CHARACTERIZATION OF ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF *Brucea javanica* (L.) Merr. SEEDS

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CHARACTERIZATION OF ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF *Brucea javanica* (L.) Merr. SEEDS

ABSTRACT

Brucea javanica (L.) Merr is a medicinal plant used for hyperlipidemia and diabetes in Malay traditional medicine. This study was designed to evaluate antidiabetic and antioxidant effects of *Brucea javanica* seed (BJS) *in vitro* and *in vivo* and also identify chemical compounds that could be responsible for its pharmacological activity. The results showed that the ethyl acetate fraction (EAF) was identified as a most active GPα inhibitor with IC_{50} = 0.75 mg/ml. The EAF effectively scavenged DPPH, NO and superoxide radicals and it reduced ferric ion to ferrous, but failed to trap this ion compared with other fractions in antioxidant assays. In animal study, the EAF exhibited antihyperglycemic effect without causing weight gain and showed strong hypolipidimic effect in diabetic rats. The EAF treatment to diabetic rats also significantly increased insulin level, markedly improved glycogen metabolism and glucose tolerance which validated its use as an antidiabetic agent in traditional medicine. It also enhanced antioxidant defense by suppressing oxidative stress and exerted anti-inflammatory effect by decreasing serum TNF α, IL-6 and IL-1β in diabetic rats compared with controls. Chromatographic separation of the EAF led to the isolation of seven known compounds; vanillic acid, bruceine D, bruceine E, *para*hydroxybenzoic acid, luteolin, protocatechuic acid and gallic acid. The GPα and α-glucosidase inhibition assays resulted in identification of luteolin as the most effective compound in BJS with the IC_{50} values of 45.1 and 26.4 µM. While vanillic acid, bruceine D and bruceine E were inactive, other compounds *para*hydroxybenzoic acid, protocatechuic acid and gallic acid showed weak inhibition of GPα (IC_{50} = 357.9, 297.4 and 214.4 µM) and α-glucosidase (IC50 = 649.1, 368.7 and 277.0 µM). Overall, the EAF reduced glycemia of T2D rats through diverse

mechanisms acting as GPα and α-glucosidase inhibitors and luteolin most probably is the major contributor to the observed antidiabetic effects.

Keywords: *Brucea javanica* (L.) Merr, T2D, GPα, hyperlipidemia, luteolin

PENCIRIAN AKTIVITI ANTIDIABETIK DAN ANTIOKSIDAN BIJI *Brucea javanica* (L.) Merr.

ABSTRAK

Brucea *javanica* (L.) Merr adalah tumbuhan ubatan yang digunakan dalam merawat hyperlipidemia dan diabetes dalam perubatan Melayu tradisional. Kajian ini dirangka untuk mengkaji kesan biji benih *Brucea javanica* (BJS) terhadap aktiviti antidiabetik dan antioksidan secara *in vitro* dan *in vivo*, serta mengenalpasti sebatian kimia yang mungkin berkaitan terhadap aktiviti farmakologi mereka. Hasil menunjukkan, fraksi etil esitat (EAF) telah dikenalpasti sebagai perencat GPα yang paling aktif dengan nilai IC_{50} = 0.75 mg/ml. EAF telah secara efektif menjerap DPPH, NO dan radikal-radikal superoksida serta menurunkan ion ferik kepada ferus, tetapi gagal untuk menjerap ion ini jika dibandingkan dengan fraksi lain dalam ujian antioksidan. Dalam kajian terhadap haiwan, EAF menunjukkan kesan antihiperglisemik tanpa menyebabkan pertambahan berat badan dan menunjukkan kesan hipolipidemik yang kuat terhadap tikus diabetik. Kajian EAF terhadap tikus diabetik menunjukkan peningkatan aras insulin yang signifikan, menambahbaik metabolisma glikogen dan toleransi glukosa yang jelas yang mana menyokong penggunaannya sebagai agen antidiabetik dalam perubatan tradisional. Ia juga membantu meningkatkan pertahanan antioksidan dengan menekan tekanan oksidatif dan terdapat kesan anti-inflamasi melalui penurunan aras serum TNFα, IL-6 dan IL-1β terdadap tikus diabetik berbanding tikus kawalan. Pemisahan kromatografi EAF membawa kepada pemencilan tujuh sebatian; asid vanilik, bruseina D, bruseina E, asid *para*hidroksibenzoik, luteolina, asid protokatekuik dan asid gallik. Keputusan ujian perencatan GPα dan α-glukosida telah mengenalpasti luteolina sebagai sebatian yang paling efektif didalam BJS dengan nilai IC_{50} sebanyak 45.1 and 26.4 µM. Sementara itu, asid vanilik, bruceina D dan bruceina E adalah tidak aktif, sebatian lain seperti asid *para*hidroksibenzoik, asid protokatekuik dan asid gallik menunjukkan perencatan yang

lemah terhadap GPα (IC_{50} = 357.9, 297.4 dan 214.4 µM) dan α-glukosida (IC_{50} = 649.1, 368.7 dan 277.0 µM). Keseluruhannya, EAF menurunkan aras glysemia tikus T2D melalui pelbagai mekanisma yang bertindak sebagai perencat GPα dan α-glukosida serta sebatian luteolina adalah paling berkemungkinan memainkan peranan utama dalam kesan antidiabetik yang ditemui.

Kata kunci: *Brucea javanica* (L.) Merr, T2D, GPα, hyperlipidemia, luteolin

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ADA:	American Diabetes Association
AGA:	advanced glycation end-product
ALP:	alkaline phosphatase
ALT:	alanine aminotransferase
AMP:	adenosine monophosphate
AMPK:	adenosine monophosphate protein kinase
AST:	aspartate aminotransferase
ATP:	adenosine tree phosphate
AUC:	area under the curve
BJS:	*Brucea javanica* seed
cm:	centimeter
CVD:	cardiovascular disease
DM:	diabetes mellitus
DPP:	dipeptidyl peptidase
EAF:	ethyl acetate fraction
ELISA:	enzyme-linked immunosorbent assay
EMA:	European Medicine Agency
ESI:	electrospray ionization
FDA:	food and drug administration
FFA:	free fatty acid
FPG:	fasting plasma glucose
g:	gram
G1P:	glucose-1 phosphate
G6P:	glucose-6 phosphate
GD:	gestational diabetes

GIP: glucose-dependent insulinotropic polypeptide

GLP: glucagon-like peptide

GP: glycogen phosphorylase

GSH: glutathione

HbA1c: glycosylated haemoglobin

HDL: high density lipoprotein

IC_{50}: half maximal inhibitory activity

IDF: International Diabetes Federation

IFG: impaired fasting glucose

IGT: impaired glucose tolerance

IL: interleukin

IRS: insulin receptor substrate

Kg: kilogram

L: liter

LDL: low density lipoprotein

M: molarity

MDA: malondialdehyde

mg: milligram

min: minute

ml: milliliter

mM: millimolar

NA: nicotinamide

NBT: nitro blue tetrazolium

NFkB: nuclear factor kB

NMR: nuclear magnetic resonance

NO: nitric oxide

OAT:	oral acute toxicity
^{o}C:	degree celsius
OGTT:	oral glucose tolerance test
PCT:	proximal convoluted tubule
PGM:	phosphoglucomutase
PKC:	protein kinase C
PPARγ:	peroximose-proliferator-activated receptor γ
PTP1B:	phosphotyrosine phosphatase 1B
ROS:	reactive oxygen species
SD:	Sprague Dawley
SGLT:	sodium glucose cotransporter
STZ:	streptozotocin
SUR:	sulfonylurea receptor
T1D:	type 1 diabetes
T2D:	type 2 diabetes
TC:	total cholesterol
TG:	triglyceride
TLC:	tin layer chromatography
TNFα:	tumor necrosis factor α
TZD:	thiazolidinedione
WHO:	the World Health Organization
µg:	microgram
µl:	microliter
% :	percentage
≤ :	less than or equal to
≥ :	more than or equal to

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CHAPTER 1 : INTRODUCTION

1.1 Overview

Diabetes mellitus (DM) is becoming a major public health concern and regarded as worldwide epidemic due to rapid rise in incidence and prevalence of diabetes globally (Guariguata *et al.*, 2014). It is estimated that nearly 415 million people afflicted in 2015 live with diabetes around the world and this number is predicted to rise to 642 million by 2040 (International Diabetes Federation, 2016). The World Health Organization (WHO) predicts that diabetes is a leading cause of mortality worldwide in the 21st century. In 2015 alone, it was estimated that around 5 million people died from diabetes and its complications. The main complications of diabetes are cardiovascular diseases (CVD) which include both micro and macrovascular diseases (Huynh *et al.*, 2014). The CVD is one of the greatest risk factors of mortality among the people who has diabetes and an estimated 52% of people died from cardiovascular disease have history of type 2 diabetes (The World Health Organization, 2016).

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by hyperglycemia as result of insulin resistance in peripheral tissues and defects in insulin secretion by pancreatic β-cells (Grundy, 2012). The T2D is the dominant type of diabetes which accounts for 90 ~ 95% of all forms of diabetes. The evidences showed that T2D is greatly associated with obesity and physical inactivity that have been shown to lead to insulin resistance (Lorenzo *et al.*, 2010). The clinical study showed that people can have prediabetes for several years, and 50% of people who has T2D are unaware of its conditions at the time of diagnosis, and the most of them are found to be insulin resistance (Grundy, 2012). In fact, obesity is believed to be major risk factors of insulin resistance and it is estimated that 80% of people with T2D are overweight (Triplitt *et al.*, 2006). The rise of this global phenomenon has been believed to be

associated with substantial changes in people's lifestyle that refers to the combined detrimental effects of reduced exercise and bad diet. The strict control of obesity and lifestyle modification such as proper diet and regular exercise has been shown to slightly delay the progression of disease at early stage and reduce the rate of the onset of both T2D and prediabetes (Van Gaal & Scheen, 2015). The lifestyle modification therapy has been initiated primarily by the WHO and the American Diabetes Association (ADA) as a viable approach to prevent the onset of diabetes at earliest stage and improve glycemic control. The scientific evidences showed that such method even though is useful for controlling hyperglycemia for some period of time, it unable to reduce diabetes-specific cardiovascular complications (The Look AHEAD Research Group, 2013). Therefore, pharmaceutical therapy and metabolic surgery are remained as an alternative therapeutic approach to control T2D and its complications.

The gastrointestinal operations termed as metabolic surgery have been suggested as treatments for T2D (Cummings, 2012). The clinical trials evidenced that metabolic surgery including pancreas and islet replacement therapy even though effectively control hyperglycemia and decrease the risk factors of diabetes-mediated cardiovascular complications in overweight or obese people with T2D, the function of β-cell is progressively declined within few years after surgery (American Diabetes Association, 2017). As a result, most diabetes people have to revert to pharmacological treatments using oral anti-diabetic drugs, insulin therapy, and or combination of both within years (Meier *et al.*, 2006). Monotherapy or combination therapies with oral antidiabetic drugs improve glycemic control and preserve β-cell function at the early-stage therapies, but unable to achieve glycemic goal in long-term and the progression of disease is worsening with time (Cahn & Cefalu, 2016).

Insulin resistance and β-cell dysfunction underline the disease. In the initial stage of insulin resistance, glucose homeostasis can be controlled through high amount of

insulin secretion by β-cells. Overt diabetes only occurs when β-cells can no longer compensate for insulin resistance (AbdulGhani *et al.*, 2006). It is reported that the most newly diagnosed patients with T2D have around 50% β-cell function due to reduction of β-cell mass. In this study, increased blood glucose levels in patients treated with diet alone or with intensive sulfonylurea therapy for 6 years, was associated with a reduction of insulin levels due to progressive β-cells dysfunction (The United Kingdom Prospective Diabetes Study Group, 1995). Therefore, the improvement of the decline in β-cell function and mass are critical in altering the progressive nature of the disease.

There is no effective cure for diabetes but the progression of the disease may be retarded considerably through diet control and regular physical activity (Van Gaal *et al.*, 2015). Present therapy of diabetes is aimed at maintaining strict glycemic control and while some diabetic patients may be managed by exercise and diet, but in the most cases, one or a combination of antihyperglycemic agents are required for efficiently control glycemia (Bianchi *et al.*, 2007). However, even with current available pharmacological therapy the disease progressively worsens with time. For these reason, the development of novel drug is actively pursued.

Plants have been used traditionally to treat diabetes for generations. *Brucea javanica* (L.) Merr. is a member of Simaroubaceae family in the plant kingdom. It consists of six species and mostly originates in tropical Africa and throughout Asia (Liu *et al.*, 2009). In Malaysian Peninsula, the seeds of this plant are used by indigenous people to treat lipid disorder and diabetes. It is reported that methanol extract of *Brucea javanica* seed (BJS) exerted hypoglycemic effect in non-diabetic mouse. The compounds (Bruceine D and E) isolated from BJS exhibited antidiabetic effect through a single dose administration in both non-diabetic mouse and diabetic rats (Noorshahida *et al.*, 2009). In another study, compounds bruceajavanone B, bruceine A, and bruceatin

isolated from BJS have been demonstrated to have the ability to inhibit nuclear factor-kappa B (NFkB) activation in colon cancer and leukemia cells in vitro (Kim *et al.*, 2010). Activation of NF_kB is known to induce β-cell apoptosis by generation of free radical superoxide and develop insulin resistance and cardiovascular complications in patients with T2D (Arkan *et al.*, 2005). More recent study (Yang *et al.*, 2013) has shown that the BJS have anti-inflammatory effects in nondiabetic mouse. In diabetes, inflammation has been shown to contribute β-cell dysfunction and insulin resistance through cytokine production (Yekollu *et al.*, 2011). Because of these beneficial effects of BJS, it was selected for further study.

1.2 Research Objectives

1. To extract, isolate and determine phytochemicals from *Brucea javanica* seed.
2. To determine antidiabetic and antioxidant activities of fractions of *B. javanica* seed in *in vitro* model.
3. To determine antidiabetic, anti-inflammatory and antioxidant activities of active fraction from *B. javanica* seed in nicotinamide-streptozotocin induced diabetic rats.
4. To determine antidiabetic effects of isolated compounds of *B. javanica* seed in *in vitro* model.

CHAPTER 2 : LITERATURE REVIEW

2.1 Diabetes

Diabetes mellitus (DM) is a chronic metabolic dysfunction characterized by hyperglycemia due to absolute loss of insulin production and/or insulin resistant and impaired insulin secretion by pancreatic β-cell (Fonseca, 2009). The prevalence of diabetes is on the rise in recent years and nearly 415 million people had diabetes in 2015 (International Diabetes Federation, 2016). Diabetes is a serious public health concern that resulting increased morbidity and mortality due to diabetes-mediated cardiovascular complications. In 2015, it was estimated that around 5 million people died from diabetes and its complications, the mostly due to cardiovascular diseases, making it the seventh leading cause of death in the world (The World Health Organization, 2016).

The T2D is the most predominant among all cases of diabetes and is commonly associated with obesity, over-nutrition and physical inactivity. The mechanisms whereby obesity contributed to the development of diabetes is incompletely understood but it was estimated that 80% of people who has diabetes are overweight (Triplitt *et al.*, 2006). The insulin resistance is most common factor associated with obesity and inflammation (Osborn & Olefsky, 2012). In terms of the total number of people afflicted globally, China, India, and United States (US) are the top three countries among the highest prevalence of diabetes in 2015. In China, 109.6 million people had diabetes in 2015 and this number is predicted to increase to 150.7 million by 2040. In India, 69.2 million had diabetes in 2015 increasing to 123.5 million by 2040. In the US, 29.3 million people had diabetes in 2015 and it is estimated to rise to 35.1 million by 2040 (International Diabetes Federation, 2015).

The T2D is a metabolic syndrome which refers to several metabolic disorders characterized by hyperglycemia, obesity, hypertension and dislipidemia. Most T2D with

metabolic syndrome have abdominal obesity, excess visceral fat and also are insulin-resistant (Després & Lemieux, 2006). It was found that the development of diabetes is 5-fold greater in people with metabolic syndrome compared with people without this syndrome. In fact, the metabolic syndrome can be regarded as a pre-diabetic stage and the most people with pre-diabetes have shown insulin resistance (Stern *et al.*, 2004).

Insulin resistance and insulin deficiency are the major metabolic defects that underline much of the pathology of diabetes. Insulin deficiency occur ether from a loss of β-cell number or impairment in β-cell function. In type 1 diabetes (T1D), the disease occurs when there is absolute loss of insulin in the body and commonly believed to be inherited genetically (Lewis *et al.*, 2002). In the primary stage of insulin resistance, glucose homeostasis can be maintained through higher insulin secretion by β-cells. The T2D become overt only when β-cells can no longer compensate for insulin resistance (Bluestone *et al.*, 2010). The etiologic process underlining insulin resistance and diabetes are still unknown and mostly genetic and environmental factors are thought to be main contributor of ultimate failure of β-cell function (Talchai *et al.*, 2009). It is reported that newly diagnosed people who has T2D have approximately 50% β-cell function and due in part a 30% reduction in β-cell mass. This study showed that an elevation of blood glucose levels in patients treated with diet alone or with sulfonylureas therapy for more than 6years, was associated with a decline in insulin levels due to progressive impairment in β-cell function (The United Kingdom Prospective Diabetes Study Group, 1995). Therefore, preservation of β-cell mass and amelioration of β-cell function could be promising therapeutic approach and critical in altering nature of the disease. Unfortunately, current available therapies for diabetes are only able to control the symptoms of the disease and there is no effective way to prevent decline in β-cell mass.

Aside from human pain and suffering, diabetes imposes huge economic burdens on national health systems and countries. It was estimated that global health expenditure on diabetes was between $ 673 billion and $1197 billion in 2015 and is predicted to exceed $802 billion to $1452 billion by 2040. In terms of total spending with the diabetes-related coast, the US, China and Germany are the top three countries in the world which accounts for 60% of the total global health expenditure in 2015. Total cost in 2015 in the US alone has been estimated at $320 billion and is estimated to rise to $349 billion in 2040. The China which is number one in prevalence of diabetes in the world has spent around $51 billion in 2015 and the coast is estimated to increase to $72 billion by 2040. In 2015, Germany, even not in the list of top 10 countries for number of people with diabetes has spent $35 billion and this number is expected to increase $36 billion by 2040. Meanwhile, India which is second highest number of people with diabetes spent less than 3% the total global health expenditure on diabetes in 2015 (International Diabetes Federation, 2015). Because of the enormous prevalence of the diabetes, cost-effective therapies will be required to treat people, especially those from developing countries that cannot afford precious medication. The solution to this problem is still remains complex and will need a novel and concerted global effort that combines modern medicine with alternative medicine used around the world.

There are three types of diabetes which include type 1, type 2 and gestational diabetes. Type 1 diabetes (T1D) is recognized as an autoimmune disease caused by a destruction of β-cells by its own antibodies, leading to absolute dependence on exogenous insulin. T1D occurs in 5~10% of all cases of diabetes mostly among children and young adults (Canivell & Gomis, 2014). Since insulin can no longer be produced, T1D patients are depends on daily insulin injection for preserving their life.

It is estimated that 90~95% people with diabetes are T2D diabetes and is usually caused by ether insulin resistance in adipose and muscle tissue and also in liver or

decreased insulin production by β-cells in pancreas or a combination of both (Ashok & Singh, 2018). In contrast to T1D, T2D diabetes has no dependency on exogenous insulin. However, it may require insulin for the control of fasting hyperglycemia if normal glycemic target cannot be achieved by the diet restriction, exercise or oral agents (Van Gaal *et al.*, 2015).

Gestational diabetes (GD) is obscure and a temporary condition that occurs in pregnancy. It became overt through prenatal screening by oral glucose tolerance test (OGTT) during pregnancy (Monteiro *et al.*, 2016). GD is largely associated with intake of high colory diets during pregnancy and is also believed to be inherited genetically. In most cases, conditions of gestational diabetes are disappearing after childbirth. But several studies (Agarwal *et al.*, 2010; Seino *et al.*, 2010) presented that women with GD are at high risk to develop T2D in long term and increase the risk of T2D in offspring.

2.1.1 Diagnosis

In normal condition, fasting plasma glucose (FPG) levels are strictly maintained within 5-6.7 mmol/L and most of the time it below than 5.6 mmol/L but in T2D the patients are unable to maintain blood glucose levels within this range. Diabetes is diagnosed by a FPG of ≥ 7.0 mmol/L or by 2-hour plasma glucose (2h-PG) in response to a 75 g oral glucose tolerance test (OGTT) of ≥ 11.1 mmol/L or by glycosylated haemoglobin (HbA_{1c}) of ≥ 6.5% (Kim *et al.*, 2017). In gestational diabetes, it is diagnosed by FPG between 5.1-6.9 mmol/L and by 2h-PG of 8.5-11.0 mmol/L during pregnancy. The onset of diabetes is begun by a prediabetes with FPG between 6.1-6.9 mmol/L defined as impaired fasting glucose (IFG) and by 2h-PG during OGTT of 7.8-11.1 mmol/L referred to as impaired glucose tolerance (IGT) (Benhalima *et al.*, 2015).

More recently, the American Diabetes Association (ADA) recognized people with HbA_{1c} levels between 5.7% and 6.4% as prediabetes (American Diabetes Association,

2014). Defining prediabetes based on HbA_{1c} levels are controversial but its use for diagnosis of diabetes is recognized by the WHO, the IDF, the ADA and European Association for the Study of Diabetes. Many studies presented that person with HbA1c levels 5.7% to 6.4% have insulin resistance and most individuals have one or more metabolic syndrome. People who have IFG and IGT plus metabolic syndrome are at greater risk to develop diabetes and its complications than those with only one of them. Many reports showed that HbA1c is directly proportional to blood glucose levels and is a most accurate indicator of overall glycemic exposure because it reflects plasma glucose levels over the period of 2 to 3 month. It has less biologic variability and predicts most likely risk factors for long-term complications (Carlos *et al.*, 2007; Ferrannini *et al.*, 2011).

2.1.2 Complications of Diabetes

Hyperglycemia primarily affects the heart, blood vessels, eyes, kidney, and nerves and can cause severe micro and macro vascular complications as well as abnormalities in lipid metabolisms (Born *et al.*, 2016). Microvascular complications refer to those affecting small blood vessels in the retina, renal glomerulus, and peripheral nerves and can lead to diabetes-specific retinopathy, nephropathy and neuropathy. Diabetic retinopathy is a leading cause of blindness and severe visual loss. Diabetic nephropathy can develop end-stage of renal failure and responsible for around 20% of deaths in diabetes. Diabetic neuropathy refers to a group of diabetes-specific nerve dysfunction that affects approximately 60% people in diabetes. As a result of nerve damage, it can cause tingling, pain, numbness, and weakness in the extremities and at the end most possibly amputation (Rask-Madsen & King, 2013). Macrovascular complications refer to diseases affecting large blood vessels that supply the heart, brain, and lower extremities and leading to cardiovascular complications such as atherosclerosis, heart failure, heart attack, stroke, myocardial infarction and limb amputation. Diabetic

dyslipidemia can cause atherosclerosis due to overproduction of triglycerides in the liver. In gestational diabetes, hyperglycemia can cause heart defects through oxidative stress and myocyte apoptosis in developing embryo (Brownlee, 2001). In addition to these complications diabetes-specific vascular complications are the major cause of morbidity and mortality that responsible for 50% of deaths in people with diabetes (The World Health Organization, 2016).

It was discovered that there are four major mechanisms by which hyperglycaemia cause diabetic complications. These mechanisms are: (i) increased polyol pathway flux; (ii) increased advanced glycation end-product (AGE) formation; (iii) activation of protein kinase C (PKC) isoforms; and (iv) increased hexosamine pathway flux (Brownlee, 2001). Diabetes cause cataract through accumulation of sorbitol on the lens of the retina. Sorbitol is a product formed through overexpression of aldose reductase by polyol pathway flux. Several studies demonstrated that sorbitol induce osmotic stress to microvascular cells and cause organ damage (Hamada *et al.*, 1991; Cardoso *et al.*, 2016).

Diabetes can induce apoptosis by modifying protein function and gene expression through generation of AGE precursors by AGE pathway. AGEs are by-products formed through non-enzymatic reaction between proteins and glucose initiated by intracellular hyperglycemia. Diabetes can cause vision loss and increased urinary albumin excretion through abnormal blood flow in the retina and renal glomerulus by PKC pathway (Ishii *et al.*, 1996; Koya *et al.*, 1997). Diabetes can cause insulin resistance trough influencing regulations of insulin gene transcription (Marshall *et al.*, 1991; Kajimoto & Kaneto, 2004) and induce hepatic steatosis, tissue damage, inflammation, and atherosclerosis (Garg & Misra, 2002; James *et al.*, 2002; Sage *et al.*, 2010) by hexosamine pathway flux.

In addition to these effects, a common effect of these four pathogenic mechanisms is that they rise superoxide generation by the mitochondrial electron-transport chain. Superoxide is a reactive oxygen species (ROS) that induce oxidative stress and decrease antioxidant defence systems, and subsequently cause β-cell dysfunction, defective insulin gene expression, and increased β-cell apoptosis. It is suggested that interrupting the overproduction of superoxide by the mitochondrial electron-transport chain would normalize the effects of four pathogenic pathways involved in the progression of diabetic complications (Brownlee, 2001). Therefore, this suggests that antioxidant therapy would provide novel mechanisms to treat diabetes and prevent the development of diabetes related complications.

2.1.3 Pathogenesis of Diabetes

The pathogenesis of diabetes is unclear, but in most cases genetic factors and immune systems coupling with other metabolic disorders are believed to be involved in pathogenic process of both types of diabetes. Genetic mutation and pathological activation of the immune systems are primarily considered as pathogenic factors in T1D (Quinn *et al.*, 1996; Weiss *et al.*, 2008), but in T2D, the pathology of diabetes mostly begins with insulin resistance at target organs such as liver, muscle and adipose tissues.

In general, normal circulation of blood glucose level and regulation of glucose metabolisms are controlled by the balanced interplay between insulin action and insulin secretion (Kahn *et al.*, 2006). When insulin action is decreased, the body usually compensates this resistance by increasing insulin secretion from pancreatic β-cells, but at the same time, concentration of blood glucose is increase slightly. This phenomenon is called hyperinsulinemia. This hyperinsulinemic state is only temporary and over time insulin secretion diminishes duo to progressive decline of β-cell function. The combined effects of insulin resistance and β-cell dysfunction result in a diminished capacity to

suppress hepatic glucose production as well as decrease uptake and utilization of glucose in skeletal muscle and adipose tissue (Lorenzo *et al.*, 2010).

Insulin resistance is a complex metabolic syndrome and is the consequence of a number of defects along with insulin signaling cascade. The most likely factors that cause insulin resistance include obesity, visceral fat (Tanaka *et al.*, 2016), inflammation, oxidative stress along with increased concentration of free fatty acids (FFAs), tumor necrosis factor α (TNFα), hormone resistin, nuclear factor kB (NFkB) and phosphotyrosine phosphatase 1B (PTP1B). In obese T2D, elevation of triglyceride in visceral adipose tissue decease the action of insulin which in turn leading to increased FFAs production (Barma *et al.*, 2009). Elevated FFAs cause insulin resistance by inhibiting glucose uptake and its oxidation in skeletal muscle. FFA's also stimulate hepatic gluconeogenesis. Both TNFα and hormone resistin are produced by adipose tissue at the highest amount in obese people with diabetes. TNFα impairs insulin action while resistin is known to antagonize the effects of insulin (Winkler *et al.*, 2002). PTP1B induce insulin resistance by inactivation of insulin receptor substrate (IRS) proteins. Glucose oxidation in β-cells and inflammation increase production of reactive oxygen species (ROS) and, In turn, produce proinflammatory cytokines IL-1β. The IL-1β causes insulin resistance by impairment of insulin action (Cavelti-Weder *et al.*, 2012). Both IL-1β and ROS increase NFkB activity and ultimately cause β-cell apoptosis (Stumvoll *et al.*, 2005).

Elevated hepatic glucose production and decreased utilization of glucose in T2D are attributed to both hepatic insulin resistance and increased glucagon levels (Shiba *et al.*, 1998). Pancreatic β-cells can compensate for this resistance by subsequently secreting more insulin. This resulting 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 as a result of β-cell apoptosis. High glucose and

FFA's contribute to β-cell dysfunction, in a condition called glucolipotoxicity. When insulin resistance can no longer be overcome transition to T2D occurs. Thus, β-cell dysfunction is a critical factor in the pathogenesis of T2D (Kahn *et al.*, 2006).

2.2 Regulation of Glucose Homeostasis

Glucose is produced intracellularly and extracellularly by several mechanisms and it was used as cellular energy for maintaining the life. It is also produced through degradation of carbohydrate by activity of multiple enzymes during meal and utilized by cells as energy. Glucose is stored as in the form of glycogen mainly in liver and in skeletal muscle and acts as intracellular glucose store and is released to the bloodstream when needed. Homeostasis of blood glucose is controlled by several hormones, the most importantly by insulin and glucagon (Campbell & Drucker, 2015). Insulin is released by pancreatic β-cells in response to high glucose and reduces glucose level by two general mechanisms. These two mechanisms are inhibition of hepatic glucose production by glycogenolysis and gluconeogenesis, and increase glucose uptake into adipocytes and muscle to store it as triglycerides and glycogen. Glucagon is a hormone secreted by pancreatic α-cells in response to hypoglycaemia and is responsible for rising blood glucose levels. It antagonizes the effects of insulin by increasing glycogenolysis and gluconeogenesis and also inhibiting glycogenesis and glycolysis in the liver (Zois *et al.*, 2014).

Other hormones such as amylin, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are also responsible for maintaining normal glucose levels. Amylin is released together with insulin from pancreatic β-cells and functions in reducing gastric emptying, which restrict glucose excursions following a meal (Heptulla *et al.*, 2005). GLP-1 and GIP are incretin hormones that secreted by intestinal L cells in response to food ingestion. Increatin hormones stimulate the

synthesis and secretion of insulin and inhibit glucagon release in glucose-dependent manner (Irwin *et al.*, 2006; Vella & Cobelli, 2015). Evidences presented that any defects in the secretion of these hormones contribute to pathogenesis of diabetes and its complications.

2.3 Current Antihyperglycemic Agents

The current management of T2D is aims to achieve effective glycemic control initially using lifestyle modifications followed by pharmacologic treatment and reduce the risk of diabetes-related complications in earliest stage. Maintaining fasting blood glucose levels in nearly normal range and HbA1c levels lower than 7% has been shown to decrease the risk of microvascular complications and is became an important therapeutic goal (Pawlyk *et al.*, 2014). Weight reduction and exercise followed by monotherapy with metformin has been shown to improve insulin action in insulin sensitive tissues and reduce hyperglycemia and microvascular complications, but target glycemic goal is still not achieved in the long term, and large numbers of people with diabetes remain in very poor glycemic control (Indelicato *et al.*, 2017). When initial treatment fails to achieve glycemic goal the use of additional antidiabetic agents become essential. A variety of oral hypoglycemic drugs are currently available for the treatment of T2D and they are generally be classifies as (i) insulin secretagogues, (ii) biguanides, (iii) thiazolidinediones, (iv) α-glucosidase inhibitors, (v) sodium glucose cotransporter 2 (SGLT2) inhibitors, (vi) incretin mimetics, (vii) dipeptidyl peptidase-4 (DPP-4) inhibitors, and (viii) glycogen phosphorylase α (GPα) inhibitors (Neumiller, 2014).

2.3.1 Insulin Secretagogues

The insulin secretagogues drugs include sulfonylureas and meglitinides and both promote insulin release from pancreatic β-cells by a common mechanism. Sulfonylureas and meglitinides stimulate insulin release by binding to the sulfonylurea receptor (SUR) on the surface of pancreatic β-cells by blocking ATP-depended potassium channels.

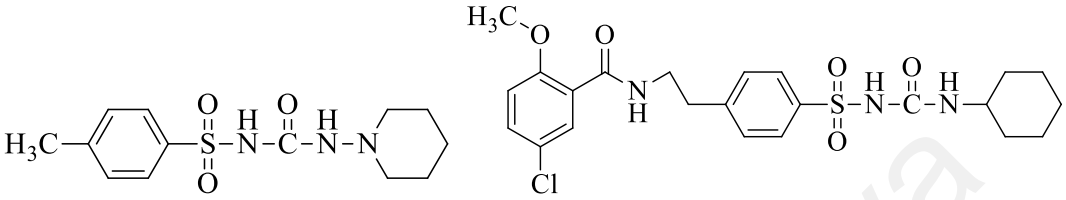
Meglitinides bind to a diffident site of sulfonylurea receptor as sulfonylureas do in β-cells and promote insulin secretion by blocking ATP-depended potassium channels (Proks *et al.*, 2002).

Sulfonylureas are the first available oral drugs for diabetes medication. Based on availability in this class, sulfonylurea drugs are further classified as first generation sulfonylureas (chlorpropamide, tolazamide, tolbutamide and acetohexamide) and second generation sulfonylureas (glibenclamide or glyburide, gliclazide, glipizide, and glimepiride). All sulfonylurea drugs have the same mechanism of action but second generation sulfonylureas are more potent and pharmacologically safer than first generation sulfonylureas (Bell, 2004).

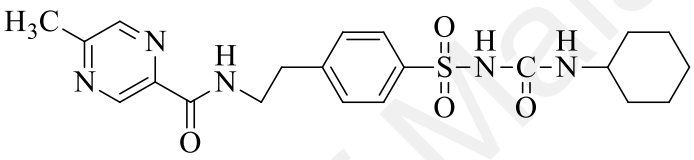
Meglitinides have efficacy similar to that of sulfonylureas, but they are distinguished from sulfonylureas by metabolic have life. The current available meglitinides include repaglinide and nateglinide. Both sulfonylureas and meglitinides release insulin from β-cell dependent of glucose concentrations, but meglitinides has little stimulatory effect on insulin secretion to that of sulfonylureas when treated in fasting state (Fonseca *et al.*, 2004). Therefore, comparing to sulfonylureas, meglitinides have probably a slight lower tendency to cause hypoglycemia.

Except this small effect, hypoglycemia is one of the most common side effects for both sulfonylurea and meglitinides and they are did not associated with decreasing microvascular or macrovascular complications and lipid levels. Another common side effect is weight gain (Del Prato & Pulizzi, 2006). This adverse effect is undesirable especially considering obese T2D patients. Despite these problems, sulfonylureas treatment is considered as one of the well-validated core therapies for T2D to maintain HbA1c levels below than 7%. Sulfonylurea can be used as monotherapy, combination with insulin and in combination with all other oral agents except meglitinides. Based on

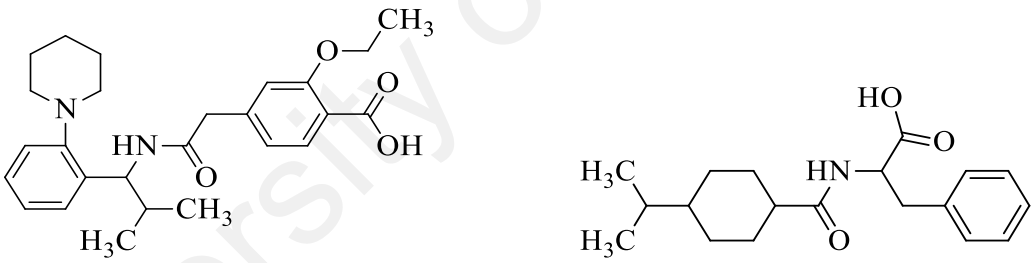
clinical data, monotherapy with a sulfonylurea for 10 years, they become gradually ineffective and most patients may require a second agent to maintain glycemic control (Turner *et al.*, 1999). Some examples of sulfonylureas are given in Figure 2.1, along with meglitinides drugs repaglinide and nateglinide.



Tolazamide Glibenclamide



Glipizide



Repaglinide Nateglinide

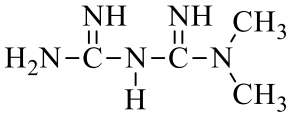
Figure 2.1: Structures of sulfonylureas and meglitinides

2.3.2 Biguanides

Biguanides include metformin and phenformin. Phenformin is the first discovered oral hypoglycemic agent among the biguanides, and it is discontinued later duo to its severe side effects. Metformin is the only available drug in this class (Pawlyk *et al.*, 2014). The mechanisms of action of metformin is not completely understood, but it is largely believed that metformin reduce blood glucose levels through inhibiting hepatic glucose output by gluconeogenesis. Metformin has been shown to decrease

intestinal glucose absorption, enhance insulin sensitivity, glucose uptake in skeletal muscle and adipose tissues. In contrast to sulfonylureas and meglitinides, metformin does not stimulate insulin release from pancreatic β-cells and does not cause hypoglycemia and hyperinsulinemia (Liepinsh *et al.*, 2011).

In addition, metformin treatment has been shown to increase insulin action and improve glucose tolerance in T2D patients who have insulin resistance and it also associated with decreasing lipid levels and macrovascular complications but does not affect microvascular complications (Nesti & Natali, 2017). Abdominal pain and diarrhea are the major side effects of metformin. Beside this, one of the important beneficial adverse effects is that it's associated with weight loss, and this makes it preferable to sulfonylureas to treat severely obese diabetes. Recently, metformin has been shown to increase concentrations of glucagon-like peptide-1 (GLP-1), a potent incretin hormone that inhibits glucagon secretion thus reduce blood glucose levels (Mannucci *et al.,* 2001). Metformin is presently a best frontline treatment option that may be used as alone or in combination with other agents.



Metformin

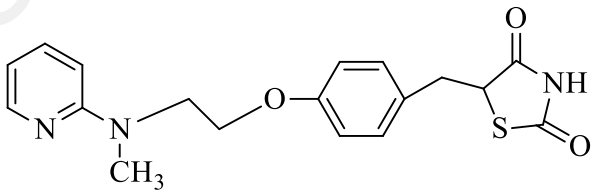
Figure 2.2: Structure of metformin

2.3.3 Thiazolidinediones

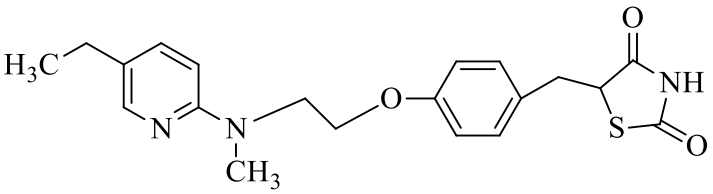
Thiazolidinediones (TZDs) are the ligands of peroximose-proliferator-activated receptor γ (PPARγ). These drugs increase insulin sensitivity in muscle, adipose tissue, fat, and liver to exogenous insulin by activating the nuclear PPARγ receptor which regulates lipid and glucose metabolisms through activation of specific genes (Kawai &

Rosen, 2010). TZD drugs include troglitazone, rosiglitazone and pioglitazone. TZD class of drugs decrease hyperglycemia by increasing insulin-stimulated glucose uptake in skeletal muscle and reduce insulin resistance. They inhibit hepatic glucose production when treated higher dose and do not stimulate β-cells to secrete insulin thus do not effect insulin levels. TZDs treatment protects β-cell survival by decreasing the levels of glucose and fatty acid that have detrimental effects on insulin secretions. TZDs also promote adipocyte differentiation and decrease lipolysis as a result cause weight gain as a main side effect (Pravenec *et al.*, 2008).

Troglitazone is the first oral agent among the TZDs approved for use the treatment of T2D, and discontinued later duo to its cause of severe hepatotoxicity. Rosiglitazone and pioglitazone in TZDs are currently available and used to counter the side effects of troglitazone. Combination product of rosiglitazone and metformin has been removed from the market duo to its associations with increased cardiovascular complications. TZDs are indicated as use for monotherapy and only pioglitazone is approved for use as combination with metformin, sulfonylurea and insulin (Inzucchi, 2002). The structure of rosiglitazone and pioglitazone are shown in Figure 2.3.



Rosiglitazone

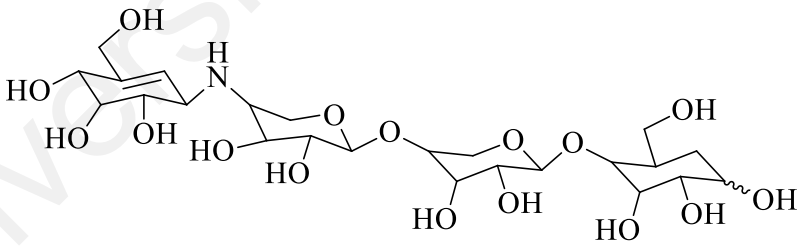


Pioglitazone

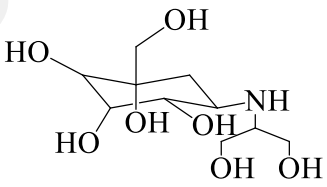
Figure 2.3: Structures of rosiglitazone and pioglitazone

2.3.4 α-Glucosidase Inhibitors

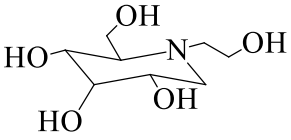
The α-glucosidase inhibitors inhibit the enzyme activities that responsible for breakdown polysaccharides and complex carbohydrates to glucose and other monosaccharides in the small intestine. They decrease hyperglycemia by preventing the digestion and delaying absorption of carbohydrates and increase postprandial glucose excursions (Casirola & Ferraris, 2006). The currently available α-glucosidase inhibitors include acarbose, voglibose and miglitol. These drugs are capable of limiting postprandial glucose levels without causing hypoglycemia and should be taken with food for optimal effect. It has been clinically observed that they have only modest antidiabetic efficacy if taken with meal. Therefore, α-glucosidase inhibitors are usually used in combination therapy. Adverse effects of α-glucosidase inhibitors include gastrointestinal discomfort such as flatulence, abdominal pain, and diarrhea (Borges de Melo *et al.,* 2006). The structure of α-glucosidase inhibitors acarbose, voglibose and miglitol, are shown in Figure 2.4.



Acarbose



Voglibose



Miglitol

Figure 2.4: Structures of acarbose, voglibose and miglitol

2.3.5 Sodium Glucose Cotransporter 2 (SGLT2) Inhibitors

Sodium glucose cotransporter 2 (SGLT2) inhibitors are a class of drugs that used for treatment of T2D. They selectively target a specific class of proteins called sodium glucose cotransporters (SGLTs) which is present in the renal proximal convoluted tubule (PCT) cells. The SGLTs are glycoproteins that have six isomers: SGLT1, SGLT2, SGLT3, SGLT4, SGLT5, and SGLT6 (Wright & Turk, 2004; Wright *et al.*, 2011). Two types of sodium glucose transporter proteins SGLT1 and SGLT2 are mainly expressed in the cell membrane of the PCT in the kidney and responsible for absorption of glucose. SGLT2 has been shown to have high capacity for glucose transport and low affinity for glucose where it is responsible for the reabsorption of 90% of the glucose while remaining 10% was reabsorbed by SGLT1 (Lee & Han, 2007). During blood circulation, glucose binds to glucose transporters where it pass through sodium gradient Na^+/K^+ ATPase channel in the membrane of the PTC and then it is transferred into the blood stream for the use of other cells as an energy sources (Mackenzie *et al.*, 1998). In people with diabetes, upregulation of SGLT2 leads to increase the rate of reabsorption of glucose in the kidney and progressively causing hyperglycemia (Rahmoune *et al.*, 2005). Therefore, it was believed that SGLT2 inhibition is crucial in glucose regulation.

Dapagliflozin is the first drug in the class of SGLT2 inhibitors and it was approved for clinical use by the European Medicine Agency (EMA) in 2012 and by the US food and drug administration (FDA) in 2014. Other drugs in this class include canagliflozin, empagliflozin, ipragliflozin, tofogliflozin and luseogliflozin. Most recently, remogliflozin atabonate was identifies as a selective SGLT2 inhibitor and is now in the late stage of clinical trial (Nakano *et al.*, 2015; Sykes *et al.*, 2015). Among the SGLT2 inhibitors, canagliflozin and empagliflozin including dapagliflozin are approved for use in Europe and the US market by both EMA and FDA while others are only available for use in Japan.

SGLT2 inhibitors reduce hyperglycemia by binding to the SGLT2 proteins in the membrane of renal PCT and causing the blockage of sodium glucose transport cycle (Vick *et al.*, 1973) and thus increase urinary glucose excretion. This class of drugs is able to decrease blood glucose levels without causing hypoglycemia and is not associated with the risk of cardiovascular complications. They do not stimulate insulin secretion from pancreatic β-cells (Nauck, 2014; Zinman *et al.*, 2015). The most possible side effects of SGLT2 inhibitors include urinary tract infection and weight loss due to high amount of glucose excretion in urine (Cefalu & Riddle, 2015). SGLT2 inhibitors are approved for use as monotherapy and in combination with insulin. Other possible combinations such as combination with sulfonylureas, metformin and with other classes are still in clinical trials and expected to be available for use soon. The structure of currently available SGLT2 inhibitors is shown in Figure 2.5.

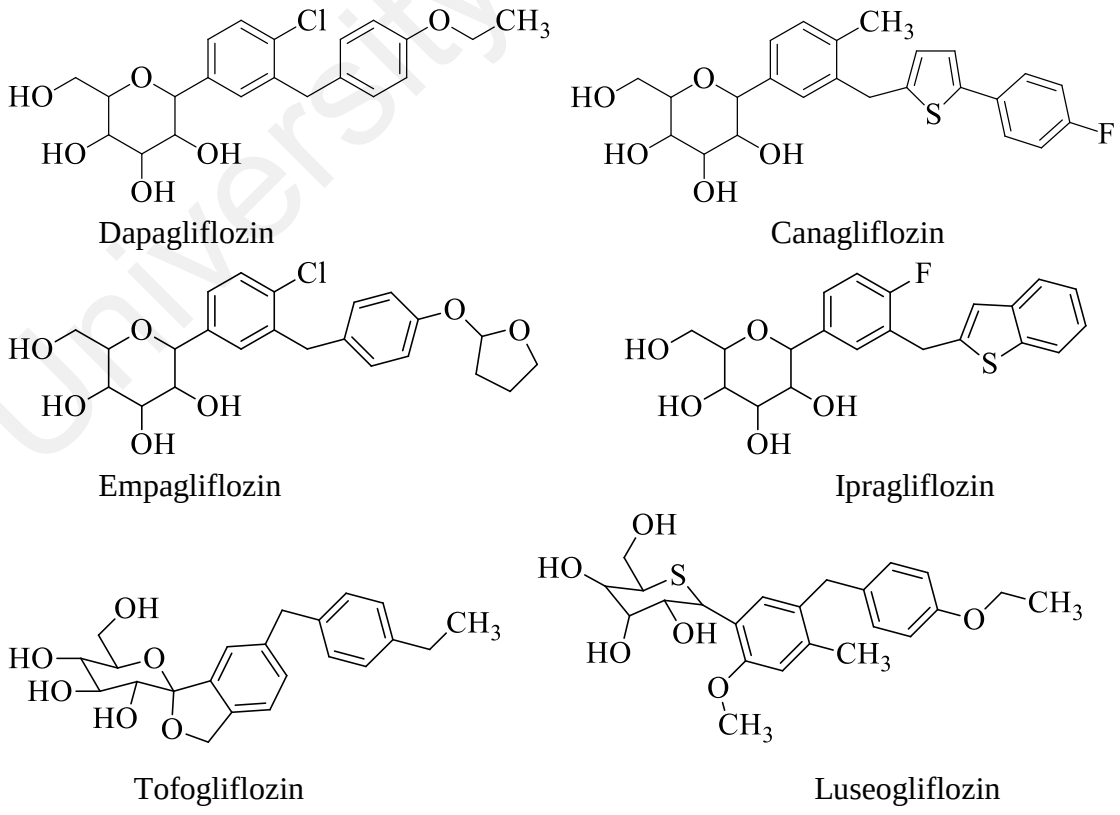


Figure 2.5: Structure of sodium glucose cotransporter inhibitors

2.3.6 Incretin Mimetics

Incretin is a gut derived hormone that released from intestinal cells into the bloodstream and regulates insulin secretion in response to meal ingestion. The role of intestinal factors in the regulation of insulin release was first proposed based on the observation that the amount of insulin released following an oral glucose dose exceeded that of a same dose administered intravenously (Mcintyre *et al.*, 1964). In diabetic and non-diabetic subjects, insulin concentrations in the blood following an intravenous glucose dose were found around 30% of that after the same dose administered orally (Godinho *et al.*, 2015). This phenomenon was called the "incretin effect" and was subsequently found to be primarily due to two incretin hormones, GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulinotropic polypeptide). Both GLP-1 and GIP are secreted by intestinal cells in response to orally ingested food and stimulate insulin secretion in a glucose dependent fashion. They are responsible for up to 70% the insulin secreted in response to glucose load (Ahren, 2009).

The incretin effect provides a rapid mechanism for glucose homeostasis by correlating immediate release of insulin with ingestion of meal. GLP-1 acts as specific G-protein-coupled receptors located not only on the pancreas, but also the brain, heart, stomach, intestine, lung and kidney (Verspohl, 2009). The incretin hormone GLP-1 therapy has been shown to rapid decrease hyperglycemia by stimulating insulin secretion through activation of the guanine nucleotide-binding protein (G protein)-coupled GLP-1 receptors in pancreatic β-cells and inhibiting glucagon secretion and decreasing appetite in patients with type 2 diabetes (Ahren, 2009). Other beneficial effects of GLP-1 therapy are that it stimulates proinsulin production, inhibit β-cell apoptosis caused by glucotoxicity, promote β-cell proliferation, and improve β-cell function by increasing β-cell mass in a few days (Drucker & Nauck, 2006). GLP-1 has been shown to have short half-life (1~2 min) due to its rapid degradation by the enzyme

DPP-4 and this limits the use of native GLP-1 as therapeutic agent. In order to overcome this problem, the GLP-1 receptor agonists or incretin mimetics that have strong affinity to the receptors and highly resistant to inactivation by DPP-4 has been developed. Incretin mimetics are functional analogous of the human incretin GLP-1 that capable of inhibit degradation by the action of DPP-4 and mimic all features of antihyperglycemic activity of native GLP-1. They reduce hyperglycemia by activation of GLP-1 receptors thus leading to increase insulin secretion, inhibit glucagon release and improve β-cell function (Barber *et al.*, 2010).

Several Incretin mimetic drugs have been developed by various pharmaceutical companies and examined in clinical trials. These classes of drugs are currently represented by exenatide, liraglutide, lixisenatide, albiglutide and dulaglutide and are approved for clinical use by both FDA and EMA. Based on clinical data, Incretin mimetics therapy has been shown to decrease overall HbA1c levels between 0.8% and 1.8% without causing hypoglycemia in people with T2D. Weight loss has been found to be a common side effect for incretin mimetic drugs due to its association with gastric appetying and reduced appetite and food intake. In addition to this, other main side effects are nausea and diarrhea (Ahren, 2009). Incretin mimetics can be used as monotherapy and in combination with other existing agents. Combination therapy with sulfonylureas would not be expected to be useful since they both function by stimulating insulin secretion from pancreatic β-cells. Another alternative strategy to enhance incretin effects is to inhibit DPP-4 activity to prevent degradation and inactivation of GLP-1 (Nauck & Smith, 2009).

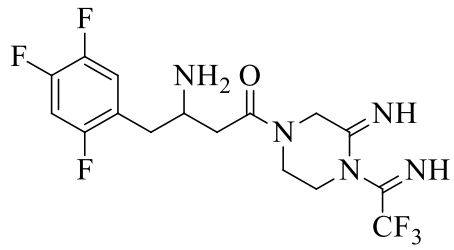
2.3.7 Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

DPP-4 is a ubiquitous enzyme that exists as both a membrane bound and plasma soluble form. It is widely expressed in many tissues with highest levels found in kidney, lung, small intestine, liver, pancreas, placenta, spleen, lymphocytes and endothelial cells

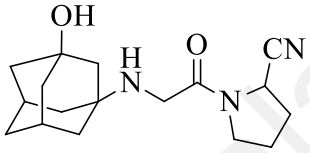
(Mentlein, 1999; De Meester *et al.*, 2004). DPP-4 is a serine peptidase that selectively degrades a number of biologically active peptides including GLP-1 and GIP with proline or alanine residue in position 2 of the aminoterminal of the peptide chain. Newly secreted active GLP-1 (7-36 amide) is extensively metabolized by DPP-4 to yield a dipeptide (His-Ala) and inactive form of GLP-1 (9-36 amide) and this metabolite has no insulinotropic effect (Thornberry & Gallwitz, 2009). To enhance incretin effects, selective inhibition of this enzyme is crucial and it was thought that this provides an alternative therapeutic option in the treatment of diabetes. Sitagliptin and vildagliptin are the first identified DPP-4 inhibitor class of drugs and are available for clinical use in many countries. Other DPP-4 inhibitors identified to date and approved for clinical use include saxagliptin, linagliptin and alogliptin (Thornberry *et al.*, 2009) and structures of these DPP-4 inhibitors are shown in Figure 2. 6.

The use of DPP-4 inhibitors have been shown to decrease hyperglycemia by preventing inactivation of active GLP-1 thus stimulate insulin secretion in response to glycemic rise, inhibit glucagon secretion and β-cell apoptosis, and improve β-cell function through stimulation of cell proliferation (Drucker *et al.*, 2006). Clinical studies indicated that these drugs can be used safely as a monotherapy and in combination with other oral antidiabetic agents in patients with T2D that unable to control blood glucose at their desired level. Monotherapy with sitagliptin and vildagliptin or their combination therapies with other oral agents such as metformin, thiazolidinediones, sulfonylureas and sulfonylureas plus metformin have been shown to decrease hyperglycemia and HbA1c levels by increasing insulin secretion without causing hypoglycemia and decrease glucagon secretion following meal ingestion, and preserve β-cell function in people with T2D (Verspohl, 2009; Karagiannis *et al.*, 2014). DPP-4 inhibitors have a number of distinct advantages over current antihyperglycemic agents. Since they function by enhancing incretin effect, they are capable of stimulating insulin secretion

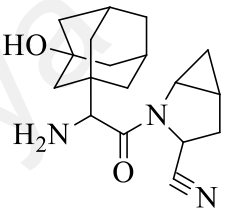
without causing hypoglycemia and inhibiting glucagon secretion though the action of native GLP-1. In addition, they have neutral effect on body weight and the potential to preserve β-cell mass via cell proliferation. Antihyperglycemic agents that are capable of restoring β-cell mass are highly desirable. Because of this effect, the search for novel DPP-4 inhibitors is an active area of research (Nauck Michael *et al.*, 2009; Ruscica *et al.*, 2017)



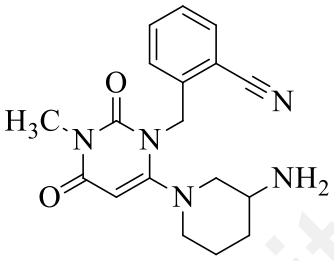
Sitagliptin



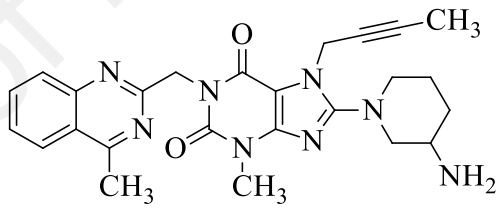
Vildagliptin



Saxagliptin



Alogliptin



Linagliptin

Figure 2.6: Structure of DPP-4 inhibitors

Monotherapy with DPP-4 inhibitors improved glycemic control and reduced HbA1c levels by 0.65-1.1% within 12 weeks and this effects is remained up to two years of duration (Pratley *et al.*, 2007; Debora *et al.*, 2008). They showed similar effect on HbA1c levels when comparison with sulfonylurea (Nauck *et al.*, 2007), metformin (Schweizer *et al.*, 2007), and TZDs (Rosenstock *et al.*, 2007) monotherapy. Monotherapy with either a sulfonylurea or metformin for 3 years, approximately 50% of patients have HbA1c above 7% and this number increases to approximately 75% after 9 years (Turner *et al.*, 1999; Brown *et al.*, 2010). When glycemic control does not achieved by monotherapy, 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. More recent combination therapy with metformin and DPP-4 inhibitors are even more attractive due to its rapid action and weight neutral effect and its association with low risk of hypoglycemia (Ahrén, 2009). Clinical studies to date showed that these combinations are able to reduce glucose level nearly normal range in short term, overall efficacies are similar as monotherapy of these agents and the risk of progression of cardiovascular complications are not achieved in long term study. In the case when two agents are no longer effective a third agent of another class might also be added. Combination therapy with more than two drugs reduced HbA1c levels lower than 7%, but resulted in more gain weight than that of patients with high glucose levels (Thornberry *et al.*, 2009). Therefore, this combination is unfavorable especially considering people who overweight in T2D therapy.

These currently available hypoglycemic agents are useful in limiting hyperglycemia ether in monotherapy or in combination with other hypoglycemic agents in some period of time but they do not reduce the progression of cardiovascular risk (Inzucchi, 2002). Therapies that can maintain blood glucose levels in normal range and minimize cardiovascular complications to its harmless level would represent a major advance. While a cure for diabetes is not currently available and both mono and combination therapy are fail in long term study, research that led to a greater understanding of the etiology of the disease, discover novel targets and identify hypoglycemic agents that have strong potency, less side effects and possible use in long term are urgent demand (DeFronzo *et al.,* 2013). GPα inhibition therapy most likely represents such a target and has already been successful in vivo diabetic animal model (Nacide *et al.,* 2005). GPα inhibitors inactivate the GPα by binding to its catalytic site thus inhibit hepatic glucose output from glycogen (Goyard *et al.*, 2016). Hepatic

glucose output is elevated in T2D patients and several studies evidenced that both glycogenolysis and gluconeogenesis is a major contributor to the abnormal hepatic glucose production by the liver. GPα is the only enzyme that well-validated today to catalyzes these processes and resulting in hyperglycemia (Treadway *et al.*, 2001; Yoon *et al.*, 2001). Therefore, inhibition of this enzyme is a promising therapeutic strategy for glycemic control and reducing its complications in T2D.

2.4 GPα: General Information and Function

Glycogen phosphorylase (GP) is an enzyme with a molecular weight of approximately 97 kDa per subunit. It is found in large number of organism including yeast, bacteria, fungi, plants, animals and mammalian tissues such as liver, muscle and brain. It has three isoforms encoded by 3 genes PYGL, PYGM, PYGB which consists of 846, 841 and 862 amino acid residues expressed in liver, muscle and brain. Amino acid sequence of GP is almost similar in humans and animals where 97% sequence homology was found between human and animal isoforms. There is 80% sequence identity between liver and muscle isoforms while brain share 83% identity with liver isoform (Newgard *et al.*, 1989; Agius, 2015). GP was first discovered from a rabbit skeletal muscle homogenate and shown to be exhibiting its regulatory properties by activation of adenosine monophosphate (AMP) protein kinase (AMPK) (Newgard *et al.*, 1989). It was subsequently purified from rabbit skeletal muscle and later from human liver. GP exists in two distinct forms α and b. The proportion that exists in the form α (GPα) and b (GPb) is controlled by phosphorylation. GPb is the less active form of GP and it is converted to more active form GPα by the action of phosphorylase kinase through phosphorylation at the position of serine-14 in its amino-terminus (Newgard *et al.*, 1989). The unphosphorylated enzyme, GPb, exhibits low activity and has less binding affinity for substrate while the phosphorylated enzyme, GPα, exhibits high activity and has greater binding affinity for substrate. The GPα is subsequently

dephosphorylated by the action of protein phosphatase 1 (PP1) whereby GPα is converted to its less active GPb form. The rate of conversion from GPb to GPα is mostly depends on the activation of AMP. Other factors including hormones such as glucagon that rise AMP concentration, inorganic phosphate and calcium also increase the rate of conversion from GPb to GPα (Stalmans & Hers, 1975; Agius, 2015).

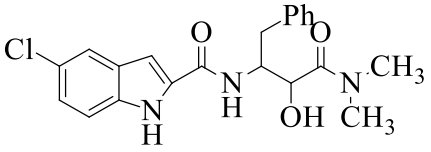
GPα is one of the major enzymes that directly involved in glycogen metabolism. It catalyzes α-1,4 glycosidic bond in glycogen by phosphorylation to yield glucose-1 phosphate (G1P) and free glucose where glycogenolysis take place. G1P is further converted to glucose-6 phosphate (G6P) by the action of phosphoglucomutase (PGM) for further metabolism and this conversion has three possible fates. (i) G1P can be metabolized through glycolytic pathway to produce pyruvate for glycolysis and generate ATP. (ii) It can be oxidized in pentose phosphate pathway and used as energy in anabolic reactions and maintains the antioxidant defences of the cells or (iii) it can be catalyzed by glucose-6-phosphatase and converted to free glucose in the liver by gluconeogenesis, and released into the bloodstream. Glycogen is synthesized again by several mechanisms in response to high glucose after meal and stored again in the liver. In diabetes, both glycogenolysis and gluconeogenesis is stimulated during fasting or starvation thus lead to increase hepatic glucose output even when blood glucose concentrations are high (Yoon *et al.*, 2001; Zois *et al.*, 2014). Therefore, inhibition of this enzyme is crucial in diabetes therapy.

2. 5 GPα Inhibition in Diabetes

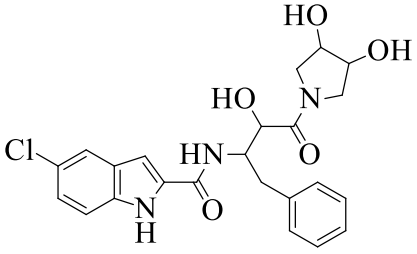
There are 4 main reasons why GPα is important in diabetes therapy (i) GPα is the first discovered enzyme that its biological activity is controlled by both allosterically and phosphorylation. (ii) Glycogen is vulnerable to rapidly degradation by GPα during prolonged fasting and starvation for providing energy for survival. (iii) GPα is the only enzyme that catalyzes the rate-limiting step in hepatic glycogenolysis and

gluconeogenesis to produce free glucose in glycogen metabolism and (iv) Glycogen is the key contributor to hepatic glucose output in diabetes (Cori & Green, 1943; Consoli *et al.,* 1989). The discovery of the primary role of GPα in glycogen metabolism led to suggest that inhibition of this enzyme may be useful in the treatment of diabetes through reduction of hepatic glucose production.

This approach was first validated in mice and in primary human and rat hepatocytes with the use of selective GPα inhibitor, CP-91149 (Figure 2.7), which exerted 20-fold high GPα inhibition potency in the presence of high glucose and resulted in rapid reduction of glucose levels within three hours without causing hypoglycemia in obese diabetic mice (Hampson & Agius, 2005). This was in direct correlation to the 3-fold increase in levels of hepatic glycogen in obese diabetic mice treated with the selective inhibitor CP-91149. Increased inhibition (80%) of glycogen breakdown by hormone stimulated glycogenolysis was also observed in primary human hepatocytes treated with this compound. Conversion of lactate to glycogen was also increased by 3-fold in rat hepatocytes treated with CP-91149. In this study, glucose lowering effect was directly related to increased levels of glycogen due to inhibition of GPα by CP-91149 (Martin *et al.*, 1998). Therefore, these studies seem to validate the notion that inhibition of GPα is a viable way of reduce hepatic glucose output and improve effective management of diabetes.



CP-91149



Ingliforib

Figure 2.7: Structure of GPα inhibitors CP-91149 and ingliforib

The evidences for the feasibility of GPα inhibition in diabetes were proved by numbers of studies using plant based extracts, isolated compounds from the plants and as well as synthetic compounds in both animal experiments and human clinical trials. Several studies reported that Corosolic acid reduced glucose AUC in a 2 hour OGTT (Fukushima *et al.*, 2006), and significantly reduced blood glucose levels in T2D patents treated with Corosolic acid for two weeks (Judy *et al.*, 2003). Another study reported that maslinic acid reduced fasting blood glucose levels and insulin resistance while significantly increased glycogen contents in genetic-type diabetic mice (Liu *et al.*, 2007). It also significantly improved antioxidant defence by suppressing oxidative stress (Teng *et al.*, 2010), and significantly reduced insulin resistance by blocking NFkB pathway in obese T2D mice (Jun *et al.*, 2014). Ingliforib prevented cardiac injury and significantly reduced fasting blood glucose levels in type 2 diabetic rabbits (Tracey *et al.*, 2004). These provide evidences for the effectiveness of GPα inhibitors therapy in diabetes. GPα inhibitors have been shown to reduce hyperglycemia without causing hypoglycemia. They have been shown to inhibit hepatic glucose output in the presence of high glucose levels (Iliana *et al.*, 2015). There are also evidences that GPα inhibitors have cardioprotective and antioxidant effects and this makes more attractive for the treatment of T2D diabetes who suffering from cardiovascular complications (Tracey *et al.*, 2004). Hypoglycemic agents that capable of reducing cardiovascular complications by increasing antioxidant defence and restoring β-cell mass are highly desirable. Because of these effects, the search for novel GPα inhibitors is an active area of research.

2.6 Traditional Medicine

In many parts of the world, plants are still used as the main source of treatment for various diseases. According to the WHO estimation, around 80% of the population in developing countries depends on traditional medicine for their primary health care

needs (Mukherjee & Wahile, 2006). The utilization of plants in the treatment of diabetes has a long and rich history. The Ebers Papyrus in ancient Egypt was written around 1550 B.C. and describes the earliest example of such use. Ayurveda in India, written in 4-5th century B.C, and Bencao Gangmu (The Compendium of Materia Medica) in China, written about 104th century B.C., record more than 2000 plant species and describe the use of plants to treat a variety of diseases including diabetes. More than 1000 plants species have been reported to treat diabetes around the world (Oubré *et al.*, 1997), and many of them originate in Malaysia (Fabricant & Farnsworth, 2001).

Traditional systems of medicine developed through experience and experimentation. Knowledge was mostly gained 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 effect of some of these herbs has been shown in various animal models of diabetes and in some instances the active constituents have even been isolated (Patel *et al.*, 2012). But, the majority of these herbs have far escaped scientific scrutiny and neither their mode of action nor the active principles are largely known.

The current prevalence of diabetes has clearly led to a need for novel drugs. Plants used traditionally to treat diabetes, particularly those that have been proven to decrease blood sugar, can potentially lead to the isolation of novel compounds with hypoglycemic effect. 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 (Fabricant *et al.*, 2001). Since humans have used these plants for generations, it can be expected that bioactive compounds isolated would have low toxicity. But this is not always true and not all of plants that

have therapeutic efficacy are entirely safe. There is also a tremendous degree of chemical diversity in a plant extract. This includes alkaloids, quassinoids, glycosides, polysaccharides, flavonoids, steroids, carbohydrates, terpenoids, amino acids and tannins. Such diversity can lead to interesting molecules that possibly useful as drug entities or more likely serve as lead molecules in a medicinal chemistry research. Metformin has its origins in the plant *Galega officinalis* L., which was used to treat diabetes in medieval Europe. Its use as hypoglycemic agent was a direct result of the isolation of galegine as an active antidiabetic agent (Oubré *et al.*, 1997). While galegine is too toxic to be used directly, it served as the template for the synthesis of metformin, which is a safer analog. The discovery of metformin emphasizes the role of the plants as a heritage for modern medicine to treat diabetes. *Gymnema sylvestre*, a plant native to the tropical forests of India, has long been used as a treatment for diabetes (Agarwal *et al.*, 2000). The use of *Gymnema sylvestre* extract and/or active constituents of this plant to treat diabetes, especially obese diabetes (Lucy & Yuan, 2002; Mall *et al.*, 2009) was an example of such use of medicinal herbs in modern medicine.

2.7 *Brucea javanica* (L.) Merr.

Brucea javanica (L.) Merr. belongs to the Simaroubaceae family and is a shrub 3-5 meter in height with drupe fruits (Bawm *et al.*, 2008). It is commonly called Melada Pahit in Malay language and kosam in Javanese (Noorshahida *et al.*, 2009). The black-grey fruit is oval shaped and up to 0.5 cm long. It originates in tropical Africa and throughout Asia. Different parts of the plant are used by indigenous healers for a number of diseases including diabetes around the world. In China, seeds are used to treat malaria, inflammation and lung cancer (Nie *et al.*, 2012). In Indonesia, the leaves are used to treat dysentery (Matsuura *et al.*, 2007). In Malaysia, the seeds are used traditionally by indigenous people in Malaysian Peninsula for lipid disorders and

diabetes (Noorshahida *et al.*, 2009). The seed is known to be reach source of quassinoids, triterpenoids, polyphenols and alkaloids (Li *et al.*, 2009; Liu *et al.*, 2012).

Aside from the hypoglycemic effect, the ethyl acetate extract of *B. javanica* seed (BJS) also exhibited anti-inflammatory effects in RAW 264.7 cells by decreasing proinflammatory cytokines such as TNFα, IL-1β and IL-6 and increasing level of anti-inflammatory cytokine IL-10 to near normal levels (Yang *et al.*, 2013). Diabetes is associated with overexpression of proinflammatory cytokines that often result in cardiovascular complication and insulin resistance. In diabetes, increased levels of proinflammatory cytokines such as TNFα, IL-1β and IL-6 have been shown to result in β-cell apoptosis and insulin resistance (Pollack *et al.*, 2016). The BJS exhibited anti-inflammatory effects and inhibited NF_kB activation that causes insulin resistance and β-cell apoptosis. However, to the best of our knowledge, no one has studied the effects of Malaysian medicinal plant BJS on inhibition of GPα. It is the purpose of this study to screen extracts of BJS for inhibition of GPα to identify novel compounds which may serve as lead molecules to treat diabetes (Kim *et al.*, 2010).

Figure 2.8: *Brucea javanica* (L.) Merr

CHAPTER 3 : METHODOLOGY

3.1 Materials

3.1.1 Plant

Brucea javanica seed was collected from Bukit Tampin reserved forest, Tampin, Negeri Sembilan, Malaysia, during the month of November. A voucher specimen (KL5794) has been retained by the Herbarium at Institute of Biological Science, University of Malaya for further reference.

3.1.2 General Chemicals and Solvents

Chemicals

Ascorbic acid, citric acid monohydrate, sodium nitrite, tri-sodium citrate dehydrate, Bismuth nitrate, potassium iodide, quercetin tannic acid, Sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate and carboxymethyl cellulose-natrium (CMC-Na) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Sodium hydroxide, α-D-Glucose, aluminum chloride, sodium chloride, Folin & Ciocalteu′s phenol reagent, sodium acetate trihydrate were purchased from Merck Chemical Co. (Malaysia).

Solvents

Ethanol, methanol, hexane, chloroform, glacial acetic acid, acetone, sulfuric acid, anisaldehyde, and 25% ammonia, 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.

NMR grade deuterated pyridine (C_5D_5N) and deuterated methanol (CD_3OD) were purchased from Merck Chemical Co. (Malaysia)

3.1.3 Chromatographic Media

Silica gel 60 F_{254} – precoated TLC plates (Merck, Germany), silica gel (0.40-0.63 µM), were purchased from Merck Chemical Co. (Malaysia). Sephadex LH-20 was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.1.4 Glycogen Phosphorylase Enzyme Assay

Glycogen phosphorylase α 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 (MgCl2), 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 (Tecan Sunrise, Austria) was used for absorbance determination.

3.1.5 α-Glucosidase Inhibition Assay

The α-glucosidase from *Saccharomyces cerevisiae*, and *p*-Nitrophenyl α-D-glucopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.1.6 Antioxidant Assays

DPPH Assay

DPPH (2,2 diphenyl-1-picrylhydrazyl) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ferric Reducing Antioxidant Power (FRAP) Assay

TPTZ (2,4,6-tripyridyl-s-triazine) and sodium acetate trihydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride, Ferrous sulfate was purchased from Merck Chemical Co. (Malaysia).

Metal Chelating Assay

Ferrozine, ferrous chloride, and ethylenediaminetetraacetic acid disodium dehydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Nitric Oxide (NO) Scavenging Activity Assay

Sodium nitroferricyanide (III) dihydrate, griess reagent, curcumin and sodium nitrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Superoxide Radical Scavenging Activity Assay

Nitro blue tetrazolium (NBT), xanthine and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.1.7 Antihyperglycemic Activity Study

Healthy adult Sprague Dawley (SD) rats 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). Rat TNF-α, rat IL-6, and rat IL-1β ELISA kits were purchased from eBioscience (San Diego, CA USA). Rat insulin ELISA kit was purchased from Mercodia AB (Uppsala, Sweden). TBARS, Glutathione, and Glycogen assay kits were purchased from Cayman chemical company (Ann Arbor, MI, USA).

3.1.8 Other Instruments

Mass spectra were carried out on Agilent 6530 Q-TOF Mass Spectrometer (California, USA). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were determined on Bruker Avance III 400 NMR spectrometer (Germany).

3.2 Phytochemistry Study

3.2.1 *Brucea javanica* Seed Extraction

The 2 kg of dried *Brucea javanica* seed (BJS) was ground using mechanical grinder and extracted three times with 95% ethanol (5 L) at room temperature for three days. The extract was filtered with Whatman filter paper and combined together. It was then concentrated under reduced pressure at 37 ^{o}C to dryness to give ethanol crude extract. This was stored at -20 ^{o}C until use.

3.2.2 Solvent Fraction of BJS

The ethanol crude extract (53.6 g) was suspended in distilled water (200 ml) and partitioned with *n*-hexane (250 ml × 3), chloroform (250 ml × 3), and ethyl acetate (250 ml × 3) in a separating funnel to obtain an *n*-hexane, chloroform, and ethyl acetate soluble fractions. All of the fractions were evaporated to dryness under reduced pressure to yield *n*-hexane fraction (HF), chloroform fraction (CF) and ethyl acetate fraction (EAF) of the ethanol crude extract. The remaining aqueous layer was then lyophilized to dryness to yield a light green powder as the water fraction (WF). The dried fractions were kept in refrigerator at 2-8 ^{o}C for further use.

3.2.3 Determination of Polyphenolic Contents in BJS

(i) Determination of Total Phenolic Content

Total phenol content (TPC) of the fractions was measured by the Folin-Ciocaltu method as described previously (Müller *et al.*, 2010). Sample solutions (1 mg/ml) were prepared by dissolving 1 mg of each fraction in 1 ml of 95% ethanol and 20 µl of these solutions was mixed with 100 µl of Folin-Ciocaltu reagent (diluted 10-fold) in a 96-well microplate, incubated for 5 min, and 75 µl of sodium carbonate solution (75 mg/ml) was added. After incubation period of 2 h in darkness at room temperature, the absorbance was measured at 740 nm using a microplate reader (Tecan Sunrise, Austria). Tannic

acid (100 – 1000 µM) was used for construction of a standard curve. The TPC was estimated as mg tannic acid equivalent (mg TAE)/g of dry extract.

(ii) Determination of Total Tannin Content

Total extractable tannins (TET) were determined according to the method (Royer *et al.*, 2011) with some modification. Sample solutions from each fraction were prepared by dissolving 10 mg of samples in water (10 ml) for water fraction and methanol-H_2O (1:1, 10 ml) for hexane, chloroform and ethyl acetate fractions. The polyvinyl polypyrrolidone (PVP, 1.1g) was added to the solutions, and the mixtures were vortexed thoroughly and centrifuged (3000 rpm, 15 min, 4^oC) to precipitate the tannin. Phenolic contents in the supernatant which is corresponds to the non-precipitable phenol (NPP) were determined by the Folin-Ciocaltu method as described (Müller *et al.*, 2010). The TET was calculated as differences using following equation: TET = TPC – NPP. The results were estimated from tannic acid standard curve and expressed as mg tannic acid equivalent (mg TAE)/g of dry extract.

(iii) Determination of Total Flavonoid Content

Total flavonoid contents (TFC) were measured according to the method (Sasipriya & Siddhuraju, 2012) with some modification. Briefly, fractions (50 µl) were added with 70 µl of distilled water and 15 µl of 5% sodium nitrite solution in a 96-well microplate. The solutions were well mixed and incubated for 5 min at room temperature. Then, 15 µl of 10% aluminum chloride solution was added into the mixture. After 6 min of incubation, 100 µl of 1 M sodium hydroxide solution was added, and the absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). The TFC was estimated from quercetin (100 – 1000 µM) standard curve, and the results were expressed as mg quercetin equivalent (mg QE)/g of dry extract.

3.2.4 Isolation of Compounds

The EAF from ethanol crude extract was subjected to chromatographic analysis in order to isolate the effective components from BJS and the structure of isolated compounds was further confirmed by HPLC/MS and NMR.

(i) Column Chromatography

The EAF (7.6g) from BJS was chromatographed over silica gel by gradient elution of *n*-Hexane and DCM (90:10-0:100) followed by DCM and MeOH (100:0 - 70:30) as mobile phase. All fractions were concentrated, monitored by TLC, and visualized under UV light. Total 91 fractions were collected and R_f values were calculated as follows:

$$R_f \text{ value} = (\text{Distance of compound spot})/(\text{Distance of solvent spot})$$

The fractions with similar R_f values were combined together to afford twelve fractions (F1-F12).

(ii) Isolation of Vanillic Acid

F1 was dried from 3 ml of a fraction 12 eluted with 10% n-Hexane: 90% DCM on silica column to give 0.012 g (0.16%) of vanillic acid, a white crystal. TLC: (silica) DCM: MeOH (9.5:0.5) R_f = 0.28 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 167.09 $[M-H]^-$.

(iii) Isolation of Bruceine D

F7 was evaporated to dryness from fractions 34-35 (6 ml) eluted with 90% DCM: 10% MeOH over silica gel column to give 0.045 g (0.59%) of Bruceine D as a light yellow solid. TLC: (silica) DCM: MeOH (8:2) R_f = 0.51 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 411.3018 $[M+H]^+$.

(iv) Isolation of Bruceine E

F9 was evaporated to dryness from fractions 54-56 (9 ml) eluted with 85% DCM: 15% MeOH over silica gel column to give 0.056 g (0.74%) of Bruceine E as a light yellow solid. TLC: (silica) DCM: MeOH (8:2) R_f = 0.39 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 435.3028 $[M+Na]^+$.

(v) Isolation of Parahydroxybenzoic Acid

F3 was chromatographed over sephadex LH-20 column with gradient elution of DCM: MeOH (65:35 – 55:45) as a mobile phase. Fractions 29-35 were dried from 10 ml of solution eluted with DCM: MeOH (45:55) to give 0.199 g of a white, amorphous solid as parahydroxybenzoic acid with 2.62% yield. TLC: (silica) DCM: MeOH (9:1) R_f = 0.65 (visualised with UV and vanillin reagent). HR-ESI-MS: m/z 137.00 $[M-H]^-$.

(vi) Isolation of Luteolin

F5 was applied to a sephadex LH20 column, eluted with DCM: MeOH (65:35 – 55:45) as mobile phase. When it was eluted with 50 ml of 65% DCM: 35% MeOH, a yellow band was observed moving down the column. When it comes down the bottom of the column it (fractions 22-27, 6 ml) was collected. These fractions were combined together and dried under reduced pressure to give 0.009 g (0.12%) of luteolin, a yellow solid. TLC: (silica) DCM: MeOH (9:1) R_f = 0.47 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 285.10 $[M-H]^-$

(vii) Isolation of Protocatechuic Acid

F6 was chromatographed over sephadex LH20 column with gradient elution of DCM: MeOH (65:35 – 55:45) as mobile phase. Fraction 27 (3 ml) was dried under reduced pressure to give 0.054 g (0.71%) of protocatechuic acid, a light brown solid. TLC: (silica) DCM: MeOH (9:1) R_f = 0.27 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 153.00 $[M-H]^-$.

(viii) Isolation of Gallic Acid

F8 was applied to a sephadex LH20 column and chromatographed with gradient elution of DCM: MeOH (60:40 – 20:80) as mobile phase. Fraction 20 (3 ml) was identified as white crystal and dried to give 0.0076 g of gallic acid with 0.1% yield. TLC: (silica) DCM: MeOH (8:2) R_f = 0.40 (visualised with UV and vanillin reagent). HR-ESI-MS: *m/z* 169.07 $[M-H]^-$.

3.2.5 HPLC-MS Analysis

All isolated compounds from EAF of BJS were analysed by Agilent 1200 HPLC-MS system. Chromatographic separation was carried out using an Agilent Zorbax Eclipse Plus C18 column Rapid Resolution HT (2.1 × 1 mm, 1.8 μm). The mobile phase (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in ACN) was eluted at a flow rate of 0.5 mL/min. The elution was gradient (90% A for 0-1 min, 50% A for 1-20 min and hold 4 min, 90% A for 25-30 min). The total run time was 30 min, and the injection volume was 10 μL.

3.2.6 Nuclear Magnetic Resonance (NMR)

^{1}H-NMR and ^{13}C- NMR (COSY, HSQC, HMBC, and NOESY) spectra for isolated compounds except for gallic acid were obtained from the samples dissolved in deuterated methanol (CD_3OD) using Bruker Avance III 400 NMR spectrometer. ^{1}H-NMR and ^{13}C- NMR for gallic acid were acquired using concentrated solution of gallic acid in pyridine (C_5D_5N). Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) are given in Hz.

3.3 Biological Assays Using *in vitro* Models

3.3.1 GP*α* inhibition Assay of the Fractions

Glycogen Phosphorylase α (GPα) activity of fractions from BJS were measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-

phosphate as described previously (Schweiker *et al.*, 2014) with slight modification. A stock solution (32 mg/ml) for each fraction was prepared by dissolving 32 mg of fractions in 1 ml of 10% DMSO in 50 mM Hepes buffer (pH 7.2) which was then serially diluted to two-fold, 4 times, to give 16, 8, 4, and 2 mg/ml solutions of each fraction.

The assay was run by mixing 10 µl solutions of each fractions at different concentrations with 40 µl of 50 mM Hepes buffer (pH 7.2) containing 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, 0.25 mM glucose-1-phosphate, and 1 mg/ml glycogen in a 96-well microplate. The reaction was initiated by adding 50 µl of enzyme (GPα) in 50 mM Hepes buffer (pH 7.2) and incubated at 22^{o}C for 30 min. The mixture was subsequently incubated with 150 µl of 1 M HCl solution containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green for 5 min, and phosphate released was measured at 620 nm using a microplate reader (Tecan Sunrise, Austria). The assay was performed in triplicate with appropriate blanks and 10% DMSO in 50 mM Hepes buffer was used as negative control. The caffeine was used as a standard. The concentration of fractions required to inhibit 50% of glycogen phosphorylase enzyme activity under the assay condition was defined as the IC_{50} value, and IC_{50} values of all fraction were calculated using the equation below.

$$\text{GP}\alpha \text{ inhibition } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The results were reported as µg/ml, and were expressed as the mean ± standard error (SE).

3.3.2 α-Glucosidase Inhibition Assay of the Fractions

The assay is a slight modification of a previously published method (Lordan *et al.*, 2013) which was designed for a 96-well plate reader. The enzyme solution was made to a concentration of 0.1unit/ml at pH 6.9 with 0.1 M phosphate buffer. A solution

of fractions (2000 μg/ml) was prepared in 10% DMSO in 0.1 M phosphate buffer at pH 6.9 which was then serially diluted two-fold, 4 times, to give 125, 250, 500, and 1000 μg/ml solutions of each fraction. 100 μl of α-glucosidase (0.1 U/ml) was premixed with 50 μl of fractions at varying concentrations and incubated for 10 min at 37°C. 50 μl of 5 mM *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG) in 0.1 M phosphate buffer (pH 6.9) was added to initiate the reaction and re-incubated at 37°C for 30 min. The reaction was terminated by addition of 50 μl of 1 M $NaCO_3$. The 10% DMSO in 0.1 M phosphate buffer (pH 6.9) was used as negative control. The quantity of *p*-nitrophenol released from *p*-NPG in the presence of α-glucosidase was determined spectrometrically at 405 nm using microplate reader (Tecan Sunrise, Austria). The percentage of α-glucosidase inhibition (αGI) was calculated as following formula:

$$\alpha\text{GI}\ (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The results were reported as μg/ml, expressed as the mean ± SE from triplicated test.

3.3.3 GPα and α-Glucosidase Inhibition Assays by Isolated Compounds

To determine antidiabetic effect of BJS, all isolated compounds were tested for their inhibition of GPα and α-glucosidase by the same assays used to test BJS fractions in the general screening as described in section 3.3.1 and 3.3.2.

In GPα inhibition assay, all isolated compounds were dissolved in 10% DMSO in 50 mM Hepes buffer at pH 7.2 and were prepared at the concentrations of 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml. To determine α-glucosidase inhibition activity, all compounds except luteolin were prepared in 10% DMSO in 0.1 M phosphate buffer (pH 6.9) at the concentrations of 62.5, 125, 250, 500 and 1000 μg/ml, while luteolin was prepared at the concentrations of 12.5, 25, 50, 100, and 150 μg/ml in 10% DMSO in 0.1 M phosphate buffer at pH 6.9, respectively. The assays were performed in triplicate

with appropriate blanks. The IC_{50} values of compounds were determined by nonlinear regression as μg/ml. The results were converted to μM and expressed as the mean ± SE.

3.3.4 Antioxidant Activity of BJS Fractions

(i) DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity of BJS was determined according to the method as described previously (Ranilla *et al.*, 2010) with some modifications. The samples of each fraction were prepared in 95% ethanol at different concentrations (25 to 400 μg/ ml). A volume of sample (40 μl) was mixed with 200 μl of 50 μM DPPH solution in 95% ethanol and incubated at 25 ^{o}C for 15 min in the darkness. The optical density was measured at 517 nm using a microplate reader (Tecan Sunrise, Austria). BHA was used as a standard and 95% ethanol (40 μl) was used as negative control. The percentage of scavenging effect of fractions was calculated as follow:

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

The assay was performed in triplicate and results were expressed as the mean ± SE

(ii) Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP activities of fractions from BJS were measured as previously described method (Müller *et al.*, 2010). The assay was run by mixing 20 μl of fraction solutions (1mg/ml and 100μg/ml) in 95% ethanol with 200 μl of freshly prepared FRAP reagent (appendix 5.7) in 96-well microplate. After 8 min of incubation time, the TPTZ-Fe^{2+} complex formed was measured at 595 nm using a microplate reader (Tecan Sunrise, Austria). Ethanol (95%) was used as blank. Ferrous sulphate ($FeSO_4$) solution (0.1 mM to 1 mM) was used for standard calibration curve. The FRAP value was determined using the corresponding regression equation and the results were estimated as mmol Fe^{2+}/g of dry extract from triplicated tests.

(iii) Metal chelating activity assay

The assay was performed according to the method (Srivastava *et al.*, 2012) as described previously. Sample solutions of all fractions were prepared in 95% ethanol at different concentrations (50 - 800 µg/ml). The fractions (100 µl) were mixed with 120 µl of distilled water and 10 µl of 2 mM $FeCl_2$ in a 96-well microplate. The reaction was initiated by addition of 5 mM ferrozine (20 µl) and the Fe^{2+}-ferrozine complex formed was measured at 562 nm using a microplate reader for 20 min. EDTA-Na_2 (5 – 80 µg/ml) was used as a standard and the 95% ethanol (100 µl) was used as a negative control. The blank reading (20 µl of distilled water instead of ferrozine) was subtracted from each well. The percent of chelating activity of fractions was calculated as follow:

$$\text{Ferrous ion chelating activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, A is absorbance. The concentration required to chelate 50% of the Fe^{2+} ion was defined as IC_{50}. The results were reported as µg/ml and were expressed as the mean ± SE from triplicated test.

(iv) Nitric Oxide Scavenging Activity Assay

Nitric oxide (NO) radical scavenging activity was measured by Griess assay as described previously (Srivastava *et al.*, 2012). All fractions from BJS were prepared at the concentrations of 100, 200, 400, 800, and 1600 µg/ml in 95% ethanol. 50 µl of fraction solutions were mixed with 50 µl of 10 mM sodium nitroferricyanide in 20 mM PBS (pH 7.4), preincubated for 150 min, and Griess reagent (125 µl) was added to the mixture. Nitrite ions formed were measured at 546 nm for 10 min with a microplate reader (Tecan Sunrise, Austria). Curcumin (10 - 160 µg/ml) was used as a standard and 95% ethanol was used as a negative control. The NO scavenging activity of the fractions was calculated using the equation bellow:

$$\text{NO radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, A is absorbance. The assay was performed in triplicate, and the results were expressed as µg/ml.

(v) Super Oxide Scavenging Activity Assay

The superoxide radical scavenging activity was measured by nitro blue tetrazolium (NBT) reduction assay as described previously (Chandrasekara *et al.*, 2012) with some modifications. The xanthine–xanthine oxidase system was used for generation of superoxide radicals. Sample solutions of all fractions were prepared at the concentrations of 125, 250, 500, 1000, and 2000 µg/ml in 10% DMSO in 50 mM PBS at pH 7.4. 50 µl of samples at different concentrations was mixed with 50 µl of NBT solution containing 1 mM xanthine, 1 mM NBT, and 0.05 mM EDTA in 50 mM phosphate buffered saline (PBS, pH 7.4). The reaction was initiated by addition of xanthine oxidase (75 µl) freshly prepared in PBS (0.25 units/ml) and incubated at 37^{o}C for 20 min. The reaction was terminated by adding 25 µl of 1 M HCl and NBT absorption was measured at 560 nm. The PBS (10% DMSO) was used as negative control. Blank reading (without enzyme) was subtracted from each well. Quercetin was used as a reference compound. The superoxide radical scavenging activity was calculated as follows.

$$\text{Superoxide radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, A is absorbance. The concentration required to scavenge 50% of superoxide radical was defined as IC_{50} and the results were expressed as µg/ml from triplicated test.

3.3.5 Determination of Antioxidant Activity of Isolated Compounds

To determine antioxidant effect of BJS, all isolated compounds were tested against NO and superoxide radical scavenging activities *in vitro* and the procedures of assays were identical to that described in sections 3.3.4 (iv) and 3.3.4 (v). In NO radical scavenging assay, isolated compounds and curcumin were dissolved in 10% DMSO in 20 mM PBS at pH 7.4. All isolated compounds were tested at 12.5, 25, 50, 100, 200, 400, and 800 µl/ml, and standard drug curcumin was tested at 3.125, 6.25, 12.5, 25, and 50 µl/ml, respectively. To determine superoxide radical scavenging activity, all compounds were dissolved in 10% DMSO in 50 mM PBS at pH 7.4 and were prepared at the concentration range of 12.5, 25, 50, 100, 200, 400, and 800 µl/ml. The assays were performed in triplicate with appropriate blanks. The IC_{50} value was calculated as µg/ml and converted to µM and the results were reported as the mean ± SE.

3.4 Biological Assays Using *in vivo* Models

3.4.1 Ethics Statement

Sprague Dawley (SD) rats were housed according to guidelines of the Institutional Animal Care and Use Committee at the University of Malaya. All procedures were carried out in compliance with standards for the Care and Use of Laboratory Animals. The animal use protocol performed in this study was approved by the Institutional Animal Care and Use Committee at the University of Malaya. Ethic No: ISB/23/05/2013/AA (R).

3.4.2 Experimental Animals

Eight-week-old male and female SD rats (200 – 230 g) were purchased from Animal Experimental Unit of the University of Malaya, housed according to Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health, USA. Total 30 SD rats were housed in polypropylene cages (3 male or 3 female in each

cage) and kept under controlled laboratory conditions at a temperature of 22 ± 3 ^{o}C, a humidity of 65 ± 5%.), and a 12 h light-dark cycle and acclimated for one week prior to treatment. The rats were offered access to Laboratory Rodent Chow (Germany) and water *ad libitum*, received human care according to the guidelines.

3.4.3 Oral Acute Toxicity Test

Oral acute toxicity (OAT) of EAF from BJS was evaluated according to the guidelines of the Organization for Economic Co-operation and Development (The Organization for Economic Co-operation and Development, 2001). The SD rats were divided into 5 groups (3 males and 3 females in each group), and fasted overnight but had been allowed to free access to water. The samples were dissolved in 0.5% CMC in distilled water. Group I: treated with in 0.5% CMC in distilled water. Group II: treated with EAF at the dose of 300 mg/kg body weight. Group III: treated with EAF at the dose of 250 mg/kg body weight. Group IV: treated with EAF at the dose of 125 mg/kg body weight. Group V: treated with EAF at the dose of 50 mg/kg body weight. All groups of rats were fed EAF in a volume of 10 ml/kg body weight using an oral gavage. Rats were observed continuously for 2 h, and then at 6 h intervals for 24 h, and finally after every 24 h up to to14 days for any physical signs of toxicity such as motor activity, sleep, urination, response to touch, and decreased respiratory rate or for any mortality.

3.4.4 Oral Glucose Tolerance Test in Non-Diabetic Rats

The selection of optimum dose of EAF was assessed by an OGTT as described previously (Veerapur *et al.*, 2012). Based on oral acute toxicity test, four doses (12.5, 25, 50 and 125 mg/kg) of EAF were selected for study. The EAF was prepared at the concentrations of 1.25, 2.5, 5.0 and 12.5 mg/ml in 0.5% CMC solution and glucose solution was prepared at the concentration of 200 mg/ml in distilled water. The standard drug, glibenclamide (GLI), was prepared at the concentration of 1mg/ml in 0.5% CMC in distilled water. The SD rats were divided into 6 groups (n = 6), fasted overnight (16

h), and were fed with EAFs (12.5, 25, 50 and 125 mg/kg) and GLI (10 mg/kg) in a volume of 10 ml/kg b.w by oral gavage, respectively. The control groups were received 0.5% CMC solution. Glucose (2.0 g/kg b.w) was fed all groups of rats 30 min after the treatment of EAFs or GLI. Blood glucose levels were measured at 0, 30, 60, 90, 120 and 180 minute after glucose load using Accu-check glucose test strips and glucose meter (Accu-check, Roche Diagnostics, USA). Data are expressed as the mean ± SE.

3.4.5 Induction of Type 2 Diabetes

The type 2 diabetes (T2D) was produced from Sprague Dawley (SD) rats as the method (Arya *et al.*, 2012) described previously with slightly modification. Sprague Dawley (SD) rats were fasted overnight (16 h) and the body weights of each rat were measured before injection. The rats were made diabetic by single intraperitoneal (*i.p*) injection of STZ (60 mg/kg b.w.) 15 minutes after injection (*i.p*) of NA (100 mg/kg b.w.) in a volume of 1 ml/kg body weight. Diabetes was confirmed 3 weeks after NA-STZ induction by measuring blood glucose levels from tail vein using glucose meter.

3.4.6 Treatment Protocol

(i) Sample Preparation

The EAF from BJS (2.5 and 5.0 mg/ml) and standard drug glibenclamide (1 mg/ml) were dissolved in 0.5% CMC solution. The solutions were prepared fresh before use.

(ii) Protocol Procedure

The non-diabetic rats (n = 6) were housed in cages (3 male and 3 female in each cage) and labelled as Group I (non-diabetic control). The T2D rats were divided into 4 groups (n = 6, 3M/3F) and labelled as Group II (diabetic control), Group III, Group IV and Group V. Group I and II were received 0.5% CMC solution. Group III and Group IV were received EAF at 25 mg/kg b.w./day and 50 mg/kg b.w./day, Group V served as

positive control and received glibenclamide (GLI) at 10 mg/kg b.w./day. All groups were treated orally by using oral gavage for 28 days.

(iii) Measurement of Fasting Blood Glucose Levels

T2D rats were treated with EAF and standard drug glibenclamide for four weeks and fasting blood glucose levels of experimental rats were measured weekly. At the end of each week, rats were fasted overnight and blood was collected from rat's tail vein using Accu-check punching device. Fasting blood glucose concentrations were measured using Accu-check glucose test strips and glucose meter (Accu-check, Roche Diagnostics, USA).

(iv) Measurement of Body Weights

During the experimental period, at the end of each week, rats were fasted overnight and body weights were recorded weekly using electronic balance.

(v) Oral Glucose Tolerance Test in T2D Rats

On the 25th day of treatment, the OGTT was carried out according to the previously reported method (Veerapur *et al.*, 2012). All animals were fasted overnight (16h) before commencing the experiments. Group I (non-diabetic control) and Group II (diabetic control) were received 0.5% CMC solution, Group III and Group IV were given EAF (25 and 50 mg/kg b.w), and Group V was given glibenclamide (10 mg/kg b.w.) using oral gavage, respectively. After 30 min, α-D-glucose (2 g/kg b.w.) was administered orally into all groups of rats. Blood samples were collected from the tail vein at 0 (immediately after glucose load), 30, 60, 90, and 120 min, and blood glucose levels were determined by glucose oxidase method using a commercial glucose meter (Roche, USA). Total glycaemic responses to OGTT were calculated from respective areas under the curve for glucose ($AUC_{glucose}$) by trapezoid rule for the 120 min.

$$\mathrm{AUC_{glucose}} = \frac{\mathrm{C_1 + C_2}}{2} \times (\mathrm{t_2 - t_1})$$

Where, C_1 and C_2 are concentrations of glucose at different time points; t_1 and t_2 are different tested time points.

(vi) Collection of Serum Samples

At the end of experiment, rats were fasted overnight. Blood samples were collected by cardiac puncture, and allowed to clot for 30 min at 25 ^{o}C. The serum samples were prepared by centrifuging the whole blood at 2000 x g at 4 ^{o}C for 15 min, and stored at -80 ^{o}C until analysis.

(vii) Determination of Serum Insulin Levels

The serum insulin levels were quantified by using rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions.

(viii) Determination of Serum Lipid Profiles

The serum total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) levels were analysed using commercial kits with biochemical analyzer.

(ix) Determination of Renal and Liver Functions

The urea and creatinine as markers of renal toxicity, and alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as markers for liver toxicity were analysed using biochemical analyzer.

(x) Determination of Lipid Peroxidation

Malondialdehyde (MDA), a marker of lipid peroxidation, was determined by a thiobarbituric acid reactive substance assay (Armstrong & Browne, 1994) for the quantitative determination of MDA in the serum of experimental rats using calorimetric assay following the instructions provided in the kit (Cayman, Ann Arbor, MI, USA).

(xi) Measurement of Glutathione

The glutathione (GSH), a marker of antioxidant defense, was measured by enzymatic recycling method (Eyer & Podhradský, 1986) for the quantification of GSH in the serum of experimental rats using calorimetric assay following the instructions provided in the kit (Cayman, Ann Arbor, MI, USA).

(xii) Determination of Cytokines

The cytokines (TNF-α, IL-6 and IL-1β) in the serum of experimental rats were measured by enzyme-linked immunosorbent assay (ELISA) using colorimetric assay kits for rat TNF-α, IL-6 and IL-1β (eBioscience, San Diego, CA USA), according to the manufacturer's instructions. Briefly, an aliquot of serum was incubated with anti-rat TNF-α, IL-6 and IL-1β antibody precoated in 96-well microplates, and the optical density was read at 450 nm in an ELISA reader (Sunrise, Austria). The cytokines levels were determined using standard curve specific for rat TNF-α, rat IL-6, and rat IL-1β and expressed as pg/ml.

(xiii) Measurement of Hepatic Glycogen Contents

Collection of Liver Samples

At the end of experiment, liver samples were carefully collected from experimental rats, and were washed in ice-cold phosphate buffered saline (PBS, pH 7.4) to remove the blood. One gram of liver samples was added with 5 ml of PBS (pH 7.4) and homogenized by using mechanical homogenizer, centrifuged at 1600 x g for 10 minutes at 4 oC. The supernatants were collected and stored at -80 oC for glycogen assay.

Assay Procedure

The assay was performed as stated by the method described in glycogen assay kit (Ann Arbor, MI, USA). The glycogen concentrations in the liver were calculated using glycogen standard curve and expressed as mg of glycogen/g of liver tissue.

3.4.7 Statistical Analysis

The difference between the groups was statistically significant as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$) using the statistical program (SPSS 16.0 version, Chicago, IL, USA).

CHAPTER 4 : RESULTS

4.1 Phytochemistry Study

4.1.1 *Brucea javanica* Seed Extractions

The dry, powdered *Brucea javanica* seeds (BJS) (2 kg) were extracted with 95% ethanol tree times at room temperature for 3 days. The filtrate were combined together and ethanol in the solution was removed under reduced pressure using rotary evaporator to give 53.6 g green oily gum in 2.68% yield as the ethanol crude extract (ECE).

4.1.2 Solvent Fraction of BJS

The ECE (53.6 g) was suspended in distilled water and partitioned with *n*-hexane. The *n*-hexane was evaporated under reduced pressure to give dark green oil as the *n*-hexane fraction (HF). The aqueous layer was continuously extracted with chloroform. The chloroform was removed under reduced pressure to yield a green solid as the chloroform fraction (CF). The remaining aqueous layer was then extracted with ethyl acetate for 3 days. The ethyl acetate layer was evaporated to dryness under reduced pressure to yield a light brown powder as the ethyl acetate fraction (EAF). The aqueous layer was lyophilized to dryness to give a light green powder as the water fraction (WF). Table 4.1 lists weights and yields of the fractions obtained after lyophilization.

Table 4.1: Weights and yields of the fractions obtained from ECE of BJS

Fractions	Weight (g)	Yield (%)
HF	18.1	33.8
CF	3.2	6.0
EAF	1.7	3.2
WF	20.4	38.1

HF, hexane fraction; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction; ECE, ethanol crude extract; BJS, *Brucea javanica* seed.

4.1.3 Determination of Polyphenolic Contents in BJS

The polyphenolic contents in the fractions from BJS were determined according to the description by Müller *et al.* (2010) and Sasipriya *et al.* (2012). The amount of total phenolic (TPC), total extractable tannin (TET) and non-precipitable phenol (NPP) contents were calculated by using tannic acid standard curve. The TPC was calculated by putting absorbance of the fractions into the equation (y = 0.0076x) obtained from tannic acid standard curve (Appendix 3). The amount of NPP in the fractions was measured in the same way as TPC after precipitation of tannins. The TET was calculated as differences using the following equation: TET = TPC – NPP and the results were reported as mg tannic acid equivalent (mg TAE)/g of dry extract. The total flavonoid contents (TFC) were determined by using equation y = 0.0002x obtained from quercetin standard curve (Appendix 4) and were reported as mg quercetin equivalent (mg QE)/g of dry extract. The results were expressed as the mean ± standard error (SE) from tree independent tests. Table 4.2 lists the amount of polyphenolic contents in BJS and it shows that EAF contained the highest amount of polyphenolic content compared to that of other fractions.

Table 4.2: Different classes of polyphenol contents in BJS

Fractions	TFC (mg QE/g)	TPC (mg TAE/g)	TET (mg TAE/g)	NPP (mg TAE/g)
HF	15.60 ± 4.82^{a}	0.82 ± 0.39^{a}	0.45 ± 1.50^{a}	0.37 ± 2.21^{a}
CF	49.45 ± 4.46^{b}	119.98 ± 2.58^{b}	15.02 ± 2.21^{b}	104.97 ± 4.72^{b}
EAF	154.73 ± 0.61^{c}	169.03 ± 3.54^{c}	107.00 ± 1.74^{c}	62.02 ± 1.50^{c}
WF	33.33 ± 5.77^{d}	46.71 ± 4.08^{d}	11.71 ± 5.12^{d}	34.99 ± 1.75^{d}

Data are mean ± SE (n = 3). The means with different lower case letters (a, b, c, and d) in the same column are significantly different at $p < 0.05$ (ANOVA, followed by Duncan's multiple comparison test). TFC, total flavonoid content expressed as mg quercetin equivalent (mg QE)/g of dry extract. TPC, total phenolic content; TET, total extractable tannin; NPP, non-precipitable phenol contents expressed as mg tannic acid equivalent (mg TAE)/g of dry extract. BJS, *Brucea javanica* seed

4.1.4 Isolation of Compounds

Column chromatography coupled with other separation methods led to the isolation of seven known compounds; vanillic acid, bruceine D, bruceine E, *para*hydroxybenzoic acid, luteolin, protocatechuic acid and gallic acid.

Isolation of Vanillic Acid

Vanillic acid was isolated from the EAF of BJS in 0.16% yield as a white crystal. It shows a single spot with R_f value of 0.28 on TLC developed by DCM: MeOH (95:5) as mobile phase. The negative mode HR-ESI-MS data showed an ion peak $[M-H]^-$ at m/z 167.09 (calcd for $C_8H_7O_4$, 167.03), suggesting a molecular formula of $C_8H_8O_4$. ^1H NMR (400 MHz, CD_3OD): δ 3.91 (3H, *s*, OCH_3-8), 4.90 (1H, *brs*, H-4), 6.85 (1H, *d*, H-5), 7.57 (1H, *brs*, H-2), 7.57 (1H, *brs*, H-6). ^{13}C NMR (100 MHz, CD_3OD): δ 55.0 (C-8), 112.4 (C-2), 114.4 (C-5), 121.7 (C-1), 123.9 (C-6), 147.3 (C-3), 151.3 (C-4), 168.8 (C-7). It was confirmed by HR-ESI-MS, ^1H NMR and ^{13}C NMR by comparison to the literature (Grieman *et al.*, 2015). The structure of vanillic acid is shown in Figure 4.1.

Isolation of Bruceine D

Bruceine D was isolated from the EAF in 0.59% yield as a light yellow solid. TLC: (silica) DCM: MeOH (80:20) R_f = 0.51.The HR-ESI-MS shows a molecular ion peak at *m/z* 411.3018 $[M+H]^+$ (calcd for $C_{20}H_{27}O_9$, 411.1655), indicating a molecular formula of $C_{20}H_{26}O_9$.. Its ^1H NMR and ^{13}C NMR data were shown in Table 4.3

Isolation of Bruceine E

Bruceine E was isolated from the EAF of BJS in 0.74% yield as a light yellow solid. TLC: (silica) DCM: MeOH (8:2) R_f = 0.39. It shows a molecular ion peak $[M+Na]^+$ at *m/z* 435.3028 (calcd for $C_{20}H_{28}O_9Na$, 435.1631) and its molecular formula was determined to be $C_{20}H_{28}O_9$. Its ^1H NMR and ^{13}C NMR data were given in Table 4.3

Table 4.3: ^{1}H and ^{13}C NMR of bruceine D and E in CD_3OD

Position	Bruceine D		Bruceine E	
	δ_H	δ_C	δ_H	δ_C
1	4.26 (1H, *s*)	81.6	3.54 (1H, *d* 7.3)	81.4
2	-	198.5	4.01 (1H, *dd* 1.3, 7.3)	72.8
3	6.05 (1H, *s*)	123.8	5.41 (1H, *d* 1.3)	123.8
4	-	164.3	-	135.3
5	2.96 (1H, *d*)	43.0	2.42 (1H, *d* 12.8)	42.4
6	2.38 (1H, *dt*), 1.85 (1H, *td*)	27.3	2.17 (1H, *dt* 2.8,), 1.70 (1H, *td*)	27.2
7	5.12 (1H, *t*)	79.8	5.08 (1H, *t* 2.7)	80.6
8	-	49.3	-	49.6
9	2.42 (1H, *d*)	44.8	2.08 (1H, *d* 4.2)	45.9
10	-	44.8	-	44.0
11	4.60 (1H, *d*)	74.1	4.60 (1H, *d* 4.4)	74.4
12	3.78 (1H, *brs*)	80.0	3.76 (1H, *brs*)	79.8
13	-	83.6	-	83.4
14	-	81.0	-	81.0
15	5.24 (1H, *s*)	69.3	5.15 (1H, *s*)	69.2
16	-	174.9	-	175.0
18	1.99 (3H, *s*)	21.2	1.67 (3H, *s*)	19.7
19	1.19 (3H, *s*)	10.1	1.24 3H, *s*)	10.8
20	4.54 (1H, *d*), 3.84 (1H, *d*)	69.0	4.63 (1H, *d* 7.3), 3.83 (1H, *d* 7.3)	69.4
21	1.44 (3H, *s*)	17.1	1.43 (3H, *s*)	17.0

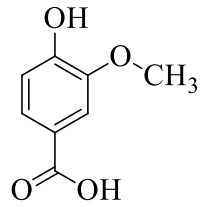
Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz.

The column chromatography showed bruceine D and E were major components of BJS. Structures of these compounds confirmed by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR are identical to this reported in the literature (Lee *et al.*, 1979) and are shown in Figure 4.1.

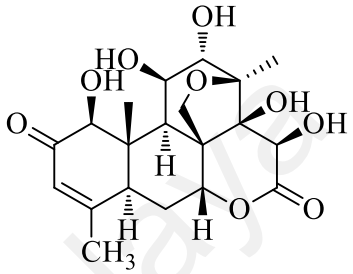
Isolation of Parahydroxybenzoic Acid

Parahydroxybenzoic acid was isolated from the EAF of BJS in 2.64% yield as a white, amorphous solid. TLC: (silica) DCM: MeOH (90:10) R_f = 0.65. The HR-ESI-MS in negative mode showed an ion peak $[M-H]^-$ at m/z 137.00 $[M-H]^-$ (calcd for $C_7H_5O_3$,

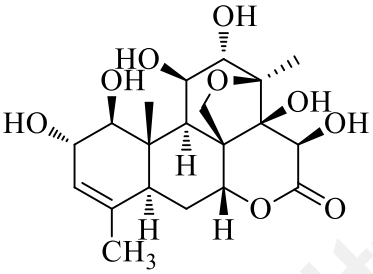
137.02) and gave a molecular of $C_7H_6O_3$. ^{1}H NMR (400 MHz, CD_3OD): δ 7.90 (2H, *d*, *J*=14 Hz, H-2, H-6), 6.83 (2H, *d*, *J*=14 Hz, H-3, H-5). ^{13}C NMR (100 MHz, CD_3OD): δ 114.6 (C-3, C-5), 121.3 (C-1), 131.6 (C-2, C-6), 162.0 (C-4), 168.7 (C-7). It was confirmed by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR in comparison with the literature (Yu *et al.*, 2006) and its structure is shown in Figure 4.1.



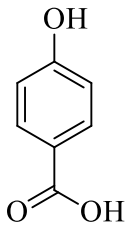
Vanillic acid



Bruceine D



Bruceine E



p-hydroxybenzoic acid

Figure 4.1: Structures of vanillic acid, bruceine D & E, and *p*-hydroxybenzoic acid

Isolation of Luteolin

Luteolin was isolated from the EAF of BJS in 0.12% yield as a yellow solid. TLC: (silica) DCM: MeOH (90:10) R_f = 0.47. The HR-ESI-MS shows a molecular ion at *m/z* 285.10 $[M-H]^-$ (calcd for $C_{15}H_9O_6$, 285.04), indicating a molecular formula of $C_{15}H_{10}O_6$. ^{1}H NMR (400 MHz, CD_3OD): δ 6.23 (1H, *s*, H-6), 6.46 (1H, *s*, H-8), 6.56 (1H, *s*, H-3), 6.92 (1H, *d*, *J*=8.4 Hz, H-5′), 7.39 (1H, *s*, H-2′), 7.40 (1H, *d*, *J*=8.4 Hz, H-6′). ^{13}C NMR (100 MHz, CD_3OD): δ 93.6 (C-8), 98.7 (C-6), 102.5 (C-3), 103.9 (C-10), 112.8 (C-2′), 115.4 (C-5′), 118.9 (C-6′), 122.3 (C-1′), 145.6 (C-3′), 149.6 (C-4′), 158.0 (C-5), 161.8 (C-9), 164.4 (C-2), 165.0 (C-7), 182.5 (C-4). Its structure was confirmed

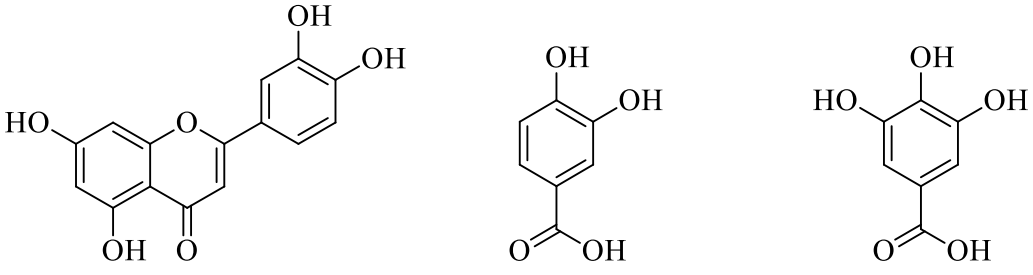
by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR and in agreement with literature (Li & Jiang, 2006). The structure of luteolin is shown in Figure 4.2.

Isolation of Protocatechuic Acid

Protocatechuic acid was isolated from the EAF of BJS in 0.71% yield as a light brown solid. TLC (silica) DCM: MeOH (90:10) R_f = 0.27. The HR-ESI-MS shows a molecular ion at *m/z* 153.00 $[M-H]^-$ (calcd for $C_7H_5O_4$, 153.01), indicating a molecular formula of $C_7H_6O_4$. ^{1}H NMR (400 MHz, CD_3OD): δ 6.82 (1H, *d*, *J*=8.2 Hz, H-5), 7.45 (1H, *dd*, *J*=14 Hz, 2.0 Hz, H-6).7.46 (1H, *d*, *J*=2.0 Hz, H-2), ^{13}C NMR (100 MHz, CD_3OD): δ 114.4 (C-5), 116.3 (C-2), 121.7 (C-1), 122.5 (C-6), 144.7 (C-), 150.1 (C-, 168.9 (C-7). It was confirmed by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR by comparison to the literature (Yu *et al.*, 2006) and Its structure is shown in Figure 4.2.

Isolation of Gallic Acid

Gallic acid was isolated from the EAF of BJS in 0.1% yield as a white solid. TLC (silica) DCM: MeOH (80:20) R_f = 0.40. The HR-ESI-MS shows a molecular ion peak at *m/z* 169.07 $[M-H]^-$ (calcd for $C_7H_5O_5$, 169.01), suggesting molecular formula of $C_7H_6O_5$. ^{1}H NMR (400 MHz, C_5D_5N): δ 8.08 (2H, *s*, H-2, H-6), ^{13}C NMR (100 MHz, C_5D_5N): δ 110.3 (C-2, C-6), 122.7 (C-1), 140.3 (C-4), 147.4 (C-3, C-5), 169.4 (C-7). It was confirmed by TLC in comparison with authentic sample and HR-ESI-MS, ^{1}H NMR and ^{13}C NMR. Its structure is identical to those reported in the literatures (Núñez Sellés *et al.*, 2002; Wang *et al.*, 2013). The structure of gallic acid is shown in Figure 4.2.



Luteolin Protocatechuic acid Gallic acid

Figure 4.2: Structures of luteolin, protocatechuic acid and gallic acid

4.2 Biological Assays Using *in vitro* Models

4.2.1 Screening Fractions for GPα Inhibition

The fractions were screened for inhibition of GPα in an assay previously published methods (Schweiker *et al.*, 2014). All fractions were tested at the concentration up to 3.2 mg/ml and the results were shown in Figure 4.3. The most potent fraction in Figure 4.3 was ethyl acetate and its IC_{50} is 0.75 ± 2.0 mg/ml. The caffeine was tested as a standard and determined to have an IC_{50} of 0.49 ± 2.3 mg/ml (Figure 4.3).

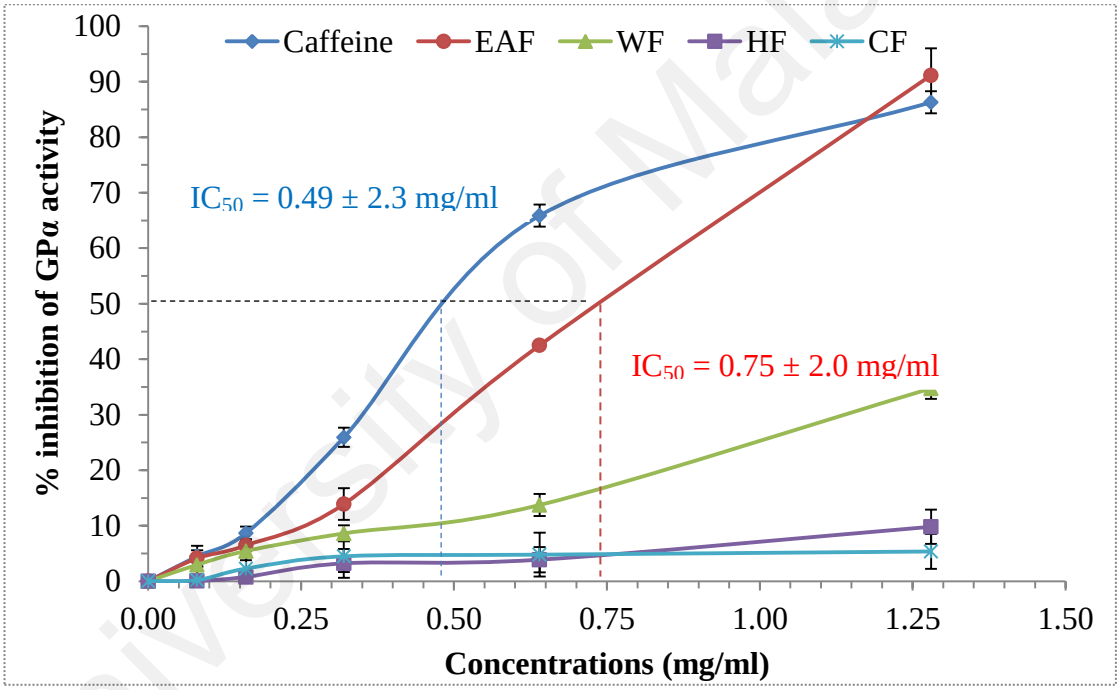


Figure 4.3: Inhibition of GPα activity by the fractions of *Brucea javanica* seed

HF: *n*-hexane fraction. CF: chloroform fraction. EAF: ethyl acetate fraction. WF: water fraction.

4.2.2 Screening Fractions for α-Glucosidase Inhibition

All fractions were tested for their inhibition of α-glucosidase. Figure 4.4 shows that the EAF is the most active inhibitor of α-glucosidase and it has an IC_{50} = 483.93 ± 0.2 µg/ml, while HF, CF, and WF showed maximal inhibition of 17.07%, 9.89%, and 2.53% at the highest concentration tested, respectively.

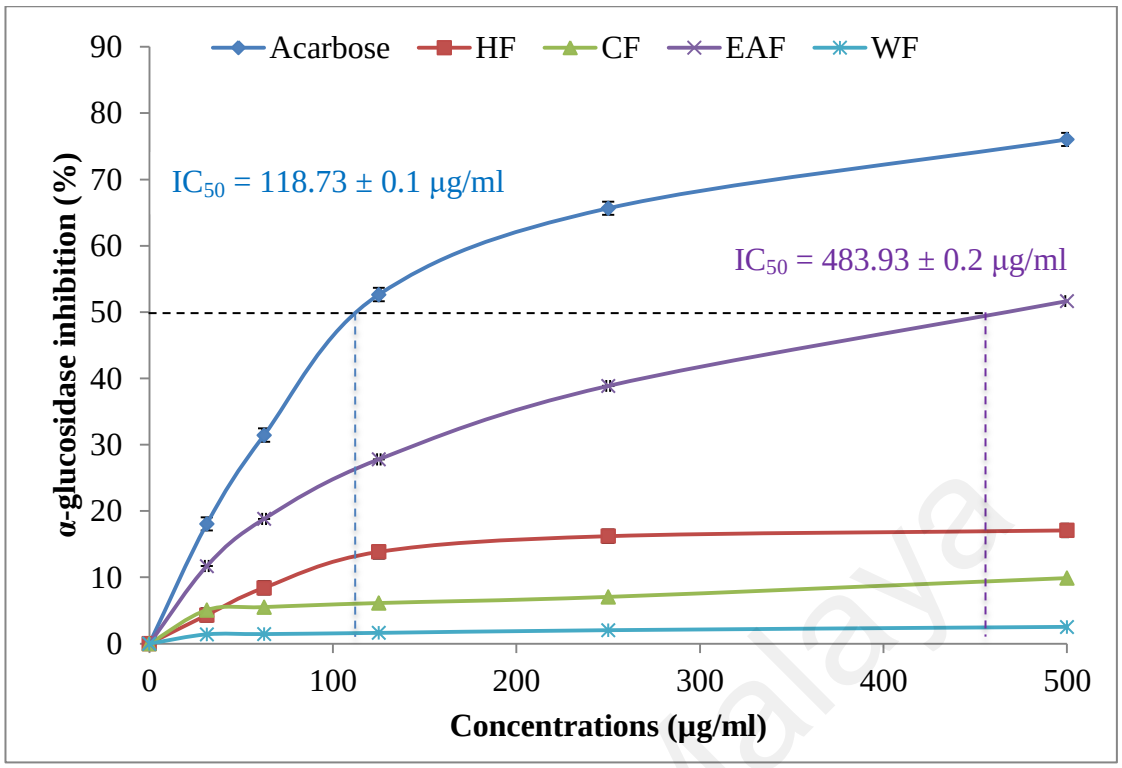


Figure 4.4: Inhibition of α-glucosidase activity by the fractions of BJS

HF: *n*-hexane fraction. CF: chloroform fraction. EAF: ethyl acetate fraction. WF: water fraction. BJS: *Brucea javanica* seed

4.2.3 GPα and α-Glucosidase Inhibition by Isolated Compounds

Seven known compounds were isolated from the EAF of BJS by various chromatographic methods and were tested for their inhibition of GPα and α-glucosidase activity *in vitro* as described in detail in materials and methods. They were each tested at various concentrations up 2000 µg/ml for GPα inhibition assay and were tested at the concentrations up to 150 µg/ml except luteolin which was tested at 150 µg/ml for α-glucosidase inhibition. The results are converted to µM and are reported as mean ± SE from triplicate test. Luteolin was identifies as the most potent inhibitor for both GPα and α-glucosidase and Table 4.4 shows that its IC_{50} is 45.08 µM and 26.41 µM.

Table 4.4: GPα and α-glucosidase inhibition by isolated compounds

Compounds	**GPα (IC_{50} = µM)**	**α-glucosidase (IC_{50} = µM)**
Vanillic Acid	ND[1]	ND[1]
Bruceine D	ND[2]	ND[2]
Bruceine E	ND[2]	ND[2]
Para-hydroxybenzoic acid	357.88 ± 0.07	649.07 ± 0.29
Luteolin	45.08 ± 0.04	26.41 ± 0.04
Protocatechuic acid	297.37 ± 0.13	368.74 ± 0.13
Gallic acid	214.38 ± 0.12	277.04 ± 0.12
Acarbose	-	145.83 ± 0.03
Caffeine	457.34 ± 0.05	-

[1]ND: IC_{50} values were not determined at concentrations below 6 mM (α-glucosidase) and 12 mM (GPα).
[2]ND: IC_{50} values were not determined at concentrations below 2.5 mM (α-glucosidase) and 5 mM (GPα).

4.2.4 Antioxidant Activity of BJS Fractions

(i) Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP activity assay was performed in a manner described previously (Müller *et al.,* 2010) with some modification. All fractions were prepared at the concentration of 1 mg/ml in 95% ethanol. The FRAP values of each fraction were calculated by putting absorbance of BJS fractions into the equation (y = 0.0016x) obtained from ferrous sulphate ($FeSO_4$) standard curve (Appendix5) and were reported as mmol Fe^{2+}/g extract. Figure 4.5 shows that the most potent fraction was EAF and it gives a FRAP value of 1.64 ± 0.1 mmol Fe^{2+}/g extract.

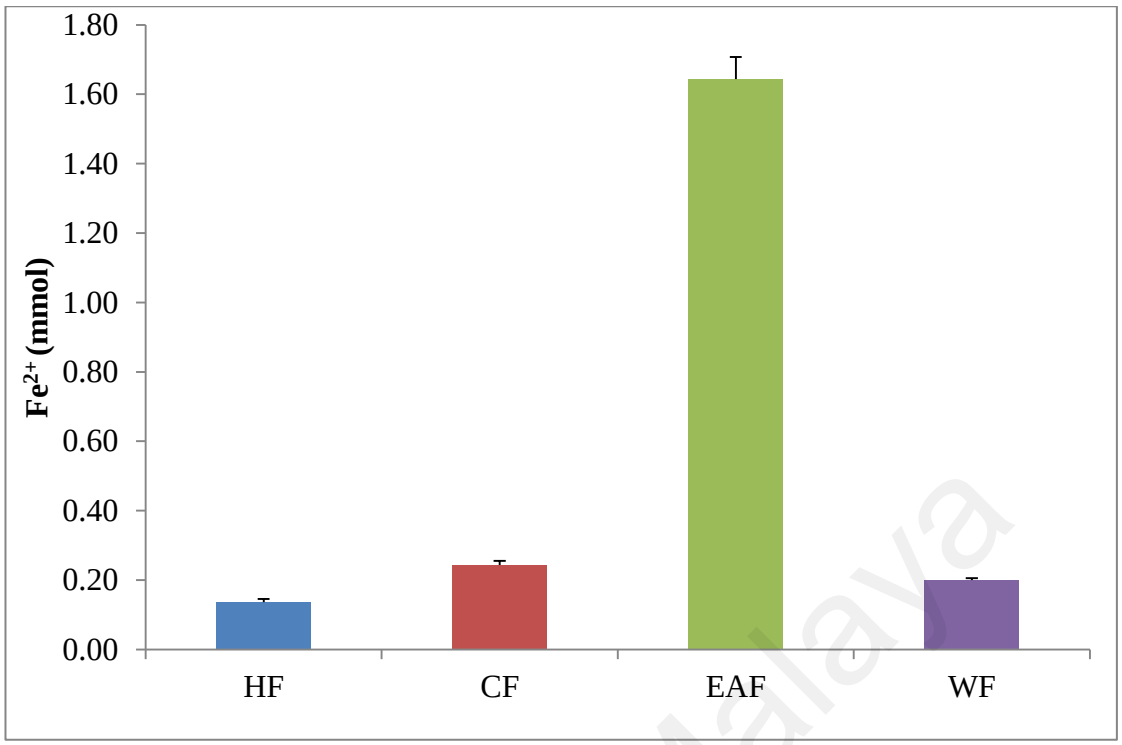


Figure 4.5: FRAP activity of BJS fractions

HF: *n*-hexane fraction; CF: chloroform fraction; EAF: ethyl acetate fraction; WF: water fraction; BJS: *Brucea javanica* seed.

(ii) DPPH Radical Scavenging Activity Assay

All fractions except EAF were tested at final concentrations from 66.67 to 333.33 µg/ml. Table 4.5 shows that WF has an IC_{50} = 184.6 ± 4.5 µg/ml, and CF and HF showed 33.9% and 22.2% inhibitions at the highest concentrations. The EAF was tested at final concentrations of 4.17, 8.33, 16.67, 33.33 and 66.67 µg/ml and determined to have an IC_{50} of 33.65 ± 3.0 µg/ml as shown in Table 4.5. The IC_{50} of the BHA was shown 5.95 ± 3.5 µg/ml (Table 4.5).

(iii) Metal Chelating Activity Assay

The assay was performed according to the method (Decker & Welch, 1990) as described in detail in materials and methods. Table 4.5 shows that the most active ferrous ion chelator was HF and it has an IC_{50} = 93.7 ± 5.9 µg/ml, and the IC_{50} values of the EAF and CF were determined to be 165.0 ± 1.6 and 314.5 ± 0.9 µg/ml. The standard drug EDTA-Na shows IC_{50} value of 10.5 ± 0.9 µg/ml as shown in Table 4.5.

(iv) Nitric Oxide Radical Scavenging Activity Assay

The assay was performed based on the method (Srivastava *et al.*, 2012) as described previously. The EAF was identified as a most active nitric oxide scavenger among the fractions tested and was determined to have an IC_{50} of 86.2 ± 3.8 µg/ml as shown in Table 4.5, and the IC_{50} of the standard drug curcumin was determined to be 9.5 ± 2.0 µg/ml (Table 4.5).

Table 4.5: DPPH and NO radicals and metal chelating activities of BJS fractions

Samples/standards	DPPH (IC_{50} µg/ml)	Metal chelating (IC_{50} µg/ml)	NO (IC_{50} µg/ml)
HF	ND	93.7 ± 5.9	ND
CF	ND	314.5 ± 0.9	ND
EAF	33.65 ± 3.0	165.0 ± 1.6	86.2 ± 3.8
WF	184.6 ± 4.5	ND	ND
BHA	5.95 ± 3.5	-	-
EDTA-Na	-	10.5 ± 0.9	-
Curcumin	-	-	9.5 ± 2.0

NO: nitric oxide. HF: *n*-hexane fraction. CF: chloroform fraction. EAF: ethyl acetate fraction. WF: water fraction. BJS: *Brucea javanica* seed. ND: not detected.

(v) Superoxide Radical Scavenging Activity Assay

The assay was performed according to the method as described in detail in materials and methods. The EAF was identified as the most active superoxide radical scavenger compared with other fractions and determined to have an IC_{50} of 251.7 ± 3.0 µg/ml, and the IC_{50} of the standard quercetin was determined to be 125.3 ± 4.4 µg/ml as shown in Figure 4.6.

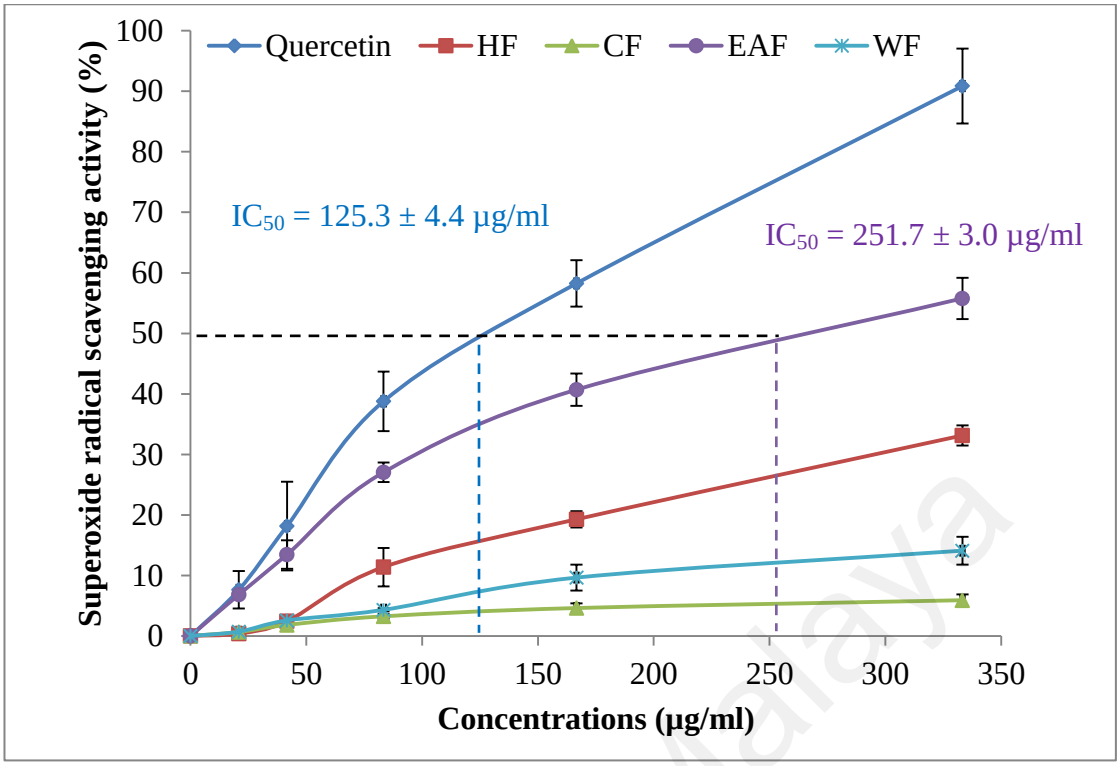


Figure 4.6: Superoxide radical scavenging activity of BJS fractions

HF: *n*-hexane fraction. CF: chloroform fraction. EAF: ethyl acetate fraction. WF: water fraction. BJS: *Brucea javanica* seed.

4.2.5 Determination of Antioxidant Activity of Isolated Compounds

For the evaluation of antioxidant effect of BJS, all isolated compounds were tested for their ability to scavenge nitric oxide (NO) and superoxide radicals *in vitro* as described in detail in materials and methods. Among them, only luteolin and gallic acid exerted NO radical scavenging activity at the concentrations tested and Table 4.5 shows that they have IC_{50} values of 425.2 and 772.4 µM. In superoxide radical scavenging assay, protocatechuic acid demonstrated the highest radical scavenging activity and determined to have an IC_{50} value of 469.5 µM. Other two compounds gallic acid and luteolin also exerted superoxide radical scavenging activity and their IC_{50} values were determined to be 591.4 and 690.3 µM (Table 4.6).

Table 4.6: Scavenging NO and superoxide radicals by isolated compounds

Compounds	Nitric oxide (IC_{50} = µM)	Superoxide (IC_{50} = µM)
Vanillic Acid	ND	ND
Bruceine D	ND	ND
Bruceine E	ND	ND
Para-hydroxybenzoic acid	ND	ND
Luteolin	425.2 ± 0.5	690.3 ± 0.6
Protocatechuic acid	ND	469.5 ± 0.7
Gallic acid	772.4 ± 0.5	591.4 ± 0.2
Curcumin	15.5 ± 0.3	-
Quercetin	-	459.5 ± 0.3

ND: IC_{50} values were not determined. NO: nitric oxide

4.3 Biological Assays Using *in vivo* Models

4.3.1 Oral Acute Toxicity

The SD rats in both sexes fasted for 16 h were used in acute toxicity test. The EAF fraction was tested at concentrations of 50, 125, 250 and 300 mg/kg body weight and observed for 14 days. At the dose of 125 mg/kg body weight or lower, EAF did not show any toxic signs or lethality. Therefore, the dose of 125 mg/kg body weight was selected as maximum dose for further study.

4.3.2 Oral Glucose Tolerance Test in Non-Diabetic Rats

The non-diabetic SD rats that fasted for 16 h were used in dose selection study as described in detail in materials and methods. Table 4.7 shows fasting glucose concentrations for control, EAF from BJS and glibenclamide treated rats during OGTT. The difference between the two groups at corresponding time was statistically significant as determined by student t-test using SPSS software. The glucose AUC for control, EAF and glibenclamide treated groups was calculated by trapezoid rule and the

results are shown in Figure 4.7. The difference in AUC between these groups was determined to be significant by ANOVA with Tukey's multiple-comparison test.

Table 4.7: Effect of EAF on OGTT in non-diabetic rats

Groups	Blood glucose level (mmol/L)				
	0 min	30 min	60 min	90 min	120 min
Group I	4.8 ± 0.2	10.8 ± 0.4	8.1 ± 0.6	6.9 ± 0.2	5.6 ± 0.2
Group II	4.7 ± 0.5	9.5 ± 1.1	8.3 ± 0.8	6.8 ± 0.5	5.8 ± 0.6
Group III	5.2 ± 0.4	8.7 ± 0.6*	7.1 ± 0.6	6.4 ± 0.7	5.5 ± 0.4
Group IV	5.4 ± 0.3	8.6 ± 0.7*	6.9 ± 0.8	6.3 ± 0.6	5.8 ± 0.4
Group V	5.2 ± 0.3	6.5 ± 0.5*	5.8 ± 0.7*	5.5 ± 0.3*	5.1 ± 0.2
Group VI	4.6 ± 0.2	6.4 ± 0.4*	5.5 ± 0.4*	4.9 ± 0.3*	4.3 ± 0.2*

The values are shown in mean ± SE (n = 6). Group I: normal control (0.5% CMC in water); Group II: treated with EAF (12.5 mg/kg); Group III: treated with EAF (25 mg/kg); Group IV: treated with EAF (50 mg/kg); Group V: treated with EAF (125 mg/kg); Group VI: treated with oral antidiabetic drug glibenclamide (10 mg/kg); All groups orally received glucose (2.0 g/kg) at 30 minutes after treatment. *p < 0.05, compared with normal control values at the corresponding time.

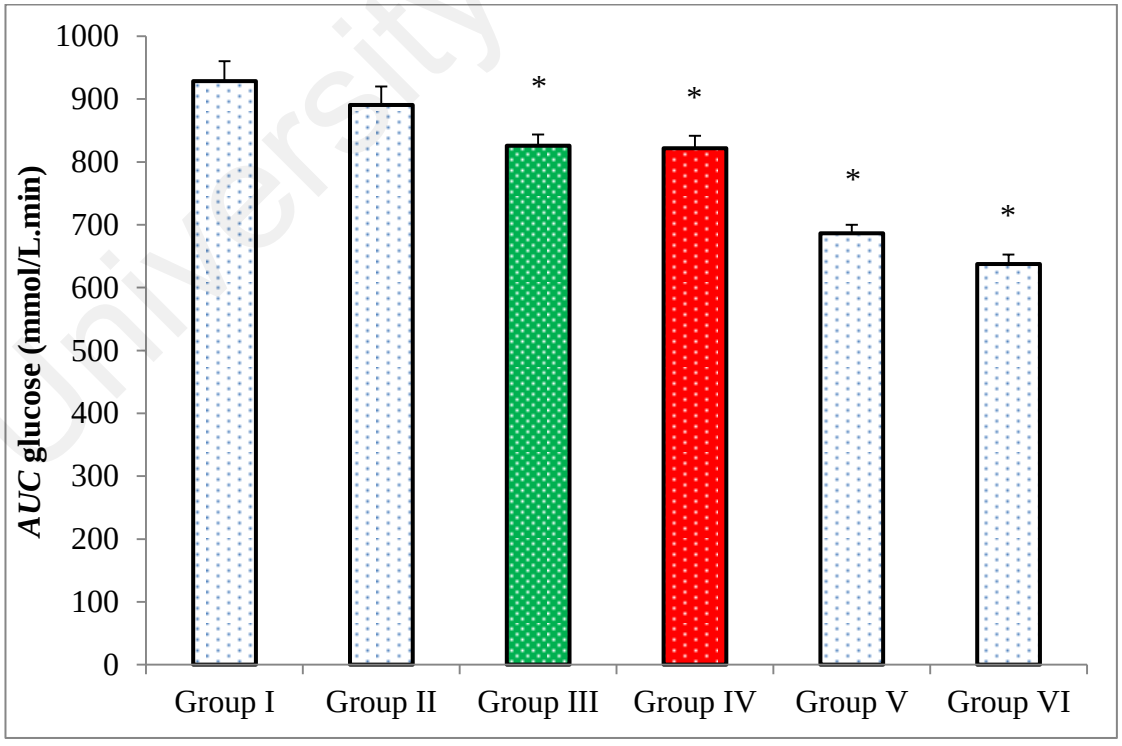


Figure 4.7: AUC for glucose tolerance in non-diabetic rats

Group I: normal control (0.5% CMC in water); Group II: treated with EAF (12.5 mg/kg); Group III: treated with EAF (25 mg/kg); Group IV: treated with EAF (50 mg/kg); Group V: treated with EAF (125 mg/kg); Group VI: treated with oral antidiabetic drug glibenclamide (10 mg/kg).

4.3.3 Induction of Type 2 Diabetes (T2D)

The SD rats were given STZ (60 mg/kg) 15 min after NA (100 mg/kg) injection. Three weeks after NA-STZ treatment, the rats having blood glucose level between 10.8 and 21.4 mmol/L were selected for study.

4.3.4 Measurement of Fasting Blood Glucose Levels

The rats in diabetic control groups had significantly ($p < 0.05$) higher fasting blood glucose levels compared with non-diabetic control groups. The diabetic rats fed with EAF at the concentrations of 25 and 50 mg/kg b.w. and glibenclamide (10 mg/kg b.w.) showed a significant ($p < 0.05$) decrease in fasting blood glucose levels compared to their corresponding 0 days (Table 4.8).

Table 4.8: Effect of EAF on fasting blood glucose levels in T2D rats

Groups	Fasting blood glucose level (mmol/L)				
	0 Day	Day 7	Day 14	Day 21	Day 28
NDC	4.4 ± 0.3	4.5 ± 0.4	4.3 ± 0.2	4.2 ± 0.1	4.2 ± 0.1
DC	12.8 ± 1.0	13.9 ± 0.8	16.2 ± 1.2	19.3 ± 1.5	22.3 ± 2.0*
D + EAF25	13.4 ± 1.0	13.3 ± 1.1	12.1 ± 1.7	11.0 ± 1.6	9.4 ± 1.1*
D + EAF50	14.3 ± 0.9	12.7 ± 0.7	11.0 ± 0.6	8.8 ± 0.7	7.9 ± 1.2*
D + GLI	16.9 ± 1.6	17.6 ± 2.1	13.0 ± 0.7	11.8 ± 1.3	9.2 ± 0.7*

The results are expressed as mean ± SE ($n = 6$). NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$, *Compared with 0 day.

4.3.5 Measurement of Body Weights

The body weights of non-diabetic control rats were significantly increased, whereas diabetic rats exhibited significant decrease in their body weights due to STZ

toxicity compared to their initial days. The diabetic rats treated with EAF and GLI exhibited no difference in body weight after four weeks of treatment when compared with their respective 0 days (Table 4.9).

Table 4.9: Effect of EAF on body weight in T2D rats

Groups	Body Weight (g)				
	0 Day	Day 7	Day 14	Day 21	Day 28
NDC	214.0 ± 10.8	236.8 ± 7.4	259.5 ± 7.7	272.2 ± 8.7	285.2 ± 8.8*
DC	232.7 ± 7.4	220.8 ± 7.6	215.3 ± 7.2	197.2 ± 9.7	188.7 ± 7.5*
D+EAF25	215.0 ± 9.9	207.0 ± 10.2	203.7 ± 10.4	198.2 ± 10.8	201.0 ± 9.9
D+EAF50	222.8 ± 10.9	218.8 ± 12.4	216.8 ± 12.7	216.7 ± 12.5	217.8 ± 12.0
D + GLI	211.0 ± 6.2	203.5 ± 8.9	205.8 ± 7.3	206.0 ± 6.6	209.3 ± 7.9

The results are expressed as mean ± SE (n = 6). NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$, *Compared with 0 day.

4.3.6 Oral Glucose Tolerance Test in T2D Rats

At the end of 4-week study, the rats were fasted overnight and glucose tolerance test was carried out as described in detail in materials and methods. Figure 4.8 shows effect of EAF on glucose tolerance and AUC for glucose (Figure 4.9) during OGTT.

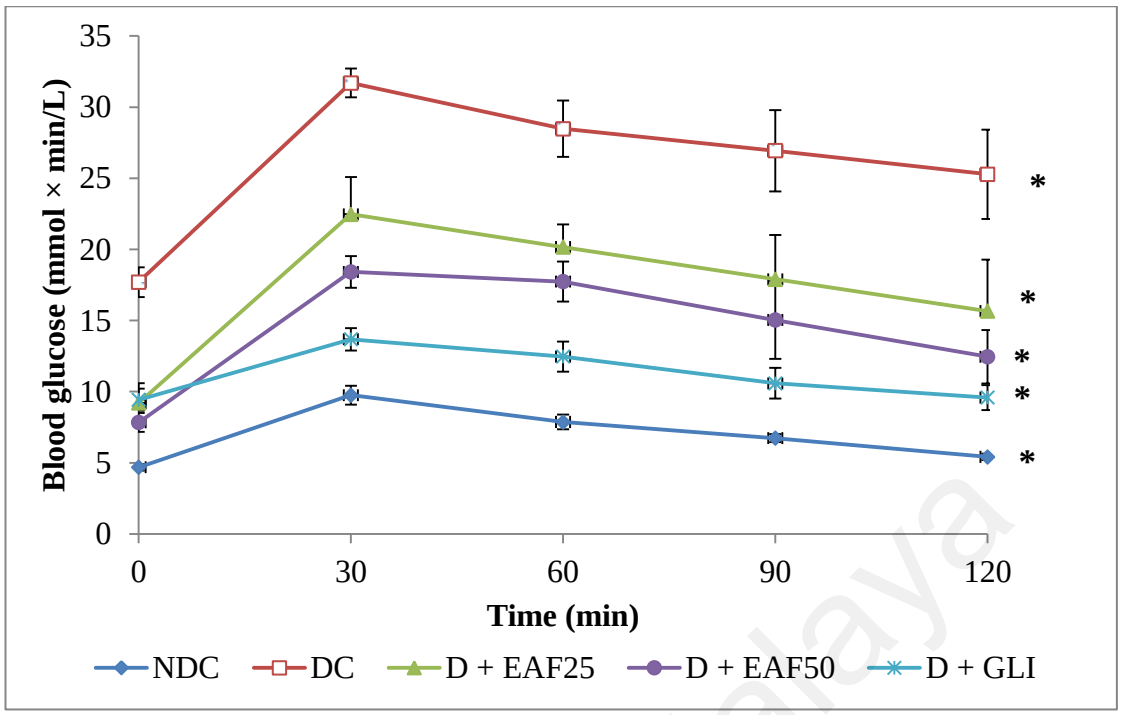


Figure 4.8: Effect of EAF on OGGT in experimental rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results represent the mean ± SE for 6 rats in each group.

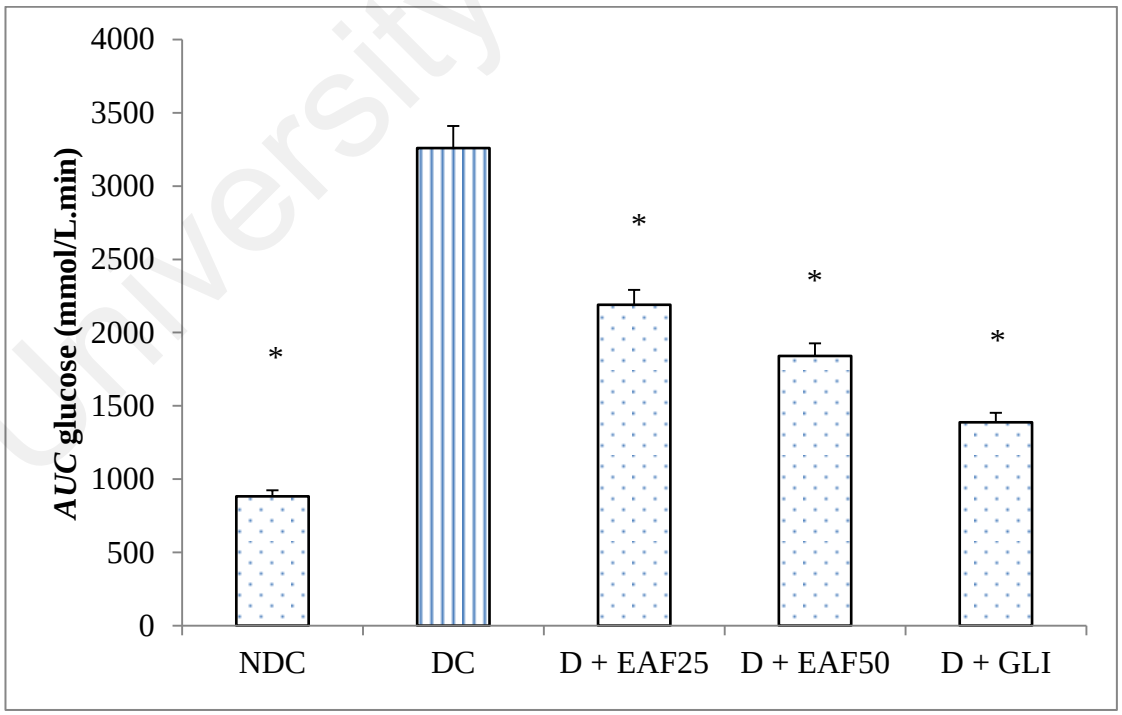


Figure 4.9: Area under the curve ($AUC_{glucose}$) for 0–120 min after glucose load

The results represent the mean ± SE for 6 rats in each group. * $P < 0.001$ compared with diabetic control group. NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w.

4.3.7 Effects of EAF on Serum Insulin Levels

The determination of insulin was performed on rat serum samples after 4-week study using rat insulin ELISA as described in the kit. Amount of insulin was quantified by using rat insulin standard curve (Figure 4.10) and the results were expressed as pmol/L insulin in the serum. Figure 4.11 showed that diabetic rats treated with EAF at the dose of 50 mg/kg b.w. had significantly higher insulin level compared with diabetic control group, while diabetic rats treated with EAF at the dose of 25 mg/kg b.w. and GLI showed a relative increase in their insulin levels, but significance was not achieved during 4-week study period compared with diabetic control group.

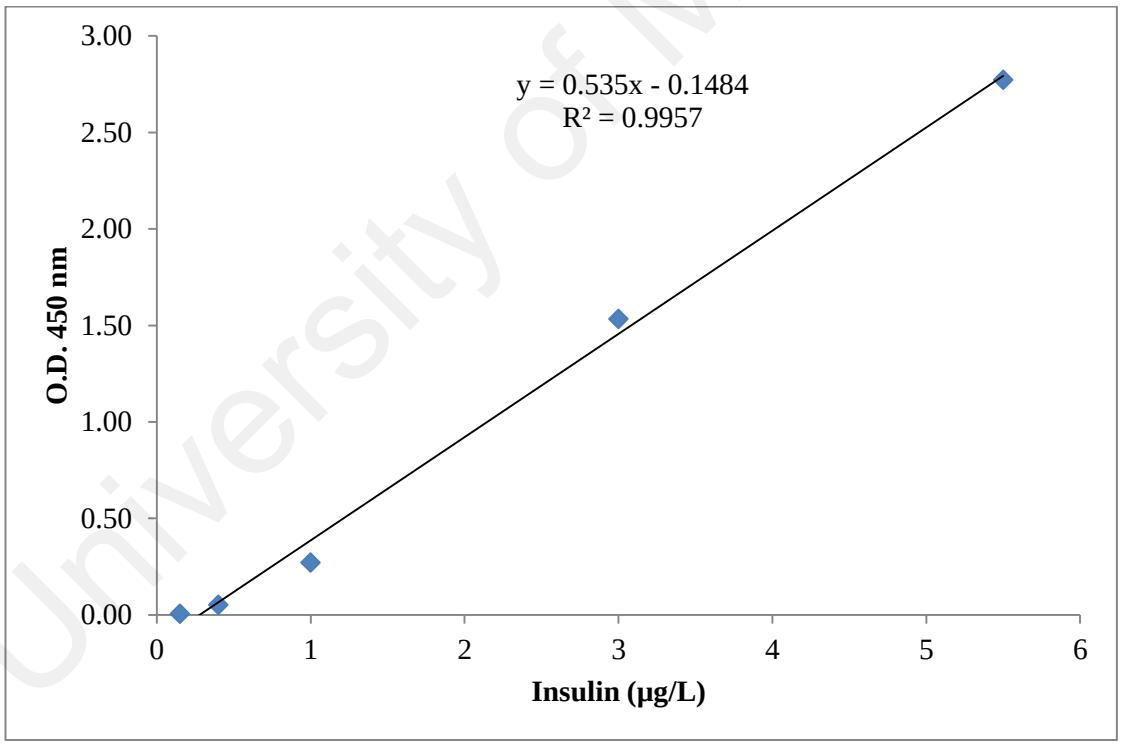


Figure 4.10: Rat insulin standard curve

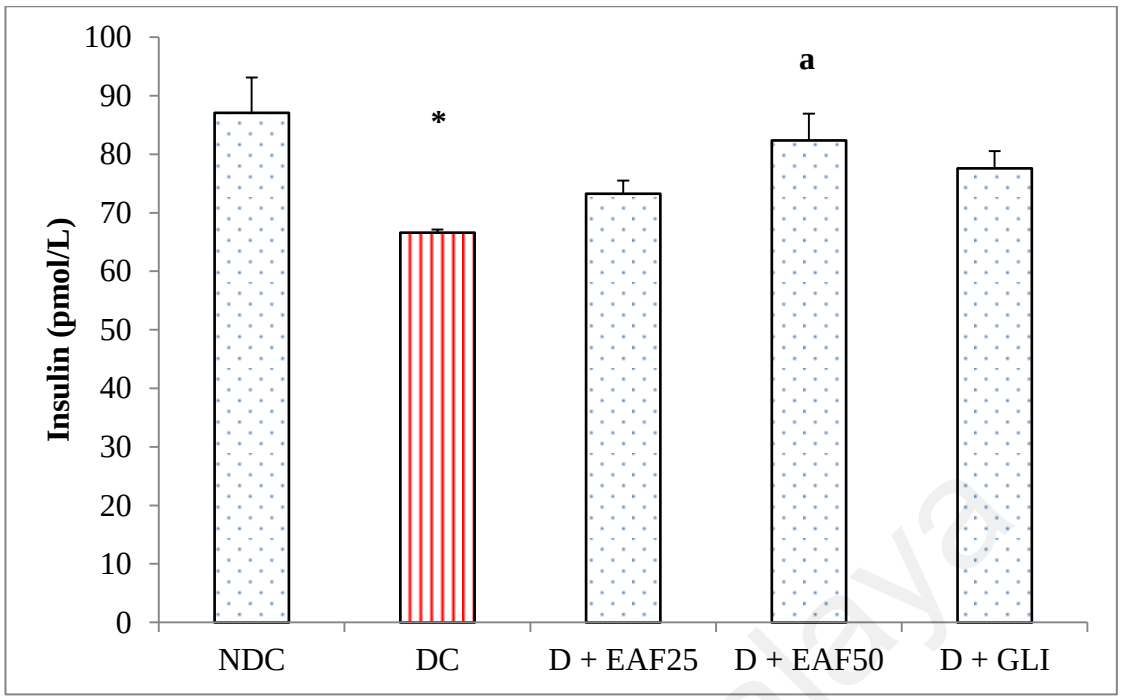


Figure 4.11: Effect of EAF on serum insulin levels in T2D rats

DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results represent the mean ± SE (n = 6). * compared with NDC group. [a] compared with DC group.

4.3.8 Effects of EAF on Serum Lipid Profiles

The lipid profiles were assessed at the end of study in all groups of experimental rats. A significant decrease in TG, TC and LDL levels, while significant increase in HDL levels were found in diabetic rats treated with EAF as well as GLI compared with diabetic control group as shown in Table 4.10.

Table 4.10: Effects of EAF on serum lipid profiles in T2D rats

Groups	Serum lipid profiles (mmol/L)			
	TG	TC	HDL	LDL
NDC	0.45 ± 0.07	1.20 ± 0.08	1.37 ± 0.02	0.52 ± 0.03
DC	0.93 ± 0.21*	1.55 ± 0.08*	0.47 ± 0.06*	0.87 ± 0.15*
D+EAF25	0.49 ± 0.01[a]	0.80 ± 0.09[b]	1.12 ± 0.05*[a]	0.44 ± 0.04[a]
D+EAF50	0.48 ± 0.04[a]	0.82 ± 0.06[b]	1.22 ± 0.04[a]	0.39 ± 0.03[a]
D + GLI	0.52 ± 0.09[a]	1.12 ± 0.05[a]	1.31 ± 0.08[a]	0.59 ± 0.04[a]

TG: triglyceride; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein. *Compared with NDC, [a] Compared with DC, [b] Compared with DC and NDC

4.3.9 Effects f EAF on Renal and Liver Functions

At the end of study, renal toxicity markers urea and creatinine, and liver toxicity markers such as ALP, ALT and AST were measured and the results were shown in Table 4.11.

Table 4.11: Effects of EAF on renal and liver functions in T2D rats

Groups	Urea (mmol/L)	Creatinine (µmol/L)	ALP(U/L)	ALT (U/L)	AST (U/L)
NDC	6.2 ± 0.6	29.2 ± 1.0	141.3 ± 16.1	45.5 ± 2.5	129.2 ± 8.9
DC	19.0 ± 2.7*	35.0 ± 2.1	319.3 ± 10.9*	172.7 ± 15.1*	290.0 ± 11.6*
D + EAF25	11.2 ± 1.3	30.5 ± 3.6	179.8 ± 19.8	81.2 ± 7.1*	159.5 ± 15.7
D + EAF50	9.5 ± 1.5	27.5 ± 1.1	159.2 ± 24.0	60.7 ± 3.4	145.3 ± 15.6
D + GLI	10.4 ± 1.8	29.0 ± 0.9	146.7 ± 10.5	58.3 ± 4.5	138.8 ± 8.3

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase. The results are presented the mean ± SE for 6 rats in each group. NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. *Compared with NDC.

4.3.10 Determination of Lipid Peroxidation

The MDA, a marker of oxidative stress, was determined in the serum of experimental rats using TBARS assay as described in detail in materials and methods. The MDA levels were quantified using MDA standard curve (Figure 4.12). The results are reported in µM as shown in Figure 4.13.

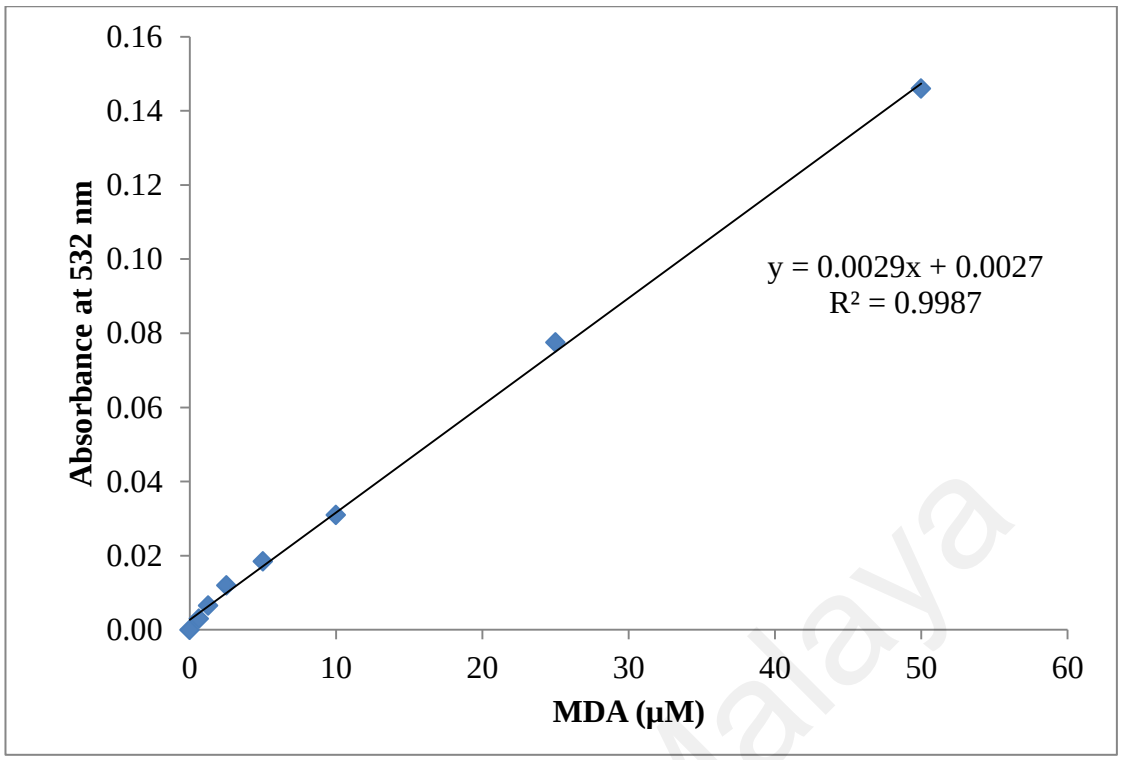


Figure 4.12: MDA standard curve

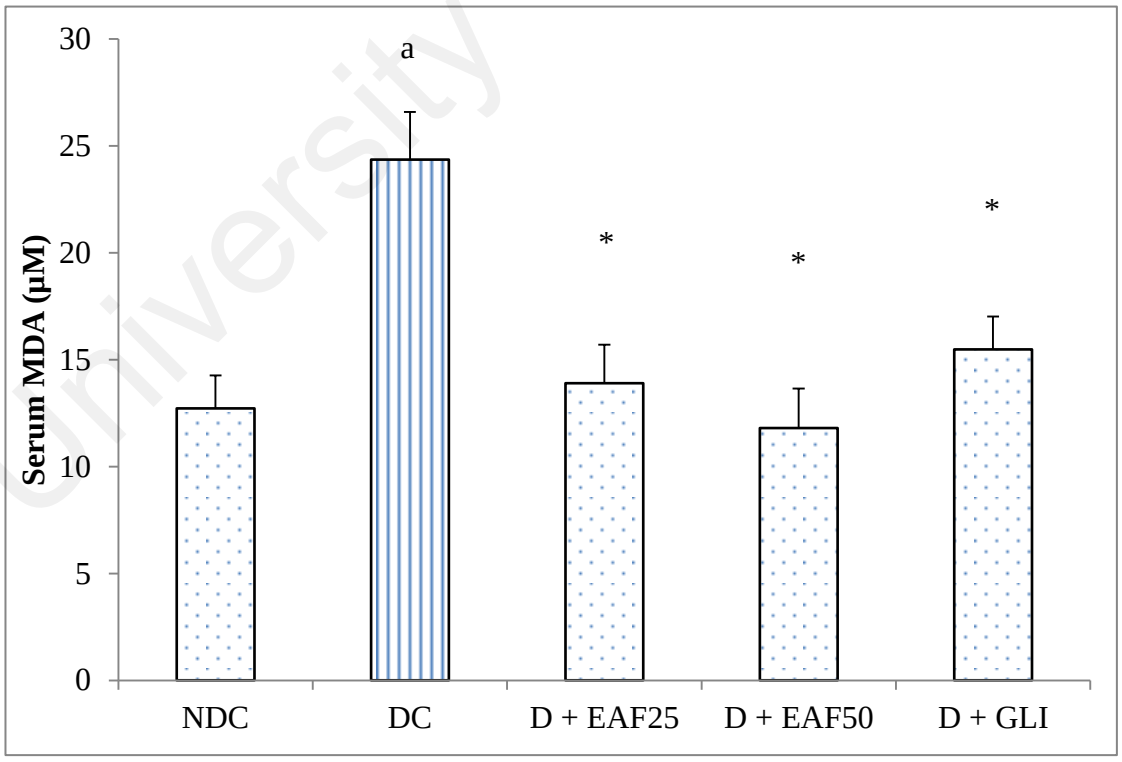


Figure 4.13: Effects of EAF on serum MDA levels in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, *Compared with DC.

4.3.12 Measurement of Glutathione

The GSH, a marker of antioxidant defense, was measured in the serum of experimental rats using glutathione assay kit as described in detail in materials and methods. The GSH levels were quantified using GSH standard curve (Figure 4.14). The results are reported as μM for serum as shown in Figure 4.15.

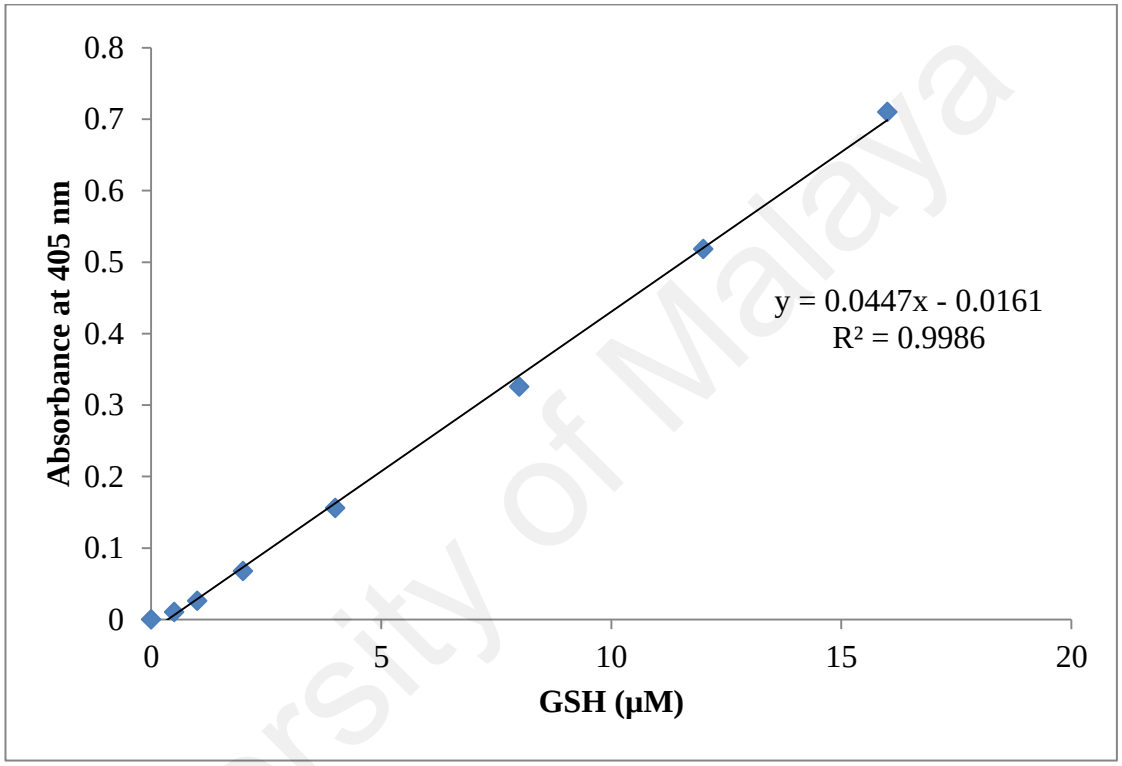


Figure 4.14: GSH standard curve

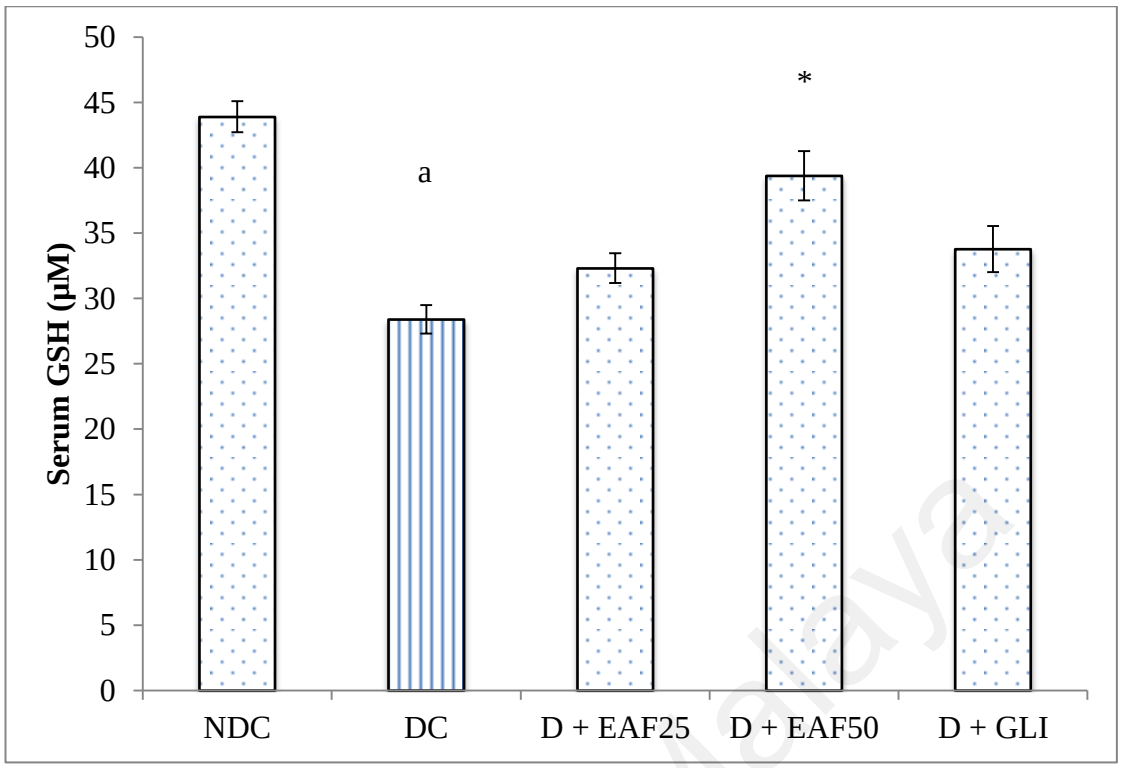


Figure 4.15: Effects of EAF on serum GSH levels in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, *Compared with DC.

4.3.13 Determination of Cytokines

The serum TNF-α, IL-6 and IL-1β levels were determined using colorimetric assay kits specific for rat TNF-α, rat IL-6 and rat IL-1β according to the manufacturer's instructions as described in detail in materials and methods. The concentrations of TNF-α, IL-6 and IL-1β were calculated by using standard curves (Figures 4.16, 4.18 and 4.20) and reported as pg/ml of TNF-α, IL-6 and IL-1β in the serum. The rats treated with EAF had a significant (p <0.05) decrease in TNF-α (Figure 4.17), IL-6 (Figure 4.19) and IL-1β (Figure 4.21) levels compared to the specific diabetic control groups.

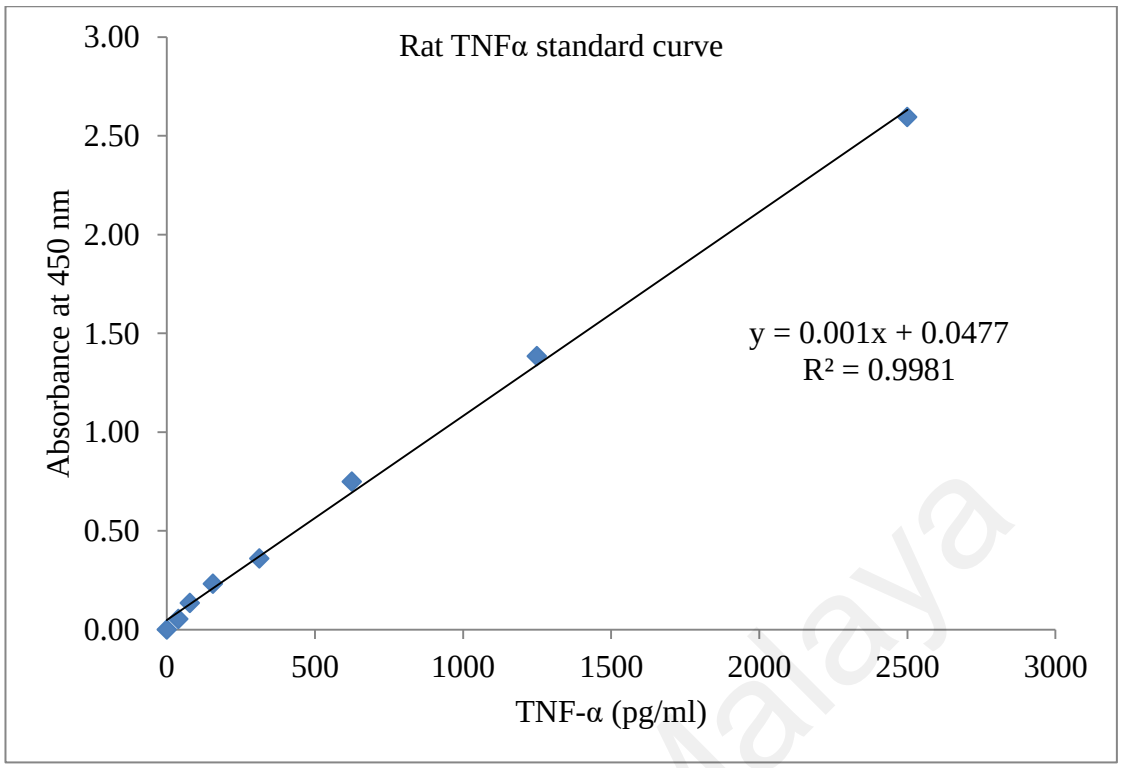


Figure 4.16: Rat TNF-α standard curve

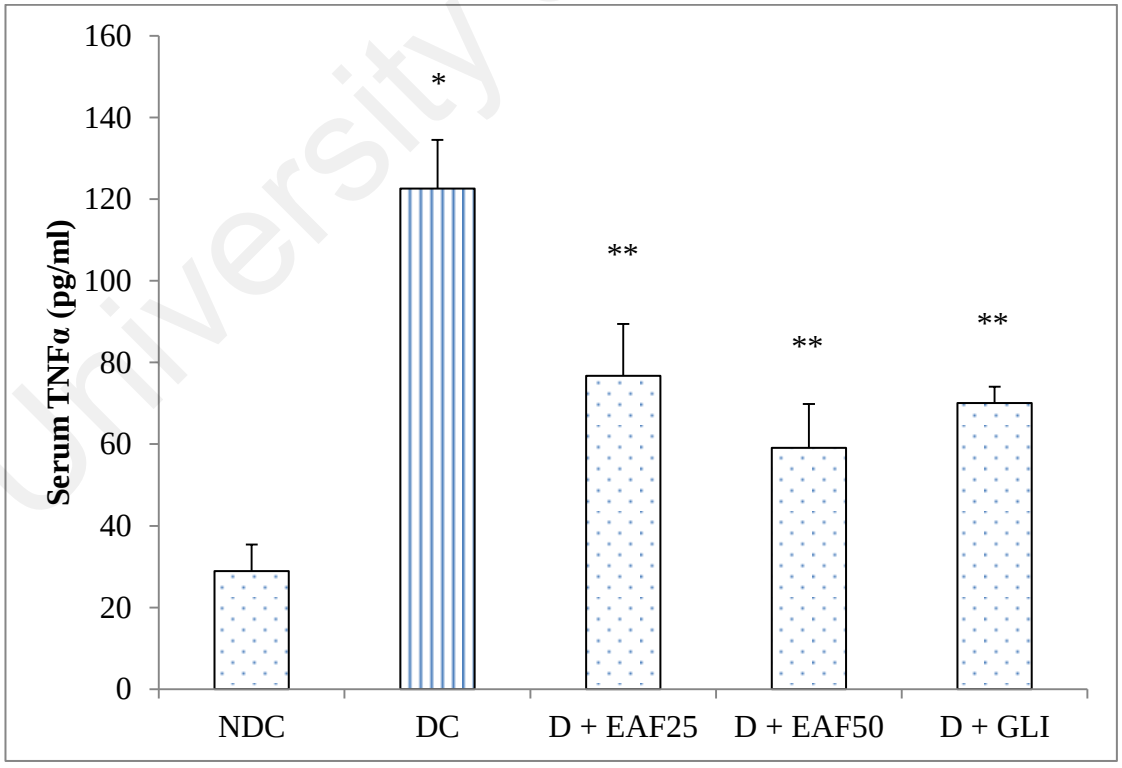


Figure 4.17: Effects of EAF on serum TNF-α level in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, **Compared with DC.

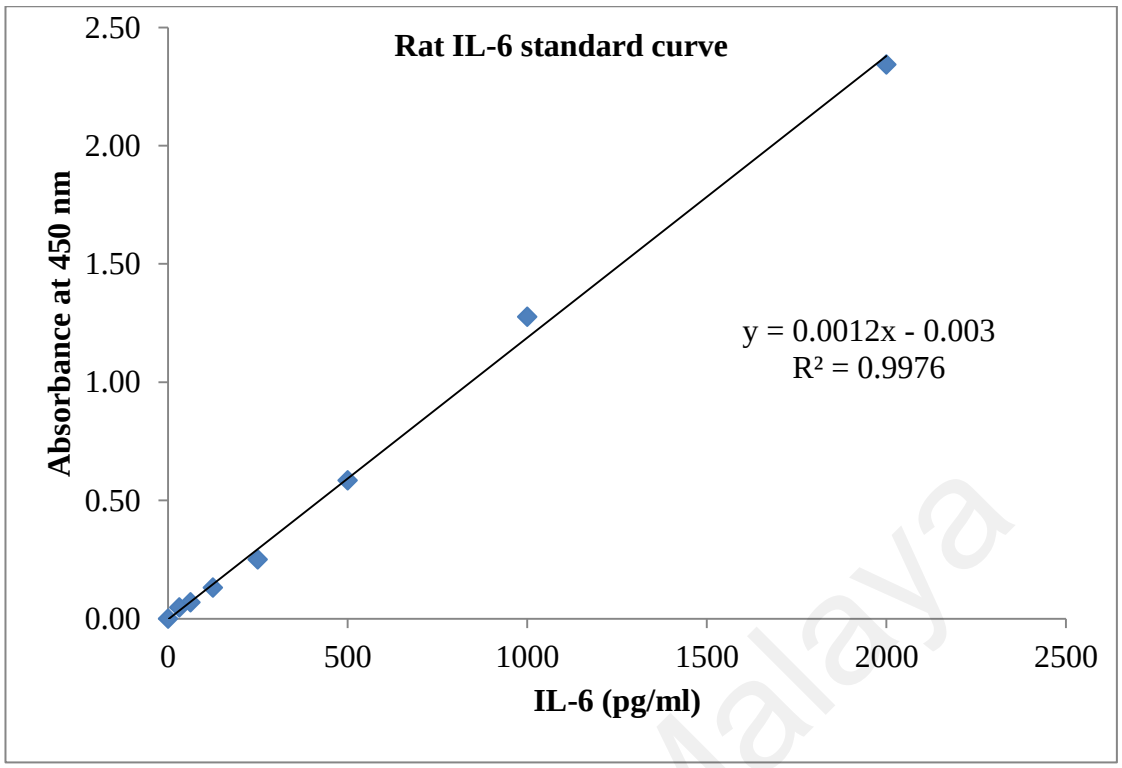


Figure 4.18: Rat IL-6 standard curve

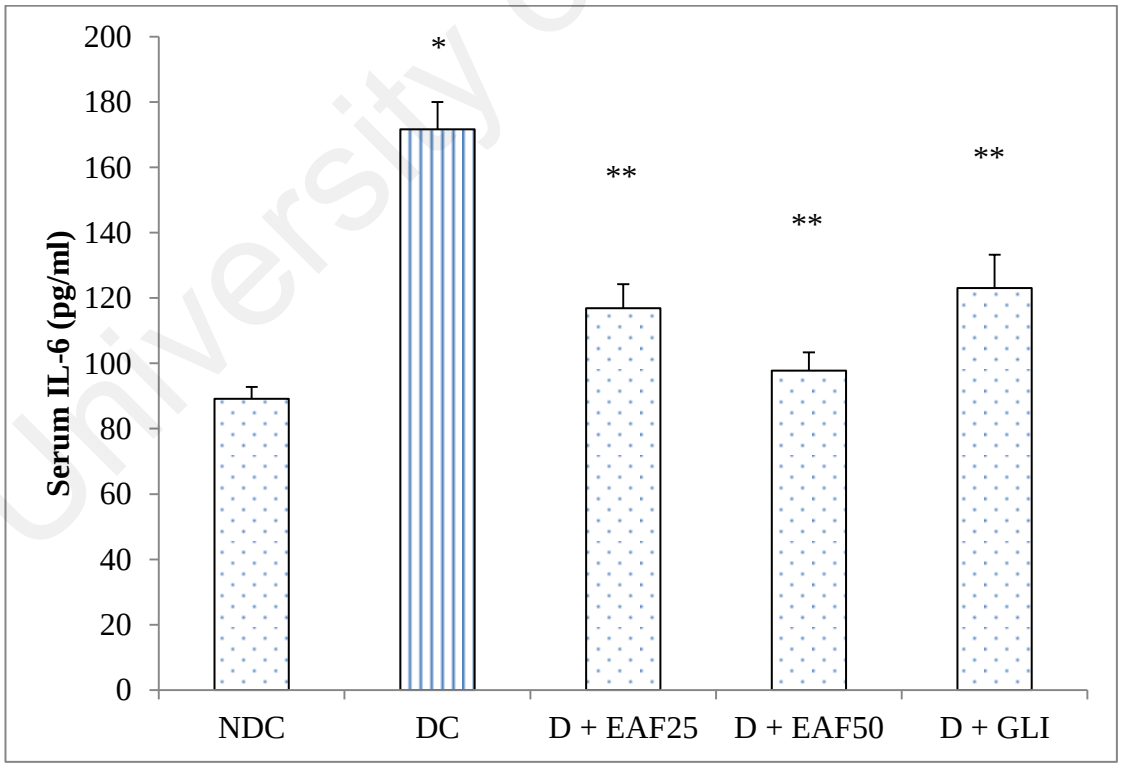


Figure 4.19: Effects of EAF on serum IL-6 level in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, **Compared with DC.

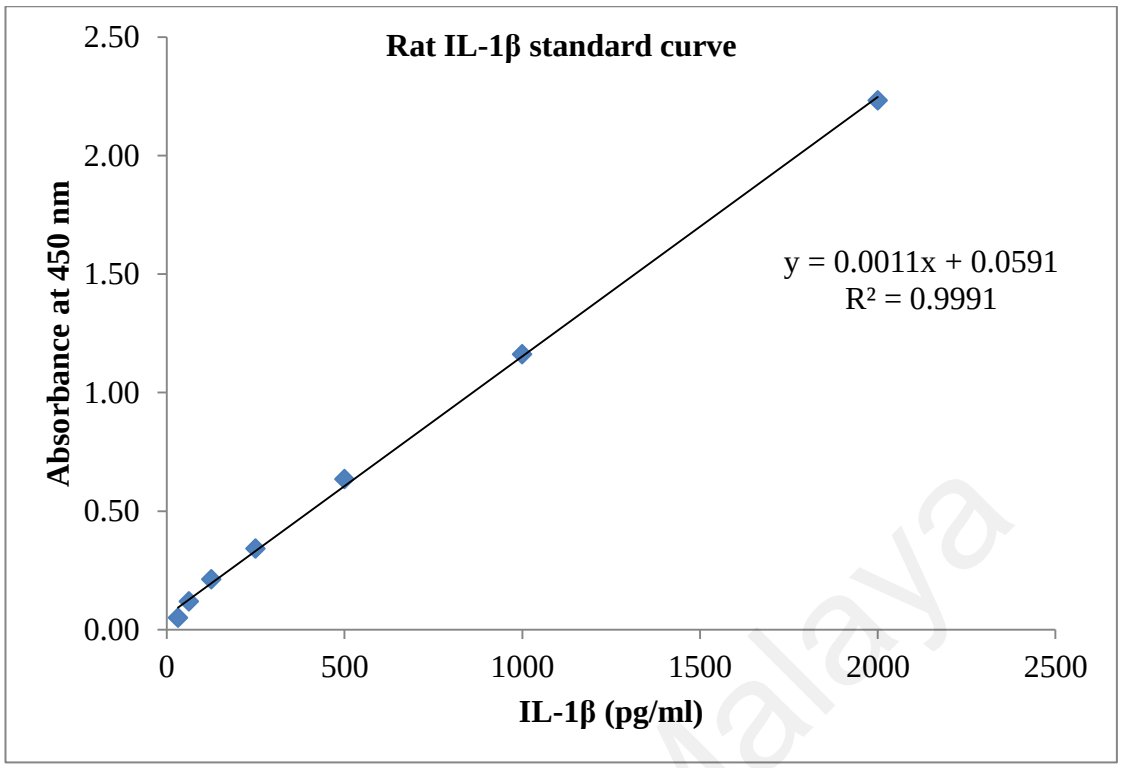


Figure 4.20: Rat IL-1β standard curve

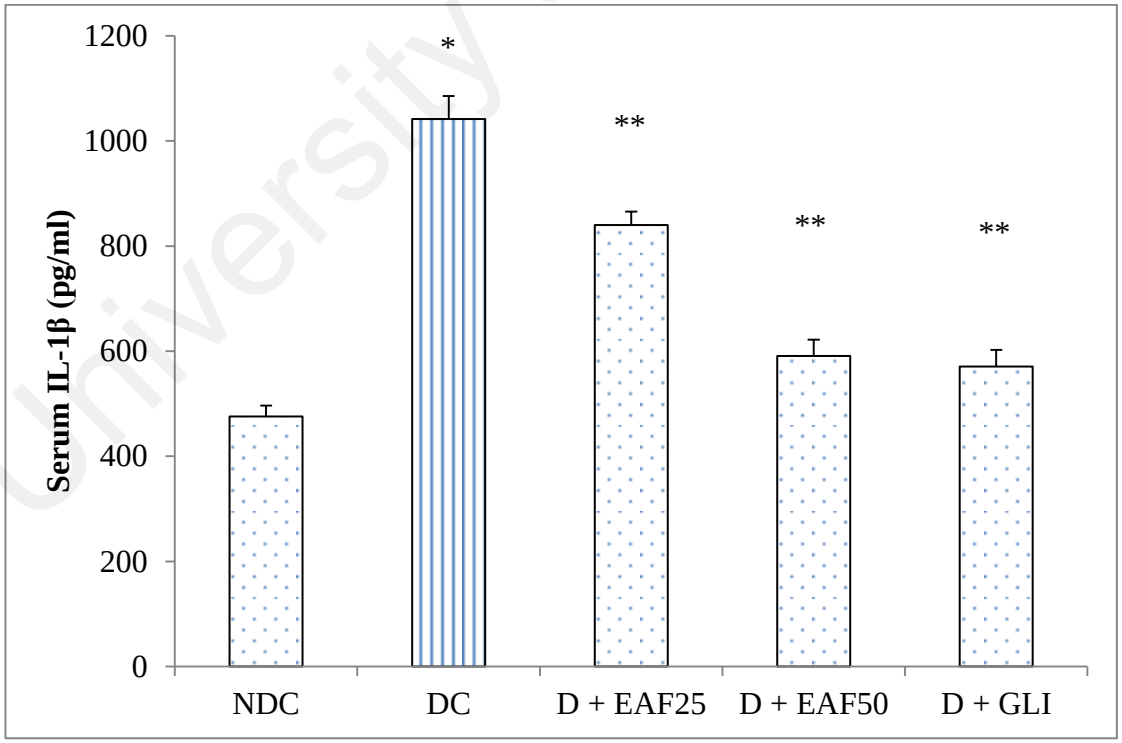


Figure 4.21: Effects of EAF on serum IL-1β level in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, **Compared with DC.

4.3.14 Measurement of Hepatic Glycogen Contents

The determination of glycogen was carried out on liver samples collected from experimental rats after 4-week study. The hepatic glycogen content was quantified using glycogen standard curve (Figure 4.22) and the results are reported as mg of glycogen/g tissue as shown in Figure 4.23.

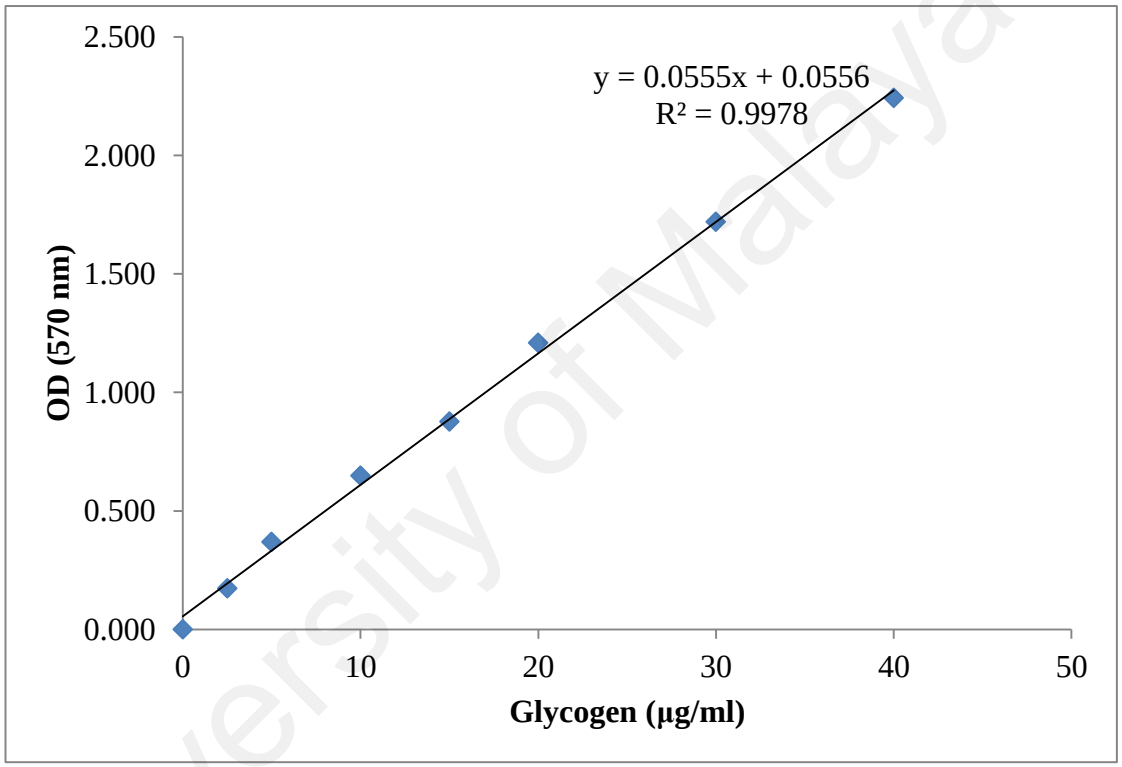


Figure 4.22: Glycogen standard curve

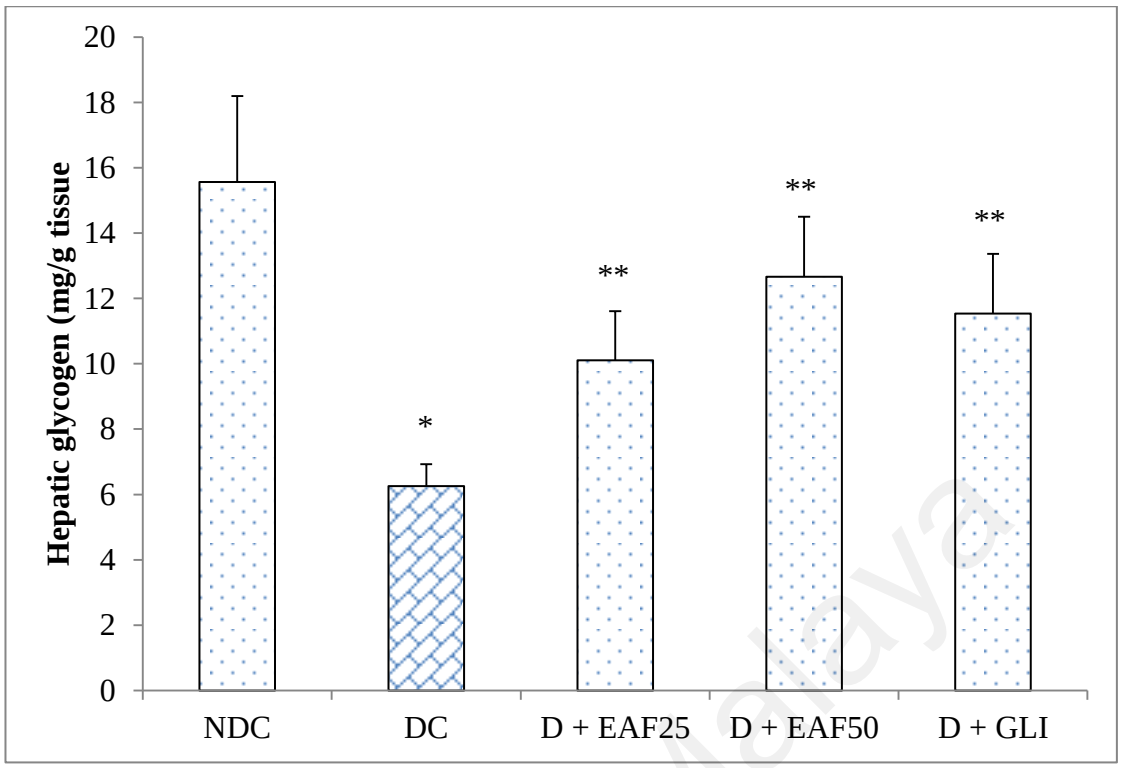


Figure 4.23: Effects of EAF on hepatic glycogen levels in T2D rats

NDC: non-diabetic control; DC: diabetic control, D + EAF25: diabetic rats treated with ethyl acetate fraction 25 mg/kg b.w.; D + EAF50: diabetic rats treated with ethyl acetate fraction 50 mg/kg b.w.; D + GLI: diabetic rats treated with glibenclamide 10 mg/kg b.w. The results are considered significant when $p < 0.05$. [a]Compared with NDC, **Compared with DC.

CHAPTER 5 : DISCUSSION

5.1 Phytochemistry Study

Brucea javanica seed (BJS) has been used to treat lipid disorders and diabetes by indigenous people in Malaysian Peninsular. Traditionally, it was taken orally by grinding 5 to 10 dried seeds daily. In the initial step of this study, BJS was extracted by maceration with 95% ethanol because this procedure was best suited for traditional preparation of this plant and it was also consideration of extracting extensive chemical compounds that may be found in the plant. In an attempt to separate out active compounds, a common liquid-liquid extraction technique was applied to fractionate a crude extract between water and organic solvents with increasing polarity. In phytochemical analysis, the highest amount of polyphenols was identified in ethyl acetate fraction (EAF), while the lowest yield of polyphenolic contents was detected in non-polar hexane fraction (HF). When comparing the yields of polyphenol contents between organic and aqueous medium, most of the polyphenols were extracted with organic solvent that has a high polarity, and amount of polyphenols were increased with the increasing polarity of the solvent. This is in agreement with previously reported result (Wang *et al.*, 2009) that polyphenolic compounds are best soluble in polar organic solvent than in aqueous medium.

In general, plant seeds contain various chemical compounds such as polyphenols, alkaloids, flavonoids, amino acids, polysaccharides, lipids, and proteins and so on. In the form of mixture, mostly lipids can be found in non-polar phase of solvent system, and proteins, amino acids, and other hydrophilic molecules such as polyphenols and polysaccharides were retained in polar phase (Wanasundara & Shahidi, 1994). Therefore, the diversity in extraction yields could be explained by a higher mass transfer between solvents employed due to hydrophobic interactions with water phase.

5.2 Biological Assays Using *in vitro* Models

Inhibition of glycogen phosphorylase α (GPα) is a novel approach to reducing blood glucose levels by inhibiting hepatic glucose output in patients with type 2 diabetes (T2D) (Agius, 2015). Inhibition of α-glucosidase is also an alternative approach to lowering blood glucose by suppressing the influx of glucose from intestinal tract to blood vessels in diabetes (Ieyama *et al.*, 2011). In this study, all fractions from BJS were tested for their inhibition of GPα and α-glucosidase under the assay conditions in colorimetric assays that are detailed in materials and methods. The results showed that the EAF fraction is the most potent inhibitor for both GPα and α-glucosidase enzymes compared to other fractions tested indicating an important clinical future of EAF of BJS in diabetes therapy. Oxidative stress plays a critical role in the development of diabetes complications including microvascular and macrovascular complications and pancreatic β-cell dysfunction (Giacco & Brownlee, 2010). Antioxidant therapy prevents these effects and exerts beneficial effects for controlling of T2D (Kajimoto *et al.*, 2004). Therefore, to determine antioxidant activity of BJS, all fractions were evaluated by several antioxidant assays including DPPH, FRAP, metal ion chelating, nitric oxide, and superoxide radical scavenging activity assays. The results showed that the EAF exhibited potent antioxidant activity to DPPH, FRAP, nitric oxide and superoxide except for against metal chelating activity compared with other fractions tested. The EAF followed by water fraction (WF) exhibited higher scavenging activities on DPPH radicals, but both of them exerted 5.5-fold and 31-fold less DPPH radical scavenging activity compared to standard antioxidant compound BHA. Chloroform fraction (CF) along with HF has almost no inhibition.

The EAF gave high FRAP value, while other fractions exhibited little or no reducing power activity. The EAF was also found the most active radical scavenger of both nitric oxide and superoxide. When comparing with specific reference compounds,

it showed 9-fold less potency than that of curcumin in nitric oxide and 2-fold less potency than that of quercetin in superoxide radical scavenging assays. The HF, CF, and WF exhibited less than 40% scavenging potency in both nitric oxide and superoxide assays. The HF, which contained significantly lower polyphenol contents than that of other fractions, was identified as the most potent ferrous ion chelator, whereas, the EA and CF are less active. When compared to standard metal chelator EDTA-Na_2, all of them showed low chelating potency.

Inhibition of GPα and α-glucosidase enzymes by polyphenols from plants as well as isolated compounds and correlation between polyphenolic content, enzyme inhibition and antioxidant potential have been studied extensively (Apostolidis *et al.*, 2011; Roy *et al.*, 2011). In this study, strong correlations were observed between polyphenolic contents and GPα and α-glucosidase inhibition activities, suggesting that polyphenols are most likely responsible for GPα and α-glucosidase inhibitory activities of BJS. In antioxidant assays, the ferrous ion chelating activity of the fractions of BJS did not correlated with its polyphenolic contents indicating that chemical components in BJS exert its antioxidant effects by scavenging free radicals rather than trapping ferrous ions. Based on initial screening, the EAF was the most active for inhibition of GPα and α-glucosidase, and also possess potent antioxidant efficiency among other fractions tested. Therefore, it was selected for evaluation of its hypoglycemic activity *in vivo* and isolation of chemical compounds that responsible for antidiabetic effects of BJS.

The chromatographic analysis of the active fraction EAF afforded seven known compounds; vanillic acid, bruceine D, bruceine E, *para*hydroxybenzoic acid, luteolin, protocatechuic acid and gallic acid. All isolated compounds from BJS were evaluated for inhibition of GPα and α-glucosidase and its antioxidant effects was also evaluated by nitric oxide and superoxide radical scavenging assays.

Luteolin exhibited strong inhibition potency to both GPα and α-glucosidase enzymes, where its GPα inhibition activity was first reported in this study. Luteolin has been shown in previous study (Yan *et al.*, 2014) to have α-glucosidase inhibition potency, but its effect is 6.6-fold less potent compared to this study. The deference between this result and other studies could be due to the techniques applied and nature of assay conditions used.

Gallic acid, protocatechuic acid and *para*-hydroxybenzoic acid which are phenolic derivatives of benzoic acid exhibited moderate to low inhibition activity against GPα and α-glucosidase by increasing number of hydroxyl substituent on the skeleton of benzoic acid, indicating hydroxyl substituent of these compounds determine their biological activity. Vanillic acid**,** which is a direct structural analogue of para-hydroxybenzoic acid without the methoxy group, exhibited little to no inhibition against GPα, and did not inhibit α-glucosidase at the concentrations tested up to 6 mM, suggested that the methoxy functionality in vanillic acid was influential on its biological effect. Both bruceine D and E isolated from EAF of BJS as major compounds exerted no inhibitory activity against GPα and α-glucosidase. Previously, antihyperglycemic activity of bruceine D and E have been reported (Noorshahida *et al.*, 2009), but possible mechanisms of action of these compounds are still unknown.

In antioxidant activity assays, luteolin and gallic acid exhibited scavenging efficiency against both nitric oxide and superoxide radicals, while protocatechuic acid exerted its potency only in superoxide radical scavenging assay. Several studies have been reported to have reactive oxygen species (ROS) scavenging activities of luteolin (Madhesh & Vaiyapuri, 2012; Naso *et al.*, 2016; Reddy *et al.*, 2016), gallic acid (Yilmaz & Toledo, 2004; Roidoung *et al.*, 2016), and protocatechuic acid (Varì *et al.*, 2011; Liu *et al.*, 2016; Erukainure *et al.*, 2017). Therefore, the results of the GPα and α-glucosidase inhibition and antioxidant activity studies strongly suggest that luteolin is

the major contributor for antidiabetic effect and antioxidant activity of BJS could be due to the synergistic effect of luteolin, protocatechuic acid, and gallic acid existed in BJS. Due to the poor yield of the most active compound luteolin, the EAF that contains luteolin was selected for further study in *in vivo* animal model.

5.3 Biological Assays Using *in vivo* Models

In the first step of *in vivo* study, the EAF was assessed for acute oral toxic effect in healthy SD rats and OECD-423 guidelines for the testing of chemicals (The Organization for Economic Co-operation and Development, 2001) was applied for evaluation. The results confirmed that the EAF treatment at the dose of 125 mg/kg body weight (b.w.) or lower to the SD rats did not produce any mortality or alter the behavioral patterns of the rats during the acute oral toxicity study. In previous studies, acute toxicity of bruceine D and bruceine E isolated from BJS were reported and it showed that bruceine D exhibited LD_{50} value of 31.86 mg/kg b.w. and bruceine E showed LD_{50} value of 3.52 mg/kg b.w. in mice when treated intraperitoneally (Noorshahida *et al.*, 2009).

In this study, about 45 mg of bruceine D in 0.59% yield and 56 mg of bruceine E in 0.74 % yield were isolated from 7.6 g of EAF of BJS. Thus, 125 mg of EAF contains about 0.74 mg of bruceine D and 0.93 mg of bruceine E. Therefore, concentrations of bruceine D and bruceine E in EAF in this study were much lower than that of reported toxic effect. Another difference in this study with previous one is that differences in animal models used and rout of delivery of compounds. In this study, EAF which contained bruceine D and E was delivered orally to the SD rats, but in other study (Noorshahida *et al.*, 2009), bruceine D and E were injected intraperitoneally to the mice. Therefore, some compounds including bruceine D and E in the EAF of BJS may have become less toxic as they go through the digestion process, causing a lower

concentration of these compounds to be absorbed. Through administration via injection, these compounds bypass the gut, so they are not broken down to a great extent, thus possibly providing close to their full potential action. Therefore, the EAF was non-toxic when treated at single dose of 125 mg/kg b.w. or lower to the SD rats.

Research on *in vivo* effects of BJS on hyperglycemia has not been thoroughly evaluated in previous studies. In the first step of this study, pharmacologically active dose was selected by OGTT in nondiabetic rats. The EAF of BJS at the dose of 25 mg/kg b.w. or higher showed significance in its ability to improve glucose tolerance, thus, seems to validate the use of BJS as antihyperglycemic agent in traditional medicine. This result coupled with the initial data, suggest that the glucose reducing ability of this plant may be due to at least in part to inhibition of GPα and α-glucosidase. Therefore, on this basis the doses of 25 and 50 mg/kg were selected for evaluation of antihyperglycemic effects of the EAF in diabetic rat model.

Diabetic rat model was made by intraperitoneal (*i.p*) injection of nicotinamide (NA) and STZ into SD rats. The reason for selecting combined injection of NA-STZ in rats is that the STZ causes hyperglycemia by selective destruction of β cells in the islets of Langerhans and nicotinamide protects β-cell death caused by STZ toxicity. Diabetic animal model induced by NA-STZ was extensively used for screening antidiabetic effect of chemicals and herbal products because it provides a very good model for type 2 diabetes which is most common to humans (Masiello *et al.*, 1998).

In this study, hypoglycemic effect of the EAF from BJS was evaluated NA-STZ induced T2D rats. The rats treated with NA-STZ exhibited severe diabetes and the fasting blood glucose levels were continuously increased during 4-week study. Fasting blood glucose levels were similar at initial day among diabetic control, EAF and glibenclamide treated rats and it was significantly increased in diabetic control rats

compared to the initial day. The EAF treatment at the dose of 25 and 50 mg/kg and glibenclamide were found to significantly reduce elevated fasting blood glucose levels in diabetic rats and reduction in glucose levels were 29.78%, 44.77% and 45.44%, respectively, compared with initial day of the respective groups. The EAF treatment also improved glucose tolerance in diabetic rats. Previous study reported that bruceine D and E are the active components of BJS and have antihyperglycemic effect (Noorshahida *et al.*, 2009). Thus, the results in this study evidenced that administration of EAF which contains bruceine D and E has antidiabetic effect, but possible mechanism of action of these compounds on hyperglycemia are still unknown.

In this study, both non-diabetic and diabetic control rats exhibited greater weight change and it was found that body weight was significantly increased in non-diabetic control rats while diabetic control rats demonstrated significant weight loss at the end of 4-week study compared to the initial day. The EAF treatment (25 and 50 mg/kg) displayed a slight decrease in body weight and no significant changes in body weight was observed compared to the initial day during study. To confirm the reduction of body weight in EAF treated rats ether caused by diabetes or consequence of EAF treatment, the kidney and liver toxicity markers in the serum were evaluated and compared with non-diabetic control. The EAF treatment exhibited significant decrease of these toxic markers in diabetic rats compared with non-diabetic control and its effect is similar compared with standard drug glibenclamide treated rats. This observation suggests that the EAF is safe when treated up to 50 mg/kg b.w to T2D rats.

It was found that the insulin level in diabetic rats was significantly decreased compared to the non-diabetic control group. The EAF treatment at the dose of 50 mg/kg resulted in a significant elevation in the insulin levels of diabetic rats during 4-week study and it showed 23.7% increase in the insulin levels of diabetic rats compared with diabetic control. The EAF at the dose of 25 mg/kg and glibenclamide have only 10.0%

and 16.5% increased insulin levels and failed to show significance compared to the diabetic control. These results suggest that the EAF are able to protect β-cell from glucose toxicity in a dose-dependent manner and increase amount of insulin by increasing β-cell mass.

In the current study, it was also found that the serum TG, TC and LDL levels are significantly high while serum HDL levels are significantly low in diabetic control rats compared with non-diabetic control. The EAF treatment at different doses (25 and 50 mg/kg) exhibited a significant decrease in TG, TC and LDL levels of diabetic rats while HDL levels were increased significantly. Interestingly, this study also showed that the administration of the EAF decreased TC levels of diabetic rats significantly lower than that of normal rats had despite differences in treatment doses and insulin levels compared to the non-diabetic control. It also showed that the EAF treatment at different doses have almost similar glycemic control. These observations suggest that the EAF has potent hypolipidimic effect in T2D rats, but possible mechanism which is responsible for reducing TC levels are unknown and no literature to date on how BJS effects hyperlipidemia have been reported. The reduction of TC by the EAF of BJS in this study may have an important clinical significance, since hyperlipidemia is often associated with T2D. Therefore, more studies need to be conducted to further clarify active components of BJS that responsible for hypolipidimic effect.

In this study, antioxidant effect of the EAF was also evaluated by measuring markers for oxidative stress and antioxidant defense in the serum of experimental rats. The diabetic control rats exhibited significantly higher serum MDA level compared to the nondiabetic control rats. The EAF treatment at the doses of 25 and 50 mg/kg for 4-week resulted in a significant reduction in the serum MDA levels of diabetic rats compared to the diabetic control rats and no significance in serum MDA levels was found in the EAF treated rats in comparison to the non-diabetic control, indicating that

the EAF treatment improves oxidative stress by preventing lipid peroxidation in T2D rats. The serum GSH levels were significantly increased in diabetic rats treated with the EAF at the dose of 50 mg/kg when compared with the diabetic control rats. The EAF treatment at the dose of 25 mg/kg and glibenclamide showed increase in serum GSH levels of diabetic rats but did not have any significance in its ability to improve antioxidant defense. This result indicate that the EAF prevent oxidative stress by improving antioxidant defense in T2D rats and it also showed more potent antioxidant effect compared to the standard drug glibenclamide. Several studies (Cai *et al.*, 2004; Choi & Hwang, 2005; Kim *et al.*, 2010) reported that BJS inhibit NFκB activity which induces pancreatic β-cell apoptosis by oxidative stress. It was also reported that BJS inhibit nuclear factor-kappa B (NFkB) activation (Kim *et al.*, 2010). Activation of NF_kB is known to be cause insulin resistance, β-cell apoptosis and cardiovascular complications in type 2 diabetes patients (Arkan *et al.*, 2005). The results in the current study supported by previous reports strongly suggest that the EAF treatment protect pancreatic β-cell apoptosis by antioxidant mechanism. Antioxidant mediated protection of β-cell mass could have a considerable effect on diabetes by preventing the progression of the disease and its related complications.

In this study, diabetic control rats showed a significant increase in serum concentration of proinflammatory cytokines TNFα, IL-6, and IL-1β compared with non-diabetic control. The EAF and glibenclamide treatment exhibited a significant reduction of these cytokines in diabetic rats compared with non-diabetic control. Previous study (Yang *et al.*, 2013) revealed that EAF of BJS exhibited anti-inflammatory effect through inhibition of proinflammatory cytokines generation in lipopolysaccharide-activated RAW 264.7 macrophage. Therefore, the results in current study indicate that the EAF has anti-inflammatory effect in T2D rats.

Hepatic glycogen was severely exhausted and almost 60 % of glycogen was degraded in diabetic control rats compared with non-diabetic control. The EAF treatment inhibited glycogen degradation and glycogen accumulation in the liver was improved in the EAF and glibenclamide treated diabetic rats in comparison to the diabetic control. Hepatic glycogen levels were positively correlated with amount of insulin secreted in the EAF treated rats. These results coupled with *in vitro* study suggest that EAF of BJS exerts its antihyperglycemic effects possibly due at least in part to inhibition of GPα, thus, reduce blood glucose level through inhibition of hepatic glucose output by glycogenolysis.

CHAPTER 6 : CONCLUSION

In this study, antihyperglycemic and antioxidant activities of BJS were evaluated *in vitro* and *in vivo*. Column chromatography and other separation methods, combined with GPα and α-glucosidase inhibition assays resulted in identification of luteolin as the most effective compound in the ethyl acetate fraction (EAF) of *Brucea javanica* seed (BJS), indicating that BJS may have a multiple mechanism of action, effects of GPα and α-glucosidase inhibitors. The major compounds Bruceine D and E isolated from the EAF of BJS did not show inhibition of either GPα or α-glucosidase, suggesting that it may exert its potency other than these mechanisms reported in this study. Thus, possible mechanisms of action of these compounds responsible for antihyperglycemic effect of BJS in previous study (Noorshahida *et al.*, 2009) remained unknown. Therefore, long term and more molecular based studies are required to further characterize the possible mechanisms of Bruceine D and E on hyperglycemia in diabetic animal models.

Animal study showed that the EAF treated diabetic rats exhibited a significant reduction in blood glucose levels compared with diabetic control and improved glucose tolerance in diabetic rats, indicating that the EAF of BJS has antihyperglycemic effect. The EAF treatment did not alter body weight or show toxic signs in diabetic rats compared to the diabetic control. These effects were further supported by measuring liver and renal function of experimental animals at the end of study. The results revealed that EAF treatment for 4-week showed normal liver and renal function suggesting that administration of EAF to these animals, at the doses and frequency used, is safe. The EAF treatment also exhibited a significant decrease in total cholesterol levels in diabetic rats compared to non-diabetic and diabetic control animals. This result strongly suggests that the EAF has hypolipidimic effect, but it is presently unclear how EAF reduced cholesterol levels in diabetic rats.

The study showed that the EAF treated diabetic rats at dose of 50 mg/kg has slightly higher insulin levels than that of standard drug glibenclamide treated diabetic rats and it also showed consistency in improved antioxidant defense on the EAF treated diabetic rats, indicating that the EAF has the effect of preserving β-cell to produce more insulin through antioxidant mechanism. This study also showed greater hepatic glycogen level in the EAF treated diabetic rats compared to the standard drug glibenclamide treated control, suggesting that the glycogenolysis was inhibited by the EAF in diabetic rats.

In overall, the EAF exhibited antihyperglycemic effect without causing weight gain and showed strong hypolipidimic effect in diabetic rats. These are highly desirable characteristics that current antidiabetic drugs have never achieved. Therefore, this study suggests that development of herbal supplement from the EAF of BJS has beneficial effects for the management of T2D. However, it remains unknown as to which component of BJS is responsible for its antihyperglycemic and hypolipidimic effects. In bioassay study, two beneficial mechanisms as inhibitors of GPα and α-glucosidase were discovered in a single compound luteolin isolated from the EAF of BJS. Further *in vivo* studies are needed to clearly identify the precise mechanisms of luteolin behind observed effects.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Publications

Ablat, A., Halabi, M. F., Mohamad, J., Hasnan, M. H. H., Hazni, H., Teh, S.-h., . . . Awang, K. (2017). Antidiabetic effects of *Brucea javanica* seeds in type 2 diabetic rats. *BMC Complementary and Alternative Medicine, 17*(1), 94.

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B. Papers presented in international conference

Ablat, A., Mohamad, J., & Awang, K. (2016). *Antidiabetic, anti-inflammatory, and antioxidant effects of Brucea javanica seeds in type 2 diabetic rats* Paper presented at the 21st Biological Science Graduate Congress Kuala Lumpur, Malaysia.

Ablat, A., Mohamad, J., & Awang, K. (2014). *Antidiabetic and antioxidant activities of Malaysian medicinal herb Brucea javanica seed*. Paper presented at the 18th Biological Sciences Graduate Congress, Kuala Lumpur, Malaysia.

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RESEARCH ARTICLE Open Access

Antidiabetic effects of *Brucea javanica* seeds in type 2 diabetic rats

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Abstract

Background: *Brucea javanica* (*B. javanica*) seeds, also known as "*Melada pahit*" in Indo-Malay region are traditionally used to treat diabetes. The objective of this study was to determine antidiabetic, antioxidant and anti-inflammatory effects of *B. javanica* seeds on nicotinamide (NA)-streptozotocin (STZ) induced type 2 diabetic (T2D) rats and to analyze its chemical composition that correlate with their pharmacological activities.

Methods: A hydroethanolic extract of *B. javanica* seeds was fractionated with n-hexane, chloroform and ethyl acetate. An active fraction was selected after screening for its ability to inhibit α-glucosidase and glycogen phosphorylase α (GP-α). Isolation and characterization were carried out by using column chromatography, NMR and LCMS/MS. All isolates were assayed for inhibition of GP-α and α-glucosidase. Antidiabetic effect of active fraction was further evaluated in T2D rat model. Blood glucose and body weight were measured weekly. Serum insulin, lipid profile, renal function, liver glycogen and biomarkers of oxidative stress and inflammation were analyzed after 4-week treatment and compared with standard drug glibenclamide.

Results: Ethyl acetate fraction (EAF) exerted good inhibitory potential for α-glucosidase and GP-α compared with other fractions. Chromatographic isolation of the EAF led to the identification of seven compounds: vanillic acid (1), bruceine D (2), bruceine E (3), parahydroxybenzoic acid (4), luteolin (5), protocatechuic acid (6), and gallic acid (7). Among them, Compound (5) was identified as the most potent inhibitor of GP-α and α-glucosidase and its GP-α inhibitory activity (IC_{50} = 45.08 μM) was 10-fold higher than that of caffeine (IC_{50} = 457.34 μM), and α-glucosidase inhibitory activity (IC_{50} = 26.41 μM) was 5.5-fold higher than that of acarbose (IC_{50} = 145.83 μM), respectively. Compounds (4), (6), and (7) inhibited GP-α activity in a concentration-dependent manner with IC_{50} values of 357.88, 297.37, and 214.38 μM, and their inhibitory effect was higher than that of caffeine. These compounds exhibited weak potency on α-glucosidase compared with acarbose. Compounds (1), (2), and (3) showed no inhibition on both GP-α and α-glucosidase. In vivo study showed that EAF treatment significantly reduced blood glucose level, increased insulin and glycogen contents, decreased markers of oxidative stress and inflammation, and lipid levels in T2D rats compared with untreated group.

Conclusions: The EAF has potential therapeutic value for the treatment of T2D via acting as GP-α and α-glucosidase inhibitors by improving hepatic glucose and carbohydrate metabolism, suppressing oxidative stress, and preventing inflammation in T2D rats. According to the results, the efficacy of EAF could be due to the presence of luteolin along with synergistic effect of multiple compounds such as parahydroxybenzoic acid, protocatechuic acid, and gallic acid in *B. javanica* seeds.

Keywords: *Brucea javanica*, T2D, α-glucosidase, GP-α, Cytokines, Luteolin

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Research Article

Evaluation of Antidiabetic and Antioxidant Properties of *Brucea javanica* Seed

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The ethanol extract of *B. javanica* seed was fractionated with solvents of different polarities and tested for antioxidant activities by several assays including DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP), ferrous ion chelating activity (FCA), and nitric oxide radical scavenging activity (NORSA) along with their polyphenolic contents. Antidiabetic activity was evaluated both in vitro and in vivo using a glycogen phosphorylase α (GPα) inhibition assay and oral glucose tolerance test (OGTT) in nondiabetic rats. The ethyl acetate fraction (EAF), rich in tannin, exhibited the strongest antioxidant activities to DPPH, FRAP, and NORSA, except for FCA. The EAF also exerted a dose-depended inhibition of GPα (IC_{50} = 0.75 mg/ml). Further evaluation of hypoglycemic effect on OGGT indicated that rats treated with EAF (125 mg/kg bw) showed a 39.91% decrease ($P < 0.05$) in blood glucose levels at 30 min, and continuous fall ($P < 0.05$) of 28.89% and 20.29% was observed in the following hours (60 and 90 min) compared to the normal control during OGTT. The EAF was applied to polyamide column chromatography, and the resulting tannin-free fraction was tested for both GPα inhibition and antioxidant (DPPH only) activity. The GPα inhibitory activity was retained, while antioxidant activity was lost (4.6-fold) after tannin removal. These results concluded that the GPα inhibitory activity initially detected was primarily due to the compounds other than tannins, whereas antioxidant activity was mainly due to the tannins.

1. Introduction

Diabetes is one of the most common chronic diseases characterized by hyperglycemia as a result of impaired insulin secretion by pancreatic β cells and by cellular resistance to insulin [1]. Diabetes mellitus is recognized by the World Health Organization (WHO) as a tremendously increasing global epidemic with more than 285 million people around the world afflicted in 2010 and it is estimated that the number of people with diabetes will increase to 439 million by 2030 [2]. The current pharmacological treatment of diabetes is aimed at maintaining strict control of glycemia using oral hypoglycemic agent and insulin or combination of both. However, currently available oral antihyperglycemic agents, even when used intensively, are often unable to control the hyperglycemia and the disease progressively worsens with time. Oxidative stress has been suggested to be critically involved in the pathogenesis and progression of diabetes, including cardiovascular diseases [3], chronic kidney disease [4], ageing [5], and diabetes [6].

The ethnopharmacological evidence has proven that the use of herbal medicine is a viable alternative for the control of diabetes and other diseases. The beneficial properties of herbal medicine include significant efficiency, fewer side effects, relative safety, and especially low cost for a patient who cannot afford precious medication [7]. In fact, the medicinal plants are important natural sources of molecules with potential antidiabetic effects. Many plant species have been reported to have hypoglycemic effect, which may act through different mechanisms, including inhibition of α-glucosidase [8], inhibition of DPP-IV [9], and inhibition of glycogen phosphorylase and/or enhancement of insulin secretion,

APPENDIX

Appendix 1: Reagent preparation for total phenolic content determination

Preparation of 75 mg/ml sodium carbonate solution

For the preparation of 50 ml, 3.75 g of sodium carbonate was dissolved in 50 ml of distilled water, and this solution was kept in a colonial flask until use.

Preparation of 10-fold diluted Folin-Ciocaltu reagent

To prepare 100 ml, 10 ml of Folin-Ciocaltu reagent purchased from Merck Chemical Co. (Malaysia) was diluted to a final volume of 100 ml with distilled water and it was kept in a colonial flask for further use. This solution was prepared fresh before use.

Preparation of 1 mM tannic acid solution for preparation of tannic acid standard curve

For the preparation of 10 ml, 0.017 g of tannic acid was dissolved in 10 ml of distilled water and it was kept in a glass container for further use.

Appendix 2: Reagent preparation for total flavonoid content determination

Preparation of 5% sodium nitrite solution

For the preparation of 100 ml, 5 g of sodium nitrite was dissolved in 100 ml of distilled water and the solution was kept in a colonial flask.

Preparation of 10% aluminum chloride solution

For the preparation of 100 ml, 10 g of aluminum chloride was dissolved in 100 ml of distilled water and the solution was kept in a colonial flask.

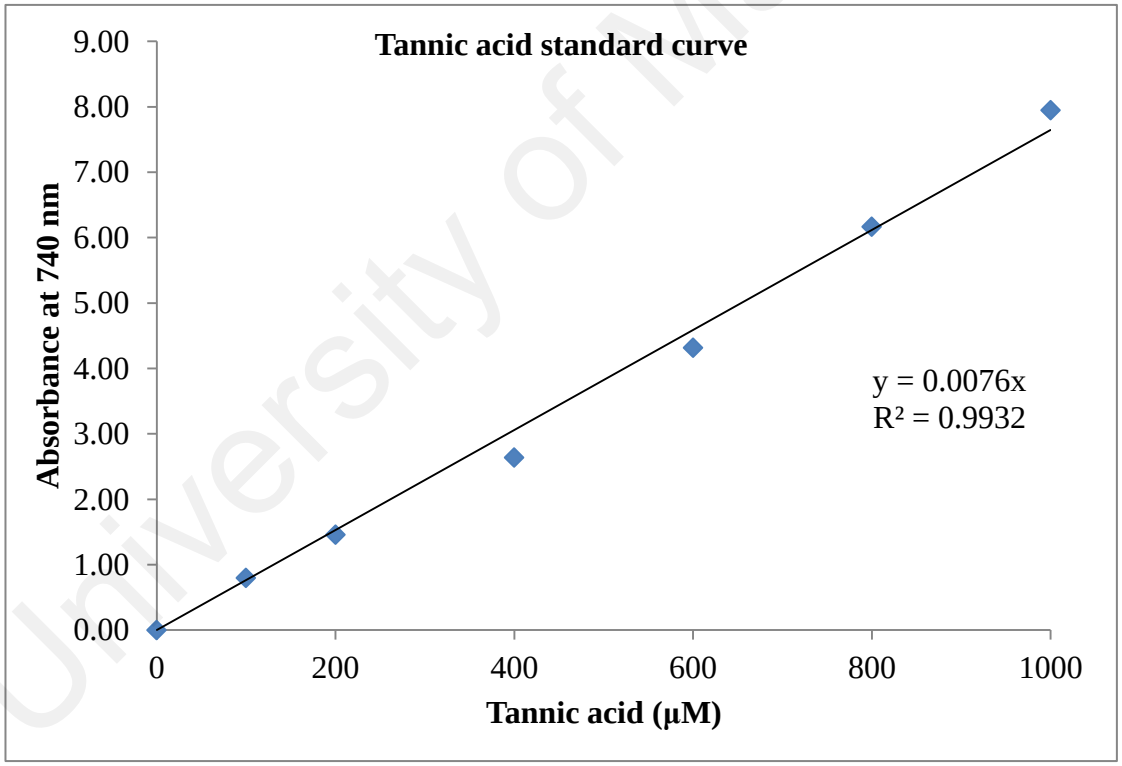
Preparation of 1M sodium hydroxide solution

The following procedure is for the preparation of 1liter. 40 g of sodium hydroxide was dissolved in 1 liter of distilled water to give a solution of 1M NaOH. This can be stored in the refrigerator for several months.

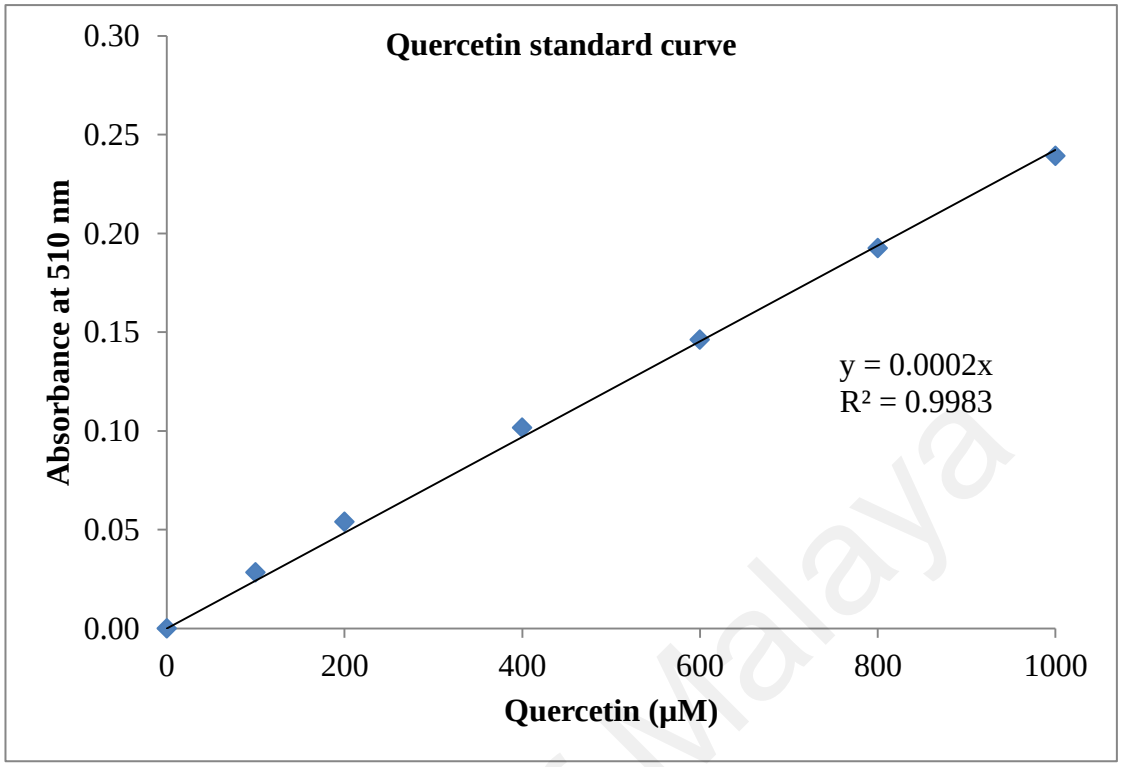
Preparation of 1 mM quercetin solution for preparation of quercetin standard curve

For the preparation of 10 ml, 0.003 g of quercetin was dissolved in 10 ml of 95% ethanol and it was kept in a glass container for further use.

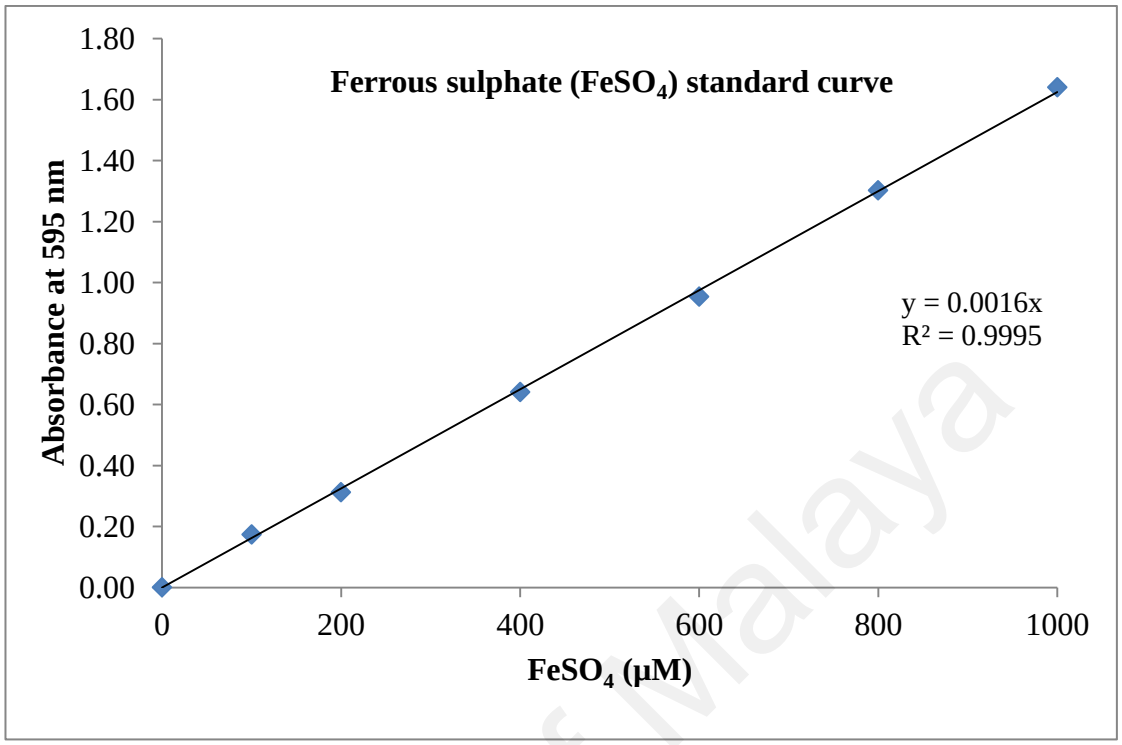
Appendix 3: Tannic acid standard curve



Appendix 4: Quercetin standard curve



Appendix 5: Ferrous sulphate ($FeSO_4$) standard curve



Appendix 6: Reagent preparation for Glycogen phosphorylase α enzyme assay

1. Preparation of 0.1 M NaOH solution

The following procedure is for the preparation of 250 ml 0.1 M NaOH solution. 1 g of NaOH (MW = 40 g/mole) was dissolved in 250 ml HPLC grade water by stirring with a magnetic stir-bar. This solution is stable for several months while stored at 4 ^{o}C.

2. Preparation of 0.1 M HEPES buffer stock solution

Hepes buffer was prepared as previously described method (Dawson *et al.*, 1986). To prepare 100 ml, 0.1M Hepes stock solution, 2.3838g Hepes was dissolved in 100 HPLC grade water and stored in a flask.

3. Preparation of 50 mM HEPES buffer (pH 7.2)

The following procedure is for the preparation of 200 ml. 100 ml Hepes 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 with pH 7.2. This solution is stable for several months while stored at 4 ^{o}C.

4. Preparation of 0.5 mg/ml GPα enzyme solution

10 mg of Glycogen phosphorylase α obtained from Sigma (St. Luis, MO) was dissolved in 20 ml of 50 mM Hepes buffer (pH 7.2) in a flask to give a solution of 0.5 mg/ml. The GPα enzyme solution was prepared fresh before use.

5. Preparation of 50 mM HEPES buffer (pH 7.2) solution containing 100 mM KCI, 2.5 mM EGTA, 2.5 mM MgCI2, 0.25 mM glucose-1-phosphate, and 1mg/ml glycogen.

To prepare 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) and mixed until all the chemicals are completely dissolved. This solution is stable for several weeks when stored in a refrigerator at 4 ^{o}C.

6. Preparation of 1M HCl solution

For the preparation of 500 ml, add 41.1 ml concentrated HCl (37%, SG = 1.19 kg/L) into a glass bottle containing 125 ml HPLC grade water, and adjust the final volume of solution to 500 ml with HPLC grade water (333.9 ml) to give a 1M HCl solution. This solution can be stored in the refrigerator for several months.

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

To prepare 50 ml solution, 0.5 g of ammonium molybdate and 0.019 g of malachite green were dissolved in 50 ml of 1M HCI, mixed well until it is completely dissolved, and filtered with Whatman filter paper. The solution was kept in a flask wrapped with aluminum foil and prepared fresh before use.

Appendix 7: Reagent preparation for α-glucosidase enzyme assay

1. Preparation of 0.1 M sodium phosphate buffer (pH 6.9)

A. Preparation of 0.2 M sodium phosphate monobasic stock solution.

For the preparation of 250 ml, weigh out 7.8 g of sodium phosphate monobasic dihydrate (156.01 g/mol) in a colonial flask and dissolve it into 250 ml of HPLC grade water by stirring with a magnetic stir-bar to give a 0.2M sodium phosphate monobasic stock solution.

B. Preparation of 0.2 M sodium phosphate dibasic stock solution.

For the preparation of 250 ml, weigh out 8.9 g of sodium phosphate dibasic dihydrate (MW=177.99 g/mol) in a colonial flask and dissolved it into 250 ml of HPLC grade water by stirring with a magnetic stir-bar to give a 0.2M sodium phosphate dibasic stock solution.

C. Preparation of 0.1 M sodium phosphate buffer (pH 6.9)

The following procedure is for the preparation of 500 ml. 130 ml of 0.2 M sodium phosphate dibasic stock solution was mixed with 120 ml of 0.2 M sodium phosphate monobasic stock solution. Then final volume of this solution was diluted to 500 ml with HPLC grade water to give a 0.1M sodium phosphate buffer with pH 6.9. This solution is stable for several months while stored at 4 ^{o}C.

2. Preparation of 5 mM p-nitrophenyl- a-D-glucopyranoside solution

The following procedure is for the preparation of 50 ml. 0.0753g of *p*-nitrophenyl- a-D-glucopyranoside was dissolved in sodium phosphate buffer (0.1M, pH 6.9), and mixed until it is completely dissolved. The substrate should be prepared fresh before use.

3. Preparation of 10 U/mL of α-glucosidase enzyme stock solution and 0.1 U/mL of solution for α-glucosidase enzyme assay

100 units of α-glucosidase from *Saccharomyces cerevisiae* obtained from Sigma (St. Louis, MO) was diluted to a final volume of 10 ml with sodium phosphate buffer (0.1M, pH 6.9) in a volumetric flask to give a stock solution of 10 U/ml. The assay required 0.1 U/ml. Therefore, the stock was diluted hundred-fold. For the preparation of 100 ml, 1 ml of stock enzyme solution was diluted with 99 ml of sodium phosphate buffer (0.1M, pH 6.9). This enzyme solution should be prepared fresh just before use.

Appendix 8: Preparation of 50 µM DPPH reagent

For the preparation of 50 ml, 0.001 g of DPPH was dissolved in 50 ml of methanol and it was kept in a flask wrapped with aluminum foil. This solution was prepared immediately before use.

Appendix 9: Reagent preparation for FRAP assay

1. Preparation of 0.04M HCI solution

For the preparation of 100 ml, 4 ml of 1M HCl stock solution was diluted to final volume of 100 ml with distilled water to give a 0.04M HCl solution. When stored in the refrigerator at 4°C it was stable for several months.

2. Preparation of 10 mM TPTZ stock solution in 0.04M HCl

For the preparation of 5 ml, 0.0156g of TPTZ (MW = 312.33g/mole) was dissolved in 5 ml of 0.04M HCl solution, and it was kept in a container wrapped with aluminum foil. This solution was prepared fresh immediately before use.

3. Preparation of 20 mM ferric chloride (FeCl3) stock solution

This procedure is for the preparation of 5 ml. Weigh out 0.0162 g of ferric chloride (MW = 162.21 g/mole) in a small glass container and dissolve it into 5 ml of HPLC grade water. This solution can be prepared fresh before use.

4. Preparation of 0.3M acetic acid stock solution

To prepare a 0.3 M, 500 ml solution, slowly add 8.6 ml concentrated acetic acid (CH_3CO_2H, 99.7%w/w, ρ = 1.049 g/ml, MW=60.05 g/mol) into a glass bottle containing 125 ml HPLC grade water, and adjust the final volume of solution to 500 ml with HPLC grade water (366.4 ml) to give a 0.3M acetic acid stock solution. This solution can be stored in the refrigerator for several months.

5. Preparation of 0.3M sodium acetate stock solution

To prepare 100 ml of 0.3 M sodium acetate stock solution, weigh out 4.083g of sodium acetate trihydrate in a glass bottle and dissolve it into 100 ml of HPLC grade water. This solution is also stable for several months when stored in a refrigerator at 4°C.

6. Preparation of 0.3M acetate buffer (pH 3.6)

For the preparation of 300 ml, 270 ml of 0.3M acetic acid stock solution was mixed with 30 ml of sodium acetate (0.3M) stock solution dropwise to give a 0.3 M acetate buffer with pH 3.6. This can be stored in the refrigerator for several months.

7. Preparation of FRAP reagent

To prepare FRAP reagent, 50 ml of 0.3M acetate buffer (pH 3.6) was mixed 5 ml of 20 mM $FeCl_3$ and 5 ml of 10 mM TPTZ stock solution. This reagent should be prepared fresh immediately before use.

8. Preparation of 1mM ferrous sulphate solution for standard curve

For the preparation of 10 ml, 0.0028 g of ferrous sulphate ($FeSO_4.7H_2O$, MW = 278.02 g/mol) was dissolved in 10 ml of distilled water to give a 1 mM ferrous sulphate solution. This solution was kept in a glass container and prepared fresh before use.

Appendix 10: Reagent preparation for metal chelating assay

1. Preparation of 2 mM ferrous chloride solution

For the preparation of 50 ml, weigh out 0.0199 g of ferrous chloride ($FeCl_2 \cdot 4H_2O$, MW = 198.81 g/mol) in a suitable container and dissolve it into 50 ml of 95% ethanol. This solution is stable for several days when stored in a refrigerator at 4°C

2. Preparation of 5 mM ferrozine solution

The following procedure is for the preparation of 25 ml. Weigh out 0.0616 g of ferrozine (MW = 492.45 g/mol) and dissolve it into 25 ml of HPLC grade water. This solution should be prepared fresh before use.

Appendix 11: Reagent preparation for nitric oxide scavenging assay

1. Preparation of 10 mM sodium nitroferricyanide solution

For the preparation of 25 ml, 0.0745 g of sodium nitroferricyanide (III) dihydrate (MW = 297.95 g/mol) was dissolved in 25 ml of HPLC grade water. This solution should be prepared fresh immediately before use.

Appendix 12: Reagent preparation for super oxide radical scavenging assay

1. Preparation of NTB solution

For the preparation of 50 ml, weigh out 0.041g of nitro blue tetrazolium, 0.0076 g of xanthine, 0.0008 g of EDTA, and dissolve it into 50 ml of 0.1M Tris-HCl buffer. This solution should be prepared fresh immediately before use.

2. Preparation of 0.25 units/ml xanthine oxidase solution

5 units of xanthine oxidase were diluted to a final volume of 5 ml with 0.1 M Tris-HCl buffer (pH 7.5) in a container to give a stock solution of 1 unit/ml. The assay required 0.25 units/ml. The stock solution was diluted four-fold. For the preparation of 10 ml, 2.5 ml of xanthine oxidase stock solution was diluted with 10 ml of 0.1 M Tris-HCl buffer. This enzyme solution should be prepared fresh immediately before use.

Appendix 13: Reagent preparation for induction of type 2 diabetic rats

A. Preparation of 0.9% saline solution.

To prepare 100 ml, 0.9 g sodium hydroxide was dissolved in 100 ml distilled water and this solution was kept in a refrigerator at 2- 4 ^{o}C for future use.

B. Preparation of 0.1 M citrate buffer (pH 4.5)

Citrate buffer was prepared according to the method described previously (Dawson *et al.*, 1986).

0.1M citric acid solution.

To prepare 100 ml, 2.10 g citric acid monohydrate (MW = 210.14 g/mole) was dissolved in 100 ml distilled water.

0.1 M trisodium citrate solution

To prepare 100 ml, 2.94 g trisodium citrate dihydrate (MW = 294.12 g/mole) was dissolved in 100 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 2- 4 ^{o}C for future use.

C. Preparation of 100 mg/ml nicotinamide (NA) solution

For the preparation of 10 ml, 1 g of nicotinamide was dissolved in 10 ml of 0.9 % saline solution. This solution was prepared fresh immediately before use.

D. Preparation of 60 mg/ml streptozotocin (STZ) solution

For the preparation of 5 ml, 300 mg of STZ was dissolved in 5 ml of 0.1 M citrate buffer (pH 4.5) in a glass container and kept on ice. This solution was prepared fresh immediately before use.

Appendix 14: Effect of the EAF on OGTT in non-diabetic rats

Multiple Comparisons

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	EAF 12.5	38.00000	27.21902	.729	-44.7892	120.7892
	EAF25	103.25000*	27.21902	.008	20.4608	186.0392
	EAF50	106.80000*	27.21902	.006	24.0108	189.5892
	EAF125	242.10000*	27.21902	.000	159.3108	324.8892
	GLI	289.25000*	27.21902	.000	206.4608	372.0392
EAF 12.5	Control	-38.00000	27.21902	.729	-120.7892	44.7892
	EAF25	65.25000	27.21902	.189	-17.5392	148.0392
	EAF50	68.80000	27.21902	.148	-13.9892	151.5892
	EAF125	204.10000*	27.21902	.000	121.3108	286.8892
	GLI	251.25000*	27.21902	.000	168.4608	334.0392
EAF25	Control	-103.25000*	27.21902	.008	-186.0392	-20.4608
	EAF 12.5	-65.25000	27.21902	.189	-148.0392	17.5392
	EAF50	3.55000	27.21902	1.000	-79.2392	86.3392
	EAF125	138.85000*	27.21902	.000	56.0608	221.6392
	GLI	186.00000*	27.21902	.000	103.2108	268.7892
EAF50	Control	-106.80000*	27.21902	.006	-189.5892	-24.0108
	EAF 12.5	-68.80000	27.21902	.148	-151.5892	13.9892
	EAF25	-3.55000	27.21902	1.000	-86.3392	79.2392
	EAF125	135.30000*	27.21902	.000	52.5108	218.0892
	GLI	182.45000*	27.21902	.000	99.6608	265.2392
EAF125	Control	-242.10000*	27.21902	.000	-324.8892	-159.3108
	EAF 12.5	-204.10000*	27.21902	.000	-286.8892	-121.3108
	EAF25	-138.85000*	27.21902	.000	-221.6392	-56.0608
	EAF50	-135.30000*	27.21902	.000	-218.0892	-52.5108
	GLI	47.15000	27.21902	.522	-35.6392	129.9392
GLI	Control	-289.25000*	27.21902	.000	-372.0392	-206.4608
	EAF 12.5	-251.25000*	27.21902	.000	-334.0392	-168.4608
	EAF25	-186.00000*	27.21902	.000	-268.7892	-103.2108
	EAF50	-182.45000*	27.21902	.000	-265.2392	-99.6608
	EAF125	-47.15000	27.21902	.522	-129.9392	35.6392

*. The mean difference is significant at the 0.05 level.

Appendix 15: Effect of EAF on serum insulin levels in T2D rats

Multiple Comparisons

Insulin

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	20.47000*	5.32669	.006	4.8262	36.1138
	D + EAF50	4.71500	5.32669	.900	-10.9288	20.3588
	D + EAF25	13.82167	5.32669	.102	-1.8221	29.4655
	D + GLI	9.48667	5.32669	.406	-6.1571	25.1305
DC	NDC	-20.47000*	5.32669	.006	-36.1138	-4.8262
	D + EAF50	-15.75500*	5.32669	.048	-31.3988	-.1112
	D + EAF25	-6.64833	5.32669	.724	-22.2921	8.9955
	D + GLI	-10.98333	5.32669	.267	-26.6271	4.6605
D + EAF50	NDC	-4.71500	5.32669	.900	-20.3588	10.9288
	DC	15.75500*	5.32669	.048	.1112	31.3988
	D + EAF25	9.10667	5.32669	.446	-6.5371	24.7505
	D + GLI	4.77167	5.32669	.896	-10.8721	20.4155
D + EAF25	NDC	-13.82167	5.32669	.102	-29.4655	1.8221
	DC	6.64833	5.32669	.724	-8.9955	22.2921
	D + EAF50	-9.10667	5.32669	.446	-24.7505	6.5371
	D + GLI	-4.33500	5.32669	.924	-19.9788	11.3088
D + GLI	NDC	-9.48667	5.32669	.406	-25.1305	6.1571
	DC	10.98333	5.32669	.267	-4.6605	26.6271
	D + EAF50	-4.77167	5.32669	.896	-20.4155	10.8721
	D + EAF25	4.33500	5.32669	.924	-11.3088	19.9788

*. The mean difference is significant at the 0.05 level.

Appendix 16: Effect of EAF on serum lipid profiles in T2D rats

1. Effect of EAF on serum TG levels

Multiple Comparisons

TG

Tukey HSD

(I) Lipid profile	(J) Lipid profile	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-.48333*	.15170	.029	-.9289	-.0378
	D + EAF50	-.03333	.15170	.999	-.4789	.4122
	D + EAF25	-.03667	.15170	.999	-.4822	.4089
	D + GLI	-.06667	.15170	.992	-.5122	.3789
DC	NDC	.48333*	.15170	.029	.0378	.9289
	D + EAF50	.45000*	.15170	.047	.0045	.8955
	D + EAF25	.44667*	.15170	.049	.0011	.8922
	D + GLI	.41667	.15170	.075	-.0289	.8622
D + EAF50	NDC	.03333	.15170	.999	-.4122	.4789
	DC	-.45000*	.15170	.047	-.8955	-.0045
	D + EAF25	-.00333	.15170	1.000	-.4489	.4422
	D + GLI	-.03333	.15170	.999	-.4789	.4122
D + EAF25	NDC	.03667	.15170	.999	-.4089	.4822
	DC	-.44667*	.15170	.049	-.8922	-.0011
	D + EAF50	.00333	.15170	1.000	-.4422	.4489
	D + GLI	-.03000	.15170	1.000	-.4755	.4155
D + GLI	NDC	.06667	.15170	.992	-.3789	.5122
	DC	-.41667	.15170	.075	-.8622	.0289
	D + EAF50	.03333	.15170	.999	-.4122	.4789
	D + EAF25	.03000	.15170	1.000	-.4155	.4755

*. The mean difference is significant at the 0.05 level.

2. Effect of EAF on serum TC levels

Multiple Comparisons

TC

Tukey HSD

(I) Lipid profile -TC	(J) Lipid profile -TC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-.35000*	.10403	.019	-.6555	-.0445
	D + EAF50	.38333*	.10403	.009	.0778	.6889
	D + EAF25	.40000*	.10403	.006	.0945	.7055
	D + GLI	.08333	.10403	.928	-.2222	.3889
DC	NDC	.35000*	.10403	.019	.0445	.6555
	D + EAF50	.73333*	.10403	.000	.4278	1.0389
	D + EAF25	.75000*	.10403	.000	.4445	1.0555
	D + GLI	.43333*	.10403	.003	.1278	.7389
D + EAF50	NDC	-.38333*	.10403	.009	-.6889	-.0778
	DC	-.73333*	.10403	.000	-1.0389	-.4278
	D + EAF25	.01667	.10403	1.000	-.2889	.3222
	D + GLI	-.30000	.10403	.056	-.6055	.0055
D + EAF25	NDC	-.40000*	.10403	.006	-.7055	-.0945
	DC	-.75000*	.10403	.000	-1.0555	-.4445
	D + EAF50	-.01667	.10403	1.000	-.3222	.2889
	D + GLI	-.31667*	.10403	.040	-.6222	-.0111
D + GLI	NDC	-.08333	.10403	.928	-.3889	.2222
	DC	-.43333*	.10403	.003	-.7389	-.1278
	D + EAF50	.30000	.10403	.056	-.0055	.6055
	D + EAF25	.31667*	.10403	.040	.0111	.6222

*. The mean difference is significant at the 0.05 level.

3. Effect of EAF on serum HDL levels

Multiple Comparisons

HDL

Tukey HSD

(I) HDL	(J) HDL	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	.90000*	.07706	.000	.6737	1.1263
	D + EAF50	.15000	.07706	.320	-.0763	.3763
	D + EAF25	.25000*	.07706	.025	.0237	.4763
	D + GLI	.05833	.07706	.940	-.1680	.2847
DC	NDC	-.90000*	.07706	.000	-1.1263	-.6737
	D + EAF50	-.75000*	.07706	.000	-.9763	-.5237
	D + EAF25	-.65000*	.07706	.000	-.8763	-.4237
	D + GLI	-.84167*	.07706	.000	-1.0680	-.6153
D + EAF50	NDC	-.15000	.07706	.320	-.3763	.0763
	DC	.75000*	.07706	.000	.5237	.9763
	D + EAF25	.10000	.07706	.695	-.1263	.3263
	D + GLI	-.09167	.07706	.757	-.3180	.1347
D + EAF25	NDC	-.25000*	.07706	.025	-.4763	-.0237
	DC	.65000*	.07706	.000	.4237	.8763
	D + EAF50	-.10000	.07706	.695	-.3263	.1263
	D + GLI	-.19167	.07706	.126	-.4180	.0347
D + GLI	NDC	-.05833	.07706	.940	-.2847	.1680
	DC	.84167*	.07706	.000	.6153	1.0680
	D + EAF50	.09167	.07706	.757	-.1347	.3180
	D + EAF25	.19167	.07706	.126	-.0347	.4180

*. The mean difference is significant at the 0.05 level.

4. Effect of EAF on serum LDL levels

Multiple Comparisons

LDL

Tukey HSD

(I) LDL	(J) LDL	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-.34833*	.09508	.009	-.6276	-.0691
	D + EAF50	.13333	.09508	.632	-.1459	.4126
	D + EAF25	.07667	.09508	.926	-.2026	.3559
	D + GLI	-.07000	.09508	.946	-.3492	.2092
DC	NDC	.34833*	.09508	.009	.0691	.6276
	D + EAF50	.48167*	.09508	.000	.2024	.7609
	D + EAF25	.42500*	.09508	.001	.1458	.7042
	D + GLI	.27833	.09508	.051	-.0009	.5576
D + EAF50	NDC	-.13333	.09508	.632	-.4126	.1459
	DC	-.48167*	.09508	.000	-.7609	-.2024
	D + EAF25	-.05667	.09508	.974	-.3359	.2226
	D + GLI	-.20333	.09508	.236	-.4826	.0759
D + EAF25	NDC	-.07667	.09508	.926	-.3559	.2026
	DC	-.42500*	.09508	.001	-.7042	-.1458
	D + EAF50	.05667	.09508	.974	-.2226	.3359
	D + GLI	-.14667	.09508	.546	-.4259	.1326
D + GLI	NDC	.07000	.09508	.946	-.2092	.3492
	DC	-.27833	.09508	.051	-.5576	.0009
	D + EAF50	.20333	.09508	.236	-.0759	.4826
	D + EAF25	.14667	.09508	.546	-.1326	.4259

*. The mean difference is significant at the 0.05 level.

Appendix 17: Effect of EAF on renal function in T2D rats

1. Effect of EAF on urea levels

Multiple Comparisons

Urea

Tukey HSD

(I) Renal function	(J) Renal function	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-12.75000*	2.56954	.000	-20.2964	-5.2036
	D + EAF50	-3.28333	2.56954	.707	-10.8298	4.2631
	D + EAF25	-4.98333	2.56954	.324	-12.5298	2.5631
	D + GLI	-4.13333	2.56954	.506	-11.6798	3.4131
DC	NDC	12.75000*	2.56954	.000	5.2036	20.2964
	D + EAF50	9.46667*	2.56954	.009	1.9202	17.0131
	D + EAF25	7.76667*	2.56954	.041	.2202	15.3131
	D + GLI	8.61667*	2.56954	.020	1.0702	16.1631
D + EAF50	NDC	3.28333	2.56954	.707	-4.2631	10.8298
	DC	-9.46667*	2.56954	.009	-17.0131	-1.9202
	D + EAF25	-1.70000	2.56954	.963	-9.2464	5.8464
	D + GLI	-.85000	2.56954	.997	-8.3964	6.6964
D + EAF25	NDC	4.98333	2.56954	.324	-2.5631	12.5298
	DC	-7.76667*	2.56954	.041	-15.3131	-.2202
	D + EAF50	1.70000	2.56954	.963	-5.8464	9.2464
	D + GLI	.85000	2.56954	.997	-6.6964	8.3964
D + GLI	NDC	4.13333	2.56954	.506	-3.4131	11.6798
	DC	-8.61667*	2.56954	.020	-16.1631	-1.0702
	D + EAF50	.85000	2.56954	.997	-6.6964	8.3964
	D + EAF25	-.85000	2.56954	.997	-8.3964	6.6964

*. The mean difference is significant at the 0.05 level.

2. Effect of EAF on creatinine levels

Multiple Comparisons

Creatinine

Tukey HSD

(I) Renal Function	(J) Renal Function	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-5.83333	2.90708	.292	-14.3711	2.7044
	D + EAF50	1.66667	2.90708	.978	-6.8711	10.2044
	D + EAF25	-1.33333	2.90708	.990	-9.8711	7.2044
	D + GLI	.16667	2.90708	1.000	-8.3711	8.7044
DC	NDC	5.83333	2.90708	.292	-2.7044	14.3711
	D + EAF50	7.50000	2.90708	.105	-1.0377	16.0377
	D + EAF25	4.50000	2.90708	.543	-4.0377	13.0377
	D + GLI	6.00000	2.90708	.267	-2.5377	14.5377
D + EAF50	NDC	-1.66667	2.90708	.978	-10.2044	6.8711
	DC	-7.50000	2.90708	.105	-16.0377	1.0377
	D + EAF25	-3.00000	2.90708	.838	-11.5377	5.5377
	D + GLI	-1.50000	2.90708	.985	-10.0377	7.0377
D + EAF25	NDC	1.33333	2.90708	.990	-7.2044	9.8711
	DC	-4.50000	2.90708	.543	-13.0377	4.0377
	D + EAF50	3.00000	2.90708	.838	-5.5377	11.5377
	D + GLI	1.50000	2.90708	.985	-7.0377	10.0377
D + GLI	NDC	-.16667	2.90708	1.000	-8.7044	8.3711
	DC	-6.00000	2.90708	.267	-14.5377	2.5377
	D + EAF50	1.50000	2.90708	.985	-7.0377	10.0377
	D + EAF25	-1.50000	2.90708	.985	-10.0377	7.0377

Appendix 18: Effect of EAF on liver function in T2D rats

1. Effect of EAF on ALP levels

Multiple Comparisons

ALP

Tukey HSD

(I) Liver function	(J) Liver function	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-178.00000*	24.17538	.000	-249.0000	-107.0000
	D + EAF50	-17.83333	24.17538	.945	-88.8333	53.1666
	D + EAF25	-38.50000	24.17538	.516	-109.5000	32.5000
	D + GLI	-5.33333	24.17538	.999	-76.3333	65.6666
DC	NDC	178.00000*	24.17538	.000	107.0000	249.0000
	D + EAF50	160.16667*	24.17538	.000	89.1667	231.1666
	D + EAF25	139.50000*	24.17538	.000	68.5000	210.5000
	D + GLI	172.66667*	24.17538	.000	101.6667	243.6666
D + EAF50	NDC	17.83333	24.17538	.945	-53.1666	88.8333
	DC	-160.16667*	24.17538	.000	-231.1666	-89.1667
	D + EAF25	-20.66667	24.17538	.910	-91.6666	50.3333
	D + GLI	12.50000	24.17538	.985	-58.5000	83.5000
D + EAF25	NDC	38.50000	24.17538	.516	-32.5000	109.5000
	DC	-139.50000*	24.17538	.000	-210.5000	-68.5000
	D + EAF50	20.66667	24.17538	.910	-50.3333	91.6666
	D + GLI	33.16667	24.17538	.650	-37.8333	104.1666
D + GLI	NDC	5.33333	24.17538	.999	-65.6666	76.3333
	DC	-172.66667*	24.17538	.000	-243.6666	-101.6667
	D + EAF50	-12.50000	24.17538	.985	-83.5000	58.5000
	D + EAF25	-33.16667	24.17538	.650	-104.1666	37.8333

*. The mean difference is significant at the 0.05 level.

2. Effect of EAF on ALT levels

Multiple Comparisons

ALT

Tukey HSD

(I) Liver Function	(J) Liver Function	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-127.16667*	11.23200	.000	-160.1536	-94.1797
	D + EAF50	-15.16667	11.23200	.664	-48.1536	17.8203
	D + EAF25	-35.66667*	11.23200	.029	-68.6536	-2.6797
	D + GLI	-12.66667	11.23200	.791	-45.6536	20.3203
DC	NDC	127.16667*	11.23200	.000	94.1797	160.1536
	D + EAF50	112.00000*	11.23200	.000	79.0131	144.9869
	D + EAF25	91.50000*	11.23200	.000	58.5131	124.4869
	D + GLI	114.50000*	11.23200	.000	81.5131	147.4869
D + EAF50	NDC	15.16667	11.23200	.664	-17.8203	48.1536
	DC	-112.00000*	11.23200	.000	-144.9869	-79.0131
	D + EAF25	-20.50000	11.23200	.382	-53.4869	12.4869
	D + GLI	2.50000	11.23200	.999	-30.4869	35.4869
D + EAF25	NDC	35.66667*	11.23200	.029	2.6797	68.6536
	DC	-91.50000*	11.23200	.000	-124.4869	-58.5131
	D + EAF50	20.50000	11.23200	.382	-12.4869	53.4869
	D + GLI	23.00000	11.23200	.274	-9.9869	55.9869
D + GLI	NDC	12.66667	11.23200	.791	-20.3203	45.6536
	DC	-114.50000*	11.23200	.000	-147.4869	-81.5131
	D + EAF50	-2.50000	11.23200	.999	-35.4869	30.4869
	D + EAF25	-23.00000	11.23200	.274	-55.9869	9.9869

*. The mean difference is significant at the 0.05 level.

3. Effect of EAF on AST levels

Multiple Comparisons

AST

Tukey HSD

(I) LiverFunction	(J) LiverFunction	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-160.83333*	17.59867	.000	-212.5184	-109.1483
	D + EAF50	-16.16667	17.59867	.887	-67.8517	35.5184
	D + EAF25	-30.33333	17.59867	.438	-82.0184	21.3517
	D + GLI	-9.66667	17.59867	.981	-61.3517	42.0184
DC	NDC	160.83333*	17.59867	.000	109.1483	212.5184
	D + EAF50	144.66667*	17.59867	.000	92.9816	196.3517
	D + EAF25	130.50000*	17.59867	.000	78.8150	182.1850
	D + GLI	151.16667*	17.59867	.000	99.4816	202.8517
D + EAF50	NDC	16.16667	17.59867	.887	-35.5184	67.8517
	DC	-144.66667*	17.59867	.000	-196.3517	-92.9816
	D + EAF25	-14.16667	17.59867	.927	-65.8517	37.5184
	D + GLI	6.50000	17.59867	.996	-45.1850	58.1850
D + EAF25	NDC	30.33333	17.59867	.438	-21.3517	82.0184
	DC	-130.50000*	17.59867	.000	-182.1850	-78.8150
	D + EAF50	14.16667	17.59867	.927	-37.5184	65.8517
	D + GLI	20.66667	17.59867	.766	-31.0184	72.3517
D + GLI	NDC	9.66667	17.59867	.981	-42.0184	61.3517
	DC	-151.16667*	17.59867	.000	-202.8517	-99.4816
	D + EAF50	-6.50000	17.59867	.996	-58.1850	45.1850
	D + EAF25	-20.66667	17.59867	.766	-72.3517	31.0184

*. The mean difference is significant at the 0.05 level.

Appendix 19: Effect of EAF on serum MDA levels in T2D rats

Multiple Comparisons

MDA

Tukey HSD

(I) Serum MDA	(J) Serum MDA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-11.63833*	2.56680	.001	-19.1767	-4.1000
	D + EAF50	.92000	2.56680	.996	-6.6184	8.4584
	D + EAF25	-1.17667	2.56680	.990	-8.7150	6.3617
	D + GLI	-2.75833	2.56680	.818	-10.2967	4.7800
DC	NDC	11.63833*	2.56680	.001	4.1000	19.1767
	D + EAF50	12.55833*	2.56680	.000	5.0200	20.0967
	D + EAF25	10.46167*	2.56680	.003	2.9233	18.0000
	D + GLI	8.88000*	2.56680	.015	1.3416	16.4184
D + EAF50	NDC	-.92000	2.56680	.996	-8.4584	6.6184
	DC	-12.55833*	2.56680	.000	-20.0967	-5.0200
	D + EAF25	-2.09667	2.56680	.923	-9.6350	5.4417
	D + GLI	-3.67833	2.56680	.613	-11.2167	3.8600
D + EAF25	NDC	1.17667	2.56680	.990	-6.3617	8.7150
	DC	-10.46167*	2.56680	.003	-18.0000	-2.9233
	D + EAF50	2.09667	2.56680	.923	-5.4417	9.6350
	D + GLI	-1.58167	2.56680	.971	-9.1200	5.9567
D + GLI	NDC	2.75833	2.56680	.818	-4.7800	10.2967
	DC	-8.88000*	2.56680	.015	-16.4184	-1.3416
	D + EAF50	3.67833	2.56680	.613	-3.8600	11.2167
	D + EAF25	1.58167	2.56680	.971	-5.9567	9.1200

*. The mean difference is significant at the 0.05 level.

Appendix 20: Effect of EAF on serum GSH levels in T2D rats

Multiple Comparisons

GSH

Tukey HSD

(I) Serum GSH	(J) Serum GSH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	15.51000*	2.05965	.000	9.4611	21.5589
	D + EAF50	4.52000	2.05965	.214	-1.5289	10.5689
	D + EAF25	11.59000*	2.05965	.000	5.5411	17.6389
	D + GLI	10.12667*	2.05965	.000	4.0778	16.1756
DC	NDC	-15.51000*	2.05965	.000	-21.5589	-9.4611
	D + EAF50	-10.99000*	2.05965	.000	-17.0389	-4.9411
	D + EAF25	-3.92000	2.05965	.342	-9.9689	2.1289
	D + GLI	-5.38333	2.05965	.098	-11.4322	.6656
D + EAF50	NDC	-4.52000	2.05965	.214	-10.5689	1.5289
	DC	10.99000*	2.05965	.000	4.9411	17.0389
	D + EAF25	7.07000*	2.05965	.016	1.0211	13.1189
	D + GLI	5.60667	2.05965	.079	-.4422	11.6556
D + EAF25	NDC	-11.59000*	2.05965	.000	-17.6389	-5.5411
	DC	3.92000	2.05965	.342	-2.1289	9.9689
	D + EAF50	-7.07000*	2.05965	.016	-13.1189	-1.0211
	D + GLI	-1.46333	2.05965	.952	-7.5122	4.5856
D + GLI	NDC	-10.12667*	2.05965	.000	-16.1756	-4.0778
	DC	5.38333	2.05965	.098	-.6656	11.4322
	D + EAF50	-5.60667	2.05965	.079	-11.6556	.4422
	D + EAF25	1.46333	2.05965	.952	-4.5856	7.5122

*. The mean difference is significant at the 0.05 level.

Appendix 21: Effect of EAF on serum TNFα in T2D rats

Multiple Comparisons

TNFα

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-93.66667*	13.78558	.000	-134.1531	-53.1802
	D + EAF50	-30.16667	13.78558	.217	-70.6531	10.3198
	D + EAF25	-47.83333*	13.78558	.015	-88.3198	-7.3469
	D + GLI	-41.16667*	13.78558	.045	-81.6531	-.6802
DC	NDC	93.66667*	13.78558	.000	53.1802	134.1531
	D + EAF50	63.50000*	13.78558	.001	23.0135	103.9865
	D + EAF25	45.83333*	13.78558	.021	5.3469	86.3198
	D + GLI	52.50000*	13.78558	.007	12.0135	92.9865
D + EAF50	NDC	30.16667	13.78558	.217	-10.3198	70.6531
	DC	-63.50000*	13.78558	.001	-103.9865	-23.0135
	D + EAF25	-17.66667	13.78558	.705	-58.1531	22.8198
	D + GLI	-11.00000	13.78558	.929	-51.4865	29.4865
D + EAF25	NDC	47.83333*	13.78558	.015	7.3469	88.3198
	DC	-45.83333*	13.78558	.021	-86.3198	-5.3469
	D + EAF50	17.66667	13.78558	.705	-22.8198	58.1531
	D + GLI	6.66667	13.78558	.988	-33.8198	47.1531
D + GLI	NDC	41.16667*	13.78558	.045	.6802	81.6531
	DC	-52.50000*	13.78558	.007	-92.9865	-12.0135
	D + EAF50	11.00000	13.78558	.929	-29.4865	51.4865
	D + EAF25	-6.66667	13.78558	.988	-47.1531	33.8198

*. The mean difference is significant at the 0.05 level.

Appendix 22: Effect of EAF on serum IL-6 in T2D rats

Multiple Comparisons

IL6

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-82.50000*	10.41397	.000	-113.0845	-51.9155
	D + EAF50	-8.61000	10.41397	.920	-39.1945	21.9745
	D + EAF25	-27.77667	10.41397	.088	-58.3612	2.8078
	D + GLI	-33.88833*	10.41397	.025	-64.4728	-3.3038
DC	NDC	82.50000*	10.41397	.000	51.9155	113.0845
	D + EAF50	73.89000*	10.41397	.000	43.3055	104.4745
	D + EAF25	54.72333*	10.41397	.000	24.1388	85.3078
	D + GLI	48.61167*	10.41397	.001	18.0272	79.1962
D + EAF50	NDC	8.61000	10.41397	.920	-21.9745	39.1945
	DC	-73.89000*	10.41397	.000	-104.4745	-43.3055
	D + EAF25	-19.16667	10.41397	.374	-49.7512	11.4178
	D + GLI	-25.27833	10.41397	.141	-55.8628	5.3062
D + EAF25	NDC	27.77667	10.41397	.088	-2.8078	58.3612
	DC	-54.72333*	10.41397	.000	-85.3078	-24.1388
	D + EAF50	19.16667	10.41397	.374	-11.4178	49.7512
	D + GLI	-6.11167	10.41397	.976	-36.6962	24.4728
D + GLI	NDC	33.88833*	10.41397	.025	3.3038	64.4728
	DC	-48.61167*	10.41397	.001	-79.1962	-18.0272
	D + EAF50	25.27833	10.41397	.141	-5.3062	55.8628
	D + EAF25	6.11167	10.41397	.976	-24.4728	36.6962

*. The mean difference is significant at the 0.05 level.

Appendix 23: Effect of EAF on serum IL-1β in T2D rats

Multiple Comparisons

IL-1B

Tukey HSD

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	-565.75500*	45.98408	.000	-700.8043	-430.7057
	D + EAF50	-114.99667	45.98408	.123	-250.0460	20.0527
	D + EAF25	-363.78500*	45.98408	.000	-498.8343	-228.7357
	D + GLI	-94.69500	45.98408	.269	-229.7443	40.3543
DC	NDC	565.75500*	45.98408	.000	430.7057	700.8043
	D + EAF50	450.75833*	45.98408	.000	315.7090	585.8077
	D + EAF25	201.97000*	45.98408	.002	66.9207	337.0193
	D + GLI	471.06000*	45.98408	.000	336.0107	606.1093
D + EAF50	NDC	114.99667	45.98408	.123	-20.0527	250.0460
	DC	-450.75833*	45.98408	.000	-585.8077	-315.7090
	D + EAF25	-248.78833*	45.98408	.000	-383.8377	-113.7390
	D + GLI	20.30167	45.98408	.992	-114.7477	155.3510
D + EAF25	NDC	363.78500*	45.98408	.000	228.7357	498.8343
	DC	-201.97000*	45.98408	.002	-337.0193	-66.9207
	D + EAF50	248.78833*	45.98408	.000	113.7390	383.8377
	D + GLI	269.09000*	45.98408	.000	134.0407	404.1393
D + GLI	NDC	94.69500	45.98408	.269	-40.3543	229.7443
	DC	-471.06000*	45.98408	.000	-606.1093	-336.0107
	D + EAF50	-20.30167	45.98408	.992	-155.3510	114.7477
	D + EAF25	-269.09000*	45.98408	.000	-404.1393	-134.0407

*. The mean difference is significant at the 0.05 level.

Appendix 24: Effect of EAF on hepatic glycogen levels in T2D rats

Multiple Comparisons

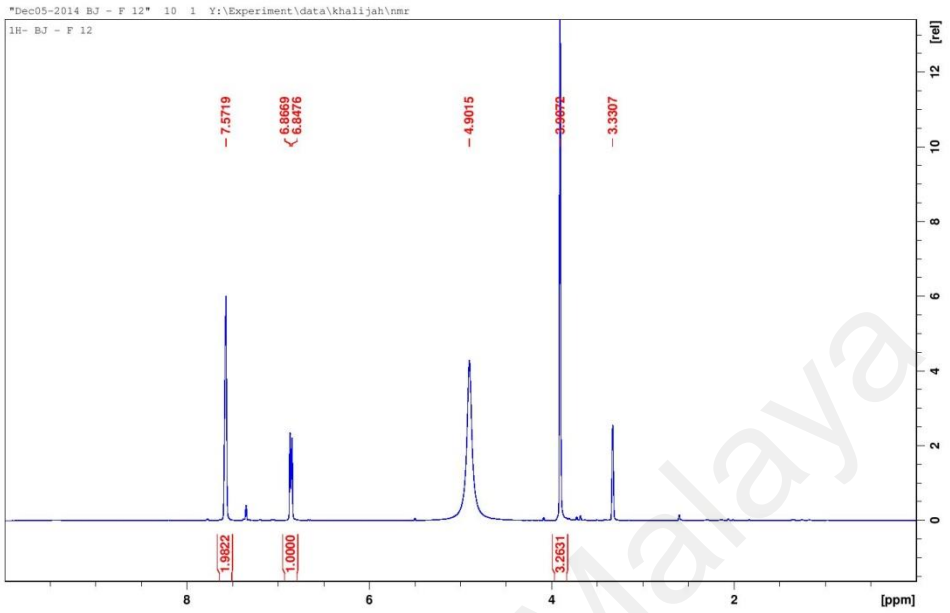
LG

Tukey HSD

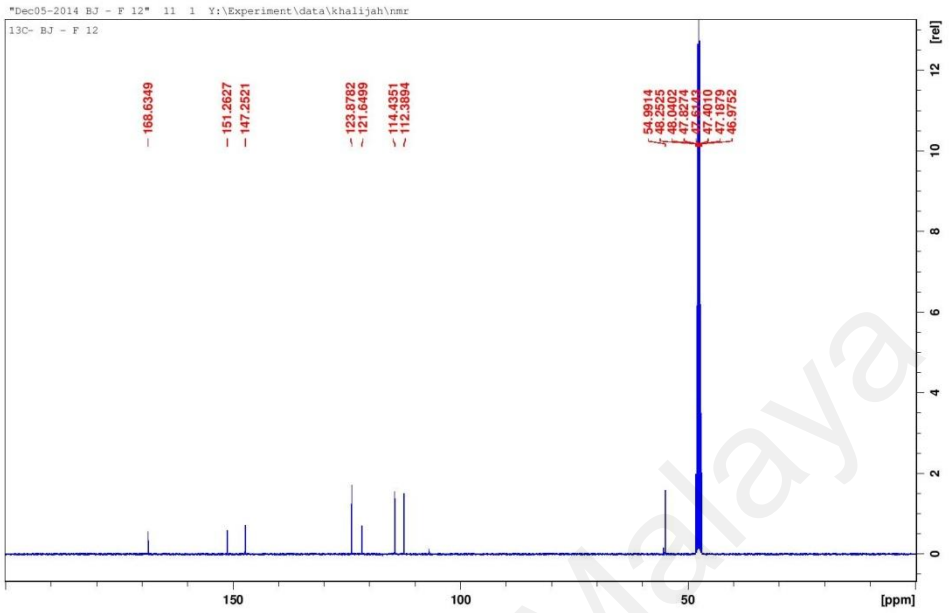
(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NDC	DC	9.30167*	2.54948	.010	1.8142	16.7892
	D + EAF50	2.89333	2.54948	.787	-4.5942	10.3808
	D + EAF 25	5.45000	2.54948	.236	-2.0375	12.9375
	D + GLI	4.01833	2.54948	.525	-3.4692	11.5058
DC	NDC	-9.30167*	2.54948	.010	-16.7892	-1.8142
	D + EAF50	-6.40833	2.54948	.120	-13.8958	1.0792
	D + EAF 25	-3.85167	2.54948	.565	-11.3392	3.6358
	D + GLI	-5.28333	2.54948	.263	-12.7708	2.2042
D + EAF50	NDC	-2.89333	2.54948	.787	-10.3808	4.5942
	DC	6.40833	2.54948	.120	-1.0792	13.8958
	D + EAF 25	2.55667	2.54948	.852	-4.9308	10.0442
	D + GLI	1.12500	2.54948	.992	-6.3625	8.6125
D + EAF 25	NDC	-5.45000	2.54948	.236	-12.9375	2.0375
	DC	3.85167	2.54948	.565	-3.6358	11.3392
	D + EAF50	-2.55667	2.54948	.852	-10.0442	4.9308
	D + GLI	-1.43167	2.54948	.979	-8.9192	6.0558
D + GLI	NDC	-4.01833	2.54948	.525	-11.5058	3.4692
	DC	5.28333	2.54948	.263	-2.2042	12.7708
	D + EAF50	-1.12500	2.54948	.992	-8.6125	6.3625
	D + EAF 25	1.43167	2.54948	.979	-6.0558	8.9192

*. The mean difference is significant at the 0.05 level.

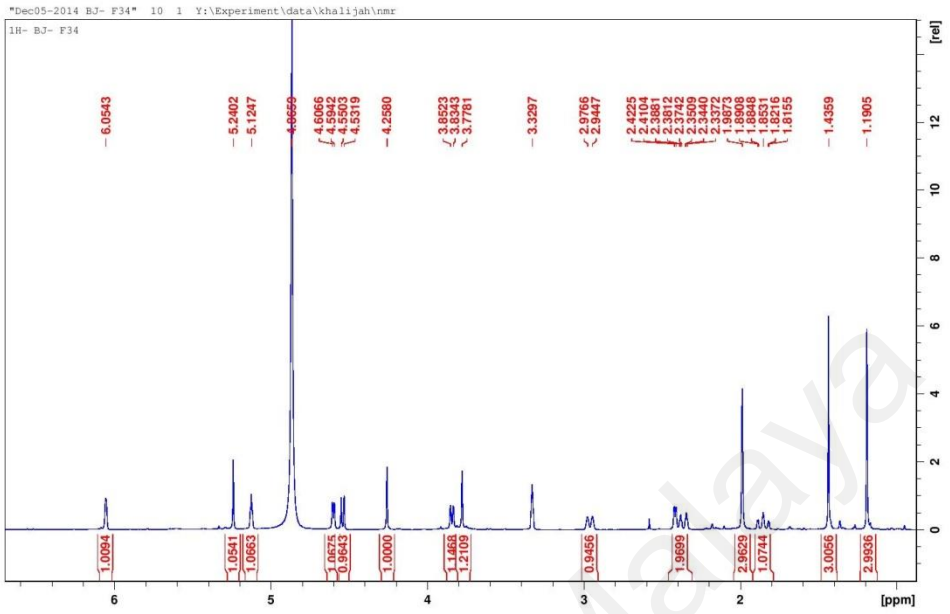
Appendix 25: ^{1}H NMR spectrum of vanillic acid



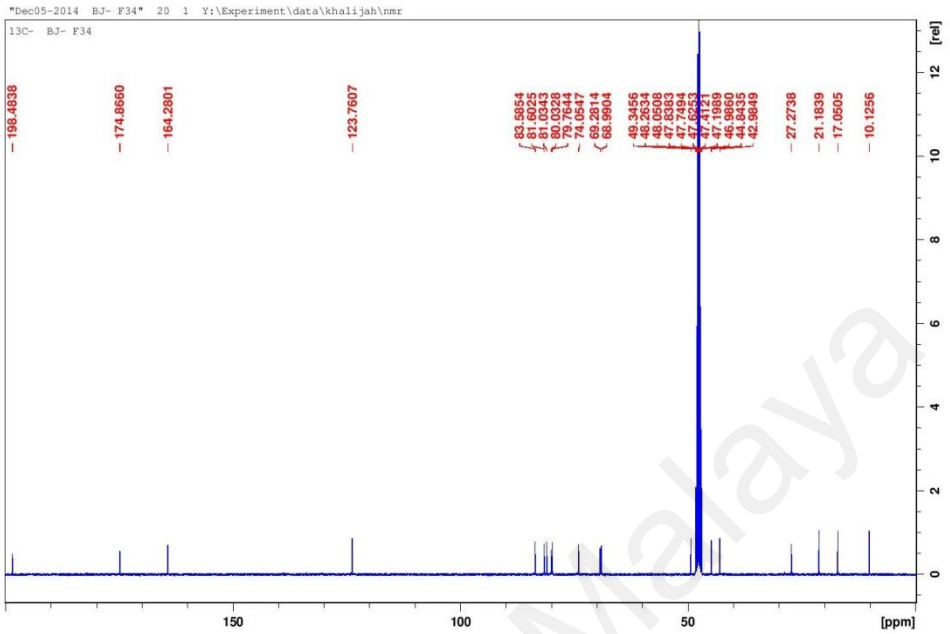
Appendix 26: ^{13}C NMR spectrum of vanillic acid



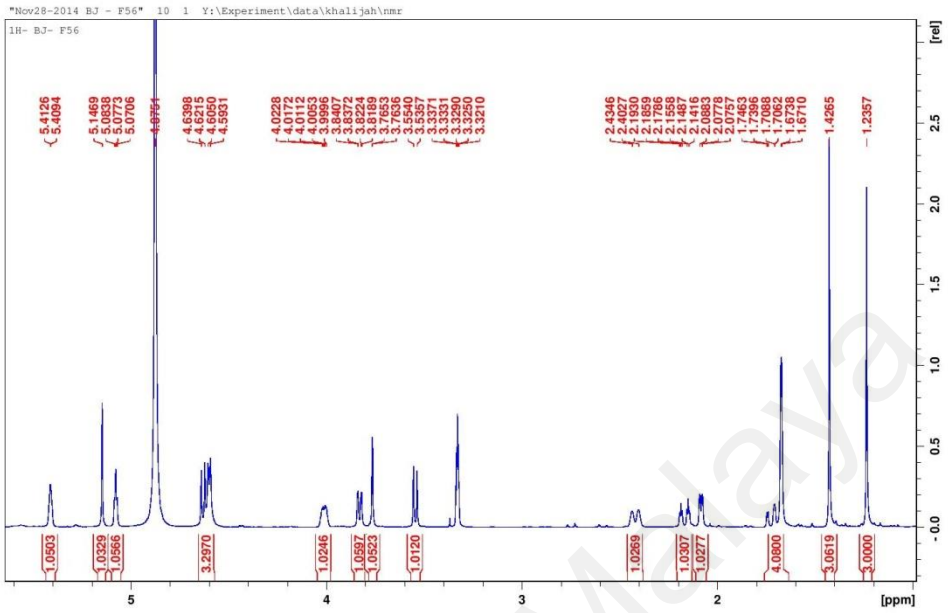
Appendix 27: ^{1}H NMR spectrum of bruceine D



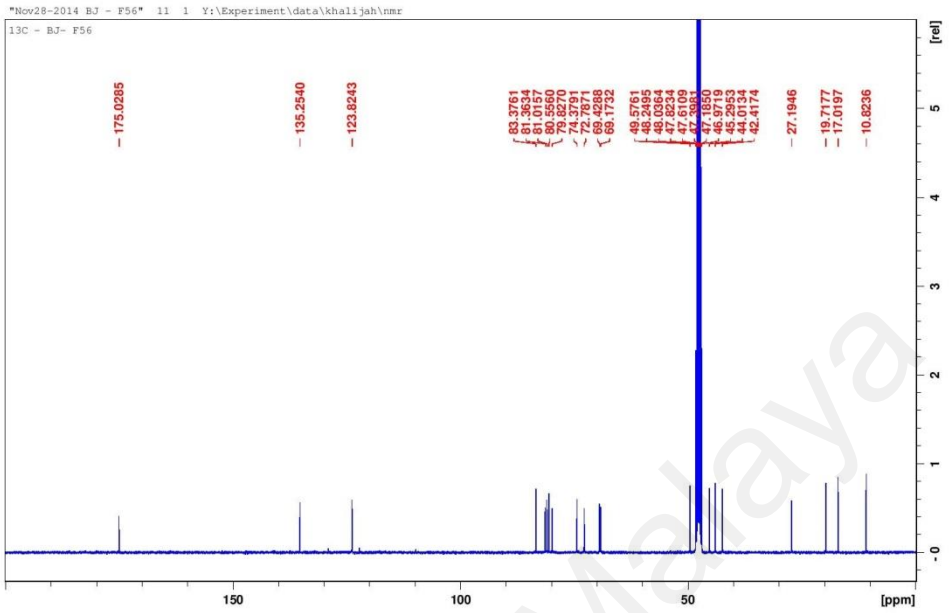
Appendix 28: ^{13}C NMR spectrum of bruceine D



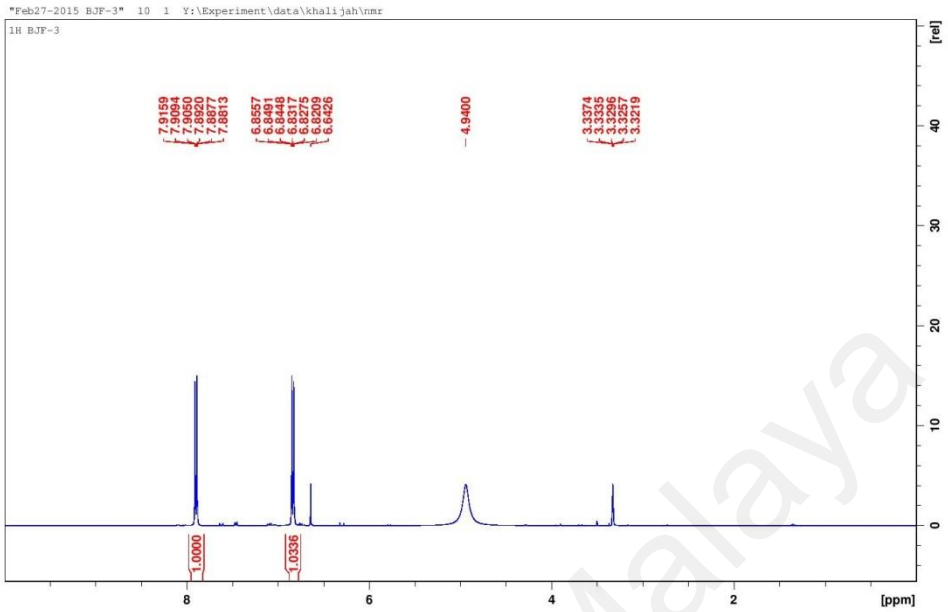
Appendix 29: ^{1}H NMR spectrum of bruceine E



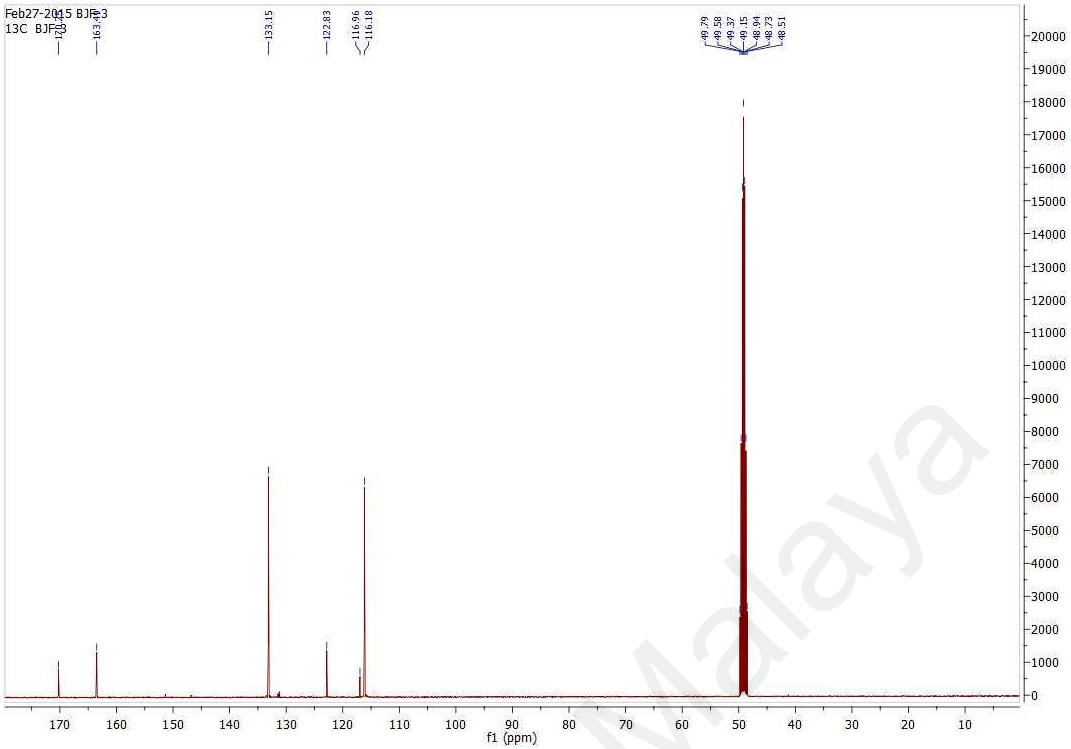
Appendix 30: ^{13}C NMR spectrum of bruceine E



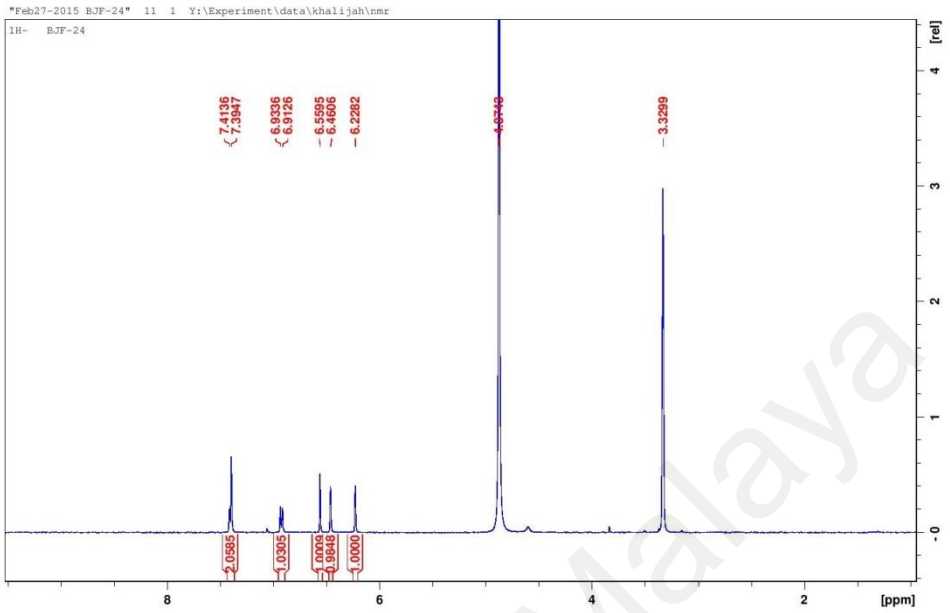
Appendix 31: ^{1}H NMR spectrum of parahydroxybenzoic acid



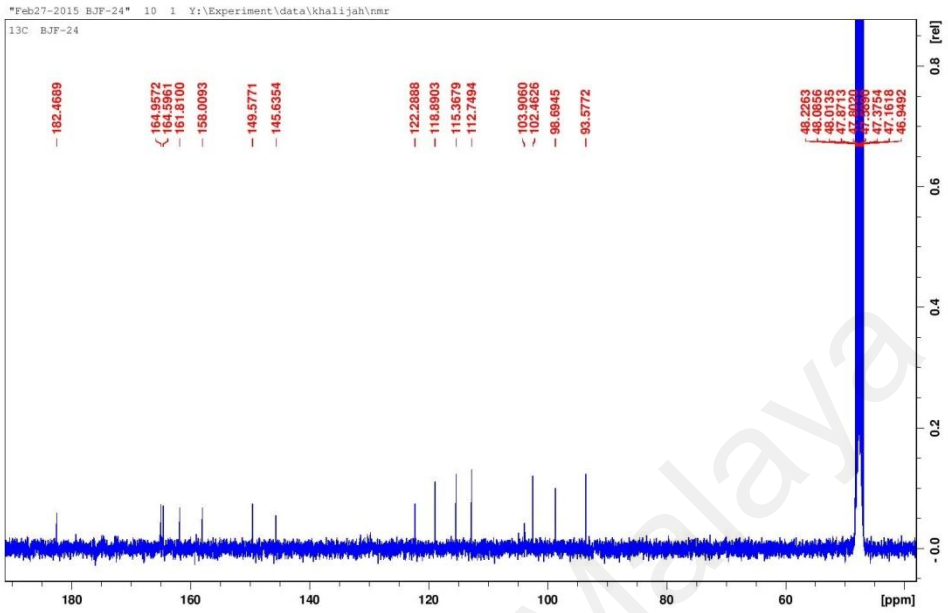
Appendix 32: ^{13}C NMR spectrum of parahydroxybenzoic acid



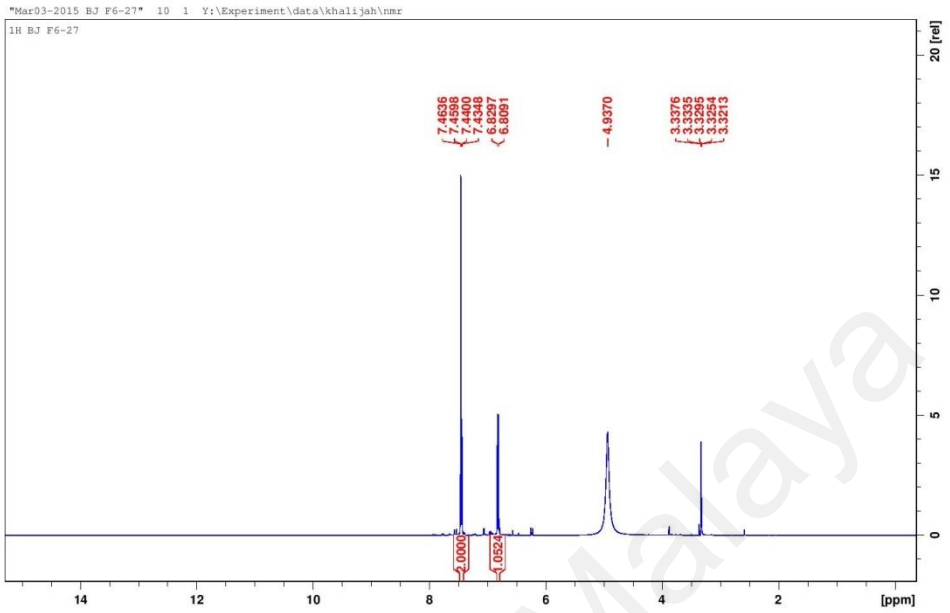
Appendix 33: ^{1}H NMR spectrum of luteolin



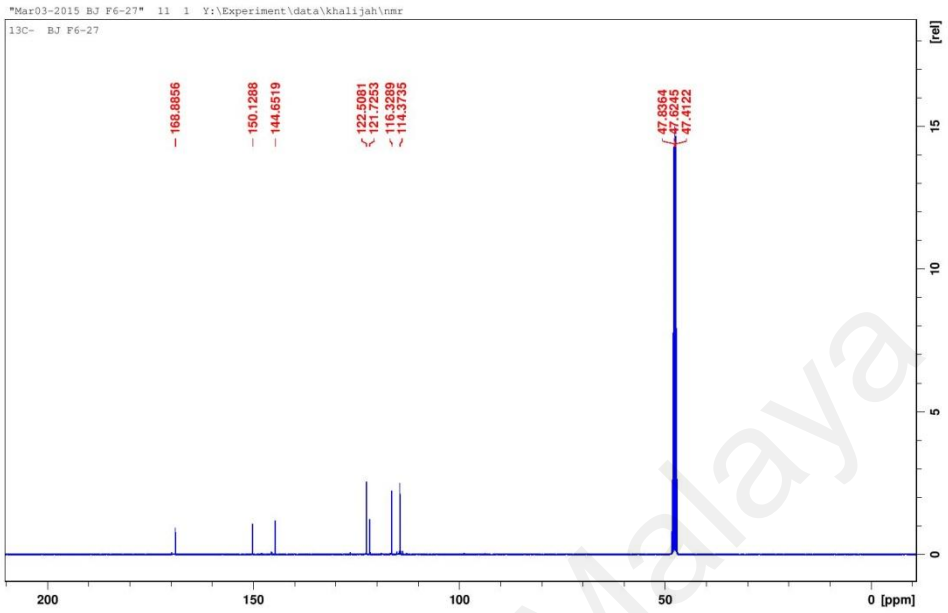
Appendix 34: ^{13}C NMR spectrum of luteolin



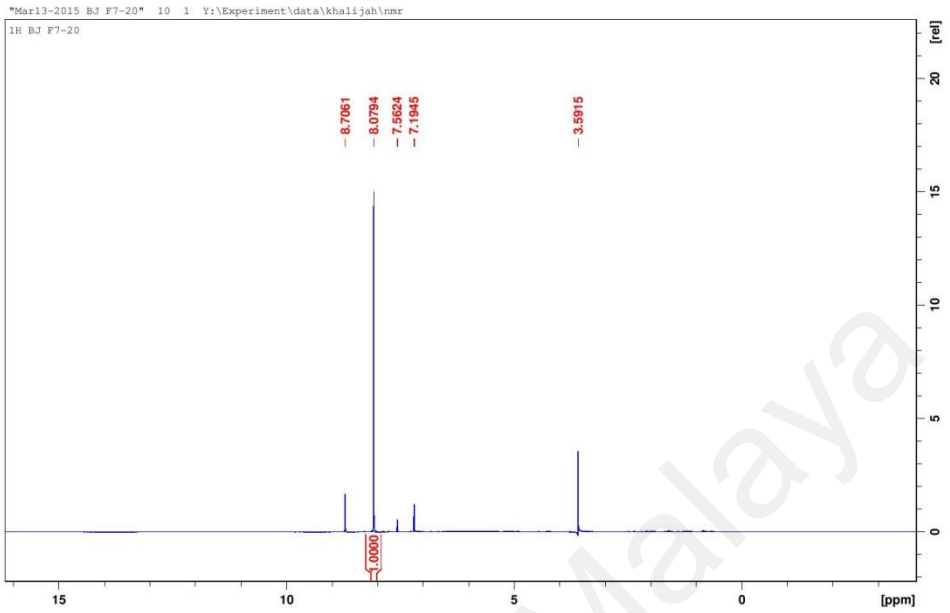
Appendix 35: ^{1}H NMR spectrum of protocatechuic acid



Appendix 36: ^{13}C NMR spectrum of protocatechuic acid



Appendix 37: ^{1}H NMR spectrum of gallic acid



Appendix 38: ^{13}C NMR spectrum of gallic acid