; Saito et al., 2000).

4.4.4 Metabolites involved in gut microbiota metabolism

Recently, changes in the gut microbiota have been linked to obesity and diabetes progression (Conterno et al., 2011; Won et al., 2013). Hippurate is a normal endogenous urinary metabolite that has long been associated with the gut microbial degradation of certain dietary components (Lees et al., 2013). Meanwhile, trigonelline

is a byproduct of niacin metabolism (vitamin B3) that is later excreted in urine (Wishart et al., 2009). The urinary levels of hippurate and trigonelline in this study were significantly decreased in Hfd rats compared to Nd rats, ND and ND+STZ. It has been reported that the long-term consumption of Hfd, which is low in dietary fiber, can cause an alteration in the gut microbiome (Kim et al., 2009). This finding is in agreement with Won et al. (2013), where they also noted that hippurate levels were significantly decreased in the ob/ob mouse.

4.4.5 Metabolites involved in other metabolism

Creatinine and creatine are metabolized in the kidney, muscle, liver and pancreas (Wishart et al., 2009). It is well known that creatinine and creatine are highly associated with each other as creatine is converted to creatinine before being transported out from the muscle and excreted in urine (Wyss & Kaddurah, 2000). The decreased level of creatinine in the OB+STZ and ND+STZ groups also implies reduced levels of creatine in these groups. This decrease might be due to several factors, including creatine reabsorption by the kidney, cell leakage and changes in the muscle mass and caloric intake (low protein diet) (Salek et al., 2007). Previously, Diao et al. (2014) and Zhao et al. (2011) also found that STZ-induced diabetic rats have creatine depletion, and Zhao et al. (2011) suggested that altered renal tubular function and morphology might be one of the causes. This suggestion was further supported by the increased urinary allantoin excretion in the diabetic rats, which indicates an increased glomerular filtration rate (Briggs et al., 1977).

In addition to creatinine, other interesting metabolites that should be highlighted here include allantoin, the end product of the oxidation of uric acid from purine metabolism (Liu et al., 2014). Allantoin has been considered one of the oxidative stress markers as it also can be produced through non-enzymatic processes, especially when the level of reactive oxygen species (ROS) is high (Saito et al., 2000). High ROS have been associated with diabetes progression and kidney dysfunction (Zhao et al., 2011). In this study, increased an allantoin level was observed in the OB rats followed by OB+STZ and ND+STZ. This result indicates that ROS production might be influenced by dietary effects as the rats fed with Hfd had higher allantoin levels than the Nd fed rats.

A low concentration of DMA was observed in OB, OB+STZ and ND+STZ and indicates perturbations in methylamine metabolism. Acting as osmoregulatory compounds, methylamines are produced from the degradation of dietary choline to trimethylamine (TMA) and di- and monoamine metabolites by gut microbiota (Asatoor & Simenhoff, 1965). In addition, polyamines also can be produced from choline breakdown into betaine and dimethylglycine (DMG). Therefore, perturbations of methylamine can also reflect the choline availability in the body. The accumulation of liver fat has been observed in choline-deficient Hfd mice (Raubenheimer et al., 2006), and the increased serum lipid in the OB, OB+STZ and ND+STZ groups seems to support this.

4.5 Conclusion

Metabolite changes in OB, ND+STZ and OB+STZ vary from each other. After diabetes induction, metabolic disturbance can be seen in carbohydrate, amino acid and lipid metabolism. Several similarities in metabolites between OB, ND+STZ and OB+STZ, such as acetate, DMA, TRP, lysine, hippurate and TCA intermediates emphasizes the link between obesity and diabetes. The dietary effect also influenced the metabolite levels of OB and OB+STZ, where the metabolite concentration of BCAA was higher and trigonelline was lower compared to the other groups. In both OB+STZ and ND+STZ, metabolites such as glucose were found to be higher, while creatinine was lower. In comparisons between these two groups, metabolites such as ketone bodies and trigonelline were higher in ND+STZ and OB+STZ had a higher level of BCAA (leucine). OB+STZ group also found to be sensitive towards a T2DM drug. All these results suggest that ND+STZ is similar to the T1DM model, and OB+STZ is similar to the T2DM model. Hence, OB+STZ model which mimics T2DM in human was chosen as animal model for diabetes treatment using *I. aquatica* extract in the next chapter.

CHAPTER 5

INTERVENTION OF *Ipomoea aquatica* EXTRACT IN STREPTOZOTOCIN (STZ)-INDUCED DIABETIC RATS VIA ^{1}H NMR-BASED METABOLOMICS APPROACH

5.1 Introduction

As DM is often linked to aspects of an inactive lifestyle such as obesity, dietary intervention has been proposed as one of the solutions in preventing and treating DM (Villegas et al., 2008). By mainly focusing on carbohydrate metabolism and fiber, several intervention studies have demonstrated the relationship between a high intake of fruits and vegetables (especially green leafy vegetables) and a reduced risk of type 2 diabetes mellitus (T2DM) as well as reduced post prandial glucose in T2DM patients (Carter et al., 2010; Liu et al., 2004). Nevertheless, there is no firm conclusion that a high intake of fruits and vegetables can decrease the risk of T2DM as there is also conflicting evidence within the literature with regard to this matter. Additionally, the exact protective mechanism by which these fruits and vegetables works are not precisely known.

In Chapter 3, ethanolic extracts of leafy vegetable *I. aquatica* had shown a promising result in inhibiting α-glucosidase activity which is associated with elevated post prandial glucose. Therefore, the best extract of *I. aquatica* from Chapter 3 was tested on T2DM animal model which was previously confirmed in Chapter 4. Thus, the aim of the present chapter was to find potential biomarkers in urine that were altered by oral gavage of *I. aquatica* extracts and to improve the understanding of the therapeutic efficacy and mechanism of *I. aquatica* extracts.

5.2 Materials and methods

5.2.1 Solvents and chemicals

All the solvents and chemicals were same as previously mention in 4.2.1 (Solvent and chemicals), Chapter 4.

5.2.2 Preparation of *I. aquatica* sample extract

In brief, an oven dried (OD) sample of *I. aquatica* that was previously planted at University Agricultural Park was extracted with absolute ethanol four times, each time for 30 minutes as previously explained in section 3.2.3 (Sampling and extract preparation), Chapter 3.

5.2.3 Animal study

5.2.3.1 Acclimatization

Acclimatization was conducted on five-week-old male Sprague-Dawley (SD) rats weighing 100-150g as previously described in section 4.2.2 (Experimental design for *in vivo* study), Chapter 4. Acclimatization was conducted first for both the preliminary and actual studies before proceeding. The same approval that been used in Chapter 4, (IACUC No. UPM/FPSK/PADS/BRUUH/00470, Appendix A) was used in this chapter.

5.2.3.2 Preliminary study

For the preliminary study of *I. aquatica* dosage, diabetes was induced in twenty SD rats via the single intravenous (iv) injection of streptozotocin (STZ) (55 mg/kg; 150 mg/ml of citrate buffer, 0.1 M, pH 4.5). The fasting blood glucose was checked after 7 days of injection using a glucometer (Accucheck Performa, Roche, Mannheim, Germany), where rats with 13.9 mmol/L were considered diabetic (Maulidiani et al., 2016). The diabetic rats were then randomly divided into five groups, with 4 rats in each group. Group I (control diabetic rats) was given vehicle, 0.03% carboxymethylcellulose (CMC) (Jalil et al., 2008; Adam et al., 2010), while groups II, III and IV were treated with an ethanolic extract of *I. aquatica* suspended in the vehicle, 0.03% CMC at doses of 150, 250 and 500 mg/kg. Group V was treated with metformin at a dose of 300 mg/kg (Sartoretto et al., 2005).

An oral glucose tolerance test (OGTT) was then conducted on the rats to determine the final dosage for the actual study. First, the fasting blood glucose was checked in 12-hour fasted diabetic rats (-30 min) followed by the oral administration of *I. aquatica* ethanolic extracts or metformin suspended in 0.03% CMC orally using intragastric gavage. Thirty minutes later (at 0 hour), rats in all of the groups were given glucose (1.5 g/kg; 100 mg/mL in distilled water) orally using intragastric gavage. Blood samples (tail prick) were collected just prior to glucose administration (0 min) and 30, 90 and 120 minutes after glucose administration and checked with a glucometer (Accucheck Performa, Roche, Mannheim, Germany).

5.2.3.3 Actual study

5.2.3.3.1 Obesity induction

The study in this chapter was conducted concurrently with a previous study in Chapter 4 (Figure 4.1). After 7 days of acclimatization, the rats were divided randomly into 3 groups (Figure 4.1). The control/lean group (ND, n = 6) was fed with normal rat chow (Nd) from Gold Coin, Malaysia and the other two groups, obese diabetic (OB+STZ, n =

5) and obese diabetic orally supplemented with *I. aquatica* (OB+STZ+IA, $n = 5$) were fed with a modified high fat diet (Hfd). The Hfd contained 49% fat, 32% carbohydrate, and 19% protein for the total energy, with kcal that consist of fat from ghee (milk fat) and corn oil (Jalil et al., 2008). The Nd contained 14% fat, 61% carbohydrate, and 25% protein for the total energy in kcal. All of these diets were continuously given throughout this study (until week 17), even after the induction of obesity (week 12).

5.2.3.3.2 Diabetes induction and intragastric gavage of *I. aquatica* extract

After the induction of obesity at week 12, rats for OB+STZ and OB+STZ+IA groups were injected with STZ via iv. STZ were freshly prepared as mentioned above, and the dosage of STZ for this obese diabetic group was 25 mg/kg. Supplementations of IA extracts were given (250 mg/kg) for 4 weeks after the induction of diabetes. The final sample sizes for the OB+STZ and OB+STZ+IA groups were $n = 5$ each.

5.2.4 Biofluids collection

For preliminary study (OGTT), the blood collection was made by the tail prick at 0 min (prior to glucose administration) and 30, 90 and 120 minutes after glucose administration. Meanwhile, for actual study, the urine and blood collection (serum) were accomplished in the week 12, 13 and 17 as previously mention in section 4.2.6 (Blood and urine collection), Chapter 4.

5.2.5 Biochemistry analyses

Serum glucose, total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured using an automatic biochemical analyzer (Hitachi 902, Roche, Germany). Values are expressed as the mean ± SD.

5.2.6 Biofluids preparation and NMR measurements

Biofluids preparation (urine and serum) and NMR measurements were conducted as previously mention in section 4.2.8 (Biofluids preparation and NMR measurements), Chapter 4.

5.2.7 Data processing and multivariate analysis

The phasing and baseline corrections were conducted as been previously described in section 4.2.5 (Data processing and multivariate analysis), Chapter 4. First, PCA was performed to visualize and discriminate the groups according to their metabolites, thus

giving the general idea within the dataset. Projection to the latent structure discriminant analysis (PLS-DA) models of the NMR spectral data were also carried out for class discrimination and putative biomarker selection. All models were calculated using a seven-fold cross validation method.

The quality of the models was assessed by the R^2 parameter, and the predictability of models was assessed by the Q^2 (Wheelock & Wheelock, 2013). The models were considered as effective and reliable when these values were more than 0.5 and better when these parameters were closer to 1.0. In addition, a permutation test (100 permutations) was conducted to measure the robustness of the model. The built model was considered robust if the Q^2 regression line had a negative intercept, and all the permuted R^2 values on the left were lower than the original point on the right. The robustness of the model was considered excellent if the R^2Y-intercept was < 0.3-0.4 and the Q^2Y-intercept was < 0.05 (Eriksson et al., 2006). In PLS-DA, the samples from different groups were separated into different classes and the metabolites that influence the separation were identified from the loading plot. Then, only the metabolites with variable importance in the projection (VIP) of more than 0.5 were selected as putative biomarkers for relative quantification (An et al., 2015).

5.3 Results

5.3.1 Oral glucose tolerance test (OGTT)

Table 5.1 shows the OGTT result for rats treated with different doses of *I. aquatica* extract. From the results, an increase in the glucose level after the glucose load at 30 min was observed. The glucose level starts to decrease at 90 min for all doses of *I. aquatica* extracts. However, only the 250 mg/kg and 500 mg/kg doses showed significant decreases relative to the 30 min of their respective groups at time 90 min and 120 min, respectively. There are no significance differences by comparing these two doses using Student t-test. Therefore, the minimum dose that is capable of reducing the post prandial blood glucose, 250 mg/kg was chosen for the actual study.

Table 5.1: Effect of ethanol extract of *I. aquatica* on blood glucose level during oral glucose tolerance test in streptozotocin-induced diabetic rats.

Group	-30min	0min	30min	90min	120min
Control diabetic	25.00±2.26	22.88±3.02	31.42±3.30	28.52±2.34	23.90±4.95
I. aquatica 150mg/kg b.w	29.03±4.41	28.66±3.90	30.61±1.92	27.16±4.43 (11.27%)	26.63±5.39 (13.00%)
I. aquatica 250mg/kg b.w	24.60±5.02	20.87±6.55	31.51±1.92	23.70±2.60 (24.78%)*	19.80±4.08 (37.16%)*
I. aquatica 500mg/kg b.w	18.43±1.95	17.70±0.26	28.77±0.26	21.80±2.86 (24.23%)*	17.43±1.59 (39.42%)**
Metformin 300mg/kg b.w	22.65±4.31	22.60±6.51	22.65±8.70	20.70±4.10 (8.60%)	18.65±6.85 (17.66%)

Note: Values in bracket indicate the percentage of blood glucose reduction relative to 30min of the respective treatment group. *$p < 0.05$ and **$p < 0.01$ compared with to 30min of the respective treatment group. b.w: body weight.

5.3.2 Serum biochemistry

The serum biochemistry of actual study is summarized in Table 5.2. As expected, both diabetic groups showed an increase in serum glucose (20.32-24.60 mmol/L). Although there was a reduction in the glucose level in OB+STZ+IA, these changes were not significant as there was huge variation between the individuals (rats). Meanwhile, we also note changes in the lipid profile test such as total cholesterol (TC), low density lipoprotein (LDL) and high density lipoprotein (HDL), where TC and LDL were high in both diabetic groups. In addition, the HDL level in OB+STZ+IA was significantly higher ($p < 0.05$) compared to OB+STZ rats. This probably due to the effect of the *I. aquatica* supplementation. High consumption of leafy green vegetables has been noted to increased plasma and serum HDL in human (Ferdowsian & Barnard, 2009).

Meanwhile, high levels of TC and LDL in both diabetic groups were probably due to lipemia, a condition where the serum have high accumulation of lipoprotein particles causes by diabetes progression and diet (Calmarza & Cordero, 2011; Nikolac, 2014). Lipemic sera known to cause significant interferences in analytical result of

biochemistry especially in lipid profile (e.g. TC and TG) and liver function test (aspartate transaminase (AST) and alanine transaminase (ALT) (Calmarza & Cordero, 2011). Even though the lipemic condition is hardy visually seen, high triglyceride value has known to be one of its signatures (Nikolac, 2014). Since urine specimen requires less samples pretreatment compare to serum due to their lower protein content, therefore urine was chosen for ^{1}H NMR metabolomics analysis.

Table 5.2: Serum biochemical chemistry for control/normal (ND), obese (OB), obese-diabetic (OB+STZ) and obese-diabetic treated with *I. aquatica* extract (OB+STZ+IA) at week 17.

Group	Parameters				
	Cholesterol mmol/L	Glucose mmol/L	Triglyceride umol/L	LDL mmol/L	HDL mmol/L
Control (ND)	1.27±0.21^{a}	4.81±1.09^{a}	0.57±0.18^{a}	0.28±0.11^{a}	0.84±0.12^{ab}
Obese Diabetic (OB+STZ)	7.00±1.34^{b}	24.60±1.67^{b}	1.94±1.00^{ab}	3.24±0.63^{b}	0.61±0.23^{b}
Obese Diabetic + *I. aquatica* (OB+STZ +IA)	7.06±5.33^{b}	20.32±8.79^{b}	2.67±1.97^{b}	2.92±2.57^{b}	0.97±0.29^{a}

Different letters in the column indicate there were significant differences ($p < 0.05$) using Tukey's test.

5.3.3 ^{1}H NMR spectral analysis of urine samples

The ^{1}H NMR spectra of urine obtained from the normal (ND), obese-diabetic (OB+STZ) and *I. aquatica* obese-diabetic (OB+STZ+IA) groups are shown in Figure 5.1. The endogenous metabolites were assigned according to Chenomx NMR Suite 6.1 (Chenomx Inc., Edmonton, Canada), Human metabolome database (HMDB) and previously reported assignments. Generally, the urine samples contained metabolites such as intermediates from the tricarboxylic cycle (TCA) (succinate, citrate and 2-oxoglutarate); amino acids: leucine, taurine and lysine; organic acids: formate, acetate, hippurate and others: allantoin, creatinine, and creatine, as summarized in previous chapter in the Table 4.1.

For details on metabolite changes due to the intervention of *I. aquatica* extract, urine NMR spectra were subjected to multivariate data analysis. Each of the individual rats is represented on the score scatter plot, where the groupings of the individual based on the effect of treatment or stimuli can be observed. While the loading scatter is

complimentary to the score scatter, it explains the metabolites that affected the grouping and the separation in the score scatter plot (Eriksson et al., 2006)

PLS-DA was first performed on all the samples (Figure 5.2A Model 1 (M1)), where the goodness of fit was $R^2X_{(cum)}$ = 0.96, $R^2Y_{(cum)}$ = 0.92, $Q^2_{(cum)}$ = 0.79. This model was shown to exhibit clear clustering between the normal rats from the diabetic group via PC1 in the score scatter plot. The separation between the diabetic groups also can be observed from the PC2, where the OB+STZ+IA rats located at the positive side and the OB+STZ rats located at the negative side. The group differentiation between the ND and the diabetic group (OB+STZ and OB+STZ+IA) was highly affected by glucose and other metabolites that located in the glucose region, such as *N*-phenylacetylglycine, taurine and carnitine. Meanwhile, the separation between OB+STZ and OB+STZ+IA can be identified from metabolites such as glucose, taurine and carnitine, where most of these metabolites were higher in OB+STZ compared to OB+STZ+IA.

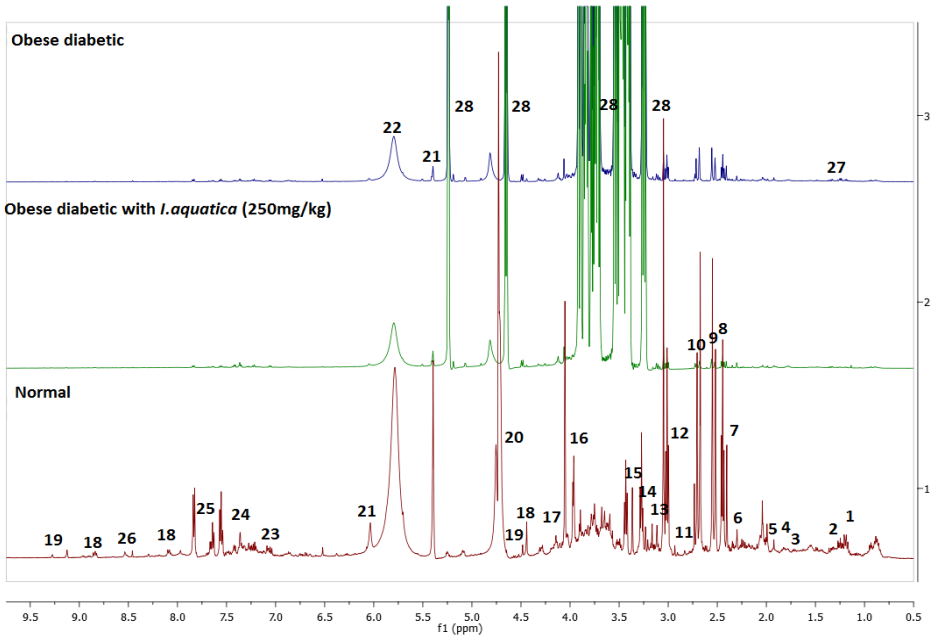


Figure 5.1: Representative of 500 MHz ^{1}H NMR spectra of urine samples for normal (ND), obese (OB), lean diabetes (ND+STZ) and obese diabetes (OB+STZ). Label : 1, Isoleucine; 2, Lactate; 3, Leucine; 4; Leucine/Lysine; 5, Acetate; 6, Acetoacetate; 7, Succinate; 8, 2-oxoglutarate; 9, Citrate; 10, Dimethylamine; 11, *N*,*N*-Dimethylglycine; 12, Creatine; 13, Carnitine; 14, Taurine; 15, Methanol; 16, Creatinine; 17, Kynurenine; 18, Trigonelline; 19, 1-Methylnicotinamide (MNA); 20, Water; 21, Allantoin; 22, Urea; 23, Tryptophan; 24, *N*-phenylacetylglycine; 25, Hippurate, 26, Formate, 27, 3-Hydroxybutryrate (3-HB) and 28; Glucose.

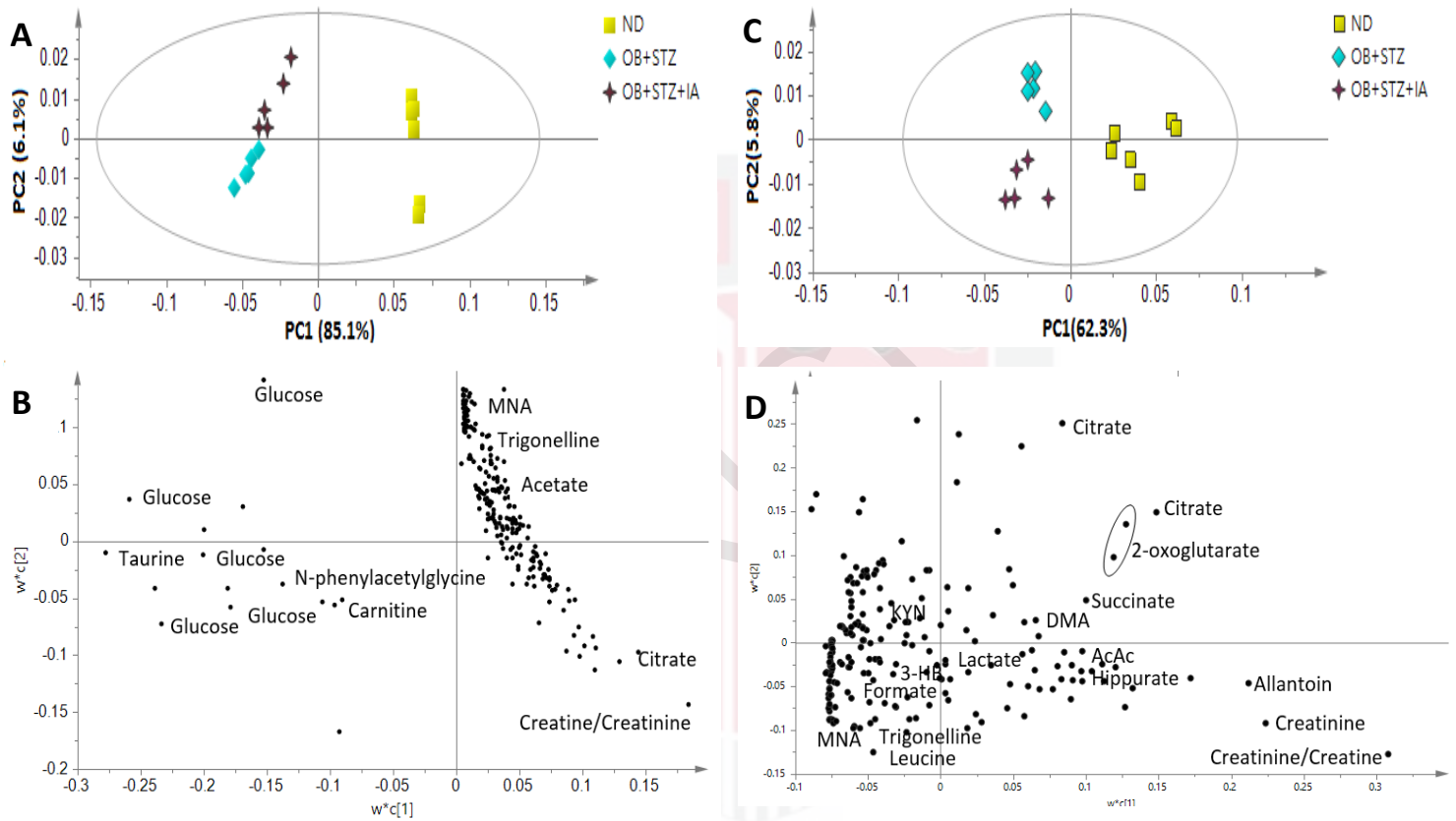


Figure 5.2: M1 PLS-DA score (A) and its scatter plot (B) before removal of glucose signals and M2 PLS-DA score (C) and its scatter plot (D) after removing glucose of ^{1}H NMR urinary spectra obtained from normal (ND), obese diabetic (OB+STZ) and obese diabetic with *I. aquatica* (OB+STZ+IA) at week 17

To filter out the obvious influence of the glucose region on the model, we decided to exclude the glucose region (δ 3.15-3.24, δ 3.31-3.52, δ 3.63-3.9, δ 5.15-5.24) from the spectra to observe the changes in other metabolites. The new spectra data were normalized again to the sum row to compensate for the variation in the total volumes. Interestingly, the new PLS-DA model (Figure 5.2 Model 2 (M2)) with goodness of fit $R^2X_{(cum)}$ = 0.87, $R^2Y_{(cum)}$ = 0.96, $Q^2_{(cum)}$ = 0.99 illustrates the same pattern as the previous PLS-DA (Fig. 5.2A), where the ND separated from the diabetic group (OB+STZ and OB+STZ+IA) with PC1 and from OB+STZ and OB+STZ+IA with PC2. In PC1, metabolites such as creatinine, allantoin, citrate, dimethylamine (DMA), acetoacetate (AcAc), TCA intermediates (succinate and 2-oxoglutarate), organic acids (hippurate and lactate) and amino acids (tryptophan (TRP) and isoleucine) were higher in the ND group, while metabolites such as 1-methylnicotinamide (MNA), lysine, leucine, formate, 3-hydroxybutyrate (3-HB), trigonelline and formate were higher in the diabetic group. Meanwhile for PC2, the metabolites that separated OB+STZ from OB+STZ+IA were TCA intermediates: citrate and 2-oxogutarate; amino acids: leucine and lysine; organic acid: formate; and others: creatinine, trigonelline, MNA and allantoin, where the TCA intermediates were higher in OB+STZ compared to OB+STZ+IA. For validity, both of the models possess excellent goodness of fit ($R^2Y_{(cum)} > 0.8$) and good predictive ability ($Q^2_{(cum)} > 0.7$). In addition, the permutation test revealed that both M1 and M2 demonstrated good and moderate model validity with R^2Y-intercepts of < 0.5 and < 0.9 and with Q^2Y-intercepts of < 0.05.

From all the metabolites that had been mentioned in M1 and M2, only metabolites with VIP values larger than 0.5 were considered as potential biomarkers (Figure 5.3). A total of 19 potential biomarkers were identified, with creatine/creatinine having the highest VIP values (> 2.0), followed by glucose, creatinine, citrate, carnitine, 2-oxoglutarate, succinate, hippurate, leucine, MNA, taurine, 3-HB, TRP, lysine, trigonelline, allantoin, formate, AcAc and lastly DMA with VIP values of 0.5. Metabolites such as creatine/creatinine, creatinine, citrate, 2-oxoglutarate, taurine, succinate, hippurate, TRP, AcAc, allantoin and DMA were found to be significantly high ($p < 0.05$) in ND. In contrast, metabolites such as glucose and carnitine were significantly high ($p < 0.05$) in OB+STZ. In OB+STZ+IA, metabolites such as leucine, lysine, formate, MNA and trigonelline were found to be significantly ($p < 0.05$) higher compared to ND and OB+STZ. In addition, Figure 5.3 also highlights metabolite levels that were significantly improved and unimproved by comparing the metabolites of OB+STZ and ND. Metabolites such as creatinine, creatine, glucose, carnitine, taurine and allantoin were improved compared to the OB+STZ group. Metabolites such as TCA intermediates (succinate, 2-oxoglutarate and succinate); amino acids: tryptophan; AcAc and DMA were found to be unimproved by comparing with the ND group.

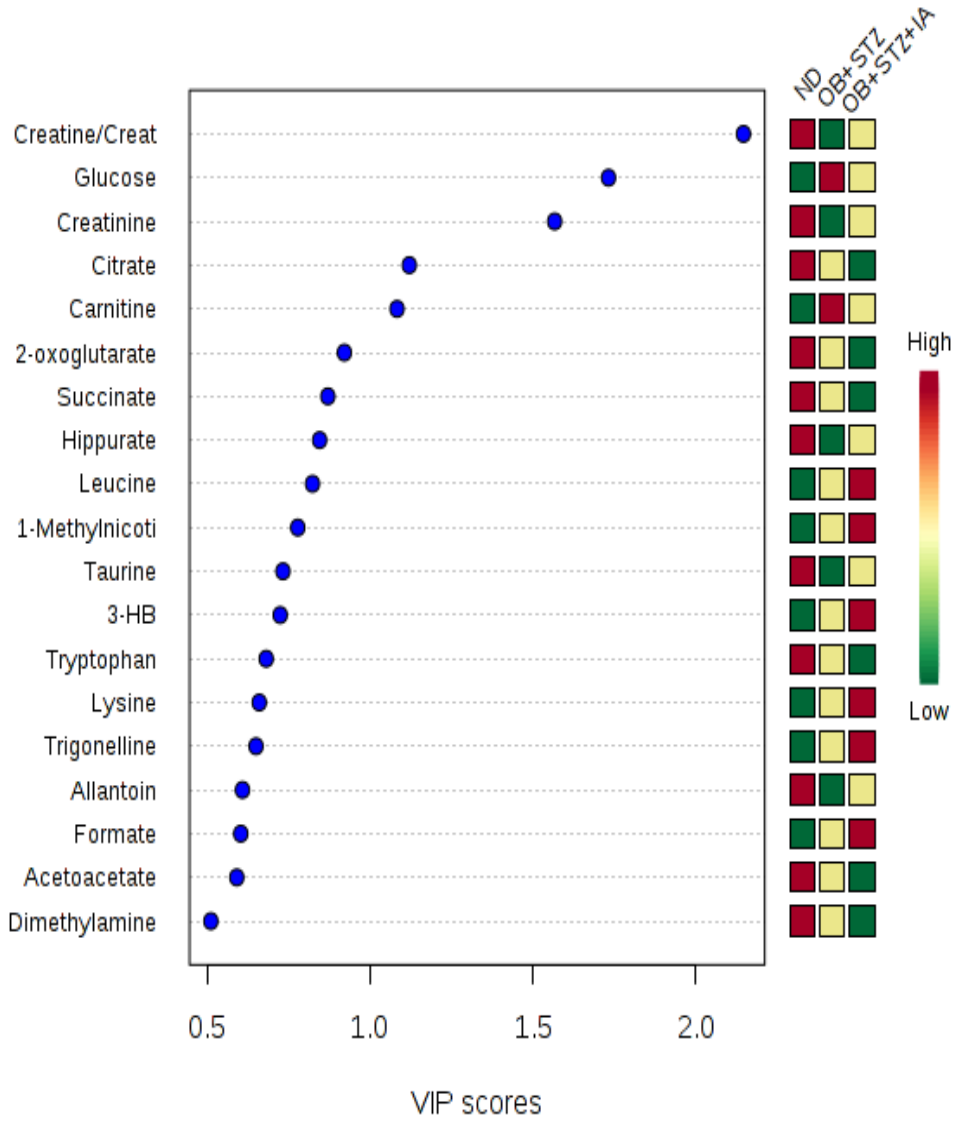


Figure 5.3: VIP score of metabolites that significantly changed in normal (ND), obese diabetic (OB+STZ) and obese diabetic with *I. aquatica* (OB+STZ+IA) at week 17

5.4 Discussion

5.4.1 Effect of *I. aquatica* extract on glucose and energy metabolism

From the serum result, *I. aquatica* supplementation is capable of promoting glycolysis by lowering the glucose level. Although *I. aquatica* extract did not significantly alter the glucose concentration in the serum biochemistry result, the PLS-DA result (Figure 5.2A) showed that lesser metabolites in the glucose region can be identified in OB+STZ+IA compared to the OB+STZ group. A high glucose content can be contributed by the endogenous glucose production (EGP) metabolites from the breakdown of glycogen through glycogenolysis and non-carbohydrate precursors via gluconeogenesis (Zhang et al., 2008). Therefore, this result suggests that *I. aquatica* reduces the EGP level in diabetes rats. In addition, lower TCA intermediates were found in OB+STZ+IA compared to OB+STZ. Lower levels of TCA intermediates indicates that there is perturbation in the energy metabolism, indicative of mitochondrial dysfunction (Diao et al., 2014). In short, *I. aquatica* extract improved the EGP level but did not improve energy production, adenosine triphosphate (ATP) via the TCA cycle.

5.4.2 Effect of *I. aquatica* extract on lipid metabolism

A higher formate level was also found in the OB+STZ+IA group compared to others (Figure 5.3). Formate is the simplest carboxylic acids and acts as an intermediate in normal metabolism. Formate production is often associated with the acetate level, as it is produced as a byproduct during the production of acetate (Wishart et al., 2009). An increase in the acetate level can be a sign of lipid accumulation, and this possibility was also supported by the lipid profile of the serum biochemistry result (Table 5.2). Lipid accumulation has been associated with mitochondrial dysfunction in insulin-resistant T2DM individuals (Diao et al., 2014). This finding can indicate that most of the acetyl-CoA in OB+STZ+IA was converted to acetate and formate rather than entering the TCA cycle.

The overproduction of free fatty acids (FFAs) is known to be an indicator of T2DM. An increase in acetyl-CoA due to FFA β-oxidation can influence the production of ketone bodies (KBs) (Filla et al., 2014). As shown in Figure 5.2 and Figure 5.3 the highest level of ketone bodies (3-HB) can be found in OB+STZ+IA compared to the other groups. Although the production of KBs has been associated with a decrease in the insulin level, AcAc and 3-HB have also been previously reported to be present in non-diabetic individuals at a 1:1 ratio, especially during fasting (Filla et al., 2014; Higashino et al., 2012). Furthermore, the increase in amino acids such as leucine may also play a role in the higher levels of acetyl-CoA and KBs (Filla et al., 2014; Newgard et al., 2009). In our results, both the production of AcAc and 3-HB were at a 1:1 ratio, which indicates that the production of KBs were likely associated with fasting and high levels of leucine.

5.4.3 Effect of *I. aquatica* extract on amino acid metabolism

Both leucine and lysine are essential amino acids, which are required to be consumed through the diet. Previously, branched chain amino acids (BCAAs) such as leucine have been reported to be one of the biomarkers for insulin resistance (Newgard et al., 2009). In contrast, there are also reports of leucine significantly improving glycemic control in multiple mouse models of obesity and diabetes with distinct etiologies (Guo et al., 2010; Zhang et al., 2007). In our study, the upregulation of leucine and lysine levels were found in OB+SZT+IA compared to OB+STZ. This upregulation is probably due to the high content of amino acids in *I. aquatica*. Previously, *I. aquatica* has been reported to contain high amounts of essential amino acids (4756 mg/100g), where leucine makes up 1365 mg/100g and lysine 628 mg/100g (Doka et al., 2014).

In addition to lysine and leucine, the carnitine urinary concentration was also changed in OB+STZ+IA rats (Figure 5.2). Carnitine is a non-essential amino acid, which means that it can be synthesized by our bodies (Wishart et al., 2009). The esterification of carnitine is important for the transportation of long fatty acyl groups for mitochondria β-oxidation. Increased acyl carnitine (mainly acetyl carnitine) in the urine and decreased of free carnitine in the serum can be found during starvation (long-term fasting) and STZ-diabetic rats due to the utilization of fat for energy (Brooks et al., 1985). Carnitine is also involved in lysine degradation pathways, where it is the end product from enzyme-substrate gamma-butyrobetaine dioxygenase and butyro-betaine (Kanehisa et al., 2016). Therefore, improved carnitine levels in OB+STZ+IA compared to OB+STZ suggests that β-oxidation and lysine degradation occur less in OB+STZ+IA. This suggestion supports the above finding, where the lysine concentration is higher in OB+STZ+IA and the production of KBs is also contributed to by leucine and not solely from the β-oxidation of FFAs.

5.4.4 Effect of *I. aquatica* extract on gut microbiota and nicotinate/nicotinamide metabolism

Recently, metabolic products from gut microbiota pathways such as choline and niacin have been associated with the development of metabolic disease such as obesity and diabetes (Nicholson et al., 2005; Palau et al., 2015). Niacin has been regarded as one of the important vitamins involved in physiological functions such as being a coenzyme in tissue respiration and carbohydrate and tissue metabolism (Palau et al., 2015). The requirements of the body are fulfilled from the diet and biosynthesis through a tryptophan-mediated metabolism (Calvani et al., 2010).

Trigonelline, also known as *N*-methylnicotinate, is a byproduct of niacin metabolism from the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine (Sun et al., 2008). In the trans-sulfuration pathways, trigonelline helps to regenerate glutathione that has been depleted by oxidative stress, where it was previously reported to decrease in obesity and type 2 diabetes (T2DM) patients and rodents (Calvani et al., 2010; Salek et al., 2007; Sun et al., 2008). In addition to trigonelline, high levels of 1-mehylnicotinamide (MNA) also support *I. aquatica* extracts altering nicotine and

nicotinamide metabolism. MNA has been cited to prolong the lifespan of diabetic rats and to have a neuroprotective mechanism (Kuchmerovska et al., 2010). From our results, the increase of trigonelline and MNA metabolites (Figure 5.3) in the urine of OB+STZ+IA suggest that *I. aquatica* extract is able to improve the gut microbiota and nicotinate/nicotinamide metabolism.

5.4.5 Effect of *I. aquatica* extract on other metabolism

Both creatinine and creatine are metabolized in the kidney, muscle, liver and pancreas (Wishart et al., 2009). Serum creatinine has long been an indicator of renal function, especially in kidney-related diseases. From our results, the creatinine depletion observed in OB+STZ indicates that there are underlying problems in either 1) kidney functions, such as cell leakage and creatinine reabsorption, 2) changes in caloric intake or 3) changes in muscle mass (Salek et al., 2007). The reduction in the allantoin urinary concentration in OB+STZ also supports kidney dysfunction, as allantoin is not reabsorbed across the proximal tubule (Salek et al., 2007). The decreased concentration of allantoin has thus been noted as indicative of a declining glomerular filtration rate (GFR), which is associated with diabetic nephropathy (DN) (Salek et al., 2007; Zhao et al., 2011). From the results shown in Fig. 5.3, an increase in the urinary allantoin concentration of OB+STZ+IA rats shows that *I. aquatica* extracts have promising effects in improving kidney functions, especially glomerular filtration rates (GFR).

5.5 Conclusion

Oral supplementation of *I. aquatica* extract manage to upregulating metabolites such as creatinine/creatine, carnitine, MNA, trigonelline, leucine, lysine, 3-HB and downregulating metabolites such as glucose and TCA's intermediate. Where it manages to promote glycolysis, gut microbiota and nicotinate/nicotinamide metabolism, improving the GF rate and reduced the β-oxidation rate. These results indicate *I. aquatica* extract possess anti-hyperglycemic activities which can postpone the glucose absorption for post prandial glucose (as been proven from their OGTT result) and lowers the glucose level after 4 weeks of supplementation. However, it also has several drawbacks as it did not improve the metabolite levels of leucine, 3-HB or TCA intermediates, metabolites which are involved in BCAAs synthesis pathways and lipid and energy metabolism.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

6.1 Summary and conclusion

Overall, this study evaluates the capabilities of *I. aquatica* as a hypoglycemic agent by using *in vitro* and *in vivo* tests. By using the ^{1}H NMR metabolomics approach, the study highlighted the effect of drying treatment and solvent on the metabolite profiles of *I. aquatica*, which later affected the bioactivities results, such as for α-glucosidase. The PCA result showed that drying gives a greater effect compared to the solvent effect, which separates oven-dried (OD) samples from others. Therefore, a separate model/analysis was conducted on the model based on solvent effects, where the best extract was found in the absolute ethanol extract from the OD method with an IC_{50} value of 203.83 ± 58.82 µg/mL and a TPC value of 22.26 ± 0.68 µg GAE/mg extract. It was revealed that the combination of metabolites such as quercetin derivatives, chlorogenic acid derivatives, sucrose, fructose and lower concentrations of organic acids such as citric acid and malic acid might influence the bioactivities result.

The capabilities of *I. aquatica* extract as a hypoglycemic agent were further tested *in vivo*. The validation of the diabetic animal model was first conducted to determine the diabetic types (Type 1 or Type 2). The rats were first acclimatized and then divided randomly into several groups, where part of the group was fed with normal chow (Nd) and the other was fed with the high fed diet (Hfd). Obesity was first induced in the rats for 4 weeks as we wanted to mimic the development of Type 2 obese-diabetes progression in humans, and streptozotocin (STZ) was injected afterward in lean (ND) and obese (OB) rats to induced diabetes. From the beginning, the lean diabetes rats (ND+STZ) exhibited higher levels of ketone bodies, hippurate, trigonelline and were also insensitive towards the Type 2 diabetes drug metformin compared to the obese diabetic group (OB+STZ). In OB+STZ, urinary metabolomics revealed that OB+STZ rats had higher branch chain amino acid (BCAA) and lower hippurate and trigonelline levels. All these results confirmed that the ND+STZ rat model is similar to Type 1 diabetes (T1DM), while OB+STZ is more similar to Type 2 diabetes (T2DM). This result also emphasizes the dietary effects of the expressed metabolites released/excreted by the rats and the link between OB and diabetes.

Further study was conducted using OB+STZ model as it mimics T2DM in human. Ethanolic *I. aquatica* extracts that were previously found to have inhibitory effects on α-glucosidase activity were given orally via intragastric gavage for 4 weeks to determine their intervention in obese-diabetic rats (OB+STZ+IA). At the 250 mg/kg dose, it was revealed that *I. aquatica* extracts improved the glycolysis, gut microbiota and nicotinate/nicotinamide metabolism, improved the glomerular filtration (GF) rate and reduced the β-oxidation rate by changing metabolites such as creatinine/creatine, glucose, allantoin, hippurate and carnitine. However, it also noted that *I. aquatica* did not improve or change the amino acid metabolism, lipid metabolism or TCA

intermediates. This result may be due to the complexity of diseases based on diabetes progression or due to the bioavailability of bioactive metabolites from *I. aquatica* after being consumed.

6.2 Recommendation for future research

As for future recommendations, we suggest using another analytical method for metabolomics studies, such as liquid chromatography (LCMS) or gas chromatography (GC-MS), in addition to nuclear magnetic resonance (NMR) for a complete identification of unknown metabolites. Further study also can be conducted on boiled *I. aquatica* samples as it was traditionally consumed, as this was previously effective for T2DM patients. For *in vivo* tests, the bioavailability of the bioactive compounds should be determined after being consumed so that the optimum dose of *I. aquatica* extract can be identified.

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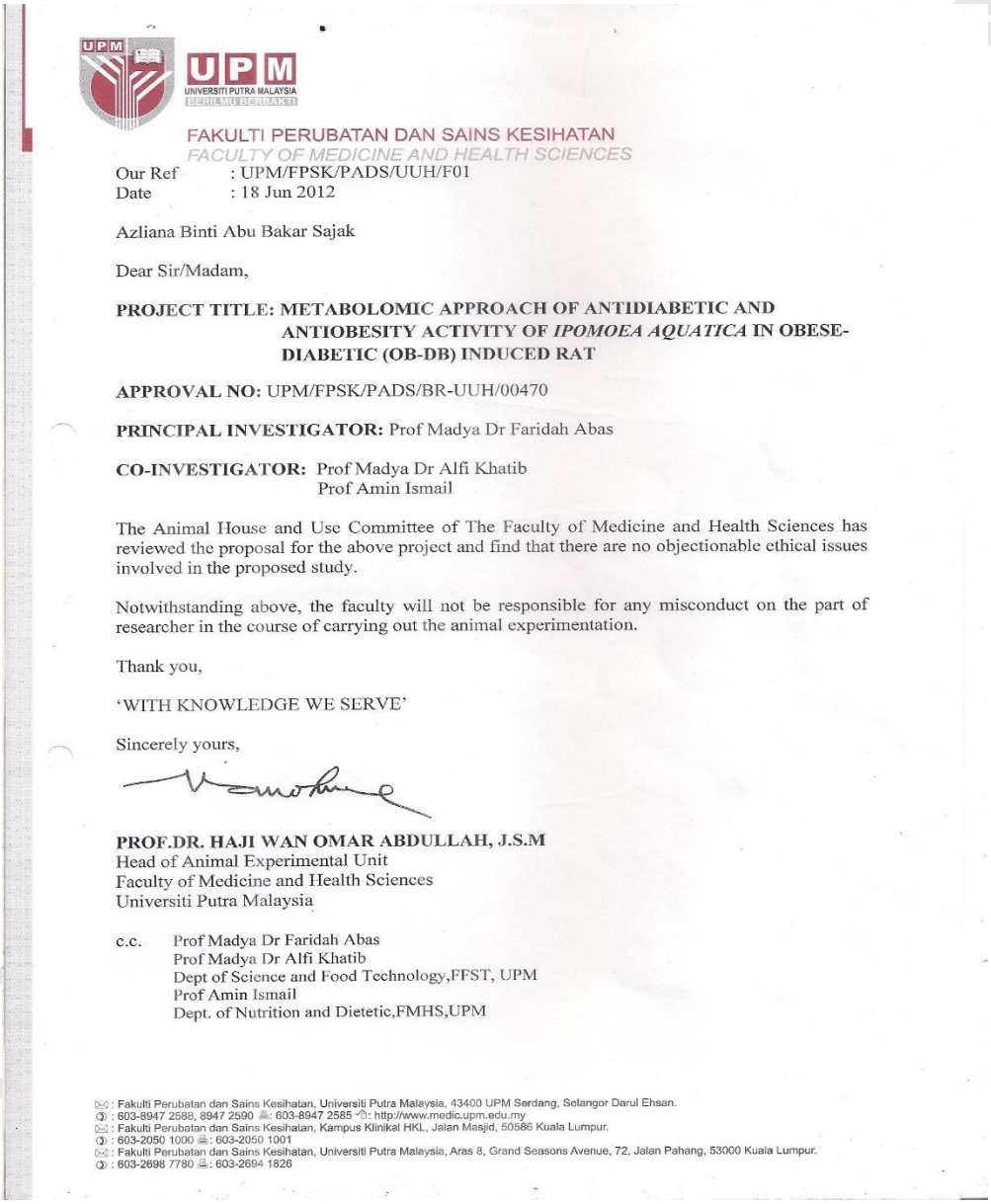
APPENDICES

APPENDIX A



FAKULTI PERUBATAN DAN SAINS KESIHATAN
FACULTY OF MEDICINE AND HEALTH SCIENCES

Our Ref : UPM/FPSK/PADS/UUH/F01
Date : 18 Jun 2012

Azliana Binti Abu Bakar Sajak

Dear Sir/Madam,

PROJECT TITLE: METABOLOMIC APPROACH OF ANTIDIABETIC AND ANTIOBESITY ACTIVITY OF *IPOMOEA AQUATICA* IN OBESE-DIABETIC (OB-DB) INDUCED RAT

APPROVAL NO: UPM/FPSK/PADS/BR-UUH/00470

PRINCIPAL INVESTIGATOR: Prof Madya Dr Faridah Abas

CO-INVESTIGATOR: Prof Madya Dr Alfi Khatib
Prof Amin Ismail

The Animal House and Use Committee of The Faculty of Medicine and Health Sciences has reviewed the proposal for the above project and find that there are no objectionable ethical issues involved in the proposed study.

Notwithstanding above, the faculty will not be responsible for any misconduct on the part of researcher in the course of carrying out the animal experimentation.

Thank you,

'WITH KNOWLEDGE WE SERVE'

Sincerely yours,

PROF.DR. HAJI WAN OMAR ABDULLAH, J.S.M
Head of Animal Experimental Unit
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia

c.c. Prof Madya Dr Faridah Abas
Prof Madya Dr Alfi Khatib
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Figure A.1. Animal ethics approval from the Institutional Animal Care and Use Committee (IACUC) of the Faculty Medicine and Health Sciences, Universiti Putra Malaysia.

BIODATA OF STUDENT

Azliana Abu Bakar Sajak was born in Pahang, Malaysia on 28th July 1986. She completed her primary education at Sekolah Kebangsaan Beserah and her junior high school at Sekolah Menengah Abdul Rahman Talib, Kuantan. After her junior high school examination (PMR), she continues her study at MARA Junior Science College Kuantan (MRSM Kuantan) in 2001. In 2004, she enrolled herself at Gambang Matriculation and continues her bachelor degree studies in Biological Sciences at Universiti Malaysia Terengganu. There, she managed to score an average CGPA 3.76. After working for few years, she decided to pursue her graduate studies at Universiti Putra Malaysia in 2012 under supervision Associate Prof. Dr. Faridah Abas.

LIST OF PUBLICATIONS

Azliana Abu Bakar Sajak, Faridah Abas, Amin Ismail & Alfi Khatib (2016). Effect of Different Drying Treatments and Solvent Ratios on Phytochemical Constituents of *Ipomoea aquatica* and Correlation with α-Glucosidase Inhibitory Activity. *International Journal of Food Properties, 19*, 2817-2831.

Azliana Abu Bakar Sajak, Ahmed Mediani, Maulidiani, Faridah Abas, Amin Ismail and Alfi Khatib. Metabolite Variation in Lean and Obese Streptozotocin (STZ)-Induced Diabetic Rats via ^{1}H NMR-Based Metabolomics Approach. *Applied Biochemistry and Biotechnology*. DOI: 10.1007/s12010-016-2352-9.

Azliana Abu Bakar Sajak, Ahmed Mediani, Maulidiani, Faridah Abas, Amin Ismail and Alfi Khatib. Intervention of *I. aquatica* Extract on Streptozotocin (STZ)-Induced Diabetic Rats via ^{1}H NMR-Based Metabolomics Approach. Submitted to Phytomedicine.

CONFERENCE

Azliana Abu Bakar Sajak, Faridah Abas, Amin Ismail & Alfi Khatib. Application of ^{1}H-NMR Based Metabolomics for Determination of Biological Activity in *Ipomoea aquatica* Extracts Prepared by Different Drying Method and Solvent Ratio. International Conference of Natural Product 2014, Putrajaya, Malaysia, 18-19 March 2014 (Poster presenter).

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