ANTI-DIABETIC ACTIVITY-GUIDED STUDIES OF *SYZYGIUM POLYANTHUM* (WIGHT) LEAF EXTRACTS AND ELUCIDATION OF THEIR MECHANISMS OF ACTION

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by

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LIST OF ABBREVIATIONS

°C	degree celius
%	percentage
α-cell	Alpha-cell
α-amylase	Alpha-amylase
β-cell	Beta-cell
δ-cell	Delta-cell
ABC	Avidin-Biotin Complex
Acarb	Acarbose
ADME	Absorption Distribution Metabolism Excretion
AIDS	Acquired Immune Deficiency Syndrome
AMPPK	Adenosine monophosphate protein-dependent protein kinase
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BGL	Blood Glucose Level
CE	Chloroform extract
CF	Chloroform fraction
cm	Centimetre
CO_2	Carbon dioxide
DAB	3,3-diamino benzidine tetrahydrochloride
DCCT	Diabetes Control and Complications Trial
DM	Diabetes Mellitus
DPP-IV	Dipeptidyl Peptidase-IV
DPX	Distrene Plasticiser Xylene
EAF	Ethyl Acetate Fraction
ELISA	Enzyme Linked Immunoassay
FPG	Fasting Plasma Glucose
G	Gram
g/kg	Gram per kilogram
GC-MS	Gas Chromatography-Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GLP-1	Glucagon-like peptide-1
GLUT	Glucose Transporter
H_2O_2	Hydrogen Peroxide
HbA1c	Glycohemoglobin
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
Hrs	Hours

IC_{50}	Inhibition Concentration 50
IDDM	Insulin-dependent Diabetes Mellitus
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
Il	islet of Langerhans
IPGTT	Intraperitoneal Glucose Tolerance Test
IU/ml	International Unit per milli litre
Kg	Kilogram
LDL	Low Density Lipoprotein
ME	Methanol Extract
mg	Milligram
mg/kg	Milligram per kilogram
mL	Milliliter
µL	Microliter
ml/kg	Milliliter per kilogram
mm	Micrometer
mmol	Millimol
n-BF	*n*-Butanol fraction
NGSP	National Glycohemoglobin Standardization Program
NIDDM	Noninsulin-dependent Diabetes Mellitus
NIST	National Institute Standard and Technique
Nm	Nanometer
OGTT	Oral Glucose Tolerance Test
PBS	Phosphate Buffer Saline
PEE	Petroleum Ether Extract
Phl	Phlorizin
POD	Peroxidase
PP	Pancreatic Polypeptide
PPAR γ	Peroxisome Proliferator Activated Receptor-gamma
Rpm	Rotation per minute
RT	Retention Time
S.polyanthum	*Syzygium polyanthum*
SD	Sprague-Dawley
SEM	Standard Error of the Mean
SF	Subfraction
SF-1	Subfraction-1
SF-2	Subfraction-2
SQ	Squalene
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus

TC	Total Cholesterol
TC/HDL	Total Cholesterol/High Density Lipoprotein
TG	Triglyceride
TMB	Tetramethylbenzidine
TZDs	Thiazolidinediones
u.v	Ultraviolet
v/v	Volume per volume
WE	Water Extract
WF	Water Fraction
WHO	World Health Organisation

KAJIAN BERPANDUKAN AKTIVITI ANTI-DIABETIK EKSTRAK DAUN *SYZYGIUM POLYANTHUM* (WIGHT) DAN ELUSIDASI MEKANISME KERJANYA

ABSTRAK

Kajian berpandukan aktiviti anti-diabetik telah dilakukan terhadap daun *Syzygium polyanthum* suatu ubatan tradisional yang popular di utara Sumatera, Indonesia. Aktiviti anti-diabetik telah dinilai dengan menentukan samaada daun tersebut dapat menurunkan paras glukosa darah (BGL) tikus normal (ujian hipoglisemik), merencat peningkatan BGL tikus yang diberikan beban glukosa secara intraperitoneal (ujian toleransi glukosa) dan menurunkan BGL tikus diabetik aruhan streptozotosin. Serbuk kering daun *S. polyanthum* telah diekstrak secara bersiri dengan eter petroleum (PEE), kloroform (CE), metanol (ME) dan air (WE). Dalam tikus diabetik, pemberian dengan dos tunggal ekstrak-ekstrak menunjukan hanya ME menurunkan BGL, sedangkan pemberian dos berulang selama 6 hari, kedua-dua PEE dan ME menurunkan BGL secara signifikan. Oleh itu, ME seterusnya difraksikan kepada fraksi kloroform (CF), etil asetat (EAF), *n*-butanol (*n*-BF) dan air (WF). Didapati pemberian dos berulang CF dan WF dapat menurunkan BGL tikus diabetik. CF kemudiannya digoncang dengan *n*-heksana menjadi fraksi tidak larut (SF-1) dan fraksi larut (SF-2). Hanya SF-1 menurunkan BGL tikus diabetik. GC-MS dapat mengenal pasti kehadiran skualena (SQ) di dalam ME, CF dan SF-1. Pemberian berulang SQ menurunkan BGL secara bergantungan dos pada tikus diabetik mencadangkan SQ adalah salah satu sebatian dalam daun *S. polyanthum* yang menyumbang kepada kesan anti-diabetiknya. Pemberian dos

berulang ME, CF, WF, SF-1 dan SQ selama 12 hari menunjukkan bahwa ME sebagai ekstrak paling aktif dalam menurunkan paras glukosa tikus diabetik. Penilaian imunohistokimia menunjukkan bahawa strepozotosin 55 mg/kg merosak struktur pulau Langerhans dan fungsi sel β tikus dan tidak ada rawatan yang dapat mengembalikan kesan kerosakan ini. Kedua-dua cerakin perencatan enzim α-glukosidase dan α-amilase menunjukan bahawa SF-1 dan SQ dapat merencat enzim-enzim ini sedangkan CF dan ME merencat aktiviti α-glukosidase. Sediaan kantung jejunum yang diterbalikkan menunjukkan ME, CF, WF, SF-1 dan SQ merencat penyerapan glukosa. Ujian toleransi glukosa oral menunjukkan ME dan CF mengurangkan luas dibawah kelok (AUC) yang berhubungan dengan perencatan aktiviti α-glucosidase dan α-amilase *in vivo*. Tambahan pula, pengambilan glukosa oleh kedua-dua otot abdomen dan soleus ditingkatkan oleh ME, CF, WF, SF-1 dan SQ. Penyelidikan ini memberikan data saintifik ekstrak metanol daun *S. polyanthum* sebagai ekstrak paling aktif. Mekanisme tindakan anti-diabetik daun *S. polyanthum* adalah melalui perencatan enzim α-glukosidase dan α-amilase, perencatan penyerapan glukosa di usus serta meningkatkan ambilan glukosa di otot-otot.

ANTI-DIABETIC ACTIVITY-GUIDED STUDIES OF *SYZYGIUM POLYANTHUM* (WIGHT) LEAF EXTRACTS AND ELUCIDATION OF THEIR MECHANISMS OF ACTION

ABSTRACT

Anti-diabetic activity guided studies were performed on the leaves of *Syzygium polyanthum*, a popular traditional medicinal herb in north Sumatera, Indonesia. The anti-diabetic activity was evaluated by determining whether the extracts lowered the blood glucose levels (BGL) of normal rats (hypoglycaemic test), inhibited the rise of BGL of intraperitoneally glucose- loaded rats (glucose tolerance test) and lowered BGL of streptozotocin-induced diabetic rats. Dried powdered *S. polyanthum* leaves were extracted serially with petroleum ether (PEE), chloroform (CE), methanol (ME) and water (WE). In diabetic rats, single-dose administration of the extracts showed that only ME reduced BGL. However, upon repeated-dose administration for 6 days both PEE and ME reduced BGL significantly. Therefore, liquid-liquid partition was used to fractionate ME into the following fractions: chloroform (CF), ethyl acetate (EAF), *n*-butanol (*n*-BF) and water (WF). Repeated administration of CF and WF decreased BGL of diabetic rats. CF was shaken with *n*-hexane to yield an undissolved fraction (SF-1) and a dissolved fraction (SF-2). Only SF-1 lowered BGL of diabetic rats. GC-MS identified the presence of squalene (SQ) in ME, CF and SF-1. Repeated administration of SQ reduced BGL of diabetic rats. This suggests that SQ is one of the compounds in *S. polyanthum* leaf that contribute to its anti-diabetic activity. Repeated-dose for 12 days of ME, CF, WF, SF-1 and SQ proved that ME was the most active blood glucose lowering agent.

Immunohistochemical staining revealed that streptozotocin 55 mg/kg obliterated the structure of the islet of Langerhans and the function of β-cells of rats and none of treatments could reverse the effect. *In vitro* α-glucosidase and α-amylase inhibition assays showed that SF-1 and SQ were able to inhibit both of these enzymes, whereas CF and ME inhibited α-glucosidase activity only. In averted jejunal sac studies, ME, CF, WF, SF-1 and SQ inhibited glucose absorption. Oral glucose tolerance tests showed that ME and CF reduced the area under the curve (AUC), which reflected α-glucosidase and α-amylase inhibition activities *in vivo*. Furthermore, glucose uptake into both abdominal and soleus muscles was increased by ME, CF, WF, SF-1 and SQ. The present work provides scientific data that indicates that the methanolic extract of *S. polyanthum* (Wight) leaf is the most active extract. The anti-diabetic mechanisms of action of *S. polyanthum* involved enzyme inhibition (α-glucosidase and α-amylase), intestinal glucose absorption inhibition and increased glucose uptake by the muscles.

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CHAPTER ONE

INTRODUCTION

1.1. Diabetes mellitus

1.1.1. Definition and historical aspect

Diabetes mellitus is a common group of metabolic disorders (American Diabetes Association[ADA], 2010) that is characterized by hyperglycaemia resulting from relative insulin deficiency or insulin resistance or both (Innes, 2009; Lenzen, 2008). Hyperglycaemia, a term denoting a high blood glucose level, has been defined by the World Health Organisation as a blood glucose level that is greater than 7.0 mmol/L (126 mg/dL) when fasting and greater than 11.0 mmol/L (200 mg/dL) 2 hours after a meal (Diabetes.co.uk, 2015).

As early as 200 years before the Christ, Greek physician Aretaeus had observed patients showing symptoms of excessive thirst and urination. He called this condition "diabetes," which means "to siphon", or " to pass through" in Greek. Afterwards, physicians added "mellitus" (Latin for "honeyed, sweet") to the disease name after observing that the diabetic patients produced urine that contained glucose. The terminology of diabetes mellitus also distinguishes this disorder from diabetes insipidus (Shu & Myers, 2004). Chronic hyperglycaemia, the main symptom of diabetes, is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association[ADA], 2012).

1.1.2. Epidemiology

Diabetes mellitus exists world-wide where 171 million people were diagnosed with diabetes in 2000, and this is expected to double by 2030. A substantial rise will most likely happen in the developing countries. Asia contributes more than 60% of the world's diabetic population, and has the greatest burden of type 2 diabetes mellitus (T2DM) (Innes, 2009; Mu et al., 2012). Furthermore, four of the top-ten countries with the largest diabetic populations are in Asia. They are Indonesia, Pakistan, Bangladesh, and the Philippines (Ramachandran, Ma, & Snehalatha, 2010).

1.1.3. Anatomy of the pancreas

The pancreas is a glandular organ that contains both exocrine and endocrine tissue (Compton et al., 2012). The exocrine portion-which constitutes 99% of the pancreatic mass-secretes bicarbonate and digestive enzymes into the gastrointestinal (GI) tract. Scattered within the exocrine tissues are nearly one million small islets of endocrine tissues that secrete hormones directly into the blood. These tiny endocrine glands are known as the islets of Langerhans (IL). They are clusters of cells, with each islet containing 3,000 to 4,000 cells (Diabetes Research Institute Foundation [DRIF], 2014). They include diverse cell types, that secrete different hormones, such as the glucagon-secreting α-cells, the insulin-secreting β-cells, the somatostatin- and gastrin-secreting δ-cells, and the pancreatic polypeptide-secreting PP or F cells. A Langerhans islet is composed in 60-80% of β-cells and forms its central core with α-, δ- and F-cells (Brunton & Parker, 2006; Shu & Myers, 2004).

1.1.4. Physiological regulation of glucose homeostasis and insulin action

Blood glucose is maintained within a narrow range by homeostatic mechanisms (Innes, 2009). The human body strictly maintains normal blood glucose levels (normoglycaemia) in the range of 4-6 mmol/L when fasting and 7.8 mmol/L 2 hrs after eating (Chew & Leslie, 2006; Diabetes.co.uk, 2015). To maintain homeostasis, the rate of glucose utilization (Wang et al., 1999) by the peripheral tissues should be matched with the rate of glucose production (Chew & Leslie, 2006). In the gastrointestinal tract, complex dietary carbohydrates are broken down to glucose by glucosidase enzymes. Glucose is absorbed by the gastrointestinal epithelial cells and transported into the blood stream. Glucose in the blood is distributed to all metabolically active tissues in the body. In pancreatic β-cells, glucose metabolism raises the level of cytosolic ATP, which stimulates insulin secretion. Then, insulin acts on the insulin receptors on the plasma membrane of target tissues, such as the muscles, adipose tissues, and liver to increase glucose uptake and glucose storage as glycogen or triglyceride (TG). Glucose is also taken up by other cells and tissues to fuel metabolism. In muscle and liver cells, insulin promotes glucose storage as glycogen; whereas in adipose cells, insulin and the peroxisome proliferator-activated receptors γ (PPARγ) promote glucose conversion to TG. If necessary, glucagon would promote both the conversion of glycogen back to glucose (glycogenolysis), and gluconeogenesis. Glucose would then be carried out of the liver cells and into the bloodstream (Figure 1.1.) (Shu & Myers, 2004). Therefore, insulin is the key hormone which regulates both the storage and the controlled release of the chemical energy obtained from food (Chew & Leslie, 2006). When intestinal glucose absorption declines between meals, hepatic glucose output is increased in response to lower insulin levels and increased levels of the counter-regulatory hormones,

glucagon and adrenaline (epinephrine). The liver produces glucose by gluconeogenesis and glycogen breakdown. After meals, blood insulin levels rise in response to a rise in blood glucose levels (Innes, 2009).

Glucagon-like peptide-1(7-36)amide (GLP-1), an incretin (gut hormone), also acts as a key determinant of blood glucose homeostasis by enhancing pancreatic insulin secretion after meal ingestion in a glucose-dependent manner, which results in delayed postprandial hyperglycaemia (Figure 1.1). GLP-1 is secreted from the L-cells of the gastrointestinal mucosa in response to meals, to activate enteric and autonomic reflexes, while also circulating as an incretin hormone to control the endocrine pancreatic functions. Its action is terminated via enzymatic degradation by dipeptidyl-peptidase-IV (DPP-IV). The glucagon-like peptide-1 receptor (GLP-1R) is a G protein-coupled receptor that is activated directly or indirectly by blood glucose-lowering agents currently in use for the treatment of type 2 diabetes mellitus (T2DM), including GLP-1R agonists and DPP-IV inhibitors (Drucker, 2006; Campbell & Drucker, 2013; Nadkarni, Chepurny & Holz 2014).

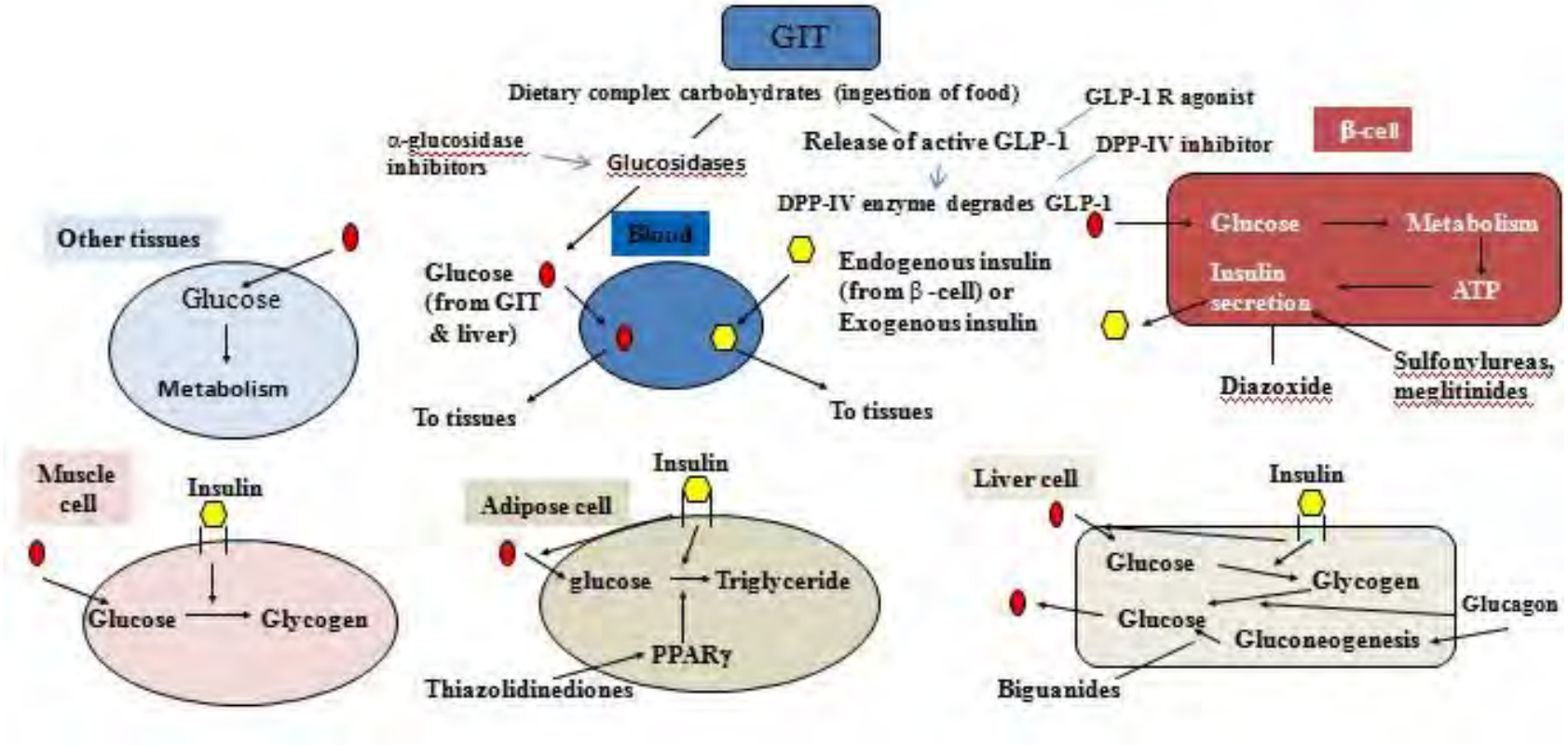

Figure 1.1. Physiological and pharmacological regulation of glucose homeostasis (Adapted from Shu and Myers, 2004 with modification).

(GIT: Gastrointestinal tract, ATP: Adenosine triphosphates, PPARγ: Peroxisome proliferator-activated receptor γ; GLP-1R: Glucagon-like peptide-1 receptor; DPP-IV: dipeptidyl-peptidase-IV; : Glucose; : Insulin)

1.1.5.Pathophysiology

Several pathogenic processes are involved in the development of diabetes (American Diabetes Association[ADA], 2012). Basically, hyperglycaemia occurs as a result of an absolute lack of insulin (Type I diabetes mellitus, also called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes) or relative insufficiency of insulin production due to insulin resistance (Type II diabetes mellitus, also called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes), or both (Innes, 2009; Lenzen, 2008; Shu & Myers, 2004). These conditions cause incapability to store glucose in the cells.

In IDDM, a cellular-mediated autoimmune response destroys the insulin producing β-cells in the pancreas. On the other hand, NIDDM, ranges in etiology from predominant insulin resistance, with relative insulin deficiency, to a predominant insulin secretory defect, with insulin resistance. Moreover, most of NIDDM patients are obese, and obesity itself causes insulin resistance (American Diabetes Association[ADA], 2012; Shu & Myers, 2004).

1.1.6. Clinical manifestation and complications

Patients with diabetes mellitus present with symptoms or clinical problems resulting from high blood glucose levels. The classical symptoms are (Chew & Leslie, 2006) polyuria, caused by the osmotic diuresis that occurs when blood glucose levels exceed the renal threshold; polyphagia, which means excessive hunger; polydipsia (thirst), caused by the loss of fluids and electrolytes; and weight loss, caused by fluid depletion and the accelerated breakdown of fat and muscles which is secondary to insulin deficiency.

Diabetes complications are categorized into microvascular and macrovascular. The microvascular complications result from damages to small blood vessels and comprise condition such as retinopathy (damage to the eyes), nephropathy (damage to the kidnesy leading to renal failure), and neuropathy (damage to the nerves leading to impotence and diabetic foot disorders). The macrovascular complications include cardiovascular events such as heart attack, stroke and insufficient blood flow to the legs (World Health Organization [WHO], 2015).

1.1.7. Classification

The clinical classification of diabetes includes :

1. Type 1 diabetes: It results from β-cell destruction and usually leads to absolute insulin deficiency. This type is underlined by a slow progessive T cell-mediated autoimmune disease (Concannon, Rich, & Nepom, 2009) that leads to the destruction of the insulin-secreting β-cells. Classical symptoms of diabetes occur when 70-90% of the β-cells have been destroyed (Sacks, 2011).
2. Type 2 diabetes: It results from a progessive insulin secretory defect or insulin resistance. In this type, patients retain some capacity to secrete insulin but there is a combination of resistance to the actions of insulin and impaired pancreatic β-cell function, which lead to „relative" insulin deficiency (Innes, 2009). The progession of type 2 diabetes is thought to begin with a state of insulin resistance (Shulman, 2000) whereby tissues that are normally insulin-responsive become relatively refractory to insulin action, and require higher levels of insulin to give appropriate responses (Shu & Myers, 2004)

3. Other specific types of diabetes which are due to other causes such as genetic defects in β-cell function, or insulin action, diseases of the exocrine pancreas (like cystic fibrosis), and drugs or chemicals (as seen in the treatment of HIV/AIDS and after organ transplantation).
4. Gestational diabetes mellitus (GDM): It is diagnosed during pregnancy and it is not a distinct form of diabetes. This type is defined as any abnormality in glucose levels noticed for the first time during pregnancy. GDM may be caused by the placenta and placental hormones, which can create insulin resistance that is pronounced in the last trimester (Nolte & Karam, 2004).

1.1.8.Diagnosis

Blood glucose levels are assessed in two ways, namely acute measurement, whereby blood glucose is measured using a glucose monitor at a single point in time, and chronic measurement, which involves the measurement of glycohemoglobin (HbA1c) (Shu & Myers, 2004). HbA1c forms when haemoglobin joins with glucose in the blood. Refered as glycated haemoglobin (A1c), this test evaluates the average plasma glucose concentration (Diabetes.co.uk, 2015).

Table 1.1. Criteria for the diagnosis of diabetes (American Diabetes Association[ADA], 2013).

HbA1c ≥ 6.5%. The test should be performed in a laboratory using the method of the National Glycohemoglobin Standardization Progam (NGSP) as certified and standardized to the Diabetes Control and Complications Trial (DCCT) assay.*
OR
Fasting plasma glucose (FPG) ≥ 126 mg/dL (7.0 mmol/L). Fasting is defined as no

caloric intake for at least 8 hour.*
OR
2-hour plasma glucose ≥ 200mg/dL (11.1mmol/L) during an oral glucose tolerance test (OGTT). The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water.*
OR
In a patient with classical symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L).

*In the absence of unequivocal hyperglycaemia, the results should be confirmed by repeated testing.

1.1.9. Treatment

The aim of the treatment of diabetes is to achieve near normal metabolism. The recommended target is HbA_{1c} ≤ 7% (Innes, 2009) to decrease the risk of long-term complications (Shu & Myers, 2004). „Anti-diabetic" denotes an agent that prevent or alleviates diabetes ("Antidiabetic", n.d.). The available currently used anti-diabetic agents are:

1. Exogenous insulin (Insulin replacement therapy).

Insulin is the mainstay for treatment in patients diagnosed with type 1 DM, and most of type 2 DM patients (Brunton & Parker, 2006). Insulin preparations are classified according to their onset of action, duration of action, and species of origin (i.e., human, pig, or cow).

2. Amylinomimetics.

Pramlintide is a synthetic amylin analog that is indicated as an adjunct to mealtime insulin therapy in patients with type 1 and type 2 diabetes. Acting as an

amylinomimetic, pramlintide delays gastric emptying time, decreases postprandial glucagon secretion, and improves satiety (Harvey, 2012).

3.Inhibitors of intestinal glucose absorption: α-Glucosidase inhibitors.

α-glucosidase inhibitors reduce intestinal absorption of starch, dextrin, and disaccharides by inhibiting the action of α-glucosidase in the intestinal brush border. Consequently, the postprandial rise in plasma glucose levels is delayed in both normal and diabetic subjects (Brunton & Parker, 2006).

4. Insulin secretagogues: sulfonylureas and meglitinides.

The major action of sulfonylureas is to increase insulin release from the pancreas. Two additional mechanisms of action for sulfonylureas have been proposed, which involve reduction of serum glucagon levels and closure of the potassium channels in extrapancreatic tissues. Meglitinides make a relatively new class of insulin secretagogues. These drugs modulate β-cell insulin release by regulating potassium efflux through the potassium channels. Unlike sulfonylureas, meglitinides have no direct effect on insulin exocytosis (Nolte & Karam, 2004).

5. Insulin sensitizers: thiazolidinediones and biguanides

Thiazolidinediones (TZDs) do not affect insulin secretion, but rather enhance the action of insulin at the target tissues. TZDs are agonists for the nuclear hormone receptor, namely the peroxisome proliferator activated receptor-ϒ (PPAR ϒ). Like TZDs, biguanides act by increasing insulin sensitivity. The molecular target of biguanides seems to be the AMP-dependent Protein Kinase (AMPPK). Biguanides activate AMPPK to block the breakdown of fatty acids and inhibit hepatic gluconeogenesis and glycogenolysis (Shu & Myers, 2004).

6. Dipeptidyl peptidase-IV inhibitors (DPP-IV inhibitors)

These drugs inhibit the enzyme DPP-IV, which is responsible for the inactivation of the incretin hormones such as glucagon-like peptide-1 (GLP-1). Prolonging the activity of the incretin hormones causes increased insulin release in response to meals and a reduction of inappropriate secretion of glucagon. A DPP-IV inhibitor may be used as monotherapy or in combination with a sulfonylurea, metformin, glitazone, or insulin (Harvey, 2012).

7. Incretin mimetics.

Glucagon-like peptide 1 (GLP-1) is an incretin hormone that is secreted by the L cells of the intestine upon meal ingestion. However, this hormone has a very short half-life as it is degraded by the ubiquitous enzyme dipeptidyl-peptidase IV (DPP-IV) (Tushuizen et al., 2006). Therefore, incretin mimetics, which are analogs of GLP-1, exert their activity by acting as GLP-1 receptor agonists. These agents improve glucose-dependent insulin secretion, slow gastric emptying time, decrease food intake, decrease postprandial glucagon secretion, and promote β-cell proliferation. Consequently, the weight gain, the postprandial hyperglycaemia and HbA_{1c} levels are reduced (Drucker & Nauck, 2006; Harvey, 2012; Tushuizen et al., 2006).

1.2. Medicinal plants of the *Syzygium* genus

Traditional medicines that are derived from medicinal plants are used by about 60% of the world‟s population (Modak et al., 2007). From the ancient times, such materials have been used for the treatment of diabetes mellitus, and are still being used extensively in present traditional of folk medicines (Evans, 2009). So far, a number of the medicinal plants that are used to treat diabetes have been proven to

possess anti-diabetic activity *in vitro*, *in vivo* and in clinical studies (Elfahmi, 2006). Some of these plants belong to *Syzygium* genus.

Herbs of the *Syzygium* genus reported to be anti-diabetic:

1. *Syzygium cumini*

Syzygium cumini (SC) (Myrtaceae) is widely used in traditional medicine to treat diabetes in India. Kumar et al. (2008) isolated a compound, mycaminose, from SC seed extract. The isolated mycaminose (50 mg/kg) together with the ethyl acetate (EA) and the methanol (ME) extracts of the plant were used to evaluate the anti-diabetic activity in STZ-induced diabetic rats. The results of this study indicated that mycaminose, and the EA and ME extracts exerted anti-diabetic effects in STZ-induced diabetic rats.

2. *Syzygium alternifolium* (Wt.) Walp.

The aqueous, ethanolic and hexane fractions of *S. alternifolium* seeds have been investigated in both normal and alloxan diabetic rats (Kameswara & Appa, 2001). The aqueous extract of *S. alternifolium* at a dose of 0.75 g/kg showed a blood glucose lowering effect in both normal and alloxan diabetic rats. The ethanol and hexane fractions also showed both hypoglycaemic and anti-hyperglycaemic activites, but the effects were significantly less than those of the aqueous extract.

3. *Syzygium malaccense*

Arumugam et al. (2014) reported the α-glucosidase and α-amylase inhibitory activities of myricetin, a compund identified in *S. malaccense* leaf extract.

4. *Syzygium aromaticum (L.) Merr. & Perry (clove)*

A *S. aromaticum* clove bud diet has been reported to attenuate hyperglycaemia, hyperlipidemia, hepatotoxicity and oxidative stress in a type 2 diabetic rat model, in which diabetes is induced by a combination of a high-fat diet and a low dose of streptozotocin (35 mg/kg) for 30 days (Adefegha, 2014).

1.3. *Syzygium polyanthum* (Wight.)

1.3.1. Synonyms and common names

Syzygium polyanthum (Wight) is synonym to *Eugenia balsamea* Ridley, *Eugenia nitida* Duthie and *Eugenia polyantha* Wight (Seidemann, 2005). This plant is also known with several common local names, such as *daun salam*, *ubar serai*, *meselengan* (in Sumatera); *samak*, *kelat samak*, *serah* (in Malaysia) and *manting* (in Jawa)(Agoes, 2010). Other names for this plants include Indonesisch laurierblad (in the Dutch language); Indian bay leaf, and Indonesian bay leaf, Indonesian laurel (in English); Pring sratoab (in the Khmer dialect); Daeng klua, Dok maeo, Mak (in Thai) and San thuyen (in Vietnamese) (Michel, 2011).

1.3.2. Taxonomy (Wasito, 2011)

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	Myrtaceae
Genus	*Syzygium*

Species *polyanthum*

1.3.3. Structural features

S. polyanthum trees are medium-sized, evergeen trees with dense crowns. They can grow up to 30 meters. The bark is grayish brown, and appears cleaved /cracked or scaly. The leaves are single and exist in an opposite formation with petioles that are up to 12 mm long. The flowers are small and fragrant with like bowl-petals. The fruits are dark red to purplish-black when ripe (Agoes, 2010).

1.3.4.Geography

S. polyanthum grows wild in forests and mountains or is usually planted in the yard and around the house. The plant can grow in low-lying areas to a height of 1,400 m above sea level. It is commonly grown for the leaves to be used as a complementary herbal remedy, and for the tree bark to be used as a dye (Wasito, 2011). It grows in thickets, bamboo forests and teak plantations. It grows in high-altitude areas up to 1,000 m of altitude in Java, 1,200 m of altitude in Sabah, Malaysia and 1,300 m of altitude in Thailand. It is widely distributed in Burma (Myanmar), Indo-China, Thailand, Malaysia, and Indonesia (Java, Sumatera, Kalimantan). *S. polyanthum* may produce flowers upon being as young as 3 years old. Flowering and fruit bearing are more or less year-round. The flowers last for 4-7 days and are usually pollinated by beetles and butterflies (Agoes, 2010).

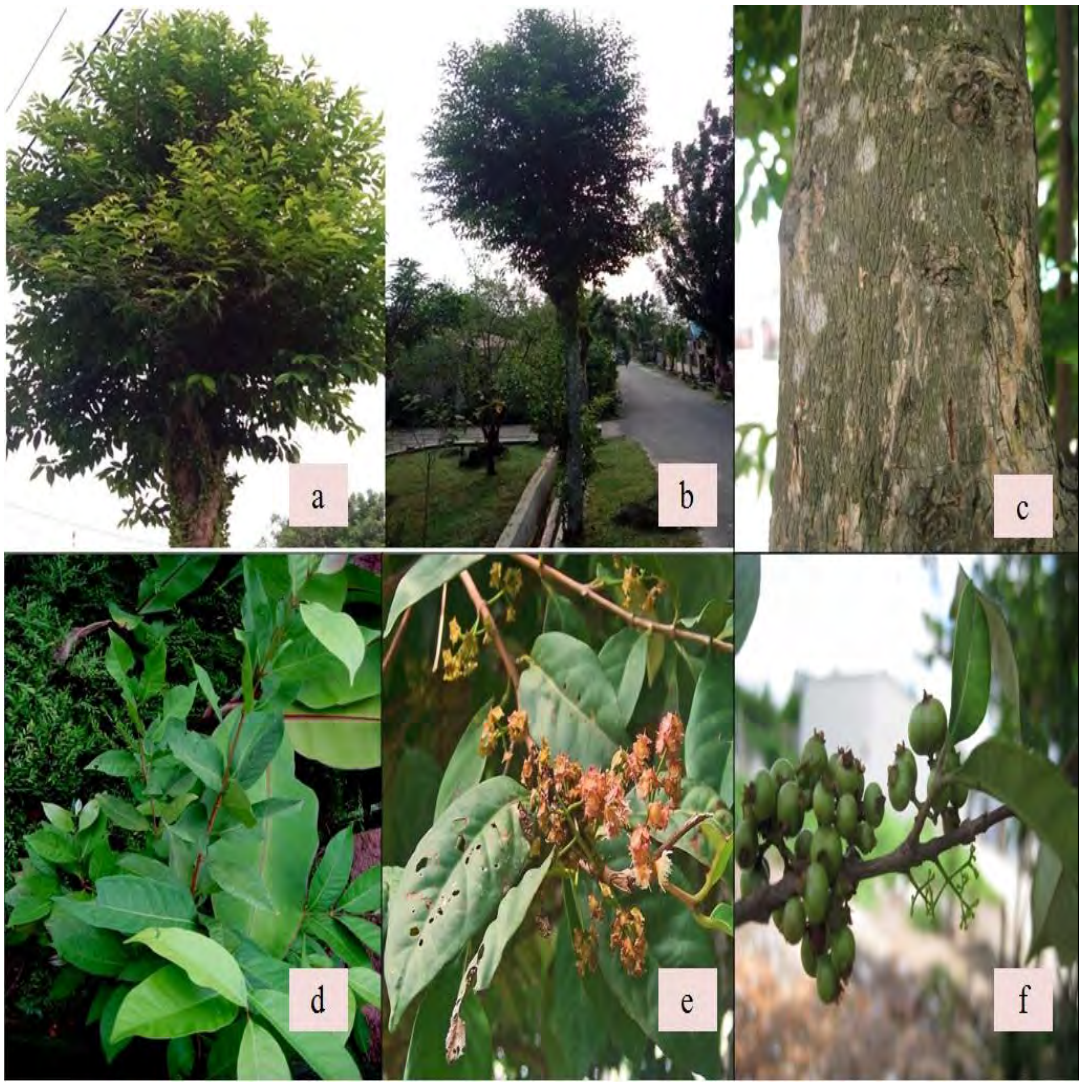

Plate 1.1. *Syzygium polyanthum* (Wight.) ("Useful Tropical Plants", n.d.)
(a-b. *S. polyanthum* tree; c.Trunk; d.Leaves; e. Flowers and developing fruits; f.Ripening fruits)

1.3.5. Phytochemical constituents

Agoes (2010) mentioned that the dry leaves of *S. polyanthum* consisted in about 0.17% of essential oils, such as eugenol and methyl chavicol. Examination of the crude extract of *S. polyanthum* leaves showed that it consisted of tannins (ethanol insoluble) at 90.95% and polar compounds at 8.8% (Anggorowati, Sukrasno, & Adnyana, 2004). The volatile components in fresh *S. polyanthum* leaves mostly consists of terpenoids (Arintawati, 2000). A phytochemical screening study conducted by Kusuma et al. (2011) reported that the ethanolic extract of *S. polyanthum* leaves contained alkaloids, carbohydrate, tannins, steroids, triterpenoids and flavonoids, with no trace of saponin.

1.3.6. Traditional uses

S. polyanthum leaves are traditionally used as a remedy for gout, stroke, high blood cholesterol levels, poor blood circulation, gastritis, diarrhea, urticaria, hypertension, and diabetes mellitus (Agoes, 2010). Widyawati et al. (2012) reported that most diabetic patient in Puskesmas Sering, Medan, Indonesia, used *S. polyanthum* as a traditional medicine for their disease.

1.3.7. Pharmacological activity

Several anti-hyperglycaemic test have been conducted on *S. polyanthum* leaf using the alloxan-induced diabetic mice model. A drug that is described as „anti-hyperglycaemic" is an agent that counteracts high levels of blood glucose or hyperglycaemia. It is a condition that describes blood glucose levels greater than 7.0 mmol/L when fasting, or greater than 11.0 mmol/L 2 hours after a meal ("Antihyperglycemic", n.d.).

A study conducted by Anggadiredja (1998) showed that the ethanol-insoluble aqueous fraction (0.7 g/kg) of the extract of *S. polyanthum* leaves reduced the blood glucose concentration in alloxan-induced diabetic mice, but the effect was less potent than insulin. Studiawan and Santosa (2005) reported that the ethanol extract of *S. polyanthum* leaves significantly decreased the blood glucose levels of alloxan-induced diabetic mice at the doses of 2.62 mg/20 g and 5.24 mg/20 g. Furthermore, 30% and 70% ethanolic extracts of *S.polyanthum* were shown to significantly inhibit the rise of blood glucose levels after glucose loading in rabbit (Wahyono and Susanti, 2008).

Riansari & Suhardjono (2008) showed that the oral administration of three doses of *S. polyanthum* ethanol extracts (0.18 g, 0.36 g and 0.72 g) daily for 15 days resulted in significant reduction of the total serum cholesterol levels in hyperlipidemic Wistar rats. A dose of 0.72 g daily for 15 days showed the greatest reduction in the observed effect.

The antioxidant and cytotoxic activities of methanolic extracts from S. *polyanthum* have also been investigated. The results revealed that the methanolic extracts of *S. polyanthum* possessed promising antiradical properties (Perumal et al., 2012). The findings were similar to those of a study conducted by Lee & Ismail (2012), which showed that the methanolic extract of *S. polyanthum* leaves demonstrated a mild anti-oxidant activity.

Despite the extensive use of *S.polyanthum* leaf by Indonesian people as traditional medicine, there has only been limited scientific data relevant to explore the mechanisms and bioactive compounds of this plan in relation to their medicinal uses as anti-diabetics. None of the previous works was based on a bioactivity-guided anti-diabetic study. The bioactivity-guided approach, when employed in initial screening of herbal extracts for a biological activity, paves the way for easy identification of the active principle(s). Therefore, the present study investigated the anti-diabetic activity of *S.polyanthum* leaf and its mechanisms of action using a bioactivity-guided approcah.

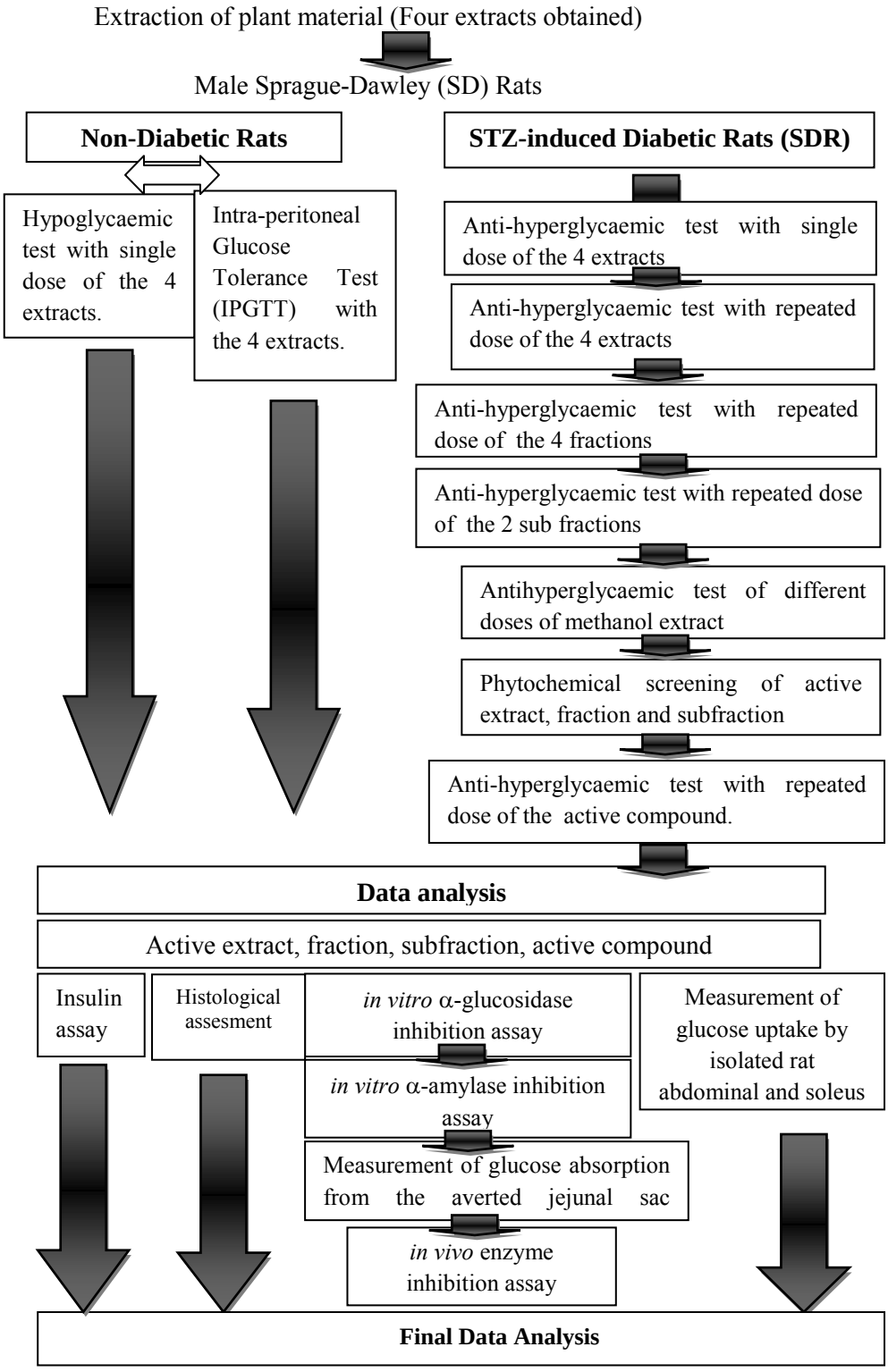

Figure 1.2. Flowchart of the research activities.

1.4. Objectives of the study

The present study was conducted with the following objectives:

1. To evaluate the hypoglycaemic and glucose tolerance effects of *Syzygium polyanthum* (Wight) leaf
2. To evaluate the anti-hyperglycaemic action of *Syzygium polyanthum* (Wight) leaf on streptozotocin-induced diabetic rats and determine the most active extract, fraction and subfraction of *Syzygium polyanthum* leaf.
3. To illucidate the phytochemistry of the most active extract, fraction and subfraction of *Syzygium polyanthum* (Wight) leaf.
4. To evaluate the anti-hyperglycaemic activity of the active compound in *Syzygium polyanthum* (Wight) leaf.
5. To examine the possible mechanisms of action of *Syzygium polyanthum* (Wight) leaf as an anti-diabetic agent.

CHAPTER TWO

HYPOGLYCAEMIC, GLUCOSE TOLERANCE, AND ANTI-HYPERGLYCAEMIC PROPERTIES OF *SYZYGIUM POLYANTHUM* (WIGHT) LEAF

2.1.Introduction

The anti-diabetic activities of plants can be assessed clinically in humans, either *in vivo,* using animal models, or *in vitro,* using a variety of test systems (Soumyanath, 2005). Preliminary testing of herbs in animal models has historically played a critical role in the exploration and characterization of disease pathophysiology, target identification, and *in vivo* evaluation of novel therapeutic agents and treatments (McGonigle & Ruggeri, 2014; Soumyanath, 2006). Since results in *in vitro* studies need to be supported and verified by *in vivo* findings, proper models must be chosen (Kasmuri, 2006). Several methods for studying oral anti-hyperglycaemic activities in humans include clinical trials involving both normal and type 2 diabetic volunteers. In animals, evaluation of a chemical‟s effects on glucose levels has been conducted in normal animals (including rabbits, rats, mice and dogs), glucose-induced hyperglycaemic animals, and also in alloxan- and streptozotocin-induced diabetic animals (Evans, 2009). In the present study, two test approaches were employed, the hypoglycaemic and the intra-peritoneal glucose tolerance tests in normal rats.

S. polyanthum is widely used in the Indonesian and the Malaysian cuisines, and it is also commonly used as a traditional medicine to treat diabetic patient in Indonesia (Agoes, 2010; Widyawati et al., 2012). The anti-hyperglycaemic activities of *S. polyanthum* extracts in different preparations and animal models have been reported (Anggadiredja, 1998; Studiawan & Santosa, 2005; Wahyono & Susanti, 2008),

however, none of the reported studies used sequential sample preparation or a bioactivity-guided approach.

Sample preparation is the most important step in the development of analytical methods for the analysis of constituents present in herbal preparations (Ong, 2004). Research on medicinal plants should be conducted beyond biological activity screening and should be tailored towards systematic standardization and must following the quality assessment and evaluation guidelines (Ali et al., 2012; Bauer & Tittle, 1996; Ong, 2004; World Health Organisation [WHO], 2000). The polarity of the solvent, the method of extraction, and the stability of the constituents may also influence the composition and quality of the extracts, and they must, therefore, be kept constant (Bauer, 1998; Handa et al., 2008). Preparation methods are tailored to the type of the natural material that is being processed, and the chosen strategy of analysis (Evans, 2009). In the present study, the dry powdered leaves were extracted sequentially using different solvents of varying polarities, starting with the non-polar and proceeding step by step using more polar solvents to obtain the crude extracts, fractions and subfractions. The activity of each extract obtained after each step of extraction was investigated to determine the most active extract, fraction and subfraction.

Basically, there are two main mechanisms for oral anti-diabetic action: which are insulin secretion induction and insulin sensitization (Brunton & Parker, 2006; Golan, 2005; Katzung, 2004; Shu & Myers, 2004). These actions result in different effects, known as hypoglycaemic and anti-hyperglycaemic.

A hypoglycemic agent, like glibenclamide, could lower high blood glucose levels to be below the normal fasting level (below 3.8 mg/dL), while an anti-hyperglycaemic agent, like metformin, albeit also being able to lower high blood glucose levels, generally cannot bring them below the normal fasting level (4-6 mmol/L) (Brunton & Parker, 2006; Nolte & Karam, 2004; Yakubovich & Gerstein, 2011).

The diabetic condition can either occur spontaneously, or be induced by chemicals, diets or surgical manipulations, and/or by a combination of these (Fröde & Medeiros, 2008; Srinivasan & Ramarao, 2012). Experimental induction of diabetes mellitus in animal models is essential for the advancement of our knowledge and understanding of the various aspects of its pathogenesis, and for, ultimately, finding new therapies and cures (Suresh et al., 2012). Streptozotocin (STZ) is often and routinely used to induce diabetes in several species, including rats. This chemical can cause fatal transitional hypoglycaemia, which typically occurs 4-8 h after injection and lasts for several hours, due to the massive β-cell islet necrosis and cell membrane rupture that produces a flood of insulin in the circulation (Srinivasan & Ramarao, 2012). The present study used STZ, which exerts its diabetogenic action when administered parenterally (i.e. intra-peritoneally). Experimental protocols recommend that administration of STZ be done in a fasting period (8-12 h), and be followed by the administration of a glucose solution (10%) to avoid hypoglycaemia, a condition in which blood glucose level fall below the lower normal level (below 3.8 mmol/L), for 24 hours as described above (Fröde & Medeiros, 2008; Wu & Huan, 2008).

Furthermore, the present study used male rats because male pancreatic β-cells islets are more prone to STZ-induced cytotoxicity in comparison with those of the female rats of the same species (Wu & Huan, 2008).

Even the most promising of novel pharmacological therapies would fail in clinical trials if it was unable to reach the target organ at a sufficient concentration to give the therapeutic effect. When dealing with animals or human subjects, the exact concentration of the drug at the receptor is unknown. However, the concentration is correlated to the administered dose as higher doses indicate greater concentrations at the receptor. Thus, all drugs must meet certain minimal requirements to achieve their clinical effectiveness (Pandit, 2007; Shu & Myers, 2004).

The pharmacodynamics of a drug can be quantified by examining the relationship between the dose or the concentration of the drug and the organism‟s response to that drug. Increased concentrations of a drug increase the magnitude of its pharmacological effect. A useful assumption is that response to a drug is proportional to the concentration of the receptors that are bound or occupied by the drug (Harvey, 2012; Shu & Myers, 2004). This study used different doses of the extracts to find the correlation between these doses and the responses, and to confirm that the previously observed pharmacological effect was genuine and not coincidental.

About 40% of prescriptions drugs are derived from herbs (Shanmuga, 2014) and many conventional drugs have been derived from prototypic molecules in medicinal plants, like the anti-diabetic biguanide class agent, metformin. This compound was obtained from a medicinal plant named *Galega officinalis* (Dey, Attele, & Yuan,