ANTIOXIDANT AND ANTIHYPERGLYCEMIC ACTIVITY OF *SOPHORA ALOPECUROIDES* SEED ON STREPTOZOTOCIN-NICOTINAMIDE INDUCED DIABETIC RATS

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

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ORIGINAL LITERARY WORK DECLARATION

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ABSTRACT

Sophora alopecuroides seed (SAS) was extracted with chloroform, ethanol and distilled water. The TLC result showed the presence of alkaloids in all of the extracts and the total alkaloid contents was 7.56%. The alkaloids were separated and identified with Q-TQF MS with known reference standard and were found that *Sophora alopecuroides* seed contained alkaloids namely sophocarpine, matrine, baptifoline, oxysophocarpine, oxymatrine, sophocarpine dimer, oxysophocarpine dimer, oxymatrine dimer and sophoranol-N-oxide dimer. The *in vitro* bioassays were performed to determine the antioxidant activity and glycogen phosphorylase enzyme inhibition activity of *Sophora alopecuroides* seed in ethanol and water extracts. In all of the bioassays, ethanol extract had showed highest activities. In DPPH assay the IC_{50} value of ethanol extract was 155.33 ± 0.06 μg/ml while in FRAP assay the IC_{50} value of ethanol extract was 9.71 ± 0.02 μg/ml. In GPa enzyme assay the IC_{50} of ethanol *Sophora alopecuroides* seed extract was 581.61 μg/ml. Acute toxicity of ethanol *Sophora alopecuroides* seed extract was tested at increasing dose level in non-diabetic rats and no toxic effects were observed in male rats at a dose of 5 g/kg body weight. The ethanol *Sophora alopecuroides* seed extract at the dose of 500 mg/kg was capable of decreasing the glycemia of non-diabetic rats during an oral glucose tolerance test (OGTT). The treatment with SAS at the dose of 500 mg/kg to the diabetic rats for 28 days decreased fasting blood glucose levels significantly compared to the 0^{th} day. The 95% ethanol SAS extract at the dose of 250 mg/kg and 500 mg/kg body weight significantly ($*P < 0.05$) decreased serum triglycerides and total cholesterol levels, and increased serum HDL levels compared to the diabetic control. Therefore, these results validate the traditional use of *Sophora alopecuroides* seed as an antidiabetic remedy.

ABSTRAK

Biji *Sophora alopecuroides* (SAS) telah diekstrakkan dengan klorofom, etanol dan air suling. Keputusan Keratan Lapisan Nipis (KLN) menunjukan kehadiran alkaloid didalam kesemua ekstrak and jumlah kandungan alkaloid adalah 7.56%. Alkaloid telah diasingkan dan dikenalpastikan menggunakan Q-TQF MS dengan rujukkan piawai dan didapati mengandungi alkaloid sophocarpine, matrine, baptifoline, oxysophocarpine, oxymatrine, sophocarpine dimer, oxysophocarpine dimer, oxymatrine dimer dan sophoranol-N-oxide dimer. Bioasei *in vitro* telah dijalankan untuk menentukan aktiviti antioksidan dan aktiviti perencatan enzim fosforilase ekstrak etanol dan air biji *Sophora alopecuroides*. Dalam kesemua bioasei, ekstrak etanol menunjukan aktivit tertinggi. Dalam asei DPPH nilai IC_{50} ekstrak etanol adalah 155.33 ± 0.06 ug/ml manakala dalam asei FRAP nilai IC_{50} ekstrak etanol adalah 9.71 ± 0.02 ug/ml. Didalam asei enzim GPa IC_{50} ekstrak etanol biji *Sophora alopecuroides* adalah 581.61 ug/ml. Ketoksikan akut ekstrak etanol biji *Sophora alopecuroides* telah diujikan pada paras dos menaik dalam tikus tak-dibetik dan tiada kesan ketoksikan dilihat di tikus jantan pada dos 5 g/kg berat badan. Ekstrak etanol biji *Sophora alopecuroides* pada dos 500 mg/kg berkebolehan menurunkan glisemia tikus tak-dibetik semasa ujian oral ketahanan glukosa (OGTT). Perlakuan dengan SAS pada dos 500mg/kg tikus diabetic signifikan merendahkan paras glukosa berbanding pada hari 0. Ekstrak 95% etanol SAS pada dos 250 mg/kg dan 500 mg/kg berat badan menurun signifikan (*$P<0.05$) dengan serum trigliserida dan jumlah paras kolesterol dan meningkatkan paras serum HDL berbanding kawalan diabetik. Oleh itu, keputusan ini mengesahkan penggunaan traditional biji *Sophora alopecuroides* sebagai remedi antidiabetik.

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ATP	adenosine tree phosphate
$CHCl_3$	Chloroform
DMSO	dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DPP-IV	dipeptidyl peptidase-IV
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EtOH	ethanol
Ext	extract
FFA	free-fatty acid
FPG	Fasting plasma glucose
FRAP	ferric reducing antioxidant power
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
GDM	gestational diabetes mellitus
GIP	glucose-dependent insulinotropic polypeptide

GLP-1	glycogen like peptide-1
GP	glycogen phosphorylase
Gpi	glycogen phosphorylase inhibitor
H_2O	distilled water
H_2SO_4	sulphuric acid
HbA1c	glycosylated haemoglobin
HCI	hydrochloric acid
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory activity
IGT	impaired glucose tolerance
KCI	potassium chloride
L	litter
ml	milliliter
µl	microliter
m	meter
cm	centimeter
Kg	kilogram
g	gram
mg	milligram

µg	microgram
M	molarity
mM	millimolar
µM	micromolar
mmol	millimole
min	minute
MW	molecular weight
MeOH	methanol
MgCI2	magnesium chloride
NaOH	sodium hydroxide
NF-kB	nuclear factor kappa B
NIDDM	non-insulin dependent diabetes mellitus
OGTT	oral glucose tolerance test
PKC	protein kinase C
PPAR-γ	peroxisome proliferation activated receptor-gamma
Q-TOF MS	quadruple time-of-flight mass spectrometer
Rf	relative mobility
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute

SAS	*Sophora alopecuroides* seed
SD	standard deviation
SEM	standard error of the mean
SGLT	sodium glucose co-transporter
STZ	streptozotocin
SUR	sulfonylurea receptor
TC	total cholesterol
TCA	trichloroacetic acid
TG	triglycerides
TLC	thin layer chromatography
TZD	thiazolidinediones
TNF-a	tumour necrosis factor-a
UV	ultraviolet
WKY	Wistar Kyoto rats
WHO	World Health Organization
^{o}C	degree Celsius
%	percentage
≤	Less than or equal to
≥	More than or equal to

CHAPTER 1

INTRODUCTION

Type 2 diabetes is one of the most common chronic diseases characterized by hyperglycemia as result of impaired insulin secretion by pancreatic β-cells and by cellular resistance to insulin (American Diabetes Association, 2004). Diabetes mellitus is recognized by the World Health Organization (WHO) as a tremendously increasing worldwide epidemic with more than 285 million people worldwide afflicted in 2010 and it is estimated that the number of people with diabetes will increase to 439 million by 2030 (Shaw, *et al.*, 2010). The WHO predicts that diabetes mellitus will become one of the leading causes of death within the next century (World Health Organization, 2011). International Diabetes Federation estimated that the number of people died from diabetes and its complications were 3.8 million of total world mortality in 2007, and making it the fifth leading cause of death in the world (Dieren, *et al.*, 2010).

Type 2 diabetes accounts for 90 ~ 95% of all cases of diabetes and is largely associated with severe obesity and physical inactivity, which have been shown to lead to insulin resistance. The diets with higher in fats and sugar are significant factors to lead to obese and it is estimated that 80% of people who have diabetic are overweight (Yurgin, *et al.*, 2008). The increase in this phenomenon around the world has been largely associated with people's lifestyle, which refers to the combined detrimental effects of decreased exercise and bad diet. In terms of the total number of people afflicted globally, India, China and USA are the top three countries among the highest prevalence of diabetes for 2010. In India, 50.8 million people had diabetes in 2010 and this number is predicted to increase to 87 million by 2030. In China, 43.2 million had

diabetes in 2010 increasing to 62.6 million by 2030. In the USA, 26.8 million people had diabetes in 2010 and it is estimated to rise to 36 million by 2030 (Shaw, et al., 2010).

There is no effective cure for diabetes, but the progression of disease may be controlled considerably through proper diet and regular exercise. The current treatment of diabetes is aimed at maintaining strict control of glycaemia and while some patients may be achieved control of progression by healthy diet and regular physical activity, but in most cases, effective glycaemic control is required one or a combination of oral hypoglycemic agents. However, currently available oral antihyperglycemic agents, even when used intensively, they are often unable to control the hyperglycaemia and the disease progressively worsens with time. Therefore, there is a need for development of new anti-diabetic drugs.

Medicinal plants have been used traditionally throughout the world as remedies for the treatment of diabetes, especially in India and China where herbal medicine are extensively practiced. *Sophora* species are widely distributed in Taklimakan region, northwest of China. It can also be found in Oceania, the Pacific islands and some parts of Europe (Qiu, *et al.*, 2004). In Chinese traditional medicine, the root of *Sophora flavescens* is used to treat hepatitis B virus (HBV) infection, tumor (Abbott, *et al.*, 1966) and cancer (Fei, *et al.*, 2009). In Uyghur traditional medicine, the seeds of this plant are used for diabetes. In previous studies by (Kim, *et al.*, 2006) found that chemical compounds extracted from the roots of *Sophora flavescens* has α -glucosidase inhibitory activity. It has been shown that glycosidase inhibitors are deeply involved in several important biological processes in carbohydrate metabolism and biosynthesis of glycoprotein (Oh, *et al.*, 2010). An extensive study conducted by (Sato, *et al.*, 2007) revealed that flavonoids extracted from the roots of *Sophora flavescens* shows Na^+-glucose co-transporter (SGLT) inhibitory activity. The biological activities of SGLT

inhibitors are known as decrease glucose reabsorption in kidney, and this could result in lower blood glucose level by increase of urinary glucose excretion (Ohsumi, *et al.*, 2003). α -glucosidase inhibitors are believed to inhibit the enzymes that responsible for conversion of disaccharides to monosaccharides, and delay the digestion and intestinal absorption of carbohydrates, because only monosaccharides can be absorbed through the gut and thus result in reducing blood glucose level (Cheng and Josse, 2004). The recent studies (Li, *et al.*, 2011) has revealed that sophocarpine isolated from Sophora alopecuroides preserves myocardial function in rats by inactivation of nuclear factor-kappaB (NF-kB). NF-kB activation is known to be associated with insulin resistance in type 2 diabetes and other non-diabetic disorders such as cancer and cardiovascular diseases (Barma, *et al.*, 2009). However, to the best of our knowledge, it is noted that there is no one has examined the effects of *Sophora alopecuroides* seed on inhibition of glycogen phosphorylase and in vivo antidiabetic activities. In these properties, this medicinal plant was selected for study.

1.1 Research objective

1. To extract and determine the chemical compounds from *Sophora alopecuriodes* seed using TLC and Q-TOF Mass Spectrometer.
2. To evaluate the antioxidant activity of crude extract from *Sophora alopecuriodes* seed.
3. To determine antihyperglycemic activity of crude extract using glycogen phosphorylase enzyme assay.
4. To determine in vivo antihyperglycemic activity of *Sophora alopecuroides* seed extracts in streptozotocin - nicotinamide induced diabetic rats.
5. To determine the effect of *Sophora alopecuroides* seed extracts on serum lipid profiles in streptozotocin - nicotinamide induced diabetic rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Diabetes mellitus is one of the most common chronic diseases which lead to grow public health problem that result in reduced life expectancy in all around the world. The numbers affected are continuously increased as changing lifestyles associated with reduced physical activity and increased obesity. The disease is characterized by complex disorders of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion by pancreatic β-cell, insulin action or both (Sesti, 2006) . Type 2 diabetes is a chronic metabolic disorder that results from insulin resistance and relative insulin deficiency in patients with diabetes. Even with current therapies, disease is progressively worsens with time, and some of its specific complications include retinopathy, nephropathy, neuropathy and cardiovascular disease (Meshkani and Adeli, 2009).

Insulin resistance is described an impairment of biological response to insulin, which causes hyperinsulinaemia and leads to pancreatic β-cell failure. In the early stage of insulin resistance, glucose homeostasis can be maintained through the excess insulin secretion by β-cells. Overt diabetes only develops when β-cells can no longer compensate for insulin resistance. It is reported that newly diagnosed patients with type 2 diabetes mellitus have approximately 50% β-cell function due to reduction in β-cell mass (Lupi and Del Prato, 2008). In the studies (Lin, *et al.*, 2003; Luley, *et al.*, 2011), inadequate glycaemic control in patient with treated diet alone or with anti-diabetic agent sulfonylurea treatment more than six years, was significantly associated with a

reduction in insulin levels due to progressive β-cell dysfunction. Therefore, improving β-cell function is critical in altering the progressive nature of the disease.

The United Nations (UN) has recognized the diabetes as a worldwide epidemic and a threat to entire world (United Nations, 2007). Apart from the human pain and suffering, diabetes imposes an increasing economic burden on the individuals with diabetes, national healthcare system and economy worldwide. International Diabetes Federation indicated that the global health expenditure on diabetes has been expected to account for USD 376 billion in the world in 2010, and estimated to increase USD 490 billion by 2030. An estimated average of USD 1330 per person with diabetes is expected to be spent on diabetes-related complications in 2010 globally. In the USA alone, total cost in 2010 has been estimated at USD198 billion which account for 52.7% of the global expenditure, and it is estimated to rise to USD 264.3 billion in 2030. In India, which has the largest population with diabetes in the world has been estimated to spent USD 2.8 billion which is account for less than 1% of the world total in 2010. The total cost in 2010 in china has been estimated at USD 5 billion and is estimated to rise to USD 14 billion by 2030. Healthcare costs for nations are vary by region and it range from 5-13% of their annual healthcare budgets (Zhang, *et al.*, 2010). Because of the significant increase in the number of people with the diabetes, cost-effective therapies will be required to treat people, particularly those from developing parts of the world that cannot afford expensive medication. The solution to this problem is still remains unclear, and it will need a novel and concerted global effort that combines modern medicine with alternative medicine used throughout many parts of the world.

2.2 Diabetes

There are several pathogenic processes involved in the development of diabetes. According to the International Diabetes Federation (IDF), there are three major types of diabetes: type 1 diabetes, type 2 diabetes, and gestational diabetes.

2.2.1 Type 1 diabetes

Type 1 diabetes is one of a group of metabolic disease that largely recognized as an absolute deficiency of insulin secretion. It is caused by an autoimmune reaction whereby the β-cells are destroyed by the body's own antibodies. It is accounted for 5-10% of all cases of diabetes and no or very little insulin are produced people with type 1 diabetes. Since insulin can no longer be produced, the only effective treatment is to daily insulin injection.

2.2.2 Type 2 diabetes

Type 2 diabetes is sometimes called non-insulin dependent diabetes and it is estimated that 90-95% of diabetes are belong to this type. The disease is usually caused by a combination of resistance to insulin action, particularly in adipose tissue, muscle, liver and β-cell dysfunction. The disease can remain undetected in early stage of development. It is assumed that type 2 diabetes is mostly associated with people's lifestyle which itself can cause insulin resistance and lead to progressive elevation in plasma glucose level, and thus result in continuously worsen the disease (Luley, *et al.*, 2011).

2.2.3 Gestational diabetes

Gestational diabetes mellitus (GDM) is defined as 'carbohydrate intolerance during pregnancy (Carolan, *et al.*, 2010) and studies demonstrated that gestational diabetes mellitus were associated with defect in β-cell function and obesity in pre-pregnancy (Hak, *et al.*, 2003). It is estimated that 50% of women with GDM have been in the risk of development type 2 diabetes within five to ten years after delivery (United Nations, 2007).

The type 2 diabetes mellitus is widespread in all populations around the world and the prevalence is in a progressively increase. Prevention and treatment are still a

challenge in all types of diabetes. The World Health Organization (WHO) has chosen the use of fasting plasma glucose (FPG) and two hour oral glucose tolerance test for the diagnosis of the diabetes. Normally, fasting plasma glucose (FPG) concentrations are strictly maintained within 5 mmole/l to 6.6 mmole/l but in type 2 diabetes the person is unable to maintain glucose levels within this range. The disease is characterized by fasting plasma glucose of ≥ 7 mmole/l or by a two hour oral glucose tolerance test (OGTT) of ≥ 11.1 mmole/l. The onset of diabetes is preceded by an early diabetic state with fasting plasma glucose between 6.1 and 6.9 mmole/l and is referred to as impaired fasting glucose (IFG). Alternatively, it is recognized by a two hour OGTT between 7.8 and 11.1 mmole/l and is referred to as impaired glucose tolerance (IGT). International Diabetes Federation (IDF) has recommended reducing the threshold for IFG from 5.6 to 6.9 mmole/l. The people with IFG or IGT have been referred to as having pre-diabetes, and indicating that they have relatively high risk for development of diabetes in his future life (World Health Organization, 2006).

Aside from FPG and 2 hour OGTT, the American Diabetes Association has recently recommended the use of the glycosylated haemoglobin A1C (HbA_{1c}) test to diagnose diabetes. The report from Japan Diabetes Society showed that glucose in the body may bind to haemoglobin to give glycosylated haemoglobin (HbA_{1c}). HbA_{1c} concentration is directly proportional to blood glucose levels because it reflects blood glucose concentrations over the previous 1-2 month. When comparison of FPG levels with HbA_{1c} concentration, the FPG levels of 7.0 mmole/l correspond to HbA1c of 6.5% and results are not affected by the food intake prior to blood sampling(Shibata, *et al.*, 2005). In the criteria for the diagnosis of diabetes, present study indicates that if people with A1C of 5.7-6.4%, is considered to be pre-diabetes and lowering their A1C to below or around 7% has been shown to reduce micro-vascular and neuropathic complications in both type 1 and type 2 diabetes. According to the most recent study

results, when compare to the accuracy of a FBG of $\geq$ 7.0 mmole/L to that of an HbA1c of $\geq$ 6.5% in the detection of hyperglycaemia, the result of FPG have been observed similar to $A1_C$. The specificity and sensitivity of the test have been improved by combination measurement of FPG and HbA_{1c} in the predicting of diabetes. Therefore, HbA_{1c} test may be the most important indicator in diagnosis of diabetes and would be the ideal standards for assessing glycaemic control (Valdés, *et al.*, 2011). However, the WHO still does not consider the HbA_{1c} result alone a suitable diagnostic test for diabetes for following reasons. First, this test is not readily standardized and also not readily available many parts of the world. Secondly, HbA_{1c} results are influenced by the patient's clinical situation that completely unrelated to diabetes such as anaemia, pregnancy and uraemia (American Diabetes Association, 2010; Beard, *et al.*, 2010). Thirdly, HbA_{1c} sensitivity is vary for different age group (Tay, *et al.*, 2011). To compare conventional tests (FPG and OGTT) with HbA_{1c} test, conventional tests are the most applicable means of accessing glucose levels. Therefore, it remains most prime position as a standard for diagnosis of diabetes (World Health Organization, 2006).

2.3 Glucose metabolism

After meal, carbohydrates are degraded by enzymes and which result in formation of free glucose. Glucose is transferred to cell surfaces where it was utilized by cells as energy or stored as a glycogen in liver or in skeletal muscle. Glucose is the major energy source for cells and its concentrations are controlled by a number of hormones, the most importantly insulin and glucagon. Insulin is secreted by pancreatic β-cells when blood glucose concentration rises, and reduces glucose levels by two general mechanisms; (1) inhibition of hepatic glucose production (glycogenolysis and gluconeogenesis) and (2) increasing glucose uptake into muscle and fat tissue. Glucagon is a hormone secreted by pancreatic α-cells and hepatic glucose output is

stimulated by this hormone. Glucagon is released into blood stream in response to low concentrations of glucose and is responsible for increase in glycaemia. It acts at the liver and opposes the effects of insulin by increasing glycogenolysis and gluconeogenesis and also by inhibiting glycogenesis and glycolysis through multiple mechanisms (Jiang and Zhang, 2003). Other hormones also participate in maintain normal glucose levels. These hormones include amylin, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Amylin is actually secreted with insulin from pancreatic β-cells and functions in decreasing gastric emptying, which limits glucose excursions following a meal (Heptulla, *et al.*, 2005). GLP-1 and GIP are incretins, gut derived hormones, which is secreted by intestinal L cells. They have a multitude of effects, both of which are to promote the synthesis and secretion of insulin from pancreatic β-cells, stimulates glucose uptake in muscle, and reduce glucagon-stimulated hepatic glucose production (Irwin, *et al.*, 2006). Any change in the effect of these hormones leads to the progression of diabetes and its complications.

2.4 Glycogen metabolism

Glycogen is a polymer of glucose stored in the liver and skeletal muscle. It is degraded to yield free glucose when energy is needed for cells and is synthesized again when excess glucose is present in blood stream. Its degradation and release of glucose into the blood leads to elevate glucose level that is available between meals and is good source of energy for sudden need and starvation. The glucose is the only fuel for the brain tissue. Therefore, the role of glycogen metabolism in glucose homeostasis is extremely important (Pfeiffer-Guglielmi, *et al.*, 2007).

Glycogen degradation and synthesis are regulated by two key enzymes, glycogen phosphorylase and glycogen synthase, and these enzyme activities are

controlled by several hormones such as glucagon, insulin and adrenalin. Glycogen degradation consists of three steps: (1) the formation of glucose 1-phosphate from glycogen. Glycogen phosphorylase is activated by phosphorylation, and which catalyzes glycogen to produce glucose 1-phosphate (G1P), (2) the remodeling of the remaining glycogen for further degradation, and (3) conversion of G1P to G 6-Phosphate (G6P) for further metabolism. G1P can be converted to G6P in the presence of enzyme, phosphoglucomutase. G6P can be used (1) as energy sources for anaerobic or aerobic metabolism in muscle and brain, or further broken down to produce pyruvate and lactate in glycolysis, (2) are catalyzed by glucose 6-phosphatase and converted to glucose in the liver by gluconeogenesis, and released into the blood for the use of other tissue, and (3) oxidized in the pentose phosphate pathway. Glycogen synthesis is started with G1P by glycogen synthase catalyze to produce activated intermediate, UDP-glucose. Glycogen synthase is activated by insulin to start synthesis glycogen when blood glucose concentration is too high and it is inactivated by hormones such as glucagon in order to response insulin-induced hypoglycemia. Glycogen metabolism is regulated by reversible phosphorylation of enzymes that adjust glucose level for the needs of the entire organism (Lang, *et al.*, 2002; Toole and Cohen, 2007).

2.5 Pathogenesis and complications

The pathogenesis of diabetes is complex. Usually, destruction of insulin producing pancreatic β-cell by its own immune system is assumed to be the main pathogenic factor in type 1 diabetes (Weiss, *et al.*, 2008), but in type 2 diabetes, it typically begins with insulin resistance at target organs such as liver, muscle and adipose. Increased insulin demand for tissue and progressive impairment of β-cell function which result in insulin resistant are the pathophysiologic defects in progression of hyperglycemia in type 2 diabetes. In order to compensate for this, there is a necessity

of initial increase in insulin production. This hyperinsulinemic state is only temporary and over with time insulin secretion decrease due to progressive pancreatic β-cell deterioration. The combined effects of insulin resistance and pancreatic β-cell dysfunction results in a decreased capacity to limit hepatic glucose production as well as to decrease uptake and utilization of glucose in muscle and adipose tissue.

Insulin resistance is a complex disease that typical feature of the metabolic syndrome and is the result of a number of defects along the insulin signalling cascade (Venieratos, *et al.*, 2010). Other most likely factors include defective incretin activity, elevated concentrations of free fatty acids (Zinman, 2011), inappropriate glucagon production from the α-cells (Mayhew, 2010), activation of NF-kB (Barma, *et al.*, 2009), and tumour necrosis factor-α (TNF-α) and the hormone resistin (Winkler, *et al.*, 2002). Incretins are gut-derived peptides that responsible for higher insulin release when glucose was taken orally compared to an intravenous glucose load. Impaired release of incretin hormones, especially GLP-1 and impaired action of glucose-depended insulinotropic polypeptide (GIP) have been observed in patient with type 2 diabetes, and this phenomenon is called incretin deficiency. There are two main incretin hormones, glycogen-like peptide -1 (GLP-1) and glucose-depended insulinotropic polypeptide (GIP), both of which are released rapidly after meal in order to response elevated postmeal glucose levels. It is believed that GLP-1 control glucose homeostasis through stimulation of insulin secretion, and inhibition of glucagon and gastric emptying. Elevated FFAs produce insulin resistance by inhibiting glucose uptake and its oxidation in skeletal muscle. FFA's also increase hepatic gluconeogenesis and activate NF-kB. It is believed that activated NF-kB cause insulin resistance in skeletal muscle. Both TNF-α and resistin are produced by adipose tissue in greater amounts in obese diabetic individuals. TNF-α impairs insulin action while resistin is known to antagonize the effects of insulin (Winkler, *et al.*, 2002; Barma, *et al.*, 2009).

Increased hepatic glucose production and reduced glucose uptake in type 2 diabetes are attributed to both hepatic insulin resistance and increased glucagon levels (Shiba, *et al.,* 1998). Pancreatic β-cells can compensate for resistance by secreting more insulin. This hyperinsulinemic state is only temporary, as β-cells cannot maintain insulin levels required to maintain euglycemia. This is referred to as the "petering out" effect and occurs due to apoptosis of β-cells. High glucose and FFA's contribute to β-cell malfunction, in a condition called glucolipotoxicity. When insulin resistance can no longer be overcome transition to type 2 diabetes occurs.

Epidemiological study indicates that unregulated glucose control can lead to severe macro and micro vascular complications and it can result in long term damage to various organs and tissues. In fact, the correlation of these complications with glucose levels used to cut-offs for the diagnosis of diabetes has been mentioned above. Diabetes mainly affects the heart, blood vessels, eyes, kidney and nerves. Vascular complications are the fatal complications that lead to morbidity and mortality in patient with diabetes, and the complications in eyes and kidney are a leading cause of blindness and renal failure. Diabetes can cause gestational age in children during pregnancy. In general, micro-vascular complications refer to those affecting small blood vessels in the retina, kidney, and peripheral nerves, and can lead to retinopathy, nephropathy and neuropathy, respectively. Diabetic retinopathy occurs as a result of long-term damage to blood vessels in the retina and can lead to blindness or severe visual loss. Diabetes can also cause the development of cataract through the formation of sorbitol deposits on the lens of the eye. Sorbitol is a product of the polyol pathway formed by the action of aldose reductase, which becomes overexpressed in type 2 diabetes, and is believed to be intimately involved with organ damage. Diabetes is one of the leading causes of kidney failure and 10-20% of diabetics die from this disease. Diabetic nephropathy occurs as a result of increased in urine albumin excretion, and is a second cause of renal

replacement therapy. Diabetic neuropathy refers to a group of diabetes-related nerve disorders which was affected with 50% to 60% people in diabetes. It occurs as a result of damage to the nerves and results in tingling, pain, numbness and weakness in the extremities, which left untreated, can lead to infection, ulceration and possibly amputation. Macro-vascular complications refer to diseases affecting large blood vessels in the heart, brain and peripheral circulation leading to cardiovascular diseases such as atherosclerosis, heart attack and stroke, which are responsible for 50% of deaths of diabetics (World Health Organization, 2011).

It is highly hypothesized that there are some mechanisms by which hyperglycaemia induce glucose-mediated vascular complications. There are: (1) increased polyol pathway; (2) activation of protein kinase C (PKC); (3) increased advanced glycation end-products formation; (4) increased hexosamine pathway (Munusamy and MacMillan-Crow, 2009); (5) increased glucose flux through the aldose reductase pathway; (6) increased production of reactive oxygen species (ROS) (Nishikawa, et al., 2007) and (7) increased in fatty acid flux (Herlein, *et al.*, 2010). A common effect of each mechanism is that they increase the production of superoxide by the mitochondrial electron-transport chain. Superoxide is a reactive oxygen species that leads to oxidative stress and subsequently cause the tissue damage that is observed during diabetes. This suggests that antioxidants, as free-radical scavengers, may be used therapeutically to prevent the development of diabetes associated complications (Munusamy and MacMillan-Crow, 2009).

2.6 Current oral anti-hyperglycemic agents

The current ultimate goal to treat diabetes are aimed at maintaining fasting blood glucose levels between 4.5 and 6.6 mmole, and $HbA1_c$ levels at or below 7%. The

control of $HbA1_c$ levels at or below 7% has been shown to decrease the risk of developing micro-vascular complications (Ten Brinke, *et al.*, 2008). When proper diet and exercise fail to control hyperglycemia, the use of anti-diabetic agents becomes necessary. A variety of oral hypoglycemic agents are currently available and these can be generally classified as (1) insulin secretagogues, (2) biguanides, (3) insulin sensitizers, (4) α-glucosidase inhibitors, (5) dipeptidyl peptidase-IV (DPP-IV) inhibitors, (6) incretin mimetics, (7) glucose transporter inhibitors or (8) glycogen phosphorylase inhibitors.

2.6.1 The insulin secretagogues

The insulin secretagogues include the sulfonylureas and meglitinides and both stimulate insulin release from the pancreas by a common mechanism. Sulfonylureas and meglitinides stimulate insulin secretion by binding to the sulfonylurea receptor (SUR) of ATP sensitive potassium channel on pancreatic β-cell plasma membrane(Cyrino, *et al.*, 2003). Meglitinides bind to the sulfonylurea receptor, but also bind to an additional site on the pancreatic β-cell to induce insulin secretion by blocking ATP-depended potassium channels. Because they secrete insulin independent of glucose concentration, hypoglycemia is a serious side effect of sulfonylureas and meglitinides (Del Prato and Pulizzi, 2006). Another side effect is their tendency to cause weight gain. This is undesirable especially considering that 80% of diabetics are already overweight. Despite these problems, sulfonylureas are considered a frontline treatment regimen. Meglitinides have similar side effects but they are less pronounced. Some patients do not respond to sulfonylureas while others who have responded may fail to do so after several years. After 10 years of monotherapy with a sulfonylurea, they generally become ineffective and most patients require a second agent to maintain glucose control (Davis, 2008).

All of the sulfonylureas have the same mechanism of action but they differ in pharmacological potency and pharmacokinetics which result in considerable clinical differences among the same classes, therefore, they are further classified as first generation (tolbutamide and chlorpropamide) and second generation (glibenclamide, gliclazide, glipizide, and glimepiride) drugs based on their structural features, pharmacological potency, time of onset, and duration of efficiency (Cyrino, *et al.*, 2003). Repaglinide is one of the meglitinide classes which belong to insulin secretagogues. it is believed to has a rapid onset and a short duration of action in liver, and is differ with sulfonylureas in structure (Van Gaal, *et al.*, 2001).

2.6.2 Biguanides

Biguanides include metformin and phenformin. Their mechanism of action is generally believed that they inhibit hepatic glucose production by activation of AMP-activated protein kinase (AMPK). Both of them may effect on glucose metabolism by increasing glucagon-like peptide-1 (GLP-1) biosynthesis and secretion, thereby decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization in liver. Recently, metformin has been shown to increases AMPK activity in skeletal muscle which leads to increase GLUT-4 protein content (glucose transporter) in the plasma membrane, and resulting in insulin-independent glucose uptake (Svendsen, *et al.*, 2009; Grisouard, *et al.*, 2010).

Biguanides have been shown improves glucose tolerance, lowering both basal and postprandial plasma glucose in patients with type 2 diabetes. Among the biguanides, metformin is frontline treatment option that may be used alone or in combination with other antihyperglycemic agents. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with type 2 diabetes or normal subjects and does not cause hyperinsulinemia (Liepinsh, *et al.*, 2011). Aside this, a beneficial side effect is

that it is associated with weight loss, and this makes it preferable to sulfonylureas to treat severely obese diabetics.

2.6.3 Insulin sensitizers

Insulin sensitizers include pharmacological ligands for the peroxisome proliferation activated receptor-gamma (PPAR-γ) such as thiazolidinediones (TZD). Thiazolidinedione drugs enhance insulin sensitivity in adipose tissue, skeletal muscle, liver and in fat by stimulating the nuclear PPAR-γ receptor which control proteins required for glucose and lipid metabolism and activate the glucose transporter gene (GLUT-4) in muscle and adipose tissue. Thiazolidinediones reduce hyperglycemia by improving β-cell function, reduction insulin resistant and released free fatty acid, increasing cellular glucose consumption, glucose uptake, and insulin sensitivity in muscle and adipose tissue. They do not affect insulin levels. It was shown that thiazolidinediones increase pre-adipose differentiation and as a result cause weight gain as a side effect (Smith, 2003; Meriden, 2004). To counteract this, combination with insulin or metformin as an anti-diabetic agent is being considered. Some example of TDZs class of drugs is Pioglitazone and Rosiglitazone.

2.6.4 α-Glucosidase inhibitors

α-Glucosidase inhibitors inhibit the enzymes that responsible for conversion of disaccharides to monosaccharides in intestine. They reduce blood glucose by preventing digestion and absorption of complex carbohydrates (such as starch) because only monosaccharides can be readily absorbed through gut. After a few hours of having meal, blood concentration is high. During this time, insulin is secreted from the pancreatic β-cells and glucose is immediately transferred to the cells for as an energy sources. In type 2 (non-insulin dependent) diabetes mellitus (NIDD), insulin secretion can be normal but

cells are not sensitive to the insulin, as a result, glucose cannot be absorbed properly. One of the strategies for reducing elevated glucose levels in NIDD is to delay rate of digestion of ingested carbohydrates, thereby lowering postprandial blood glucose levels. Previous studies shows that this can be achieved by administering drugs which inhibit the activity of enzymes, such as α-amylase and α-glucosidase that responsible for hydrolyse polysaccharide to glucose and other monosaccharides in the small intestine. Currently available α-glucosidase inhibitors include acarbose, voglibose and miglitol. These drugs are capable of decrease postprandial glucose levels without risk of hypoglycemia and should be taken with food for optimal effect. It has been observed that they have only modest antihyperglycemic activity by themselves. Therefore, these drugs are usually recommended to use in combination therapy. Side effects which are known include flatulence, diarrhea and abdominal pain (Borges de Melo, *et al.*, 2006).

2.6.5 Dipeptidyl peptidase-IV (DPP-IV) inhibitors

Dipeptidyl peptidase-IV (DPP-IV) is a serine protease that exists as both a membrane bound and plasma soluble form. It is expressed in all tissues and some cells with highest levels found in kidney, intestine, liver, pancreas, spleen, synovia, mammary gland and immune cell (e.g. T cell and B cell) leukocytes (Nielsen, 2005). It is a proline specific amino peptidase that responsible for the degradation of a number of biologically active peptides including glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 and GIP are the hormones that secreted in the intestine after meal and are responsible for insulin release due to increased glucose levels and are rapidly degraded by DPP-IV. To enhance GLP-1 activity, inhibition of this enzyme is useful as a novel therapeutic approach in the treatment of diabetes through an enhancement of the incretin effects (Verspohl, 2009).

DPP-IV inhibitors decrease blood glucose levels by inhibiting the activities of enzyme DPP-IV, thus increase GLP-1's ability to release insulin in response to elevated concentrations of blood glucose. Several DPP-IV inhibitors which have been reported (Thornberry and Gallwitz, 2009) include Sitagliptin, vildagliptin, saxagliptin and alogliptin. Vildagliptin and sitagliptin have been approved for clinical use in both the United States and Europe, saxagliptin has been approved only in United States, and alogliptin has been approved in Japan. Clinical trials have been proved that these drugs can be used safely with other oral antidabetic agents such as metformin, sulfonylureas, and thiazolidinediones in patients unable to control blood glucose at desired levels. In previous studies showed that either monotherapy or combinations with other oral agent such as metformin, inhibition of DPP-IV with vildagliptin and sitagliptin were shown to reduce blood glucose and glycosylated haemoglobin (HbA1c) without significant changes on body weight in type 2 diabetes (Kendall, *et al.*, 2009; Campbell, 2011). Studies conducted by (Duttaroy, *et al.*, 2011) demonstrated that pancreatic β-cell mass was increased in animal model treated with vildagliptin, thus result in a rise in insulin release. Administration of sitagliptin has been observed to decrease in glucagon secretion following meals, decrease the rate of carbohydrate digestion, slow down the rate of gastric emptying, reduce food intake, and preserve β-cell function in type 2 diabetes (Zerilli and Pyon, 2007; Gustavson, *et al.*, 2011).

DPP-IV inhibitors have a number of distinct advantages over current hypoglycemic agents. Since they function by enhancing the effects of GLP-1, they are capable of stimulating insulin secretion without causing hypoglycemia. The insulinotropic effect of GLP-1 requires that glucose concentration be at or above normal fasting concentration. Therefore, as glucose concentration falls to the normal range, the insulinotropic effects of GLP-1 diminish. In addition, they have the potential to control weight change and to restore β-cell mass. Hypoglycemic agents that are capable of

restoring β-cell mass are highly desirable. Because of this, the search for novel DPP-IV inhibitors is an active area of research (Gustavson, *et al.*, 2011).

2.6.6 Incretin mimetics

Incretins are hormones that are secreted from intestinal cells into the blood in response to nutrient ingestion. The role of intestine in the regulation of insulin release was based on the observation that the amount of insulin secreted following an oral glucose dose higher than that of an equivalent dose administered intravenously. In diabetic and non-diabetic individuals, plasma insulin levels following an oral glucose dose were threefold higher than that after the same dose administered intravenously (Holst, *et al.*, 2008). This phenomena was termed the "incretin effect" and was subsequently found to be primarily the result of two incretin hormones, GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulinotropic polypeptide) (Verspohl, 2009). The effects of these hormones on glucose homeostasis were shown to activate GLP-1 receptor, which was located throughout the body , as a result, insulin secretion was increased and glucagon release was supressed in both patients with type 1 and type 2 diabetes (Mudaliar and Henry, 2009).

Incretin mimetics are functional analogous of the human incretin Glucagon-Like Peptide-1 (GLP-1) that capable of inhibit degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV). Incretin mimetics reduce blood glucose by stimulating insulin secretion from pancreatic β-cell in a glucose-depended manner, suppress glucagon release, slow gastric emptying, reduce apatite, and enhance pancreatic β-cell function (Barber, *et al.*, 2010). Currently available incretin mimetics include exenatide and liraglutide. The exenatide treatment in patients with type 2 diabetes showed that HbA1c has been reduced about 2% along with improved in glycemic control , and the body weight has been decreased up to 6 kg within three months without cause hypoglycemia

(Natarajan, *et al.*, 2010; Peters, 2010; Nauck, 2011). Incretin mimetics are generally used combination with other oral hypoglycemic agent such as metformin and sulfonylureas. These drugs cause weight loss. This side effect is beneficial in obese patients and those patients with other cardiovascular risk factors. Beside this, most common side effect currently reported is nausea and diarrhea (Mayhew, 2010).

2.6.7 Glucose transporter inhibitors

Glucose transporter, which was termed sodium-glucose co-transporter (SGLT), is a membrane protein that has isomers such as SGLT1, SGLT2, SGLT3, and SGLT4. It was well known that SGLT1 absorbs dietary glucose from gut, and SGLT2 responsible for maintain glucose homeostasis by reabsorption of glucose in the kidney. Therefore, it was expected that SGLT2 inhibitors could reduce elevated glucose levels by increasing glucose excretion in urine, thus, they have potential therapeutic value for the treatment of type 1 and type 2 diabetes (Derdau, *et al.*, 2010).

Development of glucose transporters as an antidiabetic agent is relatively novel in pharmaceutical industries. However, there are currently no hypoglycemic agents available for use in this area, but they are attracting more attention of scientists to look for potent compounds that useful for treatment of diabetes. In previous studies, sodium-glucose cotransporter inhibition activity of flavonoids isolated from *Sophora* species have been reported (Sato, *et al.*, 2007). Most recently, SGLT2 inhibitors synthesised by Sanofi-Aventis have been shown to reduce intestinal glucose absorption, and enhance renal glucose excretion in animal models (Derdau, *et al.*, 2010). This suggests that inhibition of glucose transporter is useful for diabetes.

2.7 Glycogen phosphorylase inhibitors and diabetes

Glycogen phosphorylase (GP) is an enzyme that catalyses the reversible phosphorolytic cleavage of glycogen to produce glucose 1-phosphate which is the first step of glycogen degradation. GP exists in two forms which is phosphorylase-a and phosphorylase-b that can be found in the muscle and in the liver. In the muscle where glucose is produced for as energy source and hepatic glucose production is take place in the liver. Pharmacological activities of this enzyme are regulated by phosphorylation. The phosphorylase-b is unphosphorylated form of GP and is essentially inactive. It is transformed to the more active form (phosphorylase-a) by phosphorylation, and transformation process is controlled by several mechanisms. The most active form (phosphorylase-a) is responsible for hepatic glucose production in the liver and it is an important contributor in diabetic hyperglycemia. Glycogen phosphorylase a form is dephosphorylated by the action of protein phosphatases, whereby glycogen phosphorylase is returned to its less active b form (Hampson, *et al.*, 2006; Bertus, *et al.*, 2008).

Glucose is produced by glycogen degradation in the liver, and glycogen degradation is regulated by glycogen phosphorylase. Glycogen phosphorylase (GP) is an enzyme that catalyses glycogen to produce glucose-1 phosphate (G1P) which is a first step for glycogen metabolism. G1P is then further metabolized to glucose, and secreted into the blood stream for the use of other tissues, especially the central nervous system that relies on glucose as its major energy sources. It is well known that glucose released from glycogen degradation is the main contributor to elevated hepatic glucose output in patient with diabetes, and glycogen degradation is directly associated with regulation of blood glucose levels in the liver. Therefore, inhibition of Glycogen phosphorylase has been thought to reduce hepatic glucose production, and thus decrease blood glucose levels, which is considered potential antidiabetic agent. Some example of

currently available GP inhibitors include Corosolic acid (Yamada, *et al.*, 2008) and Ingliforib (Bennett, *et al.*, 2010). Ingliforib (CP368296) is a potent GPa inhibitor which is in the stage of phase II clinical trials. Corosolic acid (commercially known Glucosol^{TM}) is the first drug in the class of glycogen phosphorylase inhibitor that has been released to the market in Japan and United States for reducing blood glucose levels and weight-loss.

These oral hypoglycemic agents are may be used alone or combination with insulin or combination with themselves. Combination therapy is an option when one drug is no longer particularly effective. After 2 to5 years follow-up studies, monotherapy with either a sulfonylurea or metformin, approximately 50% of patients have HbA_{1c} above 7%, and after more than 5 years treatment this number increases to approximately 75% (Ceriello, *et al.*, 2005; Brown, *et al.*, 2010). In this case, a second agent of a different class is usually added to the regimen to restore glycemic control through an additive or synergistic effect. The most common combination is metformin with a sulfonylurea. Other useful combinations include metformin and a TZD, metformin with a meglitinide, or an α-glucosidase inhibitor with either metformin or a sulfonylurea. In the case when two agents are no longer effective a third agent of another class might also be added (i.e. TZD to a combination of metformin and a sulfonylurea). Finally, when oral hypoglycemic therapy has failed to achieve therapeutic goals in type 2 diabetes, subcutaneous insulin injections are necessary to prevent hyperglycemia.

There are the reasons why glycogen phosphorylase inhibitor (GPi) is important in diabetes. (1) GPi has been shown to be more potent at reducing hepatic glucose output in the presence of high glucose concentrations (Martin, *et al.*, 1998); (2) GPi has been shown to significantly attenuate hyperglycemia without producing hypoglycemia (Oikonomakos, *et al.*, 2000); (3) GPi has cardioprotective effects (Cai, *et al.*, 2005); (4)

GPi has been shown to inhibit tumour inducer and has anticancer properties (Schnier, *et al.*, 2003); (5) GPi has antioxidants activities (Guan, *et al.*, 2010). The discovery of the primary role of glycogen phosphorylase in glycogen metabolism led to the suggestion that inhibition of this enzyme may be useful in the treatment of diabetes.

Glycogen phosphorylase inhibitors have variety of distinct advantages compare to other antihyperglycemic agent. It has been reported that potency of compound was significantly increased at higher glucose concentration and it was reduced when glucose concentration falls to the normal range (Ercan-Fang and Nuttall, 1997; Ercan-Fang, *et al.*, 2005). Thus, this property is important for better protection of patients from hypoglycemia. Corosolic acid is a potent glycogen phosphorylase inhibitor used for the treatment of type 2 diabetes. Increased glucose uptake by insulin receptor phosphorylation and decreased the level of oxidative stress have been observed in diabetic rats treated with Corosolic acid (Yamaguchi, *et al.*, 2006; Shi, *et al.*, 2008). Nuclear factor-kB is a family of transcription factors, believed to be involved in pathogenesis of several inflammatory diseases including tumour, cancer and insulin resistance. Inhibition of GP activity in patient with type 2 diabetes was shown to significantly inhibit NF-kB overactivation and reduces insulin resistance (Barma, *et al.*, 2009). Combined, these studies seem to validate the notion that inhibition of glycogen phosphorylase is a viable way of indirectly enhancing effective management of diabetes and its complications.

2.8 Antioxidants in diabetes

Oxidative stress has been suggested to be critically involved in the pathogenesis and progression of diabetic tissue damage. It has been shown that hyperglycemia is correlated with an increased production of the free radical superoxide and reactive nitrogen species (RNS). Overproduction of superoxide in the organ systems is

associated with nitric oxide which is a potent oxidant that causes nitrosative stress in the organ systems. It is an important feature of diabetic complications that can be found in both patients with type 1 and type 2 diabetes (Cai, *et al.*, 2005). Pancreatic β-Cells are exceptionally vulnerable to the toxic effects of free radicals because the pancreas is the organ that has the lowest levels of antioxidant enzymes and these levels are further diminished in diabetes. It has been reported that most potent oral antidiabetic agent metformin have ability to reduce cardiovascular complications by significantly reduction of reactive oxygen species (ROS) (Hou, *et al.*, 2010), and another novel antidiabetic agent repaglinide has been shown to prevent inflammation by significantly diminishing protein oxidation in diabetic rabbits (Gumieniczek, *et al.*, 2005). Inflammation is the earliest and most common diabetic complications that caused by oxidative stress. Metformin has its origin in the plant and it is a potent enhancer for pancreatic β-cell function. Antioxidant mediated preservation of pancreatic β-cell function have been considered to effect on diabetes by slowing the progression of the disease. Plants including Sophora species often contain considerable quantities of antioxidants such as alkaloids, α-tocopherols (vitamin E), carotenoids, ascorbic acid (vitamin C), and polyphenols such as flavonoids and tannins.

2.9 Traditional medicine

In many parts of the world plants are still the main source of treatment for disease and the WHO estimates that 80% of the population in the developing countries depend on traditional medicine for their primary health care needs (Mukherjee and Wahile, 2006). The use of plants in the treatment of diabetes has a long and rich history. Herbal medicines have been in use for thousands of years before modern medicine began, and continued to provide mankind with novel remedies.

Traditional medicine systems developed through experience and experimentation. Knowledge of this was most likely obtained by using a variety of plants to treat a particular disease and observe their effects. Plants that had a positive effect in treating the disease were recorded. In the case of diabetes, many plants have been used to help control blood sugar levels. In recent times, the hypoglycemic activity of some of these herbs has been demonstrated in various animal models of diabetes and in some instances the active principles have even been isolated (Shokeen, *et al.*, 2008; Daisy, *et al.*, 2009). But, the majority of these herbs still in use today have far escaped scientific scrutiny and neither their mode of action nor the active principles are known.

The modern prevalence of diabetes has clearly led to a need for new drugs. Plants used traditionally for treatment of diabetes, particularly those that have been proven to reduce blood sugar, can potentially lead to the isolation of novel molecules with significant hypoglycemic activity. Because of a number of factors, such an approach can have some advantages over the conventional approach to drug discovery. This approach is referred to as ethnopharmacology and utilizes the information learned from various systems of traditional medicine in the search for new drugs. Since humans have used these plants for generations, it can be expected that bioactive compounds isolated would have low toxicity, though this is not always the case. Also, there is a tremendous degree of chemical diversity in a plant extract. This includes alkaloids, glycosides, saponins, polysaccharides, flavonoids, steroids, carbohydrates, terpenoids, amino acids and tannins. Such diversity can lead to interesting molecules that may be useful drug entities themselves or more likely serve as lead molecules in a medicinal chemistry program.

Interestingly, Glucosol^{TM}, potent glycogen phosphorylase-a inhibitor has its origins in many plants such as *Lagerstroemia speciosa L*, which was used to treat

diabetes in the USA and Japan. Its use as an antidiabetic agent was as a direct result of the isolation of corosolic acid as an active antihyperglycemic agent (Judy, *et al.*, 2003).

2.10 Studied plant - *Sophora alopecuroides*

Sophora alopecuroides belong to the Leguminosae family and is a shrub with bead-shaped fruits. Its fruit is 3 to 7 cm long and contains oval-shaped yellow seed. It is locally called buya and foxtail-like Sophora in English. Plant is mostly originated in Taklimakan region which is central part of Asia and can be found some parts of the Europe. Different parts of the plant are used for throughout the world traditionally for a number of diseases. In Korea, roots are used for treatment of hair loss and fiver (Roh, *et al.*, 2002). In china, roots and leaves are used for the treatment of viral hepatitis, cancer and cardiac diseases, and seeds are used for tumour and diarrhea (Li, *et al.*, 2011). In Japan, the seeds are used for weight loss (Park, *et al.*, 2009). The seeds are known to be rich source of alkaloids (Xiu, *et al.*, 2010) and flavonoids (Guo, *et al.*, 2011). In Uyghur traditional medicine, the seeds are prescribed for diabetes.

It is generally the case that herbal drugs operate by a number of mechanisms to elicit their effects. Indeed, the root of *Sophora* has already been shown to inhibit α - glucosidase enzyme which is responsible for carbohydrate digestion (Kim, *et al.*, 2006). It was known that α-glucosidase inhibitors reduce blood glucose by preventing digestion and absorption of complex carbohydrates in intestine. Most recently, it has been reported that sophocarpine, which is an alkaloids, isolated from Sophora alopecuroides preserves myocardial function in rats by inactivation of nuclear factor-kappaB (NF-kB) (Li, *et al.*, 2011). NF-kB activation is known to be associated with insulin resistance in type 2 diabetes and other non-diabetic disorders such as cancer and cardiovascular diseases (Barma, *et al.*, 2009). Other evidence is that methanol extract

from *Sophora* roots has been shown to inhibit sodium-glucose cotransporter (SGLT) activity. SGLT was known to maintain glucose homeostasis by absorption of glucose in the kidney as a result increase glucose excretion in urine (Sato, *et al.*, 2007).

Aside from the α-glucosidase and SGLT inhibitory effects, combined administration of high-fat diet with powdered fruit of some *Sophora species* significantly decreased body weight in non-diabetic mice, and it also improved serum lipid profiles that often result in cardiovascular complications. In diabetes, increased lipolysis results in increased levels of triglyceride and cholesterol. *Sophora species* exhibited lowering triglyceride and cholesterol effects while at the same time increasing HDL cholesterol (Park, *et al.*, 2009).

Oxidative stress has been known to be involved in the pathogenesis of diabetic cardiomyopathy which is a leading cause for mortality in diabetes. Evidence from recent studies showed that the pathological changes in the heart caused by oxidative stress result in increased myocardial cell death, and increased generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Cai, *et al.*, 2005). It has been shown that alkaloids and flavonoids isolated from *Sophora species* decrease oxidative stress by increasing levels of the free radical scavenger (Tai, *et al.*, 2011). Antioxidants interfere with activation of free radicals and resulting in protecting the human body from free radicals that may cause some chronic diseases such as diabetes. (Jang, *et al.*, 2010). Most plants contain alkaloids, flavonoids, and polyphenols which are the major antioxidants that have substantial effects on the prevention of cellular damage caused by oxidative stress (Cotelle, 2001; Zhao, *et al.*, 2006).

Obesity is one of the common pathogens in patients with type 2 diabetes. As mentioned earlier (Park, *et al.*, 2009), *Sophora* fruit powder significantly reduced fat mass in non-diabetic obese mice. This evidence suggests that active compounds in this

species may be useful for controlling obese-related metabolic disease such as diabetes and its complications.

STATEMENT OF THE PROBLEM AND AIM OF THE STUDY

Type 2 diabetes has become a worldwide epidemic which is predicted to become oven worse. Currently there is no cure and until recently the available pharmacological agents were only able to control the hyperglycemia for some period of time. Traditionally, medicinal plants have been used throughout the world to treat diabetes. The literature is replete with research attempting to show the beneficial properties of the medicinal plant in the control of hyperglycemia through inhibition of α -glucosidase (Kim, *et al.*, 2006), increase of glucose uptake , enhancement of insulin secretion, inhibition of DPP-IV (Thornberry and Gallwitz, 2009), and inhibition of glycogen phosphorylase(Yamada, *et al.*, 2008). However, to the best of our knowledge, no one has studies the effects of *Sophora alopecuroides* seed on inhibition of glycogen phosphorylase enzyme. It is the purpose of this study to determine extracts of *Sophora alopecuroides* seed for inhibition of glycogen phosphorylase-a enzyme to identify novel chemical compounds, which may serve as leading molecule. In addition, my goal is to gain a clear understanding as to the mode of action of antihyperglycemic herb *Sophora alopecuroides* seed traditionally used for diabetes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant sample

Naturally-dried *Sophora alopecuroides* seeds were collected from Aqsu, North West of China. The plant seed was identified and quarantined at the plant quarantine center, department of Agriculture, Aqsu, Xinjiang, China (No: 650000582249). A voucher specimen was kept in The Unit of Biohealth Science, Institute of Biological Science, University of Malaya for further reference.

3.1.2 General chemicals and solvents

A. Chemicals

Ascorbic acid (fisher scientific), Citric acid monohydrate, tri-sodium citrate dehydrate, D-Glucose, Bismuth nitrate, potassium iodide, Sodium hydroxide and vanillin were purchased from Chemolab Supplier (Kuala Lumpur, Malaysia). Sodium chloride (NaCl) (Merck, Germany) was purchased from Merck Chemical Co. (Kuala Lumpur, Malaysia).

B. Solvents

Ethanol, methanol, hexane chloroform, glacial acetic acid, acetone, sulfuric acid (H_2SO_4), anisaldehyde, and 25% ammonia were purchased from Chemolab Supplies (Kuala Lumpur, Malaysia).

DMSO and hydrochloric acid (Merck, Germany) were purchased from Merck Chemical Co. (Malaysia). All chemicals and solvents were of analytical grade and used without further purification.

3.1.3 Chromatographic media

Silica gel 60 F_{254} – precoated TLC plates (Merck, Germany) were purchased from Merck Chemical Co. (Malaysia).

3.1.4 Antioxidant assay

DPPH (2,2 diphenyl-2-picrylhydrazyl), trichloroacetic acid, sodium phosphate monobasic, sodium phosphate dibasic were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride, methanol and water (HPLC) were purchased from Chemolab Supplies (Kuala Lumpur, Malaysia). An ELISA reader (Sunrise, Switzerland) and UV-vis spectrophotometer-1700 (Shimadzu, Japan) were used for absorbance determinations.

3.1.5 Glycogen phosphorylase enzyme assay

Glycogen phosphorylase a from rabbit muscle, Glycogen from rabbit liver (type III) , α-D Glucose-1-phosphate, HEPES [4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)], Magnesium chloride ($MgCl_2$), EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid), Ammonium molybdate, Malachite green, Caffeine and potassium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). An ELISA reader (Sunrise, Switzerland) was used for absorbance determination at 620 nm.

3.1.6 Antihyperglycemic activity study

Healthy adult male and female Wistar Kyoto rats (WKY) were purchased from University of Malaya Animal House (Kuala Lumpur Malaysia). Accu-Check Performa glucose miter, Accu-Check Performa glucose test strips and control solution (Roche Diagnostics, USA) were purchased from Roche Diagnostics, Malaysia. Streptozotocin and nicotinamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.1.7 Other instruments

Mass spectra were carried out on ABSCIEX Triple TOF 5600-1 Mass Spectrometer (California, USA).

3.2 Methods

3.2.1 Preparation of extracts

The seeds were ground to form fine powder and extracted with chloroform and 95 % ethanol using Soxhlet apparatus for 12-16 hours consecutively. The crude extract obtained from each solvent was concentrated and dried in vacuum rotary evaporator. The aqueous extract was prepared by incubating in water bath at 50 ^{o}C for 6 hours. Then the aqueous extract was filtered and the filtrate was lyophilized to dryness. The dried extract was kept in refrigerator at 2-8 ^{o}C for further used.

3.2.2 Detection of chemical compounds with thin layer chromatography

Thin layer chromatography was carried out to detect the chemical compounds present in the seed extracts of *Sophora alopecuroides*. The sample solutions were

placed as bands with capillary tube on Silica gel 60 F_{254} – precoated TLC plates, size 20 cm x 20 cm. The plates were allowed to develop a separation chromatography tanks using solvent chloroform: acetone: methanol: 25% ammonia (20:6:3:1). The developed plates were dried in air and viewed under UV-254 nm, and sprayed with Dragendroff's, Vanillin-sulphuric acid and Anisaldehyde-sulphuric acid reagents separately for detection of compounds present.

Spray Reagents

Reagents were prepared according to published procedure (Wagner, *et al.*, 2009).

A. Dragendroff's reagent

Preparation:

Solution A: 0.85g bismuth nitrate was dissolved in 10 ml glacial acetic acid and 40 ml distilled water.

Solution B: 8g potassium iodide was dissolved in 30 ml distilled water.

Stock solution: 30 ml solution A and 30 ml solution B was mixed to give 60 ml stock solution.

Spray reagent: It was prepared by mixing of 50 ml stock solution with 100 ml glacial acetic acid and 500 ml distilled water.

Application of the reagent: Detection of alkaloids.

B. Vanillin-sulphuric acid reagent

Preparation:

1 ml of concentrated sulphuric acid was added to 1 g of vanillin, and then this solution was mixed with 100 ml of ethanol and stirred. The TLC plates sprayed with this solution were heated at 110 ^{o}C for 5-10 minutes.

Application of the reagent: Detection of terpenoids and phenolic compounds.

C. Anisaldehyde-sulphuric acid reagent

Preparation:

0.5 ml of anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85 ml of methanol and 5 ml of concentrated sulfuric acid. The TLC plates sprayed with reagent were heated at 100 ^{o}C for 5-10 minutes.

Application of the reagent: Detection of terpenoids, saponins and flavonoid compounds.

3.2.3 Q-TOF MS analysis of alkaloids

Determination of alkaloid content in *Sophora alopecuroides* seed was carried out the method described by Liu (Liu, *et al.*, 2011). The direct infusion mass spectrometry technique was carried out on AB SCIEX Triple TOF 5600-1 quadruple time-of-flight mass spectrometer (AB SCIEX California, USA) with electrospray ionization (ESI) source. Data acquisition and processing were performed using Analyst TF 1.5 software. Positive ionization mode was recorded in the range of m/z 100-2000. The capillary and voltage of the ESI-MS source were maintained at 350°C and 5.5 kV, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulisation, curtain gas; 15 psi, collision gas; 10 psi, declustering potential; 80V. The scan mode was used to screen the sample profile and the product ion mode was used to determine the characteristic ions for structural information.

3.2.4 Determination of total alkaloid content

The amount of alkaloid contained *Sophora alopecuroides seed* was determined the method described by Edeoga (Edeoga, *et al.*, 2005) with slightly modification. The powdered sample (5g) was weighed into a 250 ml beaker and extracted with 200 ml of 10% acetic acid in ethanol for 10 hour. The extract was filtered with whatman filter paper and was concentrated under vacuum until 50 ml of original volume. The concentrated ammonium hydroxide was added to the extract by drop wise until the precipitation was complete. The solution was allowed to settle for one day. The precipitate was collected by filtration, washed with ammonium hydroxide and filtered again. The residue was dried and weighed. The result was expressed as percentage of total alkaloid equivalent of dried sample.

3.2.5 Antioxidant activity assays

3.2.5.1 DPPH scavenging activity assay

The DPPH scavenging activity was determined according to the method described by Lena with some modifications (Lena Galvez Ranilla, *et al.*, 2010).

Preparation of DPPH reagent

The following procedure is for the preparation of 100 ml of 60 μM DPPH reagent. 0.0024 g of DPPH was dissolved in 100 ml of methanol. The solution was kept in a flask wrapped with aluminum foil. This reagent is stable when stored in a refrigerator at 4 ^{o}C for several days.

The scavenging activity of crude extracts and ascorbic acid on DPPH assay.

The scavenging activity of each crude extract and ascorbic acid were tested at different concentrations (2000 µg/ml, 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml,). Reaction mixtures containing DPPH and crude extracts or ascorbic were prepared according to Table 3.1

Table 3. 1 Preparation of reaction mixtures of crude extracts or ascorbic acid and DPPH for DPPH assay.

Concentrations of ascorbic acid or extracts (µg/ml)	Volume of ascorbic acid or extracts (µl)	Volume of DPPH reagent (µl)
2000.00	25.00	125.00
1000.00	25.00	125.00
500.00	25.00	125.00
250.00	25.00	125.00
125.00	25.00	125.00
62.50	25.00	125.00
31.25	25.00	125.00

Assay was run by mixing 125 µl of 60 µM DPPH solution with 25 µl of each crude extract at various concentrations (31.25 to 2000µg/ml) in 96-well microplate. The reaction mixture was allowed to stand at 25 ^{o}C for 30 min. After 30 min incubation in darkness, the decrease in the absorbance at 517 nm was measured by using ELISA reader (Sunrise, Switzerland). Ascorbic acid was used as standard. The plant extract was dissolved in methanol. The methanol was used as control. The percentage of scavenging effect was calculated using the following equation:

$$\%\ \text{Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The DPPH scavenging activity of extracts were expressed as IC_{50} and compared with standard. The IC_{50} value was determined from the plotted graph of scavenging activity against the concentrations of extract, and was defined as the concentration (in

μg/ml) of extracts that inhibits the formation of DPPH radical by 50%. The entire test was performed in triplicate and results were expressed as mean ± standard error (SEM).

3.2.5.2 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing activity of crude extracts was determined using the method described by Yu with some modifications (Yu, *et al.*, 2008).

Preparations of reagents

1. Preparation of 10% trichloroacetic acid (TCA) solution

Trichloroacetic acid solution was prepared at the concentration of 100 mg/ml. 5 g of trichloroacetic acid was dissolved in 50 ml distilled water. The solution was kept in a flask wrapped with aluminum foil.

2. Preparation of 0.1% ferric chloride ($FeCI_3$) solution

Ferric chloride was prepared at the concentration of 1 mg/ml. To prepare 50 ml of solution, 0.05 g of ferric chloride was dissolved in 50 ml distilled water. The solution was kept in a flask wrapped with aluminum foil.

3. Preparation of 1% potassium ferricyanide $K_3[Fe(CN)_6]$ solution

Potassium ferricyanide was prepared at the concentration of 10 mg/ml. 0.5 g potassium ferricyanide was dissolved in 50 ml distilled water. The solution was kept in a flask wrapped with aluminum foil. The solution should be prepared immediately before use.

4. Preparation of 0.2 M sodium phosphate buffer solution

Sodium phosphate buffer were prepared according to published method (Dawson, *et al.*, 1986).

(1). Preparation of 0.4 M sodium phosphate monobasic (NaH_2PO_4) stock solution

To prepare 250 ml of 0.4 M stock solution, 12 g of sodium phosphate monobasic (MW=119.98 g/mole) was dissolved in 250 ml distilled water. The stock was kept in a flask prior to prepare 0.2 M sodium phosphate buffer.

(2). Preparation of 0.4 M sodium phosphate dibasic (Na_2HPO_4) stock solution

To prepare 250 ml of 0.4 M stock solution, 14.2 g of sodium phosphate dibasic (MW=141.96 g/mole) was dissolved in 250 ml distilled water. The stock was kept in a flask prior to prepare 0.2 M sodium phosphate buffer.

To prepare 500 ml of 0.2 M sodium phosphate buffer solution (pH = 6.6), approximately 93.75 ml of sodium phosphate dibasic (Na_2HPO_4) stock solution was mixed with 156.25 ml of sodium phosphate monobasic (NaH_2PO_4) stock solution and it was diluted to 500 ml with distilled water. This solution can be stored in the refrigerator for several months.

FRAP assay

0.5 ml of various concentration of the crude extracts (2.5 to 40 µg/ml) in distilled water was mixed with 0.5 ml of phosphate buffer (pH= 6.6) and 0.5 ml of potassium ferricyanide. The mixture was incubated at 50 ^oC for 20 min. After incubation, 0.5 ml of 10% trichloroacetic acid was added to the mixture, which was then the mixture was centrifuged at 3000 rpm for10 min. The supernatant (0.5 ml) was mixed with 0.5 ml of distilled water and 0.1 ml of freshly prepared 0.1% ferric chloride solution. The absorbance was measured at 700 nm using UV spectrophotometer (Shimadzu, Japan). The Ascorbic acid at different concentrations (2.5 to 40µg/ml) was

used as standard reference. Distilled water was used as blank. All the tests were carried out triplicates and results were expressed as mean ± standard error (SEM). Increased absorbance of the reaction mixture indicates increase in reducing power.

$$\%\ \text{Increase in reducing power} = \frac{\text{A sample} - \text{A control}}{\text{A control}} \times 100$$

The reducing power activity of *Sophora alopecuroides seed* extracts were expressed as IC_{50} and compared with standard.

3.2.6 Glycogen phosphorylase a enzyme inhibition assay

The assay is an end-point spectrophotometric determination designed for a 96-well microplate reader and is a slightly modification of a previously published method (Loughlin, *et al.*, 2008).

(1). Preparation of 0.1 M HEPES buffer stock solution

Hepes buffer was prepared according to published procedure (Dawson, *et al.*, 1986).

The following procedure is for the preparation of 200 ml stock solution. 4.766 g Hepes (MW = 238.38 g/mole) was dissolved in 200 ml HPLC grade water. The stock solution was kept in a flask. This can be stored in the refrigerator for several months.

(2). Preparation of 0.1 M NaOH solution

The following procedure is for the preparation of 200 ml 0.1 M NaOH solution. 0.4 g of NaOH (MW = 40 g/mole) was dissolved in 100 ml HPLC grade water by stirring with a magnetic stir-bar. The solution was kept in a flask.

(3). Preparation of 50 mM HEPES buffer for Glycogen phosphorylase enzyme assay

The following procedure is for the preparation of 200 ml. 100 ml Hepes (0.1 M) stock solution was added 0.1 M NaOH by dropwise (approximately 40 ml) to give a solution with pH 7.2. Then this solution was diluted with HPLC grade water (approximately 60 ml) to give 50 mM Hepes buffer (pH = 7.2).

(4). Preparation of 9 units/ml Glycogen phosphorylase enzyme stock solution and 4.5 units/ml solution for Glycogen phosphorylase enzyme assay

45 units (5 mg) of Glycogen phosphorylase enzyme obtained from Sigma (St. Luis, MO) was dissolved in 5 ml of 50 mM Hepes buffer (pH = 7.2) in a flask to give a stock solution of 9 units/ml. When this solution was kept in the refrigerator at 4 ^{o}C it was stable for several months.

The enzyme assay required 4.5units/ml. The stock solution was diluted two-fold. For preparation of 10 ml, 5 ml of stock enzyme solution was diluted with 5 ml of 50 mM Hepes buffer (pH = 7.2). This solution is stable when stored in a refrigerator at 4 ^{o}C.

(5). Preparation of 50 mM HEPES buffer (pH = 7.2) solution containing 100 mM KCI, 2.5 mM EGTA, 2.5 mM $MgCI_2$, 0.25 mM glucose-1-phosphate, and 1mg/ml glycogen.

The following procedure is for the preparation of 100 ml solution. 0.750 g KCI, 0.095 g EGTA, 0.0510 g $MgCI_2$, 0.0084g glucose-1-phosphate, and 0.1g glycogen were dissolved in 100 ml of 50 mM Hepes buffer (pH = 7.2). This solution is stable when stored in a refrigerator at 4 ^{o}C.

(6). Preparation of 1M HCI containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green.

The following procedure is for the preparation of 100 ml solution. 1 g of ammonium molybdate and 0.038 g of malachite green were dissolved in 100 ml of 1M HCI. The solution was kept in a flask wrapped with aluminum foil. This solution is stable when stored in a refrigerator.

GPa enzyme inhibition assay

The enzyme solution was made to a concentration of 4.5units/ml at pH 7.2 with 50 mM Hepes buffer. One unit will produce 1 μmol of free phosphate from glucose-1-phosphate per minute in 50 mM Hepes buffer at pH 7.2 at 22 ^{o}C. A 0.25 mM stock solution of glucose-1-phosphate was prepared at pH 7.2 with 50 mM Hepes buffer. Glycogen phosphorylase inhibition activity of crude extracts and caffeine were measured at the different concentrations (2 mg/ml to 32 mg/ml). All crude extracts and caffeine were dissolved in 10% DMSO in 50 mM Hepes buffer. Caffeine was used as a standard.

The assay was run by mixing 40 μl of 50 mM Hepes buffer (containing 100 mM KCI, 2.5 mM EGTA, 2.5 mM MgCI2, 0.25 mM glucose-1-phosphate, and 1mg/ml glycogen), 50 μl of enzyme solution and 10 μl of sample, standard or control in 96-well plate. After a 30 minute incubation period at 22 ^{o}C, 150 μl of 1M HCI solution containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green was added to the appropriate wells to commence the reaction. The absorbance at 620 nm was determined using a microplate reader. The assay was performed in triplicate with appropriate blanks and controls. 50 mM Hepes buffer was used as control. The percentage of inhibition effect was calculated using the following equation:

$$\% \text{ Inhibition } = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The concentration of extract required to inhibit 50% of glycogen phosphorylase enzyme activity under the assay conditions was defined as the IC_{50} value. The results are reported as the mean ± S.E.

3.2.7 Acute toxicity studies

The acute toxicity was carried out the method described by Sandesh with slightly modifications (Sancheti, *et al.*, 2010). Healthy adult male and female Wistar Kyoto rats (WKY) weighing 200 – 250 g purchased from University of Malaya Animal House were used in this study. The rats were kept individually in polypropylene cages in the lab for adaptation to the laboratory conditions for the period of 3 days. They were maintained under the standard laboratory conditions (21 ± 2 ^{o}C and 12 hour light/12 hour dark cycle). The rats were fed with standard rat pallet diet (University of Malaya Animal House, Kuala Lumpur, Malaysia). The protocol used in this study was approved by the University of Malaya Animal Care and Use Committee {ISB/28/09/2011/AA (R)}.

Non-diabetic rats were divided into two groups (male and female groups). Each group of these rats were divided into three groups again and five rats were used for each group. The dose for the study was fixed for the extract at 2, 4 and 5 g/kg.

Preparation of extract solutions

Ethanol extract of *Sophora alopecuroides* seed was made at different concentrations (200, 400 and 500 mg/ml) in distilled water.

Determination of acute toxicity

The acute toxicity was determined by single administration of extract to each group of rats orally in a volume of 10 ml/kg body weight using an intragastric tube. The

rats were fasted overnight (16 h) but had been allowed to free access to water. Group one was treated orally with extract 2 g/kg body weight. Group two was treated orally with extract 4 g/kg body weight. Group three was treated orally with extract 5 g/kg body weight.

The rats were observed continuously for 2 hours for their behavioural alterations and the mortality caused by the extract within 15 days of study time was noted.

3.2.8 Oral glucose tolerance test (OGTT) in non-diabetic rats

The oral glucose tolerance test was carried out the method described by Annie Shirwaikar, *et al*. (2006) with some slightly modifications. Male Wistar Kyoto rats (WKY) weighing 200 – 250 g were purchased from University of Malaya Animal House. Sixteen-hour fasted non-diabetic rats were used in this study. The activity of *Sophora alopecuroides* seed ethanol extract at the dose of 250 mg/kg body weight and 500 mg/kg body weight in non-diabetic rats were tested. Glucose (2 g/kg body weight) was used as standard reference. Ethanol extract of *Sophora alopecuroides* seed was prepared at the concentrations of 25 mg/ml and 50 mg/ml in distilled water. Glucose solution was prepared at 400 mg/ml in distilled water.

Rats were divided into three groups containing six rats each. Rats were fasted overnight (16 hours). The water was removed 2 h before the test was performed and the rats are not allowed to access food and drink during entire test. The rats of each group were given extract or glucose solution in a volume of 10 ml/kg body weight as following.

Group 1: Normal control group. Rats were treated orally with distilled water 10 ml/kg body weight.

Group 2: Rats were treated orally with ethanol extract of *Sophora alopecuroides* seed 250 mg/kg body weight.

Group 3: Rats were treated orally with ethanol extract of *Sophora alopecuroides* seed 500 mg/kg body weight.

Glucose (2g/kg) was fed 30 minute after the treatment of extract or distilled water to the rats of each group. Blood was withdrawn from the rat's tail at 0, 30, 60, 90 and 120 minute of glucose administration. Blood glucose concentrations were measured using Accu-check glucose test strips and glucose meter (Accu-check, Roche Diagnostics, USA). Data are expressed as the mean ± standard error of the mean (SEM). The difference between the groups was statistically significant as determined by one-way ANOVA followed by post hoc Dunnett's test.

3.2.9 Antihyperglycemic activity studies of *Sophora alopecuroides* seed on streptozotocin-nicotinamide induced diabetic rats

3.2.9.1 Induction of non-insulin depended diabetes mellitus (NIDDM)

NIDDM was induced previously published method with slightly modifications (Shirwaikar, *et al.*, 2006). Male Wistar Kyoto rats (WKY) weighing 180 – 250 g purchased from University of Malaya Animal House were used in this study.

Preparation of 0.1 M citrate buffer (pH = 4.5) and 0.9 % saline solution

Citrate buffer was prepared according to published procedure (Dawson, *et al.*, 1986).

1. **Preparation of 0.1 M citrate buffer (pH 4.5)**

0.1M citric acid solution.

5.26 g citric acid monohydrate (MW = 210.14 g/mole) was dissolved in 250 ml distilled water.

0.1 M trisodium citrate solution

7.36 g trisodium citrate dihydrate (MW = 294.12 g/mole) was dissolved in 250 ml distilled water.

For the preparation of 100 ml 0.1 M citrate buffer (pH = 4.5), 47 ml 0.1 M citric acid solution was mixed with 53 ml 0.1 M trisodium citrate solution to give a 0.1 M citrate buffer solution with pH = 4.5. The solution was kept in refrigerator at 1- 4 ^{o}C for future use.

2. Preparation of 0.9% saline solution.

The following procedure is for the preparation of 1000 ml 0.9% saline solution. 9 g of sodium hydroxide was dissolved in one 1itter distilled water. The solution was kept in a suitable container.

Induction of diabetes

Overnight fasted Rats (16 h) were induced diabetic by a single intraperitoneal injection of 100 mg/kg nicotinamide and 60 mg/kg streptozotocin body weight.

Nicotinamide was prepared at the concentration of 100 mg/ml in 0.9 % saline. Streptozotocin was prepared at the concentration of 60 mg/ml in ice-cold citrate buffer (pH 4.5) immediately before use. The body weight of each rat was measured before injection. Nicotinamide solution was injected into overnight fasted rats in a volume of 1 ml/kg body weight. 15 minute after intraperitoneal injection of nicotinamide, freshly

prepared streptozotocin solution was injected into each rat in a volume of 1 ml/kg body weight.

Blood glucose was tested before injection and after 10 days of streptozotocin and nicotinamide injection using Accu-check glucose test strips and glucose meter (Accu-check, Roche Diagnostics, USA).

3.2.9.2 Evaluation of *Sophora alopecuroides* seed ethanol extract on fasting plasma glucose levels and changes in body weight in streptozotocin-nicotinamide induced diabetic rats

Ten days after streptozotocin and nicotinamide injection, rats found with blood glucose levels of 9 – 20 mmol/L were used in this study. Group one (six rats) is non-diabetic rats, and was used as normal control group. Diabetic rats were divided into four groups with six rats in each of the following groups.

Group 1: Non-diabetic rats were treated with distilled water for 28 days

Group 2: Diabetic rats were treated with distilled water for 28 days

Group 3: Diabetic rats were treated with *Sophora alopecuroides* seed ethanol extract at the dose of 250 mg/kg body weight for 28 days

Group 4: Diabetic rats were treated with *Sophora alopecuroides* seed ethanol extract at the dose of 500 mg/kg body weight for 28 days

Group 5: Diabetic rats were treated with standard drug glibenclamide at the dose of 10 mg/kg body weight for 28 days

Preparation of extract and standard drug glibenclamide solutions

Ethanol extract of *Sophora alopecuroides* seed was prepared at the concentration of 25 mg/ml and 50 mg/ml in distilled water.

Standard drug glibenclamide was prepared at the concentration of 1 mg/ml in distilled water. The solutions were prepared fresh before use.

Sophora alopecuroides seed extract (250 mg/ml and 500 mg/ml) and standard drug solution (1 mg/ml) was fed to the rats of appropriate group in a volume of 10 ml/kg body weight by intragastric tube for 28 days.

Blood was withdrawn from the rat's tail by Accu-check punching device. Blood glucose concentrations of overnight fasted rats were measured in each week using Accu-check glucose test strips and glucose meter (Accu-check, Roche Diagnostics, USA). Data are expressed as the mean ± standard error of the mean (SEM).

3.2.9.3 Evaluation of *Sophora alopecuroides* seed ethanol extract on serum lipid profiles in streptozotocin-nicotinamide induced diabetic rats

At the end of study (28 days), rats were sacrificed after an overnight fasting by cervical dislocation, and blood was collected in EDTA containing tubes. Blood sample was spin 3000 rpm for 10 minute to collect serum. The serum was sent to University of Malaya Medical Center Laboratory for analysis of serum lipid profiles. Results were expressed as the mean ± standard error of the mean (SEM).

CHAPTER 4

RESULTS

4.1 Preparation of *Sophora alopecuroides* seed extracts

Dry and powdered *Sophora alopecuroides* seed was extracted with chloroform and 95% ethanol in a Soxhlet apparatus for 12-16 hours after passage through a 40-mesh sieve and the extracts were evaporated under reduced pressure. The chloroform extract give an oily dark brown residue in 4.39% yield and 95% ethanol extract give a brown powder in 14.7% yield.

The aqueous extract was prepared by incubating in water bath at 50^{o}C for 6 hours. The yield obtained after lyophilization was a yellow powder in 10.6% yield. The yields of each extract were summarized and it was shown in Table 4.1

Table 4. 1 Yields of *Sophora alopecuroides* seed extracted with specific solvent

Solvent used	Yield (%)
Chloroform	3.39
95% Ethanol	14.7
Aqueous	10.6

4.2 Detection of chemical compounds with thin layer chromatography

The kinds of compounds present in the chloroform, 95% ethanol and aqueous extracts were determined by the use of spray reagents. Solutions of each extract were spotted onto a TLC plate and sprayed with the particular reagent. Alkaloids and saponins were detected in the chloroform extract of *Sophora alopecuroides* seed (table

4.2). Alkaloids, flavonoids, saponins and terpenoids were detected in 95% ethanol extract (table 4.3), and alkaloids and flavonoids were detected in aqueous extract (table 4.4).

Table 4. 2 Thin Layer Chromatography of *Sophora Alopecuroides* Seed extract with chloroform

Label Compounds	R_f value	Visible light	UV light (254 nm)	Spraying Reagents			Remarks
				Dragendroff's	Vanillin-H_2SO_4	Anisaldehyde-H_2SO_4	
SAS-CHL1	0.99	-ve	Gray (++)	-ve	-ve	Black (+++)	Saponin
SAS-CHL2	0.95	-ve	-ve	-ve	-ve	Blue (++)	Saponin
SAS-CHL3	0.94	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-CHL4	0.84	-ve	-ve	-ve	-ve	Blue (+++)	Saponin
SAS-CHL5	0.60	-ve	-ve	-ve	-ve	Blue (++)	Saponin
SAS-CHL6	0.59	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-CHL7	0.53	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-CHL8	0.46	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-CHL9	0.38	-ve	-ve	Orange (++)	-ve	-ve	Alkaloid
SAS-CHL10	0.28	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-CHL11	0.20	-ve	-ve	-ve	-ve	Blue (+)	Saponin
SAS-CHL12	0.13	-ve	Gray (++)	-ve	-ve	Dark blue (+++)	Saponin
SAS-CHL13	0.80	-ve	-ve	Orange (+)	-ve	-ve	Alkaloid

Indication for intensity of color:

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

Table 4. 3 Thin Layer Chromatography of *Sophora Alopecuroides* Seed extract with 95% ethanol

Label Compounds	R_f value	Visible light	UV light (254 nm)	Spraying Reagents			Remarks
				Dragendroff's	Vanillin-H_2SO_4	Anisaldehyde-H_2SO_4	
SAS-EtOH1	0.95	-ve	Gray (++)	-ve	Violet Blue (+++)	Violet Blue (+++)	Saponin
SAS-EtOH2	0.90	-ve	-ve	-ve	Purple (++)	-ve	Terpenoids
SAS-EtOH3	0.85	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH4	0.71	-ve	-ve	Orange (++)	-ve	-ve	Alkaloid
SAS-EtOH5	0.63	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH6	0.60	-ve	-ve	-ve	-ve	Purple (+)	Terpenoids
SAS-EtOH7	0.59	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH8	0.45	-ve	-ve	Orange (+)	-ve	-ve	Alkaloid
SAS-EtOH9	0.36	-ve	-ve	-ve	-ve	Purple (+)	Terpenoids
SAS-EtOH10	0.33	-ve	-ve	Orange (++)	-ve	-ve	Alkaloid
SAS-EtOH11	0.21	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH12	0.20	-ve	-ve	-ve	Purple (+)	Purple (+)	Terpenoids
SAS-EtOH13	0.18	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH14	0.14	-ve	-ve	-ve	-ve	Purple (++)	Terpenoids
SAS-EtOH15	0.11	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-EtOH16	0.08	-ve	-ve	-ve	-ve	Yellow (+)	Flavonoids
SAS-EtOH17	0.03	-ve	-ve	Orange (+)	-ve	-ve	Alkaloid
SAS-EtOH18	0.00	-ve	-ve	-ve	-ve	Dark green (+++)	Flavonoids

Indication for intensity of color: +++ = Strong; ++ = medium; + = Weak; -ve = No color observed

Table 4. 4 Thin Layer Chromatography of *Sophora Alopecuroides Seed* extract with distilled water

Label Compounds	R_f value	Visible light	UV light (254 nm)	Spraying Reagents			Remarks
				Dragendroff's	Vanillin-H_2SO_4	Anisaldehyde-H_2SO_4	
SAS-H_2O 1	0.95	-ve	-ve	Orange (+)	-ve	-ve	Alkaloid
SAS-H_2O 2	0.91	-ve	Gray (+)	Orange (+++)	-ve	-ve	Alkaloid
SAS-H_2O 3	0.46	-ve	-ve	Orange (+)	-ve	-ve	Alkaloid
SAS-H_2O 4	0.26	-ve	-ve	Orange (+++)	-ve	-ve	Alkaloid
SAS-H_2O 5	0.21	-ve	Gray (+++)	Orange (+++)	-ve	-ve	Alkaloid
SAS-H_2O 6	0.15	-ve	Gray (+++)	Orange (+++)	-ve	-ve	Alkaloid
SAS-H_2O 7	0	-ve	-ve	-ve	-ve	Dark green	Flavonoids

Indication for intensity of color:

+++ = Strong; ++ = medium; + = Weak; -ve = No color observed

4.3 Q-TOF MS analysis of alkaloids

In this study, direct infusion of mass spectrometry analysis was carried out to determine presence of the alkaloid compounds in 95% ethanol and aqueous extracts of *Sophora alopecuroides* seed. Q-TOF MS spectrum for each extract was shown in figure 4.1, and compounds detected were summarized in table 4.5.

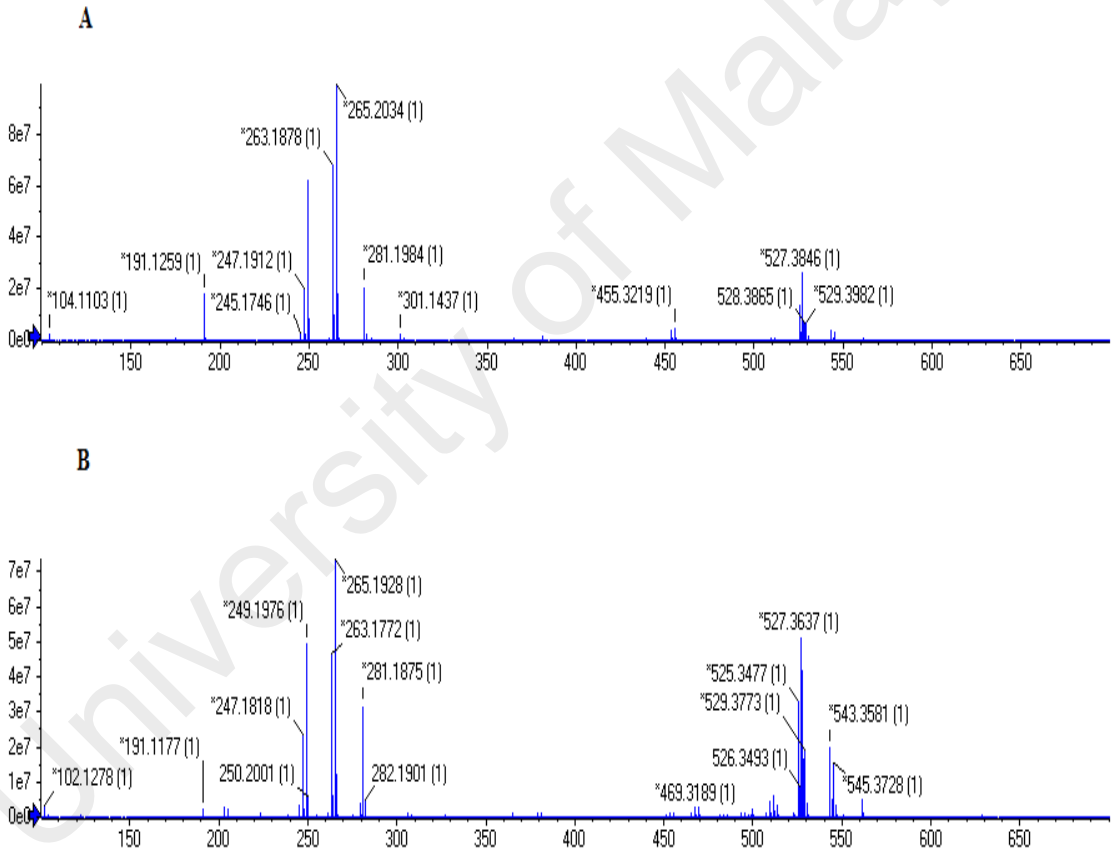

Figure 4. 1 Q-TOF MS of *Sophora alopecuroides* seed (A = Aqueous extract, B = 95% ethanol extract).

Table 4. 5 Identification of alkaloid compounds in 95% ethanol and aqueous extracts from *Sophora alopecuroides* seed by direct infusion of Q-TOF MS

No	$[M+H]^+$(m/z)	Collision energy (V)	Experimental MS^2 (m/z)	Reported MS^2 (m/z)	Identified compounds
1	247.18	55	227,179, 150, 148, 136, 108	227, 179, 150, 148, 136[a]	Sophocarpine
2	249.2	60	176, 150, 148, 134, 120,	176, 150, 148[a]	Matrine
3	261.16	45	243, 215, 187, 164, 150, 120, 114	243, 164, 114[a]	Baptifoline
4	263.18	45	245, 203, 177, 150, 136	263, 245, 150, 138[a]	Oxysophocarpine
5	265.2	45	247, 205, 177, 150, 148, 136, 122	265, 247, 205, 148[a]	Oxymatrine
6	495.38	20	265,249, 247	-	Sophocarpine dimer
7	525.35	40	281, 279, 265, 263, 245, 203, 177, 150	263, 245, 150[a]	Oxysophocarpine dimer
8	529.38	30	281, 265, 266, 247, 205, 148	265, 205, 148, 150[a]	Oxymatrine dimer
9	561.37	30	327, 297,299, 281, 265, 222, 138	-	Sophoranol-N-oxide dimer

4.4 Determination of total alkaloid content

Dry and powdered *Sophora alopecuroides* seed was suspended in 10% acetic acid in ethanol. The suspension was filtered and evaporated until ¼ of total volume under reduced pressure. Then it was precipitated with concentrated ammonium hydroxide, filtered, and dried to yield a white solid as the total alkaloid content in 7.56% yield.

4.5 Antioxidant activity assays

4.5.1 DPPH scavenging activity assay

The assay was performed according to published procedure (Lena Galvez Ranilla, *et al.*, 2010) with slightly modification which was described in detail in materials and methods. The assay was run by mixing 125 µl of DPPH reagent and 25 µl of extracts or ascorbic acid at different concentrations in 96-well microplate. The absorbance of each extracts and ascorbic acid were shown in appendix 1 and 2. Figure 4.2 shows that ethanol extract has an IC_{50} = 155.33 ± 0.06 µg/ml, aqueous extract has an IC_{50} = 167.47 ± 0.03 µg/ml, and ascorbic acid has an IC_{50} = 3.69 ± 0.01 µg/ml.

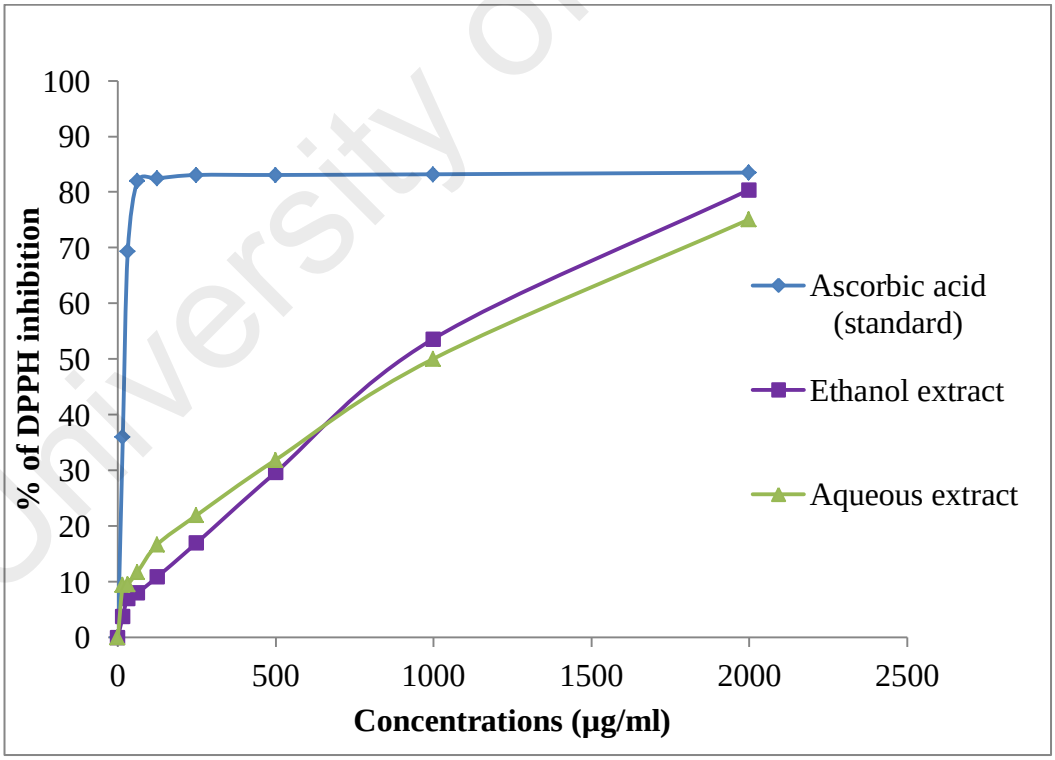

Figure 4. 2 Effect of *Sophora alopecuroides* seed extracts on DPPH assay

4.5.2 Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to published procedure (Yu, *et al.*, 2008) with some slightly modification. The standard and extracts were tested at the concentration from 2.5 to 40µg/ml. The absorbance of extracts and standard were shown in appendix 6. The similar IC_{50} values of extracts were detected at the concentrations tested. Figure 4.3 showed that IC_{50} value of ethanol extract was 9.71 ± 0.002 µg/ml, aqueous extract was 10.10 ± 0.002 µg/ml and standard ascorbic acid was 5.37 ± 0.003 µg/ml.

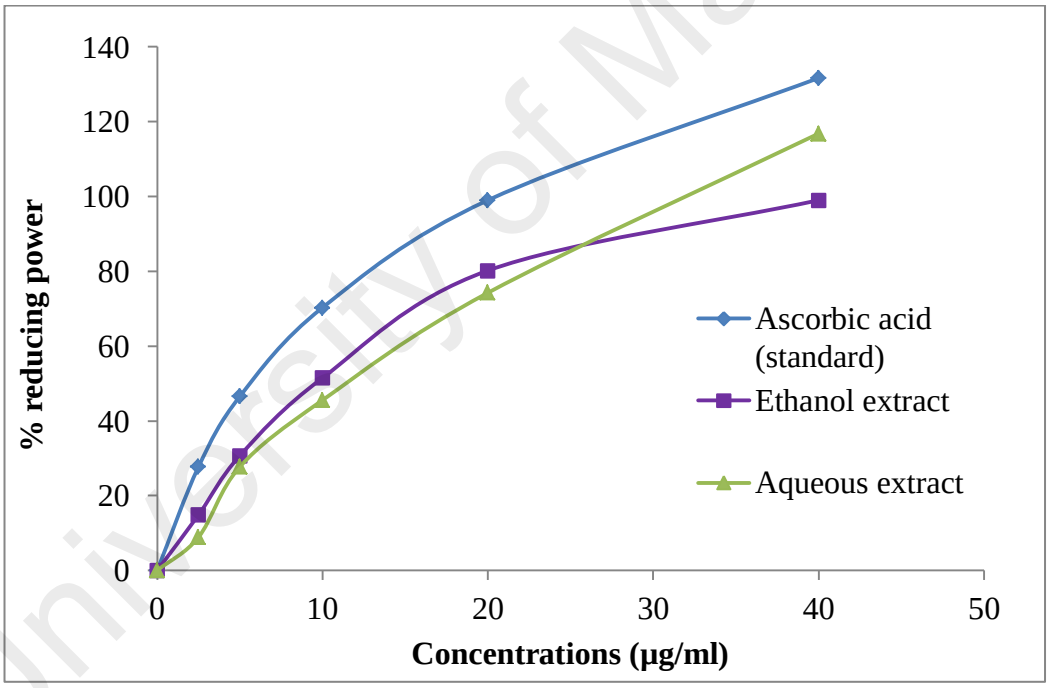

Figure 4. 3 Effect of *Sophora alopecuroides* seed extracts on FRAP assay

4.6 Glycogen phosphorylase-a enzyme inhibition assay

The assay used to screen the extracts for GPa inhibition is a modification of a previously published method (Loughlin, *et al.*, 2008) which is described in detail in materials and methods. The each extract and standard was tested at the concentration

from 2 mg/l to 32 mg/l. The absorbance of extracts and standard were shown in appendix 7and appendix 8. The GPa inhibition activity of extracts and standard are shown in Figure 4.4 and indicate a range of 0-77.52 % inhibition. Figure 4.4 shows that the most potent extract was ethanol extract and its IC_{50} is 581.61 ± 0.02 µg/ml.

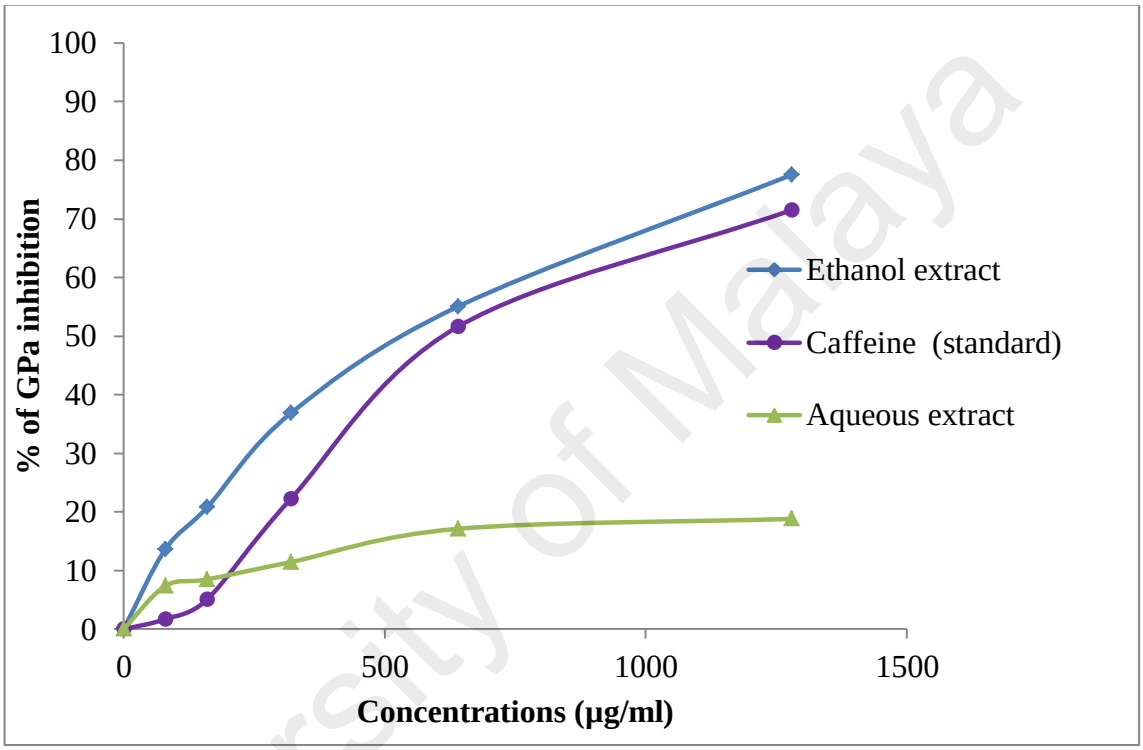

Figure 4. 4 Inhibition of GPa activity by *Sophora alopecuroides* seed extracts

4.7 Acute toxicity studies

Wistar Kyoto rats (WKY) in both sexes that were fasted for 16 hours were used in acute toxicity test. The ethanol extract of *Sophora alopecuroides* seed at the dose of 2, 4 and 5 mg/kg body weight were tested. The results revealed the toxic nature of *Sophora alopecuroides* seed ethanol extract on female rats at the dose of 5 mg/kg and it showed that two female rats died after administration of extract during 15 days of study time. There was no lethality found in male rats at any of the doses selected until the end of the study period.

4.8 Oral glucose tolerance test (OGTT) in non-diabetic rats

Male Wistar Kyoto rats (WKY) fasted for 16 hours were used in oral glucose tolerance test (OGTT). Non-diabetic rats were orally administered *Sophora alopecuroides* seed ethanol extract (250 mg/kg and 500 mg/kg) 30 minutes after glucose (2g/kg) administration. The effects of *Sophora alopecuroides* seed (SAS) extract on OGTT in non-diabetic rats at the different doses were shown in Figure 4.5. Figure 4.5 shows fasting plasma glucose concentrations between *Sophora alopecuroides* seed ethanol extract administered and control rats.

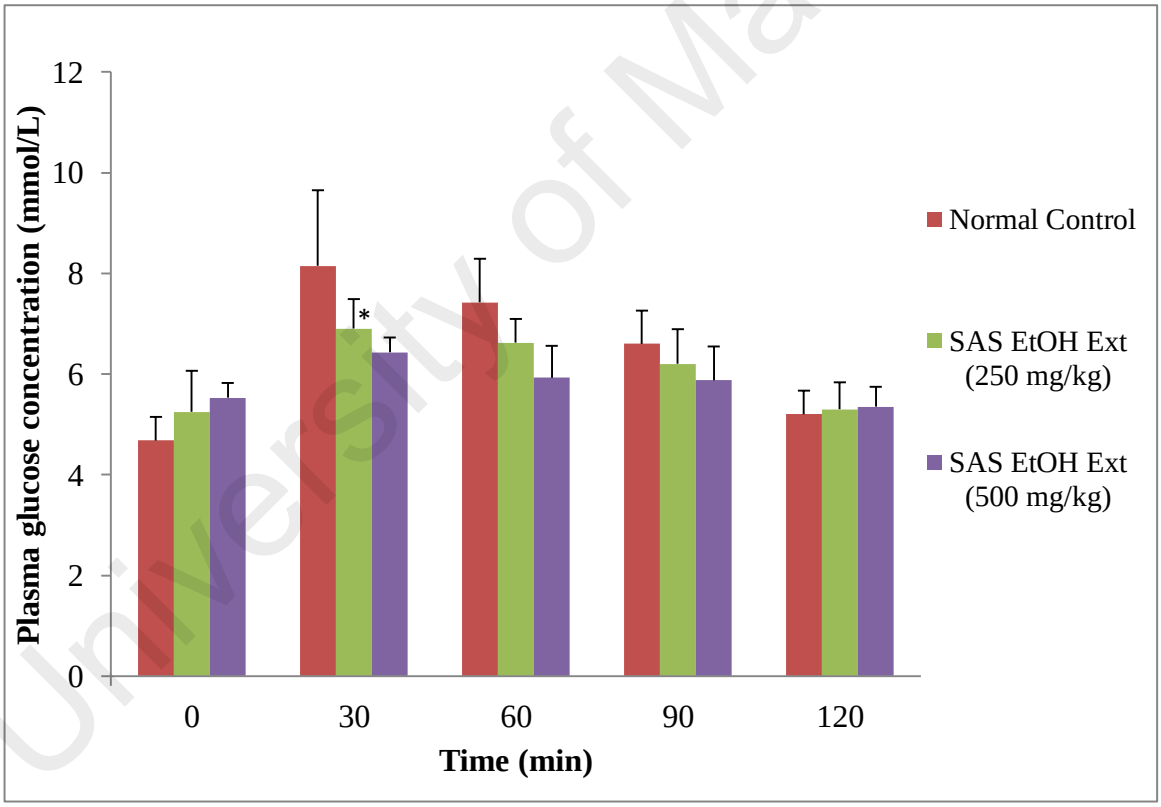

Figure 4. 5 Oral glucose tolerance test of *Sophora alopecuroides* seed ethanol extract in non-diabetic rats. Values are expressed as mean ± SEM (n = 6). *P* values were analyzed using one-way ANOVA followed by post hoc Dunnett's test. **P* < 0.05 versus normal control group with corresponding time

4.9 Antihyperglycemic activity studies of *Sophora alopecuroides* seed on streptozotocin-nicotinamide induced diabetic rats

4.9.1 Induction of non-insulin depended diabetes mellitus

NIDDM was induced previously published method (Shirwaikar, *et al.*, 2006) with slightly modifications which is described in detail in materials and methods. After 10 days streptozotocin and nicotinamide injection, rats found with blood glucose levels of 9 – 20 mmol/L were considered diabetic and selected for further studies.

4.9.2 Evaluation of *Sophora alopecuroides* seed ethanol extract on fasting plasma glucose levels and changes in body weight in streptozotocin-nicotinamide induced diabetic rats

Antihyperglycemic activity of SAS extract were evaluated in STZ-nicotinamide induced diabetic rats for 28 days. Table 4.6 shows the fasting plasma glucose concentrations between SAS treated and normal control group.

Figure 4.6 shows the changes on body weight between normal and control groups. The differences between each of these groups were determined using one-way ANOVA followed by post hoc Dunnett's test.

Table 4. 6 Effect of ethanol extract of *Sophora alopecuroides seed* on fasting plasma glucose levels in diabetic rats

Group (n=6)	Treatment	Fasting plasma glucose concentration (mmol/L)				
		0^{th} day	7^{th} day	14^{th} day	21^{th} day	28^{th} day
I	Normal control	4.20 ± 0.10	4.2 ± 0.10	4.37 ± 0.15	4.28 ± 0.15	4.13 ± 0.07
II	Diabetic control	15.37 ± 0.78*	20.13 ± 2.14	23.13 ± 1.64	25.35 ± 1.03	29.23 ± 1.93*[a]
III	Diabetic +Glibenclamide (10 mg/kg)	18.37 ± 2.15	18.53 ± 2.74	17.68 ± 1.43	13.18 ± 3.12	10.05 ± 2.38**[a]
IV	Diabetic +SAS EtOH Ext (250 mg/kg)	14.02 ± 1.74	10.68 ± 2.42	12.08 ± 2.19	11.52 ± 2.91	11.40 ± 2.81**
V	Diabetic +SAS EtOH Ext (500 mg/kg)	16.65 ± 1.71	8.38 ± 1.65	11.50 ± 1.82	10.48 ± 1.36	9.38 ± 1.47**[a]

Values are expressed as mean ± SEM (n = 6). *P* values were analyzed using one-way ANOVA followed by post hoc Dunnett's test.

*$P < 0.05$ compared with normal control. ** $P < 0.05$ compared with diabetic control. [a] $P < 0.05$ compared with 0^{th} day

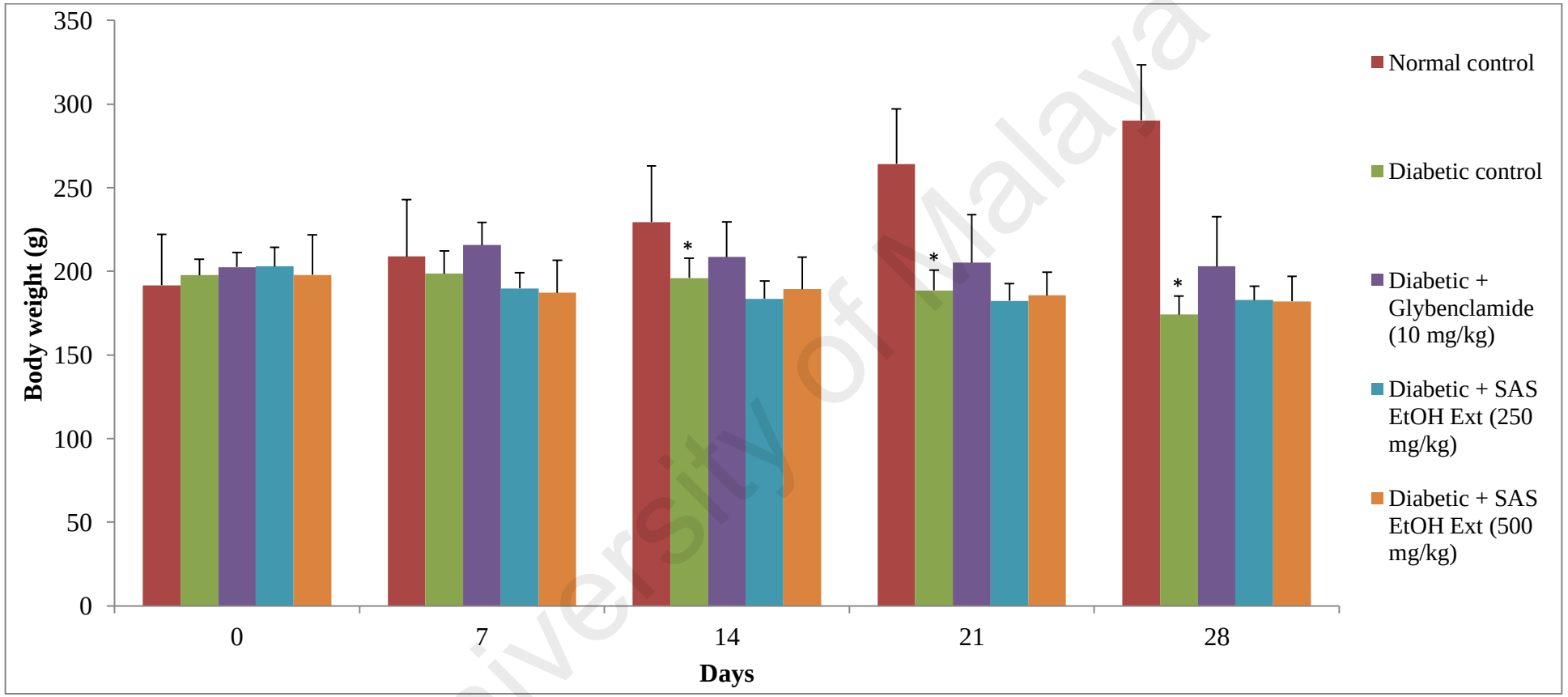

Figure 4. 6 Effect of ethanol extract of *Sophora alopecuroides* seed on body weight in diabetic rats

Values are expressed as mean ± SEM (n = 6). Significance determined by the student's t-test. *$P < 0.05$ compared with normal control.

4.9.3 Evaluation of *Sophora alopecuroides* seed ethanol extract on serum lipid profiles in streptozotocin-nicotinamide induced diabetic rats

After the period of 28 days of study, the blood samples were analysed by Clinical Diagnostic Laboratory University of Malaya Medical Center. The effect of *Sophora alopecuroides seed* ethanol extract on serum lipid profiles in streptozotocin-nicotinamide induced diabetic rats were shown in Table 4.7, and the serum lipid profiles of each rat were shown in appendix 12.

Table 4. 7 Effect of *Sophora alopecuroides seed* ethanol extract on serum lipid profiles in streptozotocin-nicotinamide induced diabetic rats

Group (n=6)	Treatment	Serum lipid profile (mmol/L)		
		TG	TC	HDL
I	Normal control	0.87 ± 0.16*	1.58 ± 0.09*	1.38 ± 0.12
II	Diabetic control	2.63 ± 0.67	1.98 ± 0.18	1.26 ± 0.12
III	Diabetic +Glibenclamide (10 mg/kg)	0.35 ± 0.06*	1.62 ± 0.12	1.34 ± 0.12
IV	Diabetic +SAS EtOH Ext (250 mg/kg)	0.92 ± 0.23*	1.48 ± 0.10*	1.31 ± 0.10
V	Diabetic +SAS EtOH Ext (500 mg/kg)	0.42 ± 0.07*	1.42 ± 0.13*	1.35 ± 0.11

Values are expressed as mean ± SEM (n = 6). *P* values were analyzed using one-way ANOVA followed by post hoc Dunnett's test. *$P < 0.05$ compared with diabetic control.

CHAPTER 5

DISCUSSION

The dried *Sophora alopecuroides* seed was ground to a fine powder with a grinder and passed through a 40-mesh sieve, extracted with chloroform, 95% ethanol and distilled water. The extracts were lyophilized, and the yields obtained are given in Table 1. The chemical compounds of *Sophora alopecuroides* seed were separated by thin layer chromatography, and the kinds of the compounds present in the extract were determined with the use of spray reagents (Table 4.2, Table 4.3, and Table 4.4). These reagents are very useful because they can confirm the presence of a particular compound by turning a specific color. The TLC revealed that the chloroform extract of *Sophora alopecuroides* seed positive for the presence of alkaloids as determined by the development of orange color with Dragendroff's reagent and saponins by blue color with anisaldehyde-H_2SO_4 reagent (Table 4.2). Saponins were also detected in the 95% ethanol extract (Table 4.3). Flavonoids were detected in the 95% ethanol extract (Table 4.3) and distilled water extract (Table 4.4) by the development of yellow color with anisaldehyde-H_2SO_4 reagent, but were not detected in the chloroform extract, probably because they were present in very low concentrations. Terpenoids were detected only in the 95% ethanol extract by the development of purple color with vanillin-H_2SO_4 reagent (Table 4.3). The color tests strongly suggest that alkaloids were present in all the extracts of *Sophora alopecuroides* seed. Alkaloids are a group of naturally occurring chemical compound that contain basic nitrogen atoms. This distinction can easily be seen based on reaction of alkaloid compounds with potassium iodide in Dragendroff's reagent. The total alkaloids content was analyzed and found 7.56 % in dried sample. The yield of chloroform extract (Table 1) was found 3.39 % of dried sample.

The alkaloids present were identified with Q-TQF MS. In Table 4.5 showed that nine alkaloid have been identified namely sophocarpine, matrine, baptifoline, oxysophocarpine, oxymatrine, sophocarpine dimer, oxysophocarpine dimer, oxymatrine dimer and sophoranol-N-oxide dimer in the seed extract of *Sophora alopecuroides.* In this study, direct infusion mass spectrometry analysis was used to determine the alkaloid profile in the 95% ethanol and distilled water extracts of *S. alopecuroides* seed. It was found that all two extracts exhibited similar spectrum as depicted in Figure 4.1. Data (pseudo molecular ion, collision energy and main fragment ions observed in MS^2) for compounds detected in all extracts along with their corresponding reported values for comparison are summarized in Table 4.5. The molecular weight and fragment ions of compound no 1, 2, 4, 5, 6, 7, 8, and 9 were in agreement with those reported in the literature, in which the fragmentation pattern of each compounds have been well described (Guo, *et al.,* 2011).

The DPPH scavenging activity assay was carried out to evaluate the ability of antioxidants to scavenge free radicals. In this assay the violet color of DPPH was changed to a pale yellow color because of the abstraction of hydrogen atom from the antioxidant compound. The more antioxidant compound in the extract, the more the DPPH reduction will occur. More complete reduction of DPPH is related to the high scavenging activity performed by particular compounds. The alkaloids possessed hydroxyl group and this indicated that the alkaloids have the potential to be good antioxidant agent. The alkaloids in Sophora species contain hydroxyl group such as hydroxymatrine and hydroxysophocarpine have been reported in the early study (Xiu, *et al.*, 2010). In this study, the scavenging activity of *Sophora alopecuroides* seed extracts was compared with ascorbic acid which is well known natural antioxidants. As shown in Figure 4.2, the scavenging activity of *Sophora alopecuroides* seed are almost 40 fold lower than that of ascorbic acid (IC_{50} = 3.69 ± 0.01 µg/ml). The IC_{50} value of ethanol

extract was found to be 155.33 ± 0.06 µg/ml and aqueous extract was 167.47 ± 0.03 µg/ml. The scavenging activity of *Sophora alopecuroides* seed increased with the increasing concentrations, and the both of the extracts shown almost the same activities. This may be due to the both of extracts contain similar compounds and its concentrations are almost the same.

The ferric reducing antioxidant power (FRAP) assay was carried out to evaluate the ability of *Sophora alopecuroides* seed extract to reduce the ferum ion in relation to its antioxidant activity. The ferric reducing activity of were analyzed based on the reduction of ferric-TPTZ (2,4,6-tri(2-pyridyl)-l,3,5-triazine) to blue ferrous-TPTZ. As shown in Figure 4.3, the extracts of *Sophora alopecuroides* seed gave significant reducing power activities when compared to standard ascorbic acid (IC_{50} = 5.37 ± 0.03 µg/ml) and its activities were increased with the increasing concentration of sample. The IC_{50} value found for ethanol extract was 9.71 ± 0.02 µg/ml and water extract was 10.10 ± 0.02 µg/ml respectively.

Enzyme reactions are typically studied under steady state conditions since under these conditions the reaction velocity remains constant. The crude extracts of *Sophora alopecuroides* seed were tested for inhibition of GPa under steady state conditions in a spectrophotometric assay that is detailed in Materials and Methods. Glycogen phosphorylase-a from rabbit muscle was used because of the expense of the human enzyme. Glucose 1-phosphate was the substrate used and it can be degraded by GPa to release phosphate, which has intensive green color when react with malachite green that absorbs light at 620 nm (Figure 4.4). Therefore, enzyme activity is directly proportional to the absorbance at 620 nm. The effect on GPa activity by any sample can easily be determined by comparing the absorbance at 620 nm to the control.

The assay was carried out at pH 7.2 because the optimal pH for GPa activity was between 6.8 and 8.2 (Loughlin, *et al.*, 2008). The enzyme and substrate were both dissolved in 50 mM Hepes buffer. The extracts were dissolved in 10% DMSO in 50 mM Hepes buffer. Appropriate controls and blanks were used. The control consisted of the solution that dissolved sample. Blanks were especially required because the extracts were colored. To account for this, the blanks contained buffer and extract. The extracts were tested for inhibition of GPa at different concentrations (2-32 mg/l). The results (Figure 4.4) show that aqueous extract exhibited little or no inhibition (< 20%) at the concentration tested and no more difference in inhibition has been observed. The standard drug, caffeine, gave 71.52% inhibition and its IC_{50} was 620.40 ± 0.01 µg/ml. The ethanol extract showed 77.52% inhibition at 32 mg/ml under the assay conditions and its IC_{50} was determined to be 581.61 ± 0.02 µg/ml. The ethanol extract of *Sophora alopecuroides* seed was the most active in inhibition of GPa, and such inhibitors are expected to be useful for treatment of diabetic. Therefore, it was selected for evaluation of its antihyperglycemic activity in diabetic rats.

In the present studies, acute toxicity test revealed that toxic reaction was found in non-diabetic rats when the ethanol extract of *Sophora alopecuroides* seed was administered to the rats at the dose of 5 mg/kg. The toxicity was observed by death of two female rats during 15 days of study period. This may be due to the possibility of low toxic alkaloid such as matrine (Dai, *et al.*, 2009) is present in *Sophora alopecuroides* seed (Table 4.5). No toxic sign such as restless, response to touch, fearfulness, urination, or death was found in male rats at any of the doses given to each rat until the end of study. This may be to the individual differences of rodents to the toxic nature. In OGTT, Glucose administration to non-diabetic rats fasted for 16 hours increased plasma glucose levels from 4.68 ± 0.19 to 8.15 ± 0.61 mmol at 30 minutes and returned to normal at 2 hours. Administration of *Sophora alopecuroides* seed

ethanol extract to glucose loaded non-diabetic rats at the dose of 500 mg/kg body weight showed significant decrease in plasma glucose levels at 30 min, and no significant differences was observed treatment with the extract at the dose of 250 mg/kg body weight compared to the normal group (Figure 4.5, $*P < 0.05$; control vs. SAS Ext 500 mg/kg). From this result it indicates that *Sophora alopecuroides* seed extract (500 mg/kg) can improve glucose tolerance in non-diabetic rats.

STZ causes hyperglycemia by selective destruction of β cells in the islets of Langerhans (Sancheti, *et al.*, 2010), and nicotinamide protects β cells caused by STZ toxicity (Huang, *et al.*, 2010). Rats were induced diabetic by injection of STZ and nicotinamide mimicking the picture of type 2 diabetes which is most common to humans (Huang, *et al.*, 2010). In this experiment, antihyperglycemic activity of the extract was evaluated streptozotocin (STZ)-nicotinamide induced diabetic rats. The diabetic rats were confirmed by the presence of high fasting plasma glucose level which is the characteristic features of diabetic mellitus. The results (Table 4.6) showed that fasting blood glucose levels were significantly increased in STZ treated group compared to the normal control group ($*P < 0.05$; diabetic control vs. normal control). This is due to the lack of insulin in STZ-nicotinamide induced rats by destructing the β cells which leads to hyperglycemia. Treatment of these diabetic rats with *Sophora alopecuroides* seed extract (500mg/kg) decreased fasting plasma glucose levels significantly ($*P < 0.05$) on the 28^{th} day by 43.7% compared to the 0^{th} day. Another group treated with SAS at the dose of 250 mg/kg body weight did not show significant ($P > 0.05$) glucose lowering effect at the end of experiment compared to the 0^{th} day, but it decreased glucose level on the 28^{th} day by 18.7%. Glucose lowering activity of the extract (250 mg/kg) was remained statistically significant compared to the diabetic control. From this result it indicates that SAS can decrease fasting plasma glucose level in a dose-dependent manner in STZ-nicotinamide induced diabetic rats. The diabetic rats treated

with standard drug glibenclamide showed significant reduction in fasting plasma glucose levels compared to diabetic control group (*$P < 0.05$; diabetic control vs. group III), and the same effect was observed SAS (500 mg/kg) treated group. It is well known that sulfonylurea causes hypoglycemia by increasing insulin secretion from pancreas (Del Prato and Pulizzi, 2006). The results in Table 4.6 showed that SAS extract in both of the doses (250 mg/kg and 500 mg/kg) produced hypoglycemia in diabetic rats. This strongly suggests that the possible mechanism by which the antihyperglycemic activity of *Sophora alopecuroides* seed due to its enhanced insulin secretion effect from pancreatic ß cells like sulfonylurea. The reducing effect of *Sophora alopecuroides* seed extract could also be due to the action of alkaloid presence in SAS. The mechanism of its action is still unknown.

Weight loss is one of the major complications in diabetes and it arises due to the impairment in insulin action caused by STZ toxicity (Sancheti, *et al.*, 2010). Due to this there is a decrease in the body weight of STZ induced diabetic animals. As shown in Figure 4.6, treatment with STZ caused a significant (*$P < 0.05$) weight loss on the 28th day compared with normal control. This may be due to the reduction of insulin release from pancreatic β cells which leads to hyperglycemia, as a result increase muscle wasting and loss of tissue protein in diabetic rats (Salahuddin and Jalalpure, 2010). In contract, rats in normal control group continued to gain weight (51.8%) during the 4-week of experimental period. Treatment with standard drug glibenclamide increased body weight by 6.1% in the first week, and declined again to nearly initial body weight in diabetic rats. Diabetic rats treated with SAS extract (250 mg/kg and 500 mg/kg) reduced body weight around 9% on the 28th day compared with their initial level. The levels of serum lipids are usually altered in diabetic mellitus (Shirwaikar, *et al.*, 2006). This was observed in diabetic rats in this study, where serum triglycerides and total cholesterol levels were significantly (*$P < 0.05$) elevated, and serum HDL cholesterol

levels were decreased 8.7% in comparison with normal control (Table 4.7). Treatment with *Sophora alopecuroides* seed extracts at the dose of 250 mg/kg and 500 mg/kg for 28 days to the diabetic rats significantly (*$P < 0.05$) decreased serum triglycerides and total cholesterol levels compared to the diabetic control. Treatment with reference drug glibenclamide significantly decreased triglyceride, but there was no significant decrease in serum total cholesterol levels compared to diabetic control. The serum HDL levels in both of the SAS and glibenclamide treated groups were restored to the control level (Table 4.7). These data consistent with previous report which was saying that administration of high-fat diet with powdered fruit of *Sophora species* significantly decreased body weight in non-diabetic mice, exhibited lowering triglyceride and cholesterol effects while at the same time increasing HDL cholesterol in hyperlipidemic and cholesterol-fed rats (Hyun, *et al.*, 2008; Park, *et al.*, 2009). In summary, the present study showed that the administration of *Sophora alopecuroides* seed ethanol extract reduced blood glucose levels, body weight gain, and improved serum lipid profiles in diabetic rats. These results provide evidence that *Sophora alopecuroides* seed and its constituents are useful for the control of body weight and preventing diabetes related metabolic diseases.

CHAPTER 6

CONCLUSION

Sophora alopecuroides seed (SAS) was extracted with chloroform, 95% ethanol and distilled water. All of the extracts showed alkaloid presence, and total alkaloid content was determined 7.56%.

Antioxidant activity of SAS extracts were evaluated by two in vitro bioassays: ferric reducing antioxidant power (FRAP) and scavenging of DPPH free radical assay. In FRAP assay, IC_{50} value of ethanol extract was 9.71μg/ml, and IC_{50} value of water extract was 10.10 μg/ml. Both of extracts showed less scavenging activity on DPPH free radical compared to standard ascorbic acid.

The crude ethanol and water extracts of *Sophora alopecuroides* seed were tested for inhibition of glycogen phosphorylase-a enzyme in an in vitro assay. The ethanol extract of *Sophora alopecuroides* seed showed the most potent inhibition of GPa enzyme and determined to have an IC_{50} = 581.61 μg/ml, however water extract was inactive.

Acute toxicity of SAS ethanol extract was tested at increasing dose level in non-diabetic rats and the toxic effects has been observed at a dose of 5 g/kg body weight.

The ethanol extract of *Sophora alopecuroides* seed significantly improved glucose tolerance at the dose of 500 mg/kg body weight and no significant difference was observed at the dose of 250 mg/kg body weight compared to control.

Treatments with ethanol extract of *Sophora alopecuroides* seed for 28 days significantly decreased plasma glucose levels at the dose of 500 mg/kg body weight and it is ineffective at the dose of 250 mg/kg body weight compared to 0 day. But glucose lowering activity of the extract (250 mg/kg) was remained significant compared to the

diabetic control on the 28th day. The results showed that SAS extract in both of the doses (250 mg/kg and 500 mg/kg) produced hypoglycemia in diabetic rats. This suggests that the possible mechanism by which the antihyperglycemic activity of *Sophora alopecuroides* seed depends on its enhanced insulin secretion effect from pancreatic β cells like sulfonylurea. It is generally believed that herbal drugs operated by a number of mechanisms to elicit their hypoglycemic effects. This experiment has shown that the crude extract of SAS possessed GPa enzyme inhibitor. Therefore, the antidiabetic effect of *Sophora alopecuroides* seed may have a multiply mechanism of action, combining the effects of sulfonylurea and GPa inhibitor.

Diabetic rats treated with SAS extract (250 mg/kg and 500 mg/kg) reduced body weight around 9% on the 28th day compared with their initial levels, significantly (*$P <$ 0.05) decreased serum triglycerides and total cholesterol levels, and the serum HDL levels were increased compared to the diabetic control.

The results obtained from this study provide scientific evidence which is supporting of the traditional use of *Sophora alopecuroides* seed as an antidiabetic remedy and its complications.

REFERENCES

Abbott, B., Hartwell, J., Leiter, J., Perdue, R. and Schepartz, S. (1966). Screening Data from the Cancer Chemotherapy National Service Center Screening Laboratories. XXXVIII. Plant Extracts. *Cancer Research, 26*(12 Part 2), 1461.

American Diabetes Association. (2004). Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care, 27*, S5-S10.

American Diabetes Association. (2010). Standards of Medical Care in Diabetes—2010. *Diabetes Care, 33*(Supplement 1), S11-S61. doi: 10.2337/dc10-S011

Barber, T. M., Begbie, H. and Levy, J. (2010). The incretin pathway as a new therapeutic target for obesity. *Maturitas, 67*(3), 197-202.

Barma, P., Bhattacharya, S., Bhattacharya, A., Kundu, R., Dasgupta, S., Biswas, A., Bhattacharya, S., Roy, S.S., Bhattacharya, S. (2009). Lipid induced overexpression of NF-[kappa]B in skeletal muscle cells is linked to insulin resistance. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1792*(3), 190-200.

Beard, E., Clark, M., Hurel, S. and Cooke, D. (2010). Do people with diabetes understand their clinical marker of long-term glycemic control (HbA1c levels) and does this predict diabetes self-care behaviours and HbA1c? *Patient Education and Counseling, 80*(2), 227-232.

Bennett, S. N. L., Campbell, A. D., Hancock, A., Johnstone, C., Kenny, P. W., Pickup, A., Plowright, A.T., Selmi, N., Simpson, L., Stocker, A., Whalley, D.P., Whittamore, P.R.O. (2010). Discovery of a series of indan carboxylic acid glycogen phosphorylase inhibitors. *Bioorganic & medicinal chemistry letters, 20*(12), 3511-3514.

Bertus, P., Szymoniak, J., Jeanneau, E., Docsa, T., Gergely, P., Praly, J.-P., Vidal, S. (2008). Synthesis of a C-glucosylated cyclopropylamide and evaluation as a glycogen phosphorylase inhibitor. *Bioorganic & medicinal chemistry letters, 18*(17), 4774-4778.

Borges de Melo, E., da Silveira Gomes, A. and Carvalho, I. (2006). [alpha]- and [beta]-Glucosidase inhibitors: chemical structure and biological activity. *Tetrahedron, 62*(44), 10277-10302.

Brown, J. B., Conner, C. and Nichols, G. A. (2010). Secondary failure of metformin monotherapy in clinical practice. *Diabetes Care, 33*(3), 501.

Cai, L., Wang, J., Li, Y., Sun, X., Wang, L., Zhou, Z., Kang, Y.J. (2005). Inhibition of superoxide generation and associated nitrosative damage is involved in metallothionein prevention of diabetic cardiomyopathy. *Diabetes, 54*(6), 1829.

Campbell, R. K. (2011). Clarifying the Role of Incretin-Based Therapies in the Treatment of Type 2 Diabetes Mellitus. *Clinical Therapeutics, 33*(5), 511-527.

Carolan, M., Steele, C. and Margetts, H. (2010). Knowledge of gestational diabetes among a multi-ethnic cohort in Australia. *Midwifery, 26*(6), 579-588.

Ceriello, A., Johns, D., Widel, M., Eckland, D. J., Gilmore, K. J. and Tan, M. H. (2005). Comparison of effect of pioglitazone with metformin or sulfonylurea (monotherapy and combination therapy) on postload glycemia and composite insulin sensitivity index during an oral glucose tolerance test in patients with type 2 diabetes. *Diabetes Care, 28*(2), 266.

Cheng, A. Y. Y. and Josse, R. G. (2004). Intestinal absorption inhibitors for type 2 diabetes mellitus: prevention and treatment. *Drug Discovery Today: Therapeutic Strategies, 1*(2), 201-206.

Cotelle, N. (2001). Role of flavonoids in oxidative stress. *Current topics in medicinal chemistry, 1*(6), 569-590.

Cyrino, F. Z. G. A., Bottino, D. A., Coelho, F. C., Ravel, D. and Bouskela, E. (2003). Effects of sulfonylureas on KATP channel-dependent vasodilation. *Journal of Diabetes and its Complications, 17*(2, Supplement 1), 6-10.

Dai, Z.-j., Gao, J., Ji, Z.-z., Wang, X.-j., Ren, H.-t., Liu, X.-x., Wu, W.-y., Kang, H.-f., Guan, H.-t. (2009). Matrine induces apoptosis in gastric carcinoma cells via alteration of Fas/FasL and activation of caspase-3. *Journal of Ethnopharmacology, 123*(1), 91-96.

Daisy, P., Jasmine, R., Ignacimuthu, S. and Murugan, E. (2009). A novel Steroid from Elephantopus scaber L. an Ethnomedicinal plant with antidiabetic activity. *Phytomedicine, 16*(2-3), 252-257.

Davis, S. N. (2008). The role of inhaled insulin in the treatment of type 2 diabetes. *Journal of Diabetes and its Complications, 22*(6), 420-429.

Dawson, R. M. C., Daphne C. Elliott, William H. Elliott and Kenneth M. Jones. (1986). pH, buffers, and physiological media *Data for biochemical research* (3rd ed., pp. 417-448). New York: Oxford University Press.

Del Prato, S. and Pulizzi, N. (2006). The place of sulfonylureas in the therapy for type 2 diabetes mellitus. *Metabolism, 55*(Supplement 1), S20-S27.

Derdau, V., Fey, T. and Atzrodt, J. (2010). Synthesis of isotopically labelled SGLT inhibitors and their metabolites. *Tetrahedron, 66*(7), 1472-1482.

Dieren, S. v., Beulens, J. W. J., van der Schouw, Y. T., Grobbee, D. E. and Neal, B. (2010). The global burden of diabetes and its complications: an emerging pandemic. *European Journal of Cardiovascular Prevention & Rehabilitation, 17*, S3.

Duttaroy, A., Voelker, F., Merriam, K., Zhang, X., Ren, X., Subramanian, K., Hughes, T.E., Burkey, B.F. (2011). The DPP-4 inhibitor vildagliptin increases pancreatic beta cell mass in neonatal rats. *European Journal of Pharmacology, 650*(2-3), 703-707.

Edeoga, H., Okwu, D. and Mbaebie, B. (2005). Phytochemical constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol, 4*(7), 685-688.

Ercan-Fang, N. and Nuttall, F. Q. (1997). The effect of caffeine and caffeine analogs on rat liver phosphorylase a activity. *Journal of Pharmacology and Experimental Therapeutics, 280*(3), 1312.

Ercan-Fang, N., Taylor, M. R., Treadway, J. L., Levy, C. B., Genereux, P. E., Gibbs, E. M., Rath, V.L., Kwon, Y., Gannon, M.C., Nuttall, F.Q. (2005). Endogenous effectors of human liver glycogen phosphorylase modulate effects of indole-site inhibitors. *American Journal of Physiology-Endocrinology And Metabolism, 289*(3), E366.

Fei, Y. P., Liu, Q., Liu, K., Yagasaki, K., Wu, E. and Zhang, G. (2009). Matrine suppresses breast cancer cell proliferation and invasion via VEGF-Akt-NF-<i>κ</i>B signaling. *Cytotechnology, 59*(3), 219-229.

Grisouard, J., Timper, K., Radimerski, T. M., Frey, D. M., Peterli, R., Kola, B., Korbonits, M., Herrmann, P., Krahenbuhl, S., Zulewski, H., Keller, U., Muller, B., Christ, C.M. (2010). Mechanisms of metformin action on glucose transport and metabolism in human adipocytes. *Biochemical Pharmacology, 80*(11), 1736-1745.

Guan, T., Qian, Y.-S., Huang, M.-H., Huang, L.-F., Tang, X.-Z., Li, Y.-M., Sun, H.-B. (2010). Neuroprotection of Maslinic Acid, a Novel Glycogen Phosphorylase Inhibitor, in Type 2 Diabetic Rats. *Chinese Journal of Natural Medicines, 8*(4), 293-297.

Gumieniczek, A., Hopkala, H., Rolinski, J. and Bojarska-Junak, A. (2005). Antioxidative and anti-inflammatory effects of repaglinide in plasma of diabetic animals. *Pharmacological Research, 52*(2), 162-166.

Guo, q. l., Dong, J., Wang, H., Hashi, Y. and Chen, S. (2011). Characterization of alkaloids in Sophora flavescens Ait. by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 54*(5), 1065-1072.

Gustavson, S. M., Dai, H., Preston, G. M., Somayaji, V., Hirshberg, B. and Calle, R. A. (2011). Effects of multiple doses of the DPP-IV inhibitor PF-734200 on the relationship between GLP-1 and glucose in subjects with type 2 diabetes mellitus. *Diabetes Research and Clinical Practice, 91*(2), e45-e49.

Hak, C., Yim, C.-H., Han, K. O., Yoon, H.-K., Han, I.-K., Kim, M.-Y., Yang, J.-H., Cho, N.-H. (2003). Gestational diabetes mellitus in Korea: prevalence and prediction of glucose intolerance at early postpartum. *Diabetes Research and Clinical Practice, 61*(2), 117-124.

Hampson, L. J., Arden, C., Agius, L., Ganotidis, M., Kosmopoulou, M. N., Tiraidis, C., Elemes, Y., Sakarellos, C., Leonidas, D.-D., Oikonomakos, N.-G. (2006). Bioactivity of glycogen phosphorylase inhibitors that bind to the purine nucleoside site. *Bioorganic & Medicinal Chemistry, 14*(23), 7835-7845.

Heptulla, R. A., Rodriguez, L. M., Bomgaars, L. and Haymond, M. W. (2005). The role of amylin and glucagon in the dampening of glycemic excursions in children with type 1 diabetes. *Diabetes, 54*(4), 1100.

Herlein, J. A., Fink, B. D. and Sivitz, W. I. (2010). Superoxide production by mitochondria of insulin-sensitive tissues: mechanistic differences and effect of early diabetes. *Metabolism, 59*(2), 247-257.

Holst, J. J., Deacon, C. F., Vilsbøll, T., Krarup, T. and Madsbad, S. (2008). Glucagon-like peptide-1, glucose homeostasis and diabetes. *Trends in Molecular Medicine, 14*(4), 161-168.

Hong, M. H., Lee, J. Y., Jung, H., Jin, D.-H., Go, H. Y., Kim, J. H., Jang, B.-H., Shin, Y.-C., Ko, S.-G. (2009). Sophora flavescens Aiton inhibits the production of pro-inflammatory cytokines through inhibition of the NF [kappa]B/I[kappa]B signal pathway in human mast cell line (HMC-1). *Toxicology in Vitro, 23*(2), 251-258.

Hou, X., Song, J., Li, X.-N., Zhang, L., Wang, X., Chen, L., Shen, Y.H. (2010). Metformin reduces intracellular reactive oxygen species levels by upregulating expression of the antioxidant thioredoxin via the AMPK-FOXO3 pathway. *Biochemical and Biophysical Research Communications, 396*(2), 199-205.

Huang, K.-H., Pai, M.-H., Wu, C.-H., Liu, J.-J. and Yeh, S.-L. (2010). Supplemental dietary arginine reduces renal RAGE expression and oxidative damage in rats with streptozotocin-induced type 2 diabetes. *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism, 5*(2), e77-e84.

Hyun, Y. K., Jeong, D. M., Jung, H. J., Jung, Y. J., Yokozawa, T. and Choi, J. S. (2008). Hypolipidemic effects of Sophora flavescens and its constituents in poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. *Biological & pharmaceutical bulletin, 31*(1), 73-78.

Irwin, N., Green, B. D., Parker, J. C., Gault, V. A., O'Harte, F. P. M. and Flatt, P. R. (2006). Biological activity and antidiabetic potential of synthetic fragment peptides of glucose-dependent insulinotropic polypeptide, GIP(1-16) and (Pro3)GIP(1-16). *Regulatory Peptides, 135*(1-2), 45-53.

Jang, I. C., Jo, E. K., Bae, M. S., Lee, H. J., Jeon, G. I., Park, E., Yuk, H.G., Ahn, G.H., Lee, S.C. (2010). Antioxidant and antigenotoxic activities of different parts of persimmon (Diospyros kaki cv. Fuyu) fruit. *J. Med. Plant Res, 4*(2), 155-160.

Jiang, G. and Zhang, B. B. (2003). Glucagon and regulation of glucose metabolism. *American Journal of Physiology-Endocrinology And Metabolism, 284*(4), E671.

Judy, W. V., Hari, S. P., Stogsdill, W., Judy, J. S., Naguib, Y. and Passwater, R. (2003). Antidiabetic activity of a standardized extract (Glucosol (TM)) from Lagerstroemia speciosa leaves in Type II diabetics:: A dose-dependence study. *Journal of Ethnopharmacology, 87*(1), 115-117.

Kendall, D. M., Cuddihy, R. M. and Bergenstal, R. M. (2009). Clinical Application of Incretin-Based Therapy: Therapeutic Potential, Patient Selection and Clinical Use. *The American Journal of Medicine, 122*(6, Supplement 1), S37-S50.

Kim, J., Ryu, Y., Kang, N., Lee, B., Heo, J., Jeong, I., Park, KH. (2006). Glycosidase inhibitory flavonoids from Sophora flavescens. *Biological & pharmaceutical bulletin, 29*(2), 302-305.

Lang, C., Schäfer, M., Varga, L., Zimmermann, A., Krähenbühl, S. and Krähenbühl, L. (2002). Hepatic and skeletal muscle glycogen metabolism in rats with short-term cholestasis. *Journal of Hepatology, 36*(1), 22-29.

Lena Galvez Ranilla, Young-In Kwon, Emmanouil Apostolidis and Kalidas Shetty. (2010). Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresource technology, 101*(12), 4676-4689.

Li, C., Gao, Y., Tian, J., Shen, J., Xing, Y. and Liu, Z. (2011). Sophocarpine administration preserves myocardial function from ischemia-reperfusion in rats via NF-[kappa]B inactivation. *Journal of Ethnopharmacology, 135*(3), 620-625.

Liepinsh, E., Skapare, E., Svalbe, B., Makrecka, M., Cirule, H. and Dambrova, M. (2011). Anti-diabetic effects of mildronate alone or in combination with metformin in obese Zucker rats. *European Journal of Pharmacology, 658*(2-3), 277-283.

Lin, B. J., Wu, H.-P., Huang, H. S., Huarng, J., Sison, A., bin Abdul Kadir, D. K., Cho, C.-G., Sridama, W. (2003). Efficacy and tolerability of acarbose in Asian patients with type 2 diabetes inadequately controlled with diet and sulfonylureas. *Journal of Diabetes and its Complications, 17*(4), 179-185.

Liu, G., Dong, J., Wang, H., Hashi, Y. and Chen, S. (2011). Characterization of alkaloids in Sophora flavescens Ait. by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 54*(5), 1065-1072.

Loughlin, W. A., Pierens, G. K., Petersson, M. J., Henderson, L. C. and Healy, P. C. (2008). Evaluation of novel Hyphodermin derivatives as Glycogen Phosphorylase a inhibitors. *Bioorganic & Medicinal Chemistry, 16*(11), 6172-6178.

Luley, C., Blaik, A., Reschke, K., Klose, S. and Westphal, S. (2011). Weight loss in obese patients with type 2 diabetes: Effects of telemonitoring plus a diet combination - The Active Body Control (ABC) Program. *Diabetes Research and Clinical Practice, 91*(3), 286-292.

Lupi, R. and Del Prato, S. (2008). [beta]-cell apoptosis in type 2 diabetes: quantitative and functional consequences. *Diabetes & Metabolism, 34*(Supplement 2), S56-S64.

Martin, W. H., Hoover, D. J., Armento, S. J., Stock, I. A., McPherson, R. K., Danley, D. E., *et al.* (1998). Discovery of a human liver glycogen phosphorylase inhibitor that lowers blood glucose in vivo. *Proceedings of the National Academy of Sciences of the United States of America, 95*(4), 1776.

Mayhew, M. S. (2010). Amylin and Incretin Enhancers for Diabetes Type 2. *The Journal for Nurse Practitioners, 6*(7), 551-552.

Meriden, T. (2004). Progress with thiazolidinediones in the management of type 2 diabetes mellitus. *Clinical Therapeutics, 26*(2), 177-190.

Meshkani, R. and Adeli, K. (2009). Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clinical Biochemistry, 42*(13-14), 1331-1346.

Mudaliar, S. and Henry, R. R. (2009). Incretin Therapies: Effects Beyond Glycemic Control. *European Journal of Internal Medicine, 20*(Supplement 2), S319-S328.

Mukherjee, P. K. and Wahile, A. (2006). Integrated approaches towards drug development from Ayurveda and other Indian system of medicines. *Journal of Ethnopharmacology, 103*(1), 25-35.

Munusamy, S. and MacMillan-Crow, L. A. (2009). Mitochondrial superoxide plays a crucial role in the development of mitochondrial dysfunction during high glucose exposure in rat renal proximal tubular cells. *Free Radical Biology and Medicine, 46*(8), 1149-1157.

Natarajan, B., Edavalath, M., Davies, J., Jenkins, L., Marshall-Richards, N., Evans, D., Price, D.E., Bain, S.C., Stephens, J.W. (2010). Clinical experience with exenatide in a routine secondary care diabetes clinic. *Primary Care Diabetes, 4*(1), 57-60.

Nauck, M. A. (2011). Incretin-Based Therapies for Type 2 Diabetes Mellitus: Properties, Functions, and Clinical Implications. *The American Journal of Medicine, 124*(1, Supplement 1), S3-S18.

Nielsen, L. L. (2005). Incretin mimetics and DPP-IV inhibitors for the treatment of type 2 diabetes. *Drug Discovery Today, 10*(10), 703-710.

Nishikawa, T., Kukidome, D., Sonoda, K., Fujisawa, K., Matsuhisa, T., Motoshima, H., Matsumura, T., Araki, E. (2007). Impact of mitochondrial ROS production on diabetic vascular complications. *Diabetes Research and Clinical Practice, 77*(3, Supplement 1), S41-S45.

Oh, K. Y., Lee, J. H., Curtis-Long, M. J., Cho, J. K., Kim, J. Y., Lee, W. S., Park, K.H. (2010). Glycosidase inhibitory phenolic compounds from the seed of Psoralea corylifolia. *Food Chemistry, 121*(4), 940-945.

Ohsumi, K., Matsueda, H., Hatanaka, T., Hirama, R., Umemura, T., Oonuki, A., Ishida, N., Kageyama, Y., Maezono, K., Kondo, N. (2003). Pyrazole-O-glucosides as novel Na+-glucose cotransporter (SGLT) inhibitors. *Bioorganic & medicinal chemistry letters, 13*(14), 2269-2272.

Oikonomakos, N. G., Schnier, J. B., Zographos, S. E., Skamnaki, V. T., Tsitsanou, K. E. and Johnson, L. N. (2000). Flavopiridol inhibits glycogen phosphorylase by binding at the inhibitor site. *Journal of Biological Chemistry, 275*(44), 34566.

Park, K. W., Lee, J.-E. and Park, K.-m. (2009). Diets containing Sophora japonica L. prevent weight gain in high-fat diet-induced obese mice. *Nutrition Research, 29*(11), 819-824.

Peters, A. (2010). Incretin-Based Therapies: Review of Current Clinical Trial Data. *The American Journal of Medicine, 123*(3, Supplement 1), S28-S37.

Pfeiffer-Guglielmi, B., Francke, M., Reichenbach, A. and Hamprecht, B. (2007). Glycogen phosphorylase isozymes and energy metabolism in the rat peripheral nervous system--An immunocytochemical study. *Brain Research, 1136*, 20-27.

Qiu, Y. Y., Ding, P. and Chen, D. (2004). Determination of quinolizidine alkaloids in Sophora medicinal plants by capillary electrophoresis. *Analytica Chimica Acta, 523*(1), 15-20.

Roh, S.-S., Kim, C. D., Lee, M.-H., Hwang, S.-L., Rang, M.-J. and Yoon, Y.-K. (2002). The hair growth promoting effect of Sophora flavescens extract and its molecular regulation. *Journal of Dermatological Science, 30*(1), 43-49.

Salahuddin, M. and Jalalpure, S. S. (2010). Antidiabetic activity of aqueous fruit extract of Cucumis trigonus Roxb. in streptozotocin-induced-diabetic rats. *Journal of Ethnopharmacology, 127*(2), 565-567.

Sancheti, S., Bafna, M. and Seo, S. Y. (2010). Antihyperglycemic, antihyperlipidemic, and antioxidant effects of Chaenomeles sinensis fruit extract in streptozotocin-induced diabetic rats. *European Food Research and Technology, 231*(3), 415-421.

Sato, S., Takeo, J., Aoyama, C. and Kawahara, H. (2007). Na+-Glucose cotransporter (SGLT) inhibitory flavonoids from the roots of Sophora flavescens. *Bioorganic & Medicinal Chemistry, 15*(10), 3445-3449.

Schnier, J. B., Nishi, K., Monks, A., Gorin, F. A. and Bradbury, E. M. (2003). Inhibition of glycogen phosphorylase (GP) by CP-91,149 induces growth inhibition correlating with brain GP expression. *Biochemical and Biophysical Research Communications, 309*(1), 126-134.

Sesti, G. (2006). Pathophysiology of insulin resistance. *Best Practice & Research Clinical Endocrinology & Metabolism, 20*(4), 665-679.

Shaw, J. E., Sicree, R. A. and Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice, 87*(1), 4-14.

Shi, L., Zhang, W., Zhou, Y.-Y., Zhang, Y.-N., Li, J.-Y., Hu, L.-H., Li, J. (2008). Corosolic acid stimulates glucose uptake via enhancing insulin receptor phosphorylation. *European Journal of Pharmacology, 584*(1), 21-29.

Shiba, Y., Yamasaki, Y., Kubota, M., Matsuhisa, M., Tomita, T., Nakahara, I., Morishima, t., Kawamori, R., Hori, M. (1998). Increased hepatic glucose production and decreased hepatic glucose uptake at the prediabetic phase in the Otsuka Long-Evans Tokushima Fatty rar model. *Metabolism, 47*(8), 908-914.

Shibata, K., Suzuki, S., Sato, J., Ohsawa, I., Goto, S., Iritani, I., Tokudome, S. (2005). Diagnostic accuracy of glycohemoglobin A1c (HbA1c) for postprandial hyperglycemia was equivalent to that of fasting blood glucose. *Journal of Clinical Epidemiology, 58*(10), 1052-1057.

Shirwaikar, A., Rajendran, K. and Barik, R. (2006). Effect of aqueous bark extract of Garuga pinnata Roxb. in streptozotocin-nicotinamide induced type-II diabetes mellitus. *Journal of Ethnopharmacology, 107*(2), 285-290.

Shokeen, P., Anand, P., Murali, Y. K. and Tandon, V. (2008). Antidiabetic activity of 50% ethanolic extract of Ricinus communis and its purified fractions. *Food and Chemical Toxicology, 46*(11), 3458-3466.

Smith, S. A. (2003). Central role of the adipocyte in the insulin-sensitising and cardiovascular risk modifying actions of the thiazolidinediones. *Biochimie, 85*(12), 1219-1230.

Svendsen, P. F., Nilas, L., Madsbad, S. and Holst, J. J. (2009). Incretin hormone secretion in women with polycystic ovary syndrome: roles of obesity, insulin sensitivity, and treatment with metformin. *Metabolism, 58*(5), 586-593.

Tai, Z., Cai, L., Dai, L., Dong, L., Wang, M., Yang, Y., Cao, Q., Ding, Z. (2011). Antioxidant activity and chemical constituents of edible flower of Sophora viciifolia. *Food Chemistry, 126*(4), 1648-1654.

Tay, T. L., Foo, J. P., Tan, E., Chen, R., Khoo, J., Soh, S. B., Au, V., Cho, L.W. (2011). HbA1c may not be a sensitive determinant of diabetic status in the elderly. *Diabetes Research and Clinical Practice, 92*(2), e31-e33.

Ten Brinke, R., Dekker, N., de Groot, M. and Ikkersheim, D. (2008). Lowering HbA1c in type 2 diabetics results in reduced risk of coronary heart disease and all-cause mortality. *Primary Care Diabetes, 2*(1), 45-49.

Thornberry, N. A. and Gallwitz, B. (2009). Mechanism of action of inhibitors of dipeptidyl-peptidase-4 (DPP-4). *Best Practice & Research Clinical Endocrinology & Metabolism, 23*(4), 479-486.

Toole, B. J. and Cohen, P. T. W. (2007). The skeletal muscle-specific glycogen-targeted protein phosphatase 1 plays a major role in the regulation of glycogen metabolism by adrenaline in vivo. *Cellular Signalling, 19*(5), 1044-1055.

United Nations. (2007). General Assembly, Resolution 61/225. *World Diabetes Day*.

Valdés, S., Botas, P., Delgado, E., Álvarez, F. and Díaz-Cadórniga, F. (2011). HbA1c in the prediction of type 2 diabetes compared with fasting and 2-h post-challenge plasma glucose: The Asturias study (1998-2005). *Diabetes & Metabolism, 37*(1), 27-32.

Van Gaal, L. F., Van Acker, K. L. and De Leeuw, I. H. (2001). Repaglinide improves blood glucose control in sulphonylurea-naive type 2 diabetes. *Diabetes Research and Clinical Practice, 53*(3), 141-148.

Venieratos, P. D., Drossopoulou, G. I., Kapodistria, K. D., Tsilibary, E. C. and Kitsiou, P. V. (2010). High glucose induces suppression of insulin signalling and apoptosis via upregulation of endogenous IL-1[beta] and suppressor of cytokine signalling-1 in mouse pancreatic beta cells. *Cellular Signalling, 22*(5), 791-800.

Verspohl, E. J. (2009). Novel therapeutics for type 2 diabetes: Incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors. *Pharmacology & Therapeutics, 124*(1), 113-138.

Wagner, H., Bladt, S. and Rickl, V. (2009). *Plant drug analysis: a thin layer chromatography atlas*: Springer Verlag.

Weiss, L., Zeira, M., Reich, S., Slavin, S., Raz, I., Mechoulam, R., Gallily, R. (2008). Cannabidiol arrests onset of autoimmune diabetes in NOD mice. *Neuropharmacology, 54*(1), 244-249.

Winkler, G., Cseh, K., Baranyi, É., Melczer, Z., Speer, G., Hajós, P., Salamon, f., Turi, Z., Kovacs, M., Vargha, P., Karadi, I. (2002). Tumor necrosis factor system in insulin resistance in gestational diabetes. *Diabetes Research and Clinical Practice, 56*(2), 93-99.

World Health Organization. (2006). Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. *World Health Organization: Geneva, Switzerland*.

World Health Organization. (2011). Fact sheet No 312. Retrieved

Xiu, J. l., Cao, M.-A., Li, W.-H., Shen, C.-S., Yan, S.-Q. and Yuan, C.-S. (2010). Alkaloids from Sophora flavescens Aition. *Fitoterapia, 81*(6), 524-527.

Yamada, K., Hosokawa, M., Fujimoto, S., Fujiwara, H., Fujita, Y., Harada, N., Yamada, C., Fukushima, M., Ueda, N., Kaneko, T., Matsuyama, F., Yamada, Y., Seino, Y., Inagaki, N. (2008). Effect of corosolic acid on gluconeogenesis in rat liver. *Diabetes Research and Clinical Practice, 80*(1), 48-55.

Yamaguchi, Y., Yamada, K., Yoshikawa, N., Nakamura, K., Haginaka, J. and Kunitomo, M. (2006). Corosolic acid prevents oxidative stress, inflammation and hypertension in SHR/NDmcr-cp rats, a model of metabolic syndrome. *Life Sciences, 79*(26), 2474-2479.

Yu, L., Zhao, M., Wang, J. s., Cui, C., Yang, B., Jiang, Y., Zhao, Q. (2008). Antioxidant, immunomodulatory and anti-breast cancer activities of phenolic extract from pine (Pinus massoniana Lamb) bark. *Innovative Food Science & Emerging Technologies, 9*(1), 122-128.

Yurgin, N., Secnik, K. and Lage, M. J. (2008). Obesity and the use of insulin: a study of patients with type 2 diabetes in the UK. *Journal of Diabetes and its Complications, 22*(4), 235-240.

Zerilli, T. and Pyon, E. Y. (2007). Sitagliptin phosphate: A DPP-4 inhibitor for the treatment of type 2 diabetes mellitus. *Clinical Therapeutics, 29*(12), 2614-2634.

Zhang, P., Zhang, X., Brown, J., Vistisen, D., Sicree, R., Shaw, J., Nichols, G. (2010). Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice, 87*(3), 293-301.

Zhao, Q., Zhao, Y. and Wang, K. (2006). Antinociceptive and free radical scavenging activities of alkaloids isolated from Lindera angustifolia Chen. *Journal of Ethnopharmacology, 106*(3), 408-413.

Zinman, B. (2011). Initial Combination Therapy for Type 2 Diabetes Mellitus: Is It Ready for Prime Time? *The American Journal of Medicine, 124*(1, Supplement 1), S19-S34.