ANTIDIABETIC ACTIVITY AND TOXICITY STUDY OF ORTHOSIPHON STAMINEUS BENTH ETHANOLIC EXTRACTS

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AKTIVITI ANTIDIABETIK DAN KAJIAN KETOKSIKAN EKSTRAK ETANOL *ORTHOSIPHON STAMINEUS* BENTH

oleh

ELSNOUSSI ALI HUSSIN MOHAMED

Tesis yang diserahkan untuk
memenuhi keperluan bagi
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ANTIDIABETIC ACTIVITY AND TOXICITY STUDY OF *ORTHOSIPHON STAMINEUS* BENTH ETHANOLIC EXTRACTS

by

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In the name of ALLAH

The most beneficent and merciful

THIS THESIS IS DEDICATED

TO

MY MOTHER WHOM ACROSS THE SEAS AND THE OCEANS TO

SUPPORT ME DURING MY STUDY

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WHO	World health organization
HDL	High-density lipoprotein
STZ	Streptozotocin
UDP	Uridine 5'-diphospho-glucuronosyltransferase
UGT	Glucoronosyltransferase
GST	Glutathione-S-transferase
RA	Rosmarinic acid
EUP	Eupatorin
TMF	3’-hydroxy-5,6,7,4’-tetramethoxyflavone
TPA	12-O-tetradecanoylphorbol-13-acetate
NDDG	National diabetes data group
IDDM	Insulin-dependent diabetes mellitus
NIDDM	Non-insulin-dependent diabetes mellitus
USA	United State of America
HLA	Human leucocyte antigen
PPARγ	Peroxisome proliferator-activated receptor γ
SD	Sprague dawley
SCGTT	Subcutaneous glucose tolerance tests
ELISA	Enzyme-linked immunoassay
TLC	Thin layer chromatography
ESF-1	Ethyl acetate fraction one
ESF-2	Ethyl acetate fraction tow
sec	Section

HPLC	High-performance liquid chromatography
OGTT	Oral glucose tolerance test
DM	Diabetes mellitus
RIA	Radio immune assays
ARASC	Animal research and service center
ESF	Ethyl acetate fraction
α	Alpha
$NaH_2PO_4.H_{2O}$	Monobasic sodium phosphate
$NaH_2PO_4.2H_{2O}$	Dibasic sodium phosphate
BSA	Bovine serum albumin
DMSO	Dimethylsulphoxide
DW	Distilled water
SS	Stock solution
Sol1	Solution 1
MeOH	Methanol
DNS	3,5-Dinitrosalicylic acid
SIN	Sinensetin
Sd	Standard division
HFE	High fat emulsion
IILI rats	Intraperitoneal injections of long-acting insulin
I.P	Intraperitoneal
OECD	Organization for Economic Cooperation and Development
CO_2	Carbon dioxide
SPSS	Statistical Package for Social Sciences
RBC	Red blood cell count

Hgb	Hemoglobin concentration
Ht	Hematocrit
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
WBC	White blood cell count
MCHC	Mean corpuscular hemoglobin concentration
NOAEL	No-observed-adverse-effect level
ADI	Acceptable daily intake
ATP	Adenosine triphosphate
S.E.M	Standard error of mean
ANOVA	Analysis of variance
CCL_4	Carbon tetrachloride
IS	Insulin sensitivity
GLP-1	Glucagon-like peptide 1
GIP	Glucose dependent insulinotropic peptide
DPP-IV	Dipeptidyl peptidase
FDA	Food and drug administration
HOMA-B	Homeostasis model of assessment of beta-cell function
AIRg	Acute insulin response to glucose
ADA	American diabetes association
NaH_2PO_4	Sodium dihydrogen phosphate
UV	Ultra violet
T_f	Tailing factor
R_s	Resolution factor
LOL	Limit of linearity

QC	Quality control
LOQ	Limit of quantification
LOD	And limit of detection
S/N	Signal-to-noise
RSD	Relative standard deviation
RP	Reversed-phase
YLL	Years of life lost
YLD	Years lived with disability
DALYs	Disability adjusted life years
NHMS-3	National health and morbidity survey

ABSTRAK

Orthosiphon stamineus ialah ubatan tempatan popular yang digunakan untuk merawat pelbagai penyakit termasuk diabetes melitus. Kajian ini dijalankan untuk mengkaji aktiviti antidiabetik, mekanisma tindak balas dan kesan toksik ekstrak piawai daun *Orthosiphon stamineus*. Daun *Orthosiphon stamineus* diekstrak menggunakan (etanol : air) 95%, 75%, 50% dan 25% v/v, masing – masing. Hanya ekstrak etanol 50% (1000 mg/kg) menurunkan paras glukosa darah tikus yang diberikan beban glukosa subkutaneus sebanyak 200 mg/kg. Walaubagaimanapun, ekstrak etanol 50% gagal menurunkan paras glukosa darah tikus diabetik aruhan-STZ. Pengekstrakan cecair – cecair ekstrak etanol 50% menghasilkan fraksi – fraksi etil asetat, n-butanol dan air. Hanya fraksi etil asetat (ESF) (1000 mg/kg) merencat kenaikan paras glukosa darah tikus yang diberi beban glukosa. ESF seterusnya disisihkan kepada dua subfraksi ESF-1 dan ESF-2, menggunakan kromatografi turus kilat kering. Keputusan menunjukkan rawatan dengan fraksi ESF-2 merencat kenaikan glukosa darah lebih daripada rawatan dengan ESF-1. Pemberian ekstrak etanol 50% pada dos 1000 mg/kg dua kali sehari selama empat belas hari menurunkan paras glukosa darah tetapi tidak meningkatkan paras insulin tikus diabetik aruhan-STZ. Kajian *in vitro* menunjukkan ekstrak etanol 50% *O. stamineus* merencat aktiviti α-glukosidase dan α-amilase dalam ciri kebergantungan kepada dos. Kajian *in vivo* menunjukkan pemberian secara oral ekstrak etanol 50% (250, 500 dan 1000 mg/kg) merencat kenaikan paras glukosa darah tikus yang diberi beban kanji dan sukrosa secara oral, masing – masing, yang seterusnya mencadangkan ekstrak mungkin merencat aktiviti α-glukosidase dan α-amilase di dalam saluran

pencernaan dan berguna dalam merawat hiperglisemia postprandial. Pembaikan dalam kerintangan terhadap insulin dengan pemberian oral ekstrak etanol 50% *O. stamineus* juga dikaji secara *in vivo*. Rawatan harian ekstrak etanol 50% (1000 mg/kg) selama empat belas hari dalam tikus rintang insulin aruhan emulsi tinggi lemak menyebabkan penurunan dalam paras glukosa dan insulin plasma. Rawatan yang sama dalam tikus diabetik aruhan-STZ menyebabkan kenaikan dalam respons pemberian eksogenus insulin tindakan jangka pendek yang mencadangkan rawatan ini telah menurunkan kerintangan terhadap insulin dalam tikus. Dalam kajian ketoksikan akut, pemberian secara oral ekstrak etanol 50% (5000 mg/kg) tidak menyebabkan sebarang tanda ketoksikan dan mortaliti yang mencadangkan bahawa LD_{50} adalah lebih tinggi daripada 5000 mg/kg. Dalam kajian ketoksikan sub-kronik, pemberian ekstrak etanol 50% pada dos 1250, 2500 dan 5000 mg/kg setiap hari selama 28 hari dalam tikus tidak menyebabkan sebarang tanda dan simptom toksik yang boleh diperhatikan, perubahan dalam parameter hematologi atau rupa secara kasar organ dalaman yang mencadangkan bahawa ekstrak ini adalah tidak toksik.

ANTIDIABETIC ACTIVITY AND TOXICITY STUDY OF *ORTHOSIPHON STAMINEUS* BENTH ETHANOLIC EXTRACTS

ABSTRACT

Orthosiphon stamineus is a popular folk medicine used for the treatment of many diseases including diabetes mellitus. This study was under taken to investigate the antidiabetic activity, mechanism of actions and toxicity study of standardized *O. stamineus* leaves extract. Leaves of *O. stamineus* were extracted with 95%, 75%, 50%, and 25% (ethanol : water) v/v respectively. Only the 50% ethanolic extract (1,000 mg/kg), reduced the blood glucose level of rats loaded subcutaneously with 200 mg/kg glucose. However, the 50% ethanolic extract failed to lower the blood glucose level of the STZ-induced diabetic rats. Liquid-liquid extraction of the 50% ethanol extract produced ethyl acetate, n-butanol and water fractions. Only ethyl acetate fraction (*ESF*) (1,000 mg/kg) inhibited the rise of blood glucose level of glucose loaded rats. *ESF* was then fractionated further into two sub-fractions *ESF-1* and *ESF-2*, using a dry flash column chromatography. The results showed that treatment with *ESF*-2 fraction inhibited the rise of blood glucose more than *ESF-1*. Administration of 50% ethanolic extract of *O. stamineus* at the doses of 1,000 mg/kg twice daily for fourteen days decreased blood glucose level but not increased the insulin level of STZ-induced diabetic rats. *In vitro* studies showed that 50% ethanolic extract of *O. stamineus* inhibited α-glucosidase and α-amylase activities in a dose-dependent manner. *In vivo* study showed that 50% ethanolic extract of (250, 500 and 1000 mg/kg) treatment inhibited the rise of blood glucose level of oral starch and sucrose loaded rats respectively which suggest the extract may be useful for treatment of postprandial hyperglycemia. The improvement of insulin resistance by an oral

administration of 50% ethanol extract of *O. stamineus* was also investigated *in vivo*. Daily treatment of 50% ethanol extract (1,000mg/kg) for 14 days in high fat emulsion-induced insulin resistance rats caused reduction in both plasma glucose and insulin levels. Similar treatment to STZ-induced diabetic rats caused an increase in the response of exogenous administration of short-acting insulin which suggests that the treatment had reduced the insulin resistance in the rats. In the acute toxicity study, oral administration of 50% ethanol extract (5,000 mg/kg) did not cause any sign of toxicity or mortality which suggest the LD50 is higher than 5,000 mg/kg. In sub-chronic toxicity study, administration of 50% ethanol extract at 1250, 2500, and 5000 mg/kg daily for 28 days in rats did not cause any observable toxic sign and symptom or changes in hematological parameters or gross appearance of the internal organs which suggest this extract is not toxic.

CHAPTER ONE: Introduction

1.1. History of traditional medicines

Traditional medicines, particularly herbal remedies, have been used for thousands of years in maintaining health as an alternative to or in conjunction with modern medicines. People in developed countries spend considerable amounts of money on herbal products. The majority of the world's populations in developing countries take herbal medicines to meet their health needs, following traditional beliefs and practices adopted by their elders and ancestors. Plants are widely used as medicines and there are a number of pure isolated compounds, such as taxol and artemisinin, which are already in use clinically. Herbal products are less potent and, in general, cause fewer adverse reactions than pharmaceuticals and their use can play an important role in the reduction of national spending (Llya *et al*., 2002).

Recent studies on the biologically active constituents of medicinal plants have made it possible to develop new drugs for clinical use (Dhawan, 1982). When natural products serve as the lead compounds in drug development, the medicinal drugs developed via this process are chemically synthesized. However, the interest shown by international bodies like the World Health Organization (WHO) towards traditional plants is a sign of global reawakening regarding the contribution such plants have as important alternatives in health care. It has been realized that if there are to be any real improvements in health care, especially in the underserved populations of the world, there will have to be an optimal use of all available resources and there is no doubt that medicinal plants are one of them. In some countries traditional herbal medicines have been placed on the same footing as

modern medicines (Fransworth and Bingel, 1977). In Asia, countries like China, Japan, Korea and India are among the foremost examples where traditional herbal medicines and remedies have been officially recognized (Dzulkifli *et al*., 1993).

1.2. The plant, *Orthosiphon stamineus* Benth

1.2.1. Botanical aspects

Scientific name : *Orthosiphon stamineus*, Benth.

Synonym : *Ocimum aristatum* Bl., *Orthosiphon aristatum* (Blume)

Common name : Java tea.

Local name : Misai Kucing, Kumis Kucing.

Part used : Leaves and stems.

Figure 1.1. The plant *O. stamineus* Benth (Mohd and Mustafa, 1994).

O. stamineus first began to interest researchers as early as the beginning of the twentieth century when this plant was introduced to Europe, where it became a

popular herbal tea. In Malaysia, it is also appreciated for its elegant, unique flowers and it is commonly seen growing in many gardens. *O. stamineus* is an herbaceous shrub that grows to a height of 1.5 m (Mohd and Mustafa, 1994). The leaves are arranged in opposing pairs. They are simple, green and glabrous, with a lancelet leaf blade and a serrated margin. The leaf apices are acuminate with an acute leaf base. The petiole is relatively short, about 0.3 cm in length, and reddish purple in color, with erect and profuse branches (Mohd and Mustafa, 1994). This plant is one of the most popular traditional folk medicines and is extensively used in Southeast Asia for the treatment of a wide range of diseases. In Malaysia, this plant is used in the form of a decoction to treat diabetes and catarrh of the bladder, and as a diuretic (Bwin and Gwan, 1967). In Vietnam, it is used for treating urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice and biliary lithiasis (Eisai, 1995). In Myanmar, the plant is used to alleviate diabetes and urinary tract and renal diseases (WHO, 1970), and in Indonesia it is used for rheumatism, diabetes, hypertension, syphilis, renal calculus and gallstones, amongst others (Bwin and Gwan, 1967).

1.2.2. Literature review on *O. stamenius* Benth.

In 2007, Abdullaha *et al*. reported that standardized extracts of *O. stamineus* have no observable acute effects on experimental animals at 5000 mg/kg and are therefore non-toxic. Olah *et al*. (2003) reported that the ethanolic extract of *O. stamineus* has high contents of sinensetin, eupatorin, rosmarinic-, cichoric- and caffeic-acids and a good pharmacological action on diuretic and uricosuric in rats. In another study, *O. stamineus* aqueous extracts were found to be useful in the control of diabetes mellitus as they markedly reduced hyperglycemia in STZ-induced diabetic rats, decreased plasma triglyceride and increased plasma high-density lipoprotein (HDL)

cholesterol concentrations (Sriplang *et al*., 2007). The methanolic extracts of this plant have been shown to inhibit nitric oxide production in macrophage-like cells (Awale *et al*., 2003a and b). A study conducted by Chin *et al*. (2009) showed that a methanolic extract of *O. stamineus* leaf exerted a selective effect on aminopyrine N-demethylase, Uridine 5'-diphospho-glucuronosyltransferase (UDP), glucoronosyltransferase (UGT) and glutathione-S-transferase (GST) activities. The activities of GST and UGT were highly sensitive and easily inducible by methanolic extracts of *O. stamineus* leaves compared to aminopyrine N-demethylase activity.

O. stamineus has been reported to contain several active constituents such as terpenoids and polyphenols (Tezuka *et al.,* 2000). Most of the therapeutic effects and health benefits of *O. stamineus* have been mainly ascribed to its polyphenolic contents (Akowuah *et al*., 2005). The latter are the most dominant constituents of the plant's leaves (Hollman and Katan, 1999). Akowuah *et al* (2005) demonstrated the presence of rosmarinic acid (RA), sinensetin (SIN), eupatorin (EUP) and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) in the leaves of *O. stamineus* (Figs. 1.2, 3, 4 and 5). Moreover, the RA contents of this plant were also reported by Tezuka *et al.* (2000). Lipophilic flavonoids isolated from *O. stamineus* have been found to possess a remarkable free radical-scavenging activity towards the diphenylpicrylhydrazyl radical, adding to their abilities to inhibit the enzyme 15-lipoxygenase from soybeans, a model for mammalian 15-lipoxygenase (Lyckander and Malterud, 1996). Researchers have also indicated that the flavones SIN and TMF, isolated from *O. stamineus*, exhibit a potent diuretic activity in rats (Schut and Zwaving, 1993). On the other hand, the diterpenes isolated from *Orthosiphon sp.* have been shown to exhibit antiproliferative activities on the rat thoracic aorta (Tezuka *et al.,* 2000;

Awale *et al*., 2001; Awale *et al*., 2002a and b). Furthermore, studies on these diterpenes revealed their substantial capabilities of antagonizing the actions of nitric oxide (Awale *et al*., 2004) and inhibiting tumor promoter of 12-O-tetradecanoylphorbol-13-acetate (TPA) induced inflammation in mouse ears (Masuda *et al*., 1992). Recent studies have also shown that *O. stamineus* extracts retain a notable hepatoprotective action against CCl_4-induced hepatopathy (Yam *et al*., 2007), anti-inflammatory and non-narcotic analgesic activities (Yam *et al*., 2008) and antipyretic effects in a yeast-induced increase in body temperature (Yam *et al*., 2009).

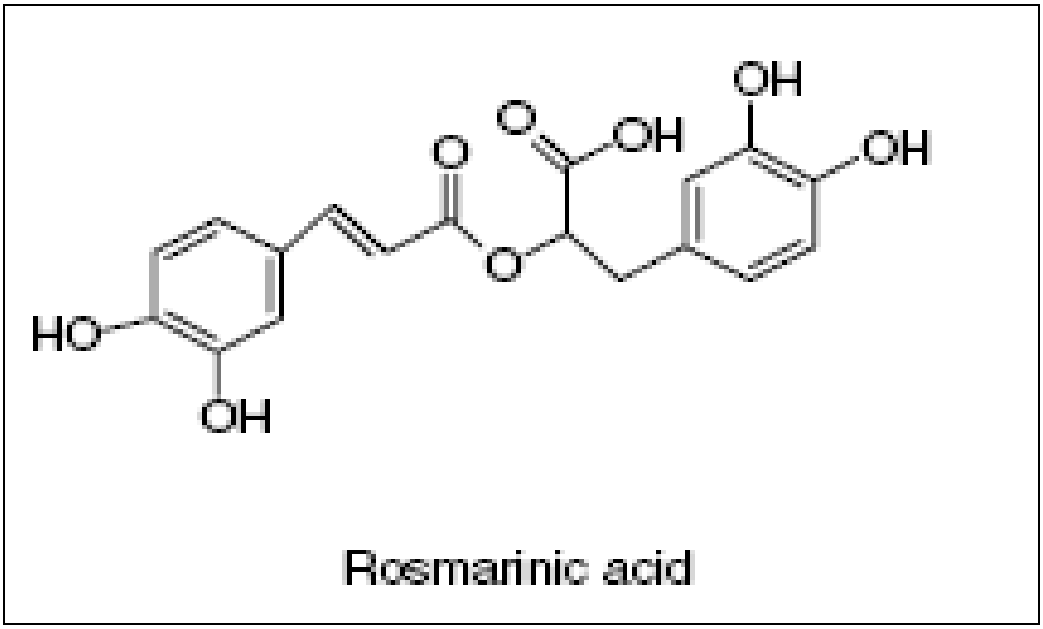

Figure 1.2. Molecules of Rosmarinic Acid (RA) (Akowuah *et al*, 2004).

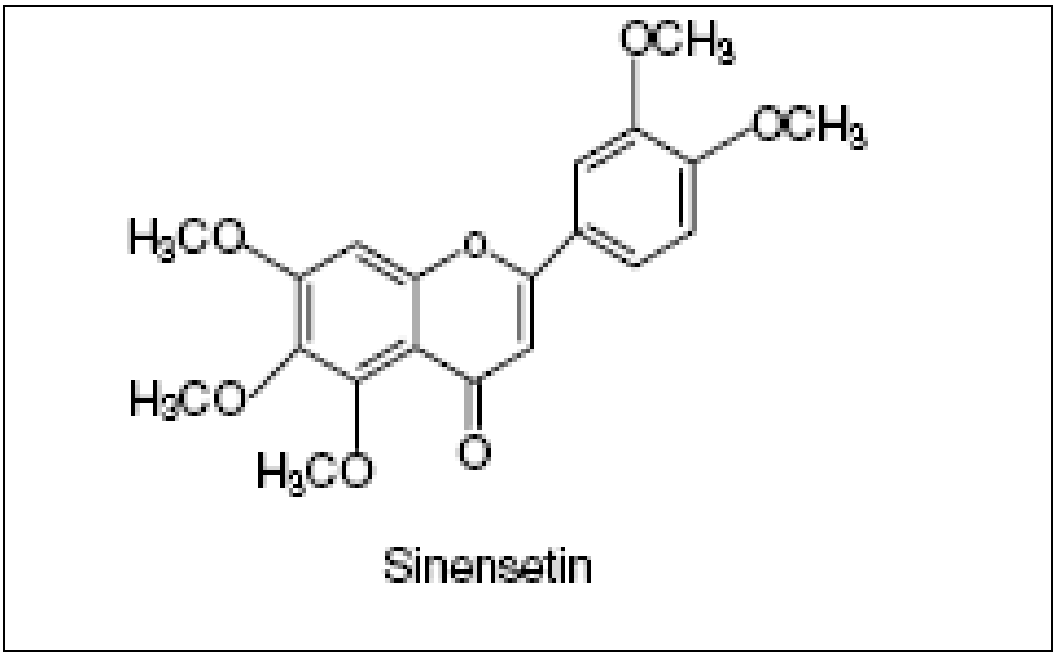

Figure 1.3 Molecules of Sinensetin (SIN) (Akowuah *et al*, 2004).

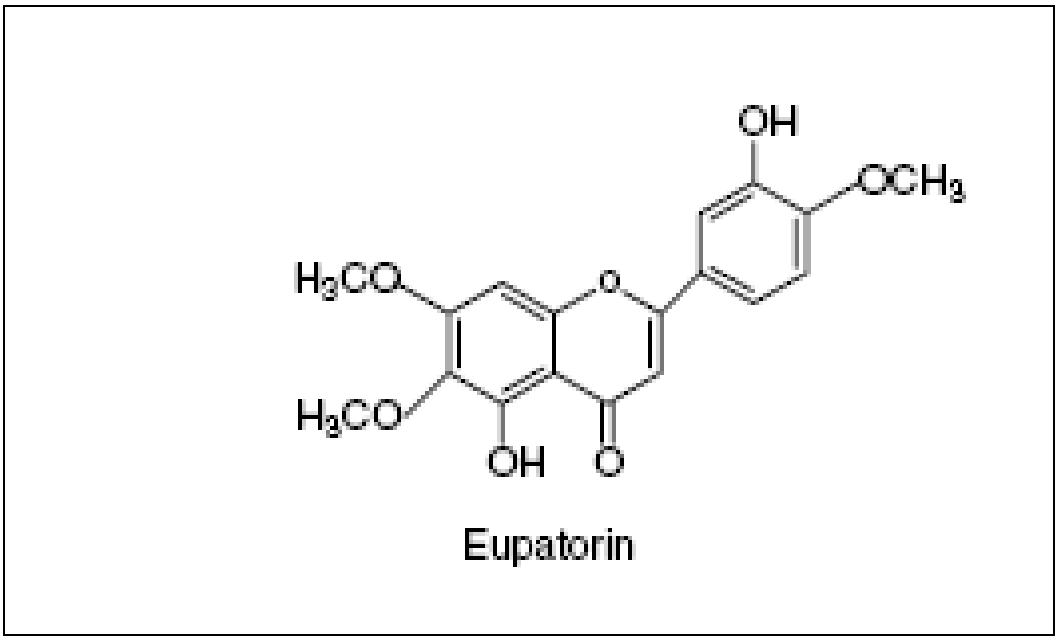

Figure 1.4. Molecules of Eupatorin (EUP) (Akowuah *et al*, 2004).

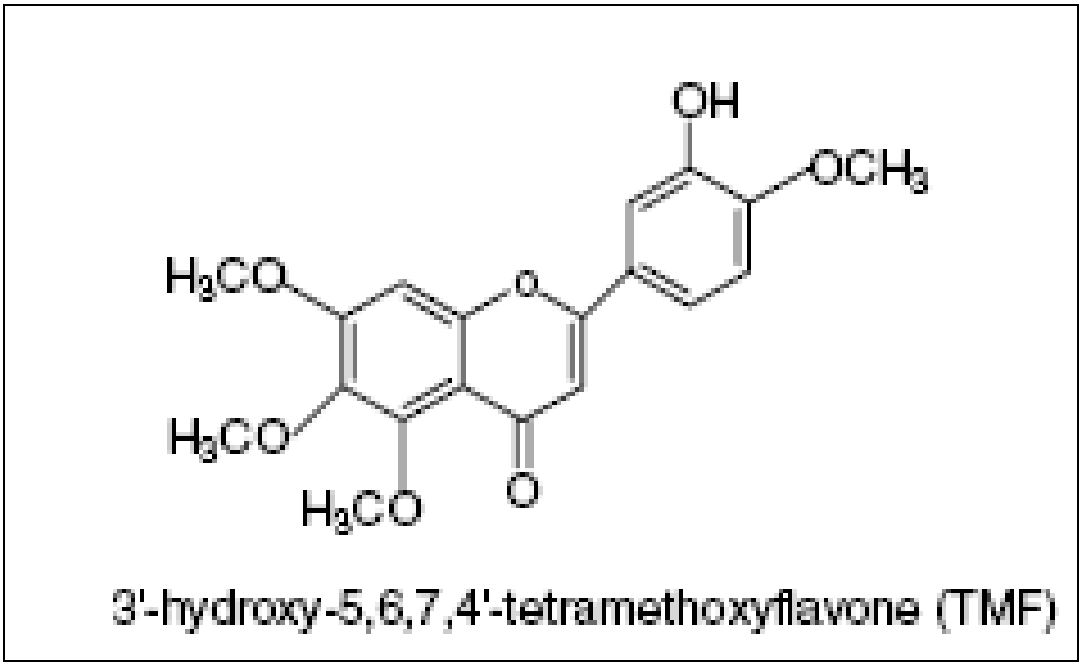

Figure 1.5. Molecules of 3-hydroxy-5,6,7,4 – tetramethoxyflavone (TMF) (Akowuah *et al*, 2004).

1.3. Diabetes mellitus

Historically, diabetes mellitus is one of the oldest diseases known to mankind. It was first mentioned in the Ebers Papyrus (Egypt 1500 BC) and '*honey urine*' was noted by Sushrutha in India in 400 BC. By the first century of the Christian era this disease was well known, both in Roman writings and in Chinese and Japanese writings (Distiller, 1980). The word '*diabetes*' was first coined by the Greeks. It means a passing-through of water. They described it as a 'melting of flesh into water', meaning urine. The Latin word '*mel*', which means honey, was used and the disease came to be known as diabetes mellitus or passing of honeyed urine. This is still the full name of the disease – *diabetes mellitus* (Distiller, 1980).

In 1997 an estimated 15.7 million Americans, or 5.9% of the population, had diabetes mellitus (Siri *et al*., 2004). Among this group, 10.3 million were diagnosed diabetics and 5.4 million were undiagnosed (Siri *et al*., 2004). Diabetes is a complex and costly disease that can affect almost every organ in the body and result in devastating consequences. Diabetes is the leading cause of non-traumatic lower extremity amputations, renal failure and blindness in working-age adults and it is also a major cause of premature mortality, stroke, cardiovascular disease, peripheral vascular disease, congenital malformation, perinatal mortality and disability (Michael and Linda, 2000).

Before 1979, no standard classification scheme or uniform diagnostic criteria existed for diabetes. In 1979-1980, the National Diabetes Data Group (NDDG) in the United States and the World Health Organization (WHO), in parallel efforts, reviewed the available scientific knowledge and developed and disseminated recommendations for

the classification and diagnosis of diabetes. These recommendations were accepted worldwide and significantly contributed to a tremendous expansion in the knowledge about diabetes (Jack *et al*., 2000). During 1995-1997, the international expert committee on the diagnosis and classification of diabetes mellitus convened and revised the NDDG-WHO recommendations for the classification and diagnosis of diabetes and also made recommendations for testing of diabetes. The committee recommended that diabetes be classified as type I and type II. The type I designation was chosen to replace the designation of insulin-dependent diabetes mellitus (IDDM), also called juvenile-onset diabetes. The type II designation replaced the designation of non-insulin-dependent diabetes mellitus (NIDDM) also called adult-onset diabetes (Jack *et al*., 2000).

1.3.1. Type I diabetes (insulin-dependent diabetes mellitus, IDDM)

Type I diabetes accounts for 5-10% of all diagnosed cases of diabetes. It is one of the most frequent chronic diseases in children. About 40% of people with type I diabetes are younger than 20 years of age, and about 30,000 new cases of type I diabetes occur each year in USA (Jack *et al*., 2000). However, about 9% of diabetics in North America and 20% of diabetics in Scandinavian countries have type I diabetes. It is a catabolic disorder in which circulating insulin is virtually absent, plasma glucagons are elevated, and pancreatic β-cells fail to respond to all known insulinogenic stimuli. In the absence of insulin the three main target tissues of insulin (liver, muscle and fat) not only fail to appropriately take up absorbed nutrients but they also fail to continue to deliver glucose, amino acids and fatty acids into the bloodstream from their respective storage depots (Francis and David, 2004).

The onset of type I diabetes is often abrupt, with symptoms of thirst, polyuria, weight loss and a tendency for the liver to produce ketones. Ketone production can be excessive and lead to ketoacidosis as the presenting clinical picture. The primary defect is insulin deficiency due to disease of the beta cells of the islets of Langerhans, and ketoacidosis results from severe insulin deficiency (John and Jeremy, 1983). Genetic factors account for about one third of the susceptibility to type I diabetes, the inheritance of which is polygenic. Over 20 different regions of the human genome show some linkage with type I diabetes, but most interest has been focused on the human leucocyte antigen (HLA) region within the major histocompatibility complex on the short arm of the chromosome. This locus is designated IDDM (Christopher *et al.*, 1999).

1.3.2. Type II diabetes (non-insulin-dependent diabetes mellitus, NIDDM)

Type II diabetes, characterized as non-insulin-dependent or insulin-resistant, can be present for months or years with few or no symptoms and is the type that occurs in middle and old age. Sufficient glucose enters cells to permit adequate energy production for most situations, and the main problem is excess glucose outside the cells rather than a shortfall inside. Such patients are often overweight (which increases cellular resistance to insulin) and do not usually become ketotic (Wiernsperger and Bailey, 1999). They can often be treated successfully with a diet that avoids immediate hyperglycemia by limiting the intake of low molecular weight carbohydrates and by losing excess weight (Derek and Andrew, 1994). Genetic factors are more important in the etiology of type II than type I diabetes, as shown by studies in monozygotic twins where concordance rates for type II diabetes were found to approach 95% (Derek and Andrew, 1994). Molecular genetics has allowed

the identification of certain specific and clinically identifiable forms of type II diabetes that are the result of single gene defects. The majority of cases of type II diabetes are multifactorial in nature, with interactions from environmental and genetic factors (Christopher *et al*., 1999). The nature of the genetic contribution is largely unknown but it is evident that several genes are involved. In this polygenic model the inheritance of abnormalities in individual genes would not be sufficient to cause type II diabetes directly, but it would confer increased (or decreased) susceptibility. Also, age is an important risk factor for type II diabetes. In Britain, over 70% of all cases of diabetes occur after the age of 50 years. Type II diabetes is principally a disease of the middle-aged and elderly, affecting 10% of the population over the age of 65 (Christopher *et al*., 1999).

1.4. Diabetes in Malaysia

Malaysia is a multiethnic country with a total population of 28.25 million (Department of Statistics Malaysia, 2010). According to the third national health and morbidity survey (NHMS-3) in Malaysia, the prevalence of type II diabetes mellitus in adults aged 30 years and over now stands at 14.9%, increased from 8.3% in 1996, with the highest prevalence among those of Indian ethnicity (National Health and Morbidity Survey III, 2006). The number of people with diabetes is expected to increase from 1,846,000 in 2010 to 3,254,994 in 2030, and the adjusted prevalence of diabetes (adjusted to the world population) in Malaysia will rise from 11.6% in 2010 to 13.8% in 2030 (International Diabetes Federation, 2009). In the Malaysian Ministry of Health, there is an increasing interest in the increasing prevalence of chronic disease, including diabetes, within the population (Lim and Lim, 2006). This increase in prevalence of diabetes is associated with many factors, including rapid

economic growth of the country in the last few decades, urbanization and industrialization which have resulted in more overweight/obese people and a sedentary population (Ismail *et al*., 2002; Kee *et al*., 2008; Mustaffa, 2004). A skyrocketing transformation in socioeconomic and demographic status over the last two decades has occurred in Malaysia as a result of massive industrialization and globalization with an improved educational system. As a result, the standard of living, quality of life, population and the concomitant ageing of the population and reduction in the death rate have improved (Yun *et al*., 2007). The percentage of Malaysia people over the age 65 of years has increased annually by an average of 2.5% between 1991 and 2000 (Poi *et al*., 2004). In this age group, around 25% to 30% of people have diabetes or glucose intolerance (Wild *et al*., 2004). In the Malaysian burden of disease and injury study (Faudzi, *et al*., 2004), it was estimated that for year 2000, there were 2,261 deaths attributed to diabetes (857 men and 1404 women). Although diabetes was not in the top 10 causes of highest years of life lost (YLL) in men, the disease incurred a huge burden in terms of non-fatal disability, i.e. morbidity measured as years lived with disability (YLD). Diabetes was ranked third at 34,750 years in the men. The scenario was worst for women; scoring higher for both indexes; 18,759 years for YLL and 37,631 years for YLD. This means, Malaysian women with diabetes die faster, and those who survived, had to endure more sufferings. The simple mathematical addition of YLL + YLD yielded yet another index; the disability adjusted life years (DALYs), is a composite measure of burden of premature mortality and non-fatal health outcome (morbidity). Based on DALYs, among the top 10 total burden of disease in Malaysia, diabetes was ranked 6th for men and 5th for women. Despite its lower ranking, diabetes probably

contributed significantly to the causality of the higher ranking diseases with the exception of unipolar major depression (Faudzi, *et al*., 2004).

1.5. Oral antidiabetic drugs

Increasing knowledge about the molecules involved in β-cell functions provides potential new targets for drug development. The aims of the treatment of type II diabetes are to alleviate symptoms through the normalization or near-normalization of fasting and postprandial blood glucose levels and to prevent acute and long-term complications. Intensive treatment of type I diabetes prevents the development of microvascular and neurological complications, and most likely will do the same in type II diabetes (Ohkubo *et al*., 1995). There are various pharmacological approaches for improving glucose homeostasis but the ones that are currently being used in clinical practice either do not succeed in restoring normoglycemia in most patients or they fail after variable periods of time. Four classes of drugs are currently available for glycemic regulation: sulphonylureas, for example glibenclamide; biguanides, for example metformin; α-glucosidase inhibitors, for example acarbose; and insulin, each of which has a different mode and site of action. These standard pharmacological treatments can be used individually for certain types of patients or they can be combined in a stepwise fashion to provide a more ideal glycemic control in these patients (Gerard *et al*., 1999).

1.5.1. Sulphonylureas

The first generation of sulphonylureas, including such compounds as chlorpropamide, acetohexamide, tolazamide and tolbutamide, were developed and introduced into clinical use in the 1950s. More potent second-generation agents, such

as glibenclamide (glyburide), glipizide and gliclazide, were developed during the late 1960s and 1970s. There are differences between the various sulphonylureas with respect to their duration of action, adverse effects, potency and metabolism (Holman and Turner, 1991). The chemical differences are responsible for pharmacokinetic differences. All sulphonylureas act by stimulating pancreatic β-cells to secrete insulin. They bind to β-cell receptors, which are closely associated with ATP-dependent K+ channels; the closure of these channels causes depolarization of the cell, an influx of Ca^{2+} and the stimulation of insulin secretion (Groop, 1992; Aguilar-Bryan *et al*., 1995). All sulphonylureas directly stimulate insulin secretion but, more importantly, they also augment glucose-induced insulin secretion. Insulin release is normally biphasic. An early peak occurs during the first few minutes of glucose-induced stimulation, after which there is a slower, more sustained second phase. In common with tolbutamide, gliclazide mainly stimulates the early peak of insulin secretion, whereas other sulphonylureas, such as glibenclamide and chlorpropamide, largely exert their insulin secretory effects during the second phase (Melander *et al*., 1989).

The timing of medication depends on the pharmacokinetics of the individual sulphonylurea, but taking a dose 30 min before breakfast will usually produce optimal control. Sulphonylureas with a short half-life, especially tolbutamide, should be taken immediately before meals. Primary failure implies that there is severe β-cell dysfunction. Patients with higher fasting blood glucose levels (usually >14 mmol/L) or ketonuria at diagnosis are unlikely to respond to initial sulphonylurea treatment and will require insulin. Mutations in the sulphonylurea receptors have yet to be implicated as a mechanism for primary drug failure (Aguilar-Bryan *et al*., 1995;

Inoue *et al*., 1996). Sulphonylurea failure is a term used to denote the presence of symptomatic hyperglycemia despite treatment or following a symptom-free period of time (secondary sulphonylurea failure) (Holman and Turner, 1991). The occurrence of secondary failure implies that the sulphonylurea is no longer effective. The clinical efficacy of sulphonylurea is greatest in patients with type II diabetes of 5 years duration or less. The secondary failure rate has been reported to be as high as 3-5% per year (Groop, 1992; Lebovitz, 1994; Pontiroli *et al*., 1994). The issue of efficacy is complicated by patient noncompliance, the use of inadequate drug doses and by other confounding factors (Groop, 1992; Pontiroli *et al*., 1994). Profound hypoglycemia is the major adverse effect associated with all sulphonylureas. This is particularly evident in the case of chlorpropamide, which has a long duration of action; for this reason, caution is needed when this drug is taken by aged patients or patients with renal insufficiency. Another adverse effect of sulphonylureas is a gain in body weight. The anabolic effects of increased insulin levels together with reduced urinary losses of glucose can contribute to such a weight increase. Sulphonylureas have no stimulatory effect on insulin secretion in patients with insulin-dependent diabetes mellitus or in patients with pancreatectomized diabetes since successful therapy requires a functioning insulin secretory system (Jackson and Bressler, 1981).

1.5.2. Biguanides

The biguanide metformin alleviates hyperglycemia from type II diabetes by inhibiting hepatic glucose production and improving peripheral insulin sensitivity. In contrast to sulphonylureas, metformin does not stimulate insulin secretion, promote weight gain, exacerbate hyperinsulinemia or cause hypoglycemia. It also favorably affects serum lipids. Metformin can be used as first-line monotherapy in type II

diabetes or in combination with a sulphonylurea when monotherapy with either agent fails (Guthrie, 1997). It can be particularly suitable when weight gain, hyperlipidemia and hypoglycemia are clinically important issues. Pharmacological studies have indicated that metformin acts by improving peripheral tissue sensitivity to insulin, reducing the gastrointestinal absorption of glucose and decreasing hepatic glucose production (Klip and Leiter, 1990; Bailey, 1992). A decrease in total plasma cholesterol, low-density lipoprotein cholesterol and triglyceride levels and an increase in high-density lipoprotein cholesterol levels were reported (Bailey, 1993; Defronzo *et al*., 1995; Stumvoll *et al*., 1995). Metformin lowers elevated blood glucose levels, but it does not normally cause hypoglycemia. It can actually reduce insulin levels and, in most cases, this is secondary to its glucose-lowering effects and not a direct action of the drug (Hermann and Melander, 1992; United Kingdom Prospective Diabetes Study Group, 1995). Tucker *et al.* (1981) reported that metformin at doses of 0.5-1.5 g has an absolute oral bioavailability of 50-60%, and that its gastrointestinal absorption is complete within 6 hours of ingestion. It has a mean plasma elimination half-life of 1.5-4.7 hours. This is prolonged in patients with impaired renal function and correlates with creatinine clearance. Drug interactions with metformin are infrequent. Metformin monotherapy (0.5-3 g/day) was shown to reduce fasting blood glucose concentrations (by 22-26% of pretreatment levels) and glycated hemoglobin (by 12-17% of pretreatment levels, which represents a fall in glycated hemoglobin from 1.4 to 1.8%) to a significantly greater extent than a placebo in controlled clinical studies of up to 8 months duration in patients with type II diabetes mellitus poorly controlled by diet alone (Dornan *et al*., 1991; Noury and Nandeuil, 1991; Defronzo *et al.,* 1995).

1.5.3. α-glucosidase inhibitors

α-glucosidase inhibitors are competitive inhibitors of small intestinal α-glucosidase enzymes that break down non-absorbable oligosaccharides (Balfour and McTavish, 1993) only commercialized α-glucosidase inhibitor is acarbose. Chemically, acarbose is an oligosaccharide produced by cultured strains of actinomycetes. It is a competitive inhibitor with a high affinity for sucrase and a lower affinity for glucoamylase and pancreatic α-amylase (Taylor, 1990). It is administered with food and must be ingested three or more times per day. When patients ingest meals containing sucrose or starch, acarbose decreases the postprandial rise in blood glucose levels (Taylor, 1990). The activity of acarbose is non-systemic. Clinical studies of acarbose in patients with both type I and type II diabetes have shown decreases in postprandial blood glucose concentrations and urinary glucose excretion (Requejo *et al*., 1990; Chiasson *et al*., 1994). The improvement in blood glucose control is modest (Taylor, 1990). Overall, acarbose has the advantage that when it is used as monotherapy it never causes hypoglycemia. The major adverse effects of acarbose are on the gastrointestinal system, particularly flatulence, abdominal bloating, borborygmus and sometimes diarrhea (Hotta *et al*., 1993).

1.5.4. Thiazolinediones

Recently, a new class of insulin-sensitizing agents, the thiazolinediones, has caused much interest. Thiazolinedions are insulin action enhancers that appear to improve glucose tolerance, decrease hepatic glucose production and increase insulin-stimulated glucose disposal (Saltiel and Horikashi, 1995). In an attempt to understand their mechanism of action, thiazolinedions were observed to bind with high affinity and activate a nuclear hormone receptor called peroxisome proliferator-

activated receptor γ (PPARγ). The ligand that binds naturally to PPARγ has not been identified, but it is believed that the PPARγ receptor is potentially important in promoting the differentiation of fat cells (Tontonoz *et al*., 1994). One of the factors that control fat cell differentiation is the switching on of genes that mediate insulin action. Accordingly, the stimulation of PPARγ has been shown to turn on the expression of several genes involved in glucose and lipid metabolism. Several thiazolinedione compounds have been developed (e.g., pioglitazone, darglitazone, BRL-49653). One of these agents, troglitazone (Rezulin®, approved by the Food and Drug Administration on January 29, 1997), was evaluated in patients with type II diabetes and found to be efficacious and safe (Ciaraldi *et al*., 1990; Colca and Morton, 1990). The efficacy of troglitazone in patients with type II diabetes has been demonstrated in a substantial number of clinical studies (Suter *et al*., 1992). Troglitazone is rapidly absorbed from the gastrointestinal tract; peak blood levels occur within 2-3 hours. It should be taken with meals because food increases its absorption by 30-85%. The use of oral troglitazone has been shown to result in a decrease in fasting and postprandial blood glucose levels (Iwamoto *et al*., 1996). Treated diabetic patients showed improved glucose tolerance test results, decreased plasma free fatty acid and triglyceride levels, decreased plasma insulin levels, decreased gluconeogenesis and increased glucose disposal (Suter *et al*., 1992; Mimura *et al*., 1994).

1.5.5. Dipeptidyl peptidase (DPP)-IV

Dipeptidyl peptidase (DPP)-IV inhibitors, which act via enhancing the incretins, represent another new therapeutic approach to the treatment of type 2 diabetes. Glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic peptide (GIP)

account for the majority of incretin action (Drucker 2003a). GLP-1 is a gut hormone that plays a key role in glucose homeostasis via its incretin effect. GLP-1 is produced from the enteroendocrine L-cell of small intestine and is secreted in response to meal and nutrients. It stimulates insulin release from the pancreatic islets in a glucose dependent manner. It restores the defective first and second phases of insulin response to glucose in type 2 diabetes patients (Fehse *et al*., 2005 and Nauck *et al*., 1993). Moreover, GLP-1 suppresses post-prandial glucagon release, delay gastric emptying and increase satiety (Drucker 2002; Nauck 1997and Kieffer and Habener 1999).

The therapeutic potential of native GLP-1 is limited by its short physiologic half-life, owing to its rapid inactivation by the enzyme DPP-IV and to renal clearance. As such, several selective inhibitors of DPP-IV are being developed for the treatment of type II diabetes mellitus (Drucker 2003b). These orally administered DPP-IV inhibitors can increase circulating levels of endogenous GLP-1 and GIP and improve glucose homeostasis in human subjects with type II diabetes mellitus (Herman *et al*., 2005 and Ahren *et al*., 2004). At the American Diabetes Association (ADA) 66th Scientific Sessions, considerable new data were presented on the actions of these agents in preclinical studies and in patients with type II diabetes mellitus (66th). This report discusses the most clinically relevant data presented at this meeting pertaining to the DPP-IV inhibitors sitagliptin (MK-0431) and vildagliptin (LAF-237).

1.5.5.1. Sitagliptin

In October 2006, sitagliptin became the first DPP-IV inhibitor to gain food and drug administration (FDA) approval for the treatment of type 2 diabetes. Sitagliptin tablets are commercially available as 100-mg (beige), 50-mg (light beige), and 25-mg (pink) tablets. Sitagliptin is also available in a combination product with metformin in doses of 50 mg sitagliptin/500 mg metformin and 50 mg sitagliptin/1,000 mg metformin (White 2008). The clinical efficacy and tolerability of sitagliptin were evaluated in monotherapy and in combination with established antidiabetic agents. A 12-week, randomized, double-blind study compared sitagliptin 100 mg once daily (n = 75) with placebo (n = 76) in Japanese patients with type II diabetes mellitus (after washout of antidiabetic medications). Mean baseline A1C was 7.5% in the sitagliptin-treated group. At week 12, there was a between-treatment group difference in A1C of -1.05% (95% confidence interval: -1.27, -0.84), favoring sitagliptin, with 58.1% of patients on sitagliptin achieving an A1C of < 7% compared with 14.5% on placebo. The incidence of adverse events was similar between the groups, with no hypoglycemia and body weight unchanged in the sitagliptin arm (Nonaka *et al*., 2006). Another monotherapy study Raz *et al* (2006) randomized 521 patients with type II diabetes mellitus (mean A1C 8.1%) to placebo, sitagliptin 100 mg once daily, or sitagliptin 200 mg once daily for 18 weeks. Compared with placebo, the reduction in A1C was 0.60% greater with sitagliptin 100 mg and 0.48% greater with sitagliptin 200 mg, respectively. Fasting glucose similarly showed significant reduction with sitagliptin. In addition, sitagliptin 100 mg was associated with improvement in both the fasting proinsulin/insulin ratio and homeostasis model of assessment of beta-cell function (HOMA-B), suggestive of beneficial effects on

beta-cell function. These data support the possibility of using this DPP-IV inhibitor in combination therapy with other antidiabetic agents (Raz *et al*., 2006).

1.5.5.2. Vildagliptin

Vildagliptin (Glavus®) is a DPP-IV inhibitor, has completed phase iii clinical development and awaiting FDA approval for marketing. A series of studies evaluated the clinical efficacy and tolerability of vildagliptin in patients with type II diabetes mellitus. In a 52-week, randomized, double-blind study (Dejager *et al*., 2006) comparing vildagliptin 50 mg twice daily (n = 526) with metformin 1000 mg twice daily (n = 254) in drug-naive patients with type II diabetes mellitus (mean baseline glycated hemoglobin [A1C], 8.7%), the adjusted mean change in A1C from baseline to endpoint was -1.0 ± 0.1% in patients receiving vildagliptin and -1.4 ± 0.1% in those receiving metformin. Although this result did not establish noninferiority of vildagliptin relative to metformin as monotherapy, it did demonstrate an early (by 12 weeks) and sustained (over 1 year) reduction in A1C with this DPP-IV inhibitor. In addition, vildagliptin therapy was not associated with weight gain and was well tolerated, with a lower incidence of gastrointestinal side effects than was observed with metformin (21.8% vs 43.7%, $P < .001$) and no significant hypoglycemia. The mechanisms underlying the glucose-lowering activity of vildagliptin were investigated in several studies. Beta-cell function was evaluated in a 12-week study (D'Alessio *et al*., 2006) comparing vildagliptin (50 mg twice daily, n = 7) and placebo (n = 5) in drug-naive patients with type II diabetes mellitus (mean A1C 6.8% ± 0.1%). Participants underwent frequently sampled intravenous glucose tolerance tests at baseline, after 12 weeks of treatment, and after 2-4 weeks of washout from study drug. Vildagliptin was associated with significant increases in the acute insulin

response to glucose (AIRg) and insulin sensitivity (IS), such that the disposition index (AIRg x IS), a measure of beta-cell compensation for ambient insulin resistance, increased by more than 4-fold during vildagliptin treatment. Furthermore, it is intriguing to note that some of the improvement in beta-cell function was maintained after the 2- to 4-week washout period, suggesting that vildagliptin exhibits the potential to exert a disease-modifying effect (D'Alessio et al., 2006).

1.6. Objective

The direction of the present study was aimed on the influence of ethanolic extract of *O. stamineus* on all major attractive targets of diabetes intended to act on decrease the hyperglycemia, countering insulin resistance, insulin sensitivity, α-glucosidase and α-amylase inhibitory potential and the safety use of *O. stamineus* by focusing on the acute and 28-days sub-chronic toxicity. This would help the research on *O. stamineus* advance further and throw some light on previously unknown details.

CHAPTER TWO: The antihyperglycemic effect of a single dose administration of ethanolic extracts of *O. stamineus* Benth in Sprague-Dawley rats and their activity-guided fractionation

2. Introduction

In vivo or whole animal models play an important part in pharmacological research, especially in preliminary bioactivity studies. Since the results of *in vitro* studies need to be supported and verified by *in vivo* findings, proper models must be chosen wisely. A scientific model is not a complete representation of human pathophysiology but merely a scientific analogy. In a preliminary screening study the model should be simple and reliable. The parameters should be objectively measurable in order to ensure easy replication and to provide a reliable guide for follow-up studies, especially in bioactivity-guided isolation studies. Two test methods were used in the present study; namely, hypoglycemic and intraperitoneal glucose tolerance tests. The latter was basically a modification of the oral glucose tolerance test in humans.

O. stamineus is traditionally used in the treatment of type II diabetes mellitus and is usually taken orally in the form of a water concoction (Bwin and Gwan, 1967). Sriplang *et al*. (2007) found that *O. stamineus* aqueous extracts reduced hyperglycemia, decreased plasma triglyceride and increased plasma HDL-cholesterol concentrations in STZ-induced diabetic rats. An antihyperglycemic effect of the 80% ethanolic extract of the plant was observed in normal and diabetic rats after acute and repeated daily dose administrations (Zhang and Tan, 2000). However, Mariam *et al*.

(1996) reported that a 1 g/kg aqueous extract of this plant reduced the blood glucose levels in both normal and STZ-induced diabetic rats. A hypoglycemic agent is an agent that has the ability to reduce blood glucose levels to below normal fasting levels, for example the antidiabetic drug glibenclamide, whereas an antihyperglycemic agent lowers the blood glucose level but not to below the normal fasting level, for example metformin (Nolte and Karam, 2001). Using these two clinically proven antidiabetic drugs in animal models as reference drugs helps to maintain the relevancy of the models.

In vivo experiments play an important role in the pharmacological screening of new drugs, especially in preliminary bioactivity studies. This is especially true where a preliminary *in vitro* result requires further *in vivo* testing to be carried out. Therefore, both *in vitro* and *in vivo* tests are important methods for screening the unknown pharmacological effects of a drug and for characterizing the range of its activities. The results of *in vivo* tests are anything but obvious because of the simple reason that *in vitro* tests are usually performed using isolated tissues or simulated conditions that do not completely relate to human pathophysiology. Thus, the effects of new drugs or plant extracts should be further demonstrated in whole live animals with simulated disease conditions.

In addition, as these two tests are capable of screening for hypoglycemic and antihyperglycemic agents they are also useful for preventing any false-negative or false-positive findings in the screening of plants with antidiabetic actions. Therefore, the aims of the present study were to examine the antihyperglycemic and hypoglycemic effects of *O. stamineus* extracts with different solvent ratios of

ethanol-water mixture. In this study different percentages of ethanol in water were used as solvents to prepare extracts *of O. stamineus* for preliminary bioactivity screening. The solvents used were 95%, 75%, 50% and 25% ethanol v/v in water. The extraction of active compounds from the plant into these solvents would be dependent on their polarity; compounds having same polarity would become concentrated in same polarity solvents.

2.1. Objectives

- To verify the validity of the hypoglycemic and antihyperglycemic screening model employed.
- To utilize the hypoglycemic and subcutaneous glucose tolerance tests (SCGTT) for screening the effects of 95%, 75%, 50% and 25% ethanol/water extracts of *O. stamineus.*
- To perform SCGTT-guided fractionation on the active extracts of *O. stamineus* in normal and STZ-induced diabetic rats.

2.2. Materials and methods

2.2.1. Plant material and preparation of the extracts

Dried leaves of *O. stamineus* were collected from Kepala Batas, Pulau Pinang Malaysia. The plant was identified at the School of Biological Sciences, Universiti Sains Malaysia, and a voucher specimen (10810) was deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The dried leaves were powdered using a milling machine and then divided into four equal portions and extracted with 95%, 75%, 50% and 25% water/ethanol, v/v, respectively. The ethanol solvent was supplied by R&M Chemical Sdn Bhd as 95% v/v denatured