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

**by**

**TRI WIDYAWATI**

**Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy**

**November 2015**

Special thank goes to Mr. Roseli, Mr. Yusuf, Mr. Hilman, Mr Basri, Mrs. Yong, Mrs. Santhini for the dedication of professional work and the valuable technical assistance in the laboratory.

Gratitude, sincere thanks and appreciation to my dearest and lovely father, Ir. H. P. F Messa and my father-in-law H. Sastro Minoto, may Allah bless both of you in peace and placed both of you in His side, my lovely mother Hj. Ummul Bariyah, my mother-in-law Hj. Suminah. May Allah always bless you in good health and condition. Aamiin Allah. Last but not least, I would like to extend my heartfelt gratitude to my lovely man, dr. H. Suci Rahmad, MKes, my dearest kids, M. Rozan Fawwaz Syariq and M. Hanif Safaraz. Please forgive me for any lack as a good wife and mother. Thank you my lovely hubby for hearing and supporting me always. You are the wind beneath my wings. My brothers and sisters, Dian Pramuka, Abu Yahya, Tyas Indah Pertiwi, my sisters- and brother-in-law, Hj. Sri Purnama Sari, Sumitri Liestari, Sri Wahyuni, Sugeng Priyono, Semi Murni and Evi Supriati.

I do believe our gathering and relationship are going exactly the way Allah s.w.t has planned it. Thank you Allah.

## TABLE OF CONTENTS

## CHAPTER THREE: ANTI-DIABETIC MECHANISMS OF ACTION OF *SYZYGIUM POLYANTHUM* [WIGHT] LEAF EXTRACTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## 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.

# 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).

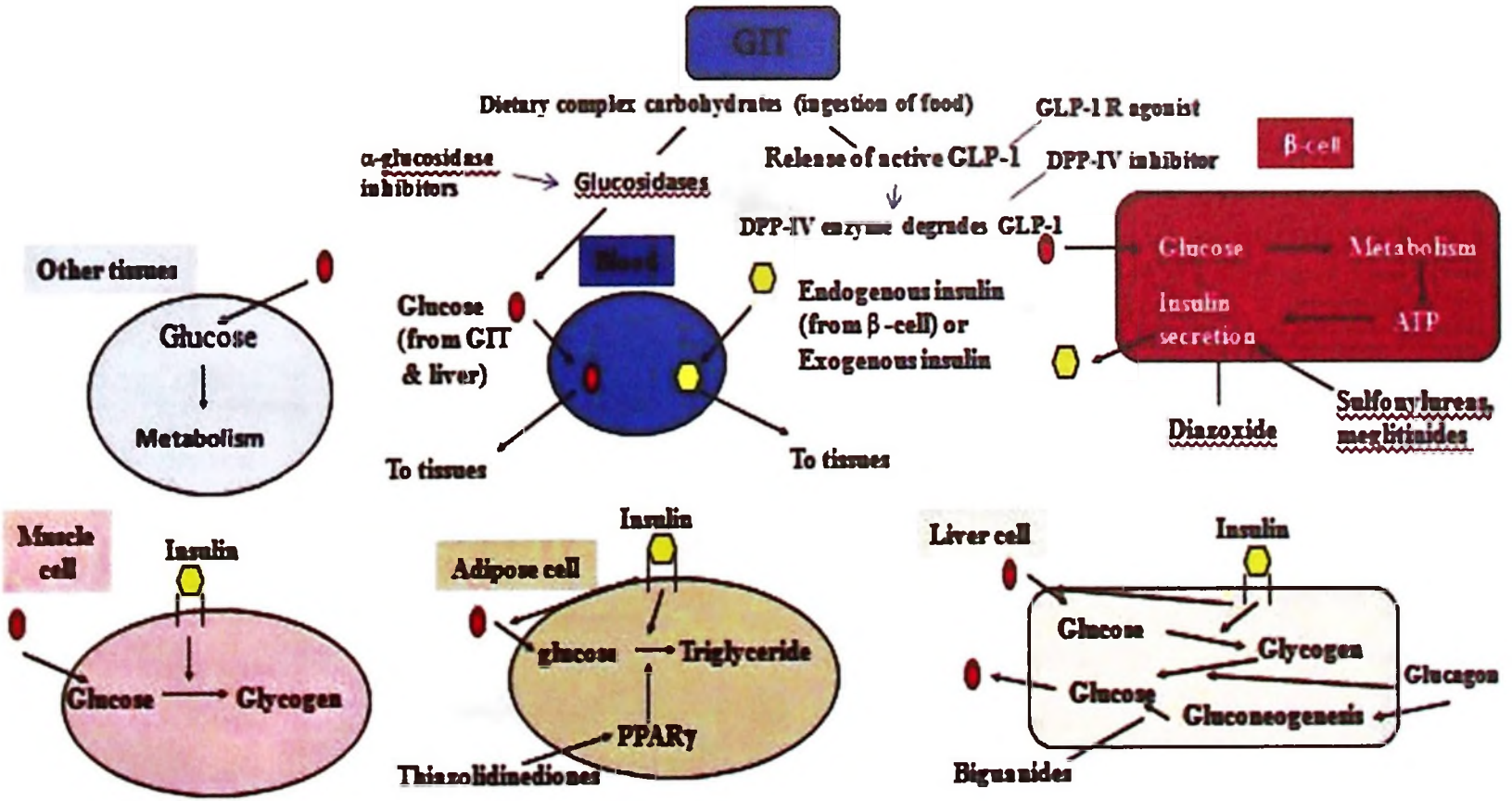


Figure 1.1. Physiological and pharmacological regulation of glucose homeostasis (Adapted from Shu and Myers, 2004 with modification).

(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 $HbA_{1c} \leq 7\%$ (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-γ (PPAR γ). 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.

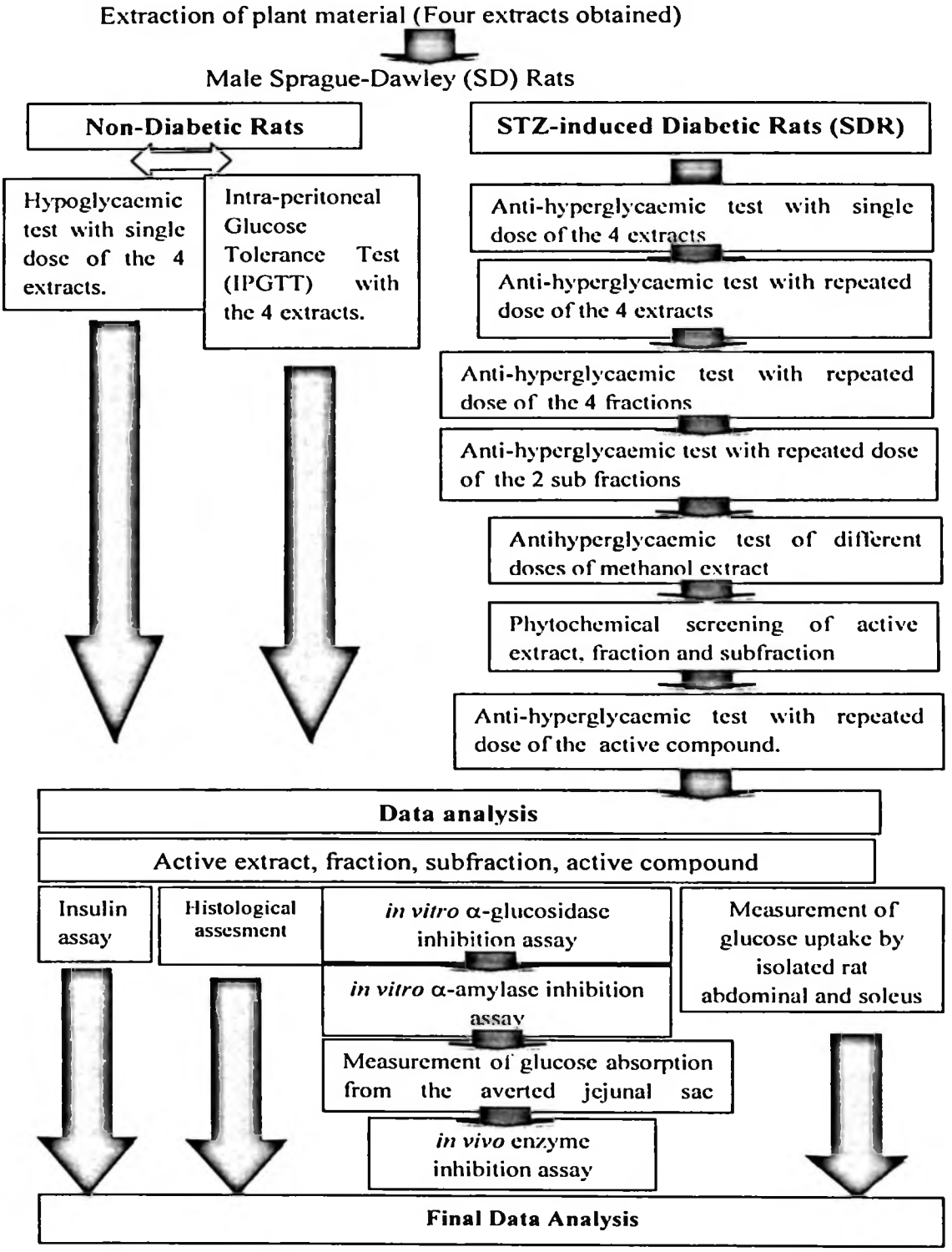


Figure 1.2. Flowchart of the research activities.

### 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,

2003; Puranik, Kammar, & Devi, 2010). Numerous chemical compounds have demonstrated activities applicable to their possible use in the treatment of DM such as alkaloids, glycosides, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and in organic ions (Puranik, 2010). To understand how plant can have hypo- or anti-hyperglycaemic effect, it is important to know its constituents as active compounds that are responsible for its pharmacological activity. Therefore, initial screening becomes a fundamental requirement to natural product development and the sourcing of therapeutic agents from medicinal plants (Ali et al, 2012).

*S. polyanthum*, a plant belonging to *Myrtaceae* (Har & Ismail, 2012; Kusuma et al., 2011), and is commonly used in the Malaysian and the Indonesian cuisine, has been traditionally used by diabetic patients in Indonesia to manage their disease (Agoes, 2010; Widyawati et al., 2012). The genus *Syzygium (Myrtaceae)* comprises about 500 species (Tian et al., 2011). Previous chemical investigations of the *Myrtaceae* family found gallic acid, phenolic acids, eugenol (Har & Ismail, 2012; Yoo et al., 2005), kaempferol and quercetin, among other flavonoids (Kuo, Yang, & Lin, 2004; Resurreccion-Magno et al., 2005; Tian et al., 2011; Wahyono & Susanti, 2008), triterpenes (Djoukeng et al., 2005), antochyanins (Nonaka et al., 1992), chromone derivatives (Toda, Kawabata, & Kasai, 2000), phenylpropanoids (Miyazawa & Hisama, 2003), and phloroglucinols (Zou et al., 2006).

## 2.2. Objectives of the study

The present study was conducted with the following objectives:

1. To evaluate the oral hypoglycaemic effect of *Syzygium polyanthum* (Wight) leaf extracts in normal rats.
2. To evaluate intra-peritoneal glucose tolerance with the administration of *Syzygium polyanthum* (Wight) leaf extracts in normal rats.
3. To evaluate the anti-hyperglycaemic activities of *Syzygium polyanthum* (Wight) leaf extracts in STZ-induced diabetic rats and determine the most active extract.
4. To evaluate the anti-hyperglycaemic activities of *Syzygium polyanthum* (Wight) leaf extract fractions in STZ-induced diabetic rats and determine the most active fraction.
5. To evaluate the anti-hyperglycaemic activities of *Syzygium polyanthum* (Wight) leaf extract subfractions in STZ-induced diabetic rats and determine the most active subfraction.
6. To evaluate the anti-hyperglycaemic dose-responses relationship of the most active extract in STZ-induced diabetic rats.
7. To identify the chemical compounds in the most active extract, fraction and sub-fraction of *Syzygium polyanthum* (Wight) leaf.
8. To evaluate the anti-hyperglycaemic activity of the active compound in *Syzygium polyanthum* (Wight) leaf.

## 2.3.Material and methods

### 2.3.1. Chemicals and equipments

Standard anti-diabetic drugs glibenclamide (Clamide®) 10 mg and metformin HCl BP 500 mg, served as positive controls; Streptozotocin was obtained from Sigma-Aldrich. Blood glucose levels were determined using an Accu-Check Advantage Clinical Glucose Meter (Roche Diagnostics).

### 2.3.2. Plant materials

The leaves of *Syzygium polyanthum* were collected from Titi Kuning, Medan, Indonesia. The plant was identified by Dr. Nursahara Pasaribu, MSc at the School of Biological Sciences, Bioteknologi no. 1 Kampus USU, University of Sumatera Utara, 20155 Medan, Indonesia (No.13/MEDA/2012).

## 2.4.Extraction procedure

### 2.4.1. Solvents for extraction

The solvents used were: petroleum ether (40-60°C) (Bendosen Laboratory Chemical); Chloroform (Trichloromethane) (QREC [Asia] Sdn Bhd); Methanol (QREC [Asia] Sdn Bhd); Ethyl acetate (QREC [Asia] Sdn Bhd); *n*-Butanol (R&M Marketing, Essex, UK); and *n*-Hexane (QREC [Asia] Sdn Bhd).

### 2.4.2. Extraction and fractionation

#### 2.4.2.1. Preparation of the extracts

The dried leaves were powdered using a milling machine. The powder, weighing about 1.5 kg was sequentially extracted by maceration (40-60°C) with four solvents ,

i.e. petroleum ether (4.9 L), chloroform (4.9 L), methanol (4.9 L) and water. Each extraction step was repeated 3 times over 3 consecutive days, yielding the extracts of petroleum ether (PEE), chloroform (CE), methanol (ME) and water (WE), respectively (Figure 2.1). The extracts obtained were filtered with Whatman No.1 filter paper and concentrated *in vacuo* by rotary evaporation (Labortechnik, AG CH-9230 Flawil, Switzerland) at reduced pressure. The concentrated extracts (PEE, CE and ME) were put in oven (40°C) until the organic solvents evaporated, except for the concentrated WE, which was freeze dried (Labconco Corporation, Kansas, MO, USA). The dried extracts were kept in the freezer (-25 °C) until further use. All of the extracts were dissolved using 5% tween®80 (Sigma, Aldrich) in 0.9% saline before administration.

**2.4.2.2. Preparation of the fractions**

The most active extract (ME), weighing about 25 g, as sequentially fractionated by liquid-liquid partition with four solvents, chloroform (250 mL), ethyl actetate (250 mL), *n*-butanol (250 mL) and water to yield the fractions of chloroform (CF), ethyl actetate (EAF), *n*-butanol (*n*-BF) and water (WF) (Figure 2.2). The fractionation procedure was repeated three times using a separatory funnel. The fractions obtained were then concentrated *in vacuo* by a rotary evaporator (Labortechnik, AG CH-9230 Flawil, Switzerland) at reduced pressure. The concentrated fractions (CF, EAF, *n*-BF) were dried in an oven (40 °C) until the organic solvents evaporated, except for the concentrated WF, which was freeze dried (Labconco Corporation, Kansas, MO, USA). The resulting completely dry fractions were kept in a freezer (-25 °C) until

further analysis. All of the fractions were dissolved using 5% tween®80 in 0.9% saline before administration.

#### 2.4.2.3. Preparation of the subfractions

The most active fraction (0.5 g CF) was further fractionated by dissolving in *n*-hexane (100 mL). The residue in remained in chloroform (100 mL) (Figure 2.3). The collected sub-fractions were filtered, and the *n*-hexane extract one was concentrated using rotary evaporation and freeze dried to obtain sub-fraction 1 (SF-1). The procedure was repeated until no color formed with the *n*-hexane used. The remaining residue, which was dissolved in chloroform, was filtered. The chloroform portion was concentrated in vacuo and yielded sub-fraction 2 (SF-2). All of the subfractions were dissolved using 5% tween®80 in 0.9% saline before administration.

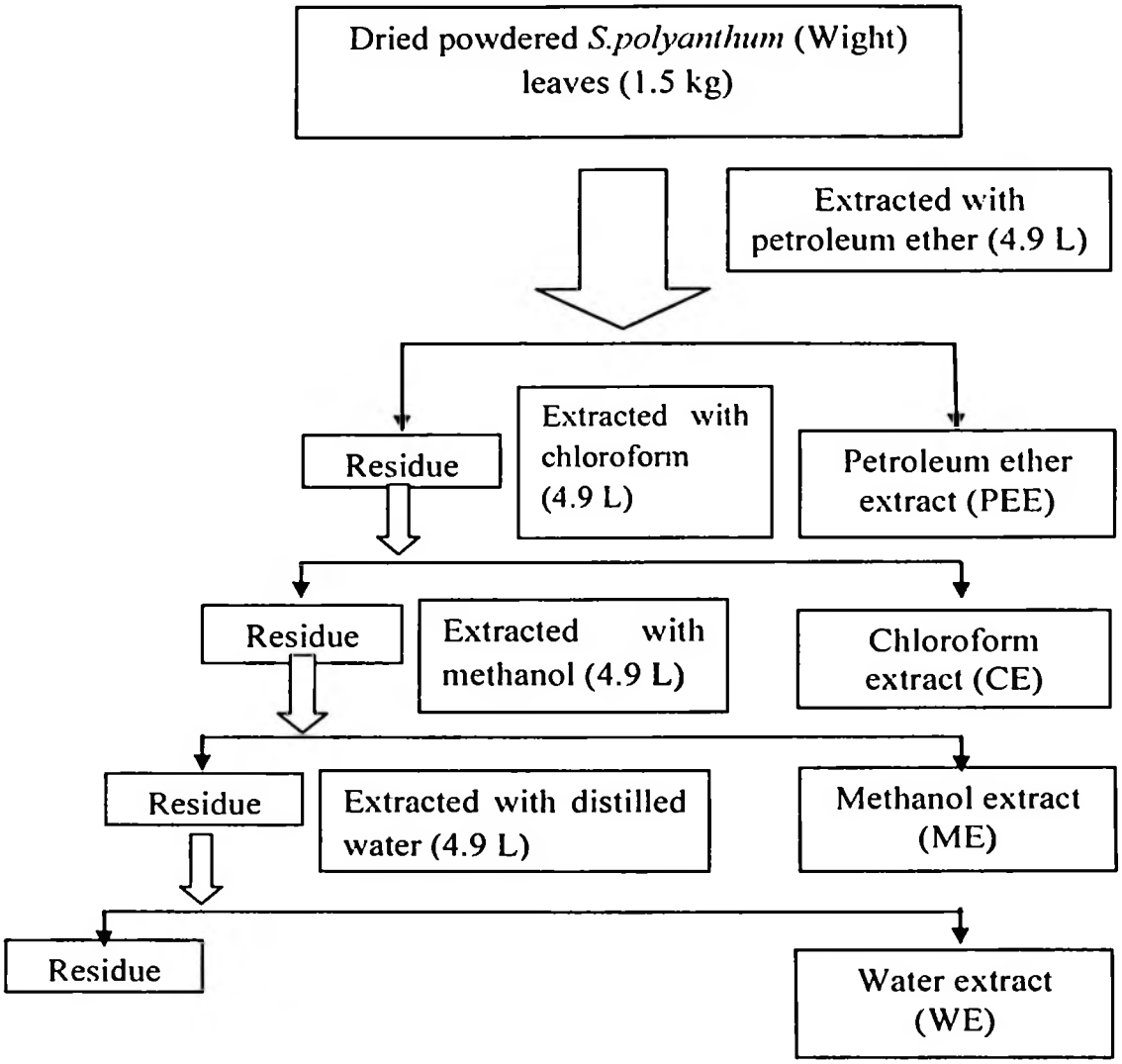


Figure 2.1. Flow chart of the serial extraction of *Syzygium polyanthum* (Wight) leaf.

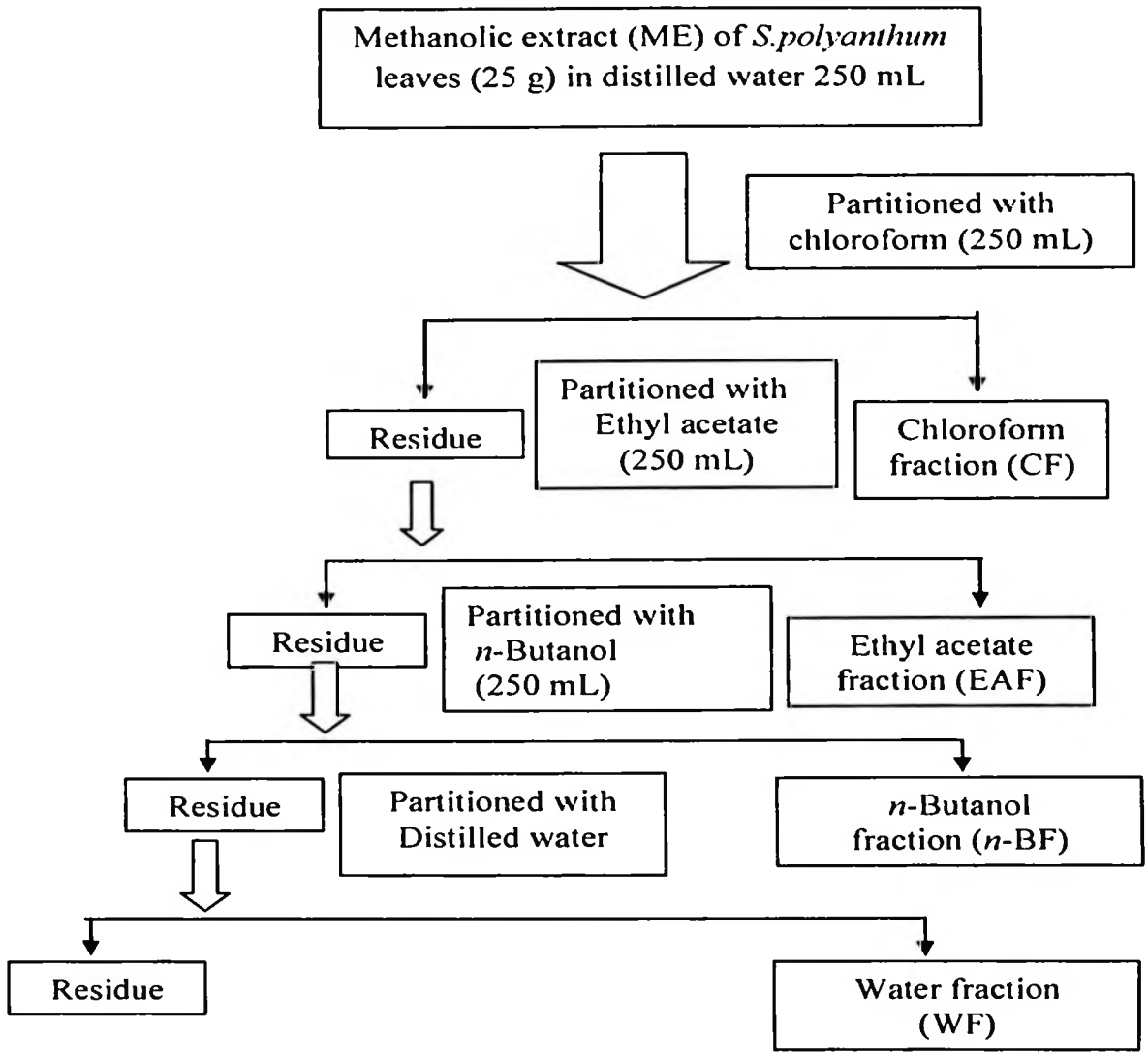


Figure 2.2. Flow chart of liquid-liquid partition of *Syzygium polyanthum* (Wight) leaf methanolic extract.

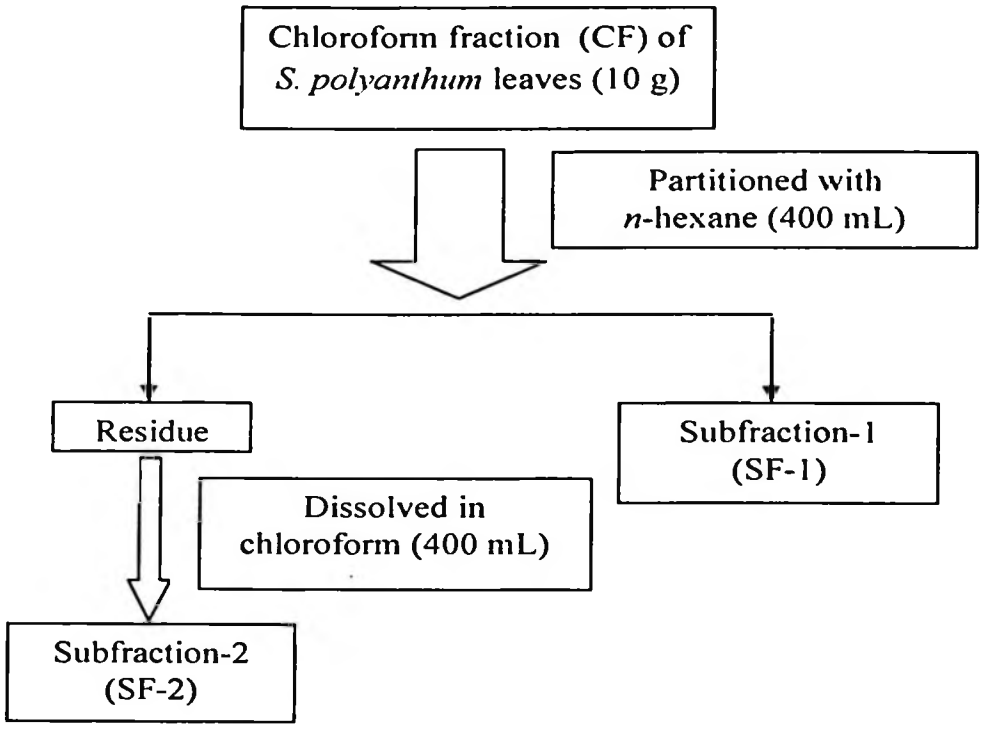


Figure 2.3. Flow chart of the fractionation of *Syzygium polyanthum* (Wight.) leaf chloroform fraction.

### 2.5.Animals

Healthy male Sprague-Dawley (SD) rats weighing between 180-250 g were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM) upon the ethical clearance of the Animal Ethics Committee of USM, Penang, Malaysia [Approval number: USM/ Animal EthicsApproval/2012/(76)(366)]. The animals were acclimatized at room temperature (25-30°C), and a 12 h dark/light cycle, and were allowed to access food (standard laboratory chow, Gold Coin SDn. Bhd., Malaysia) and water *ad libitum* for one week before being used for experimentation (Hassan et al., 2010).

### 2.6. Diabetes induction in experimental diabetic rats

STZ (prepared in a 0.9% NaCl solution, to be given at a dose of 55 mg/kg) was injected intra-peritoneally to 16-hrs-fasted rats. The rats were provided with a 10 % dextrose solution instead of drinking water for the first 24 hrs. After 72 hrs, the fasting blood glucose levels (BGL) of the rats were measured. The animals that had BGL above 11 mmol/L were considered and included for the study.

### 2.7. Experimental set up

#### 2.7.1. Hypoglycaemic test of the four extracts in normal rats

Male Sprague-Dawley rats (180-200 g) were divided into six groups (n=6) and fasted overnight. The first group was given glibenclamide (10 mg/kg) and served as the positive control group. The second, third, fourth, and fifth groups were treated with 1 g/kg of PEE, CE, ME and WE, respectively. The sixth group was treated with saline (10 mL/kg) and served as the negative control group. All treatments were

administered orally using a 16 G oral needle. The blood glucose levels (BGL) were determined before and 1, 2, 3, 5 and 7 hours after treatment.

**2.7.2.Preliminary intra-peritoneal glucose tolerance test (IPGTT) in normal rats**

Preliminary glucose tolerance test was performed using metformin. Male Sprague-Dawley rats (200-250 g) were divided into 2 groups (n=6) and fasted overnight. The first group was fed orally with saline (10 mL/kg) and served as the negative control. The second group was treated with metformin (500 mg/kg) and served as the positive control. Both of the groups were then administered with glucose (500 mg/kg) by an intra-peritoneal injection, 60 min after drug administration. The blood glucose levels of the rats were measured using blood samples from the tail vein 60 min before the oral treatment, and 0, 15, 30, 45, 60, 90 and 120 min after glucose loading (Hassan et al., 2010; Kasmuri et al., 2010). Similar procedures were conducted for two doses of glucose 1 g/kg and 1.5 g/kg.

**2.7.2.1. Intra-peritoneal glucose tolerance test (IPGTT) in normal rats**

In IPGTT, male Sprague-Dawley rats (200-250 g) were divided into six groups (n=6) and fasted overnight. The first group was fed with saline (10 mL/kg). The second group was treated with metformin (500 mg/kg) and served as the positive control. The third, fourth, fifth and sixth group were treated with 1 g/kg of PEE, CE, ME and WE, respectively. All of the rats were then administered with glucose (1 g/kg) given by an intra-peritoneal injection, 60 min after metformin/extracts administration. The blood glucose levels of the rats were measured from the tail vein 60 min before treatment and 0, 15, 30, 45, 60, 90 and 120 min after glucose loading (Hassan et al., 2010; Kasmuri et al., 2010).

### 2.7.3. Anti-hyperglycaemic test of single-dose oral administration of the four extracts in STZ-induced diabetic rats

Diabetic rats were divided into six groups (n=6). The first group was administered with saline (10 mL/kg) and served as the negative control. The second group was given metformin (500 mg/kg) to serve as the positive control. The third, the fourth, the fifth and the sixth groups were treated with 1 g/kg of PEE, CE, ME and WE, respectively. The blood glucose levels were measured prior to treatment and 1, 2, 3, 5 and 7 hours after treatment.

### 2.7.4. The effect of twice-daily oral administration of the four extracts on the blood glucose levels of STZ-induced diabetic rats

Diabetic rats were equally divided into six groups (n=6). The first group was administered with metformin (500 mg/kg). The second, third, fourth, and fifth groups were treated with 1 g/kg of PEE, CE, ME and WE, respectively. The sixth group was treated with saline (10 mL/kg). All treatment were administered orally, twice daily, for 6 days. The blood glucose levels were measured before and 6 days after treatment.

### 2.7.5. The effect of twice-daily oral administration of the four fractions on the blood glucose levels of STZ-induced diabetic rats

Diabetic rats were equally divided into six groups (n=6). The first group was administered with metformin (500 mg/kg). The second, third, fourth, and fifth groups were treated with 500 mg/kg of CF, EAF, *n*-BF and WF, respectively. The sixth group was treated with saline (10 mL/kg). All treatment were administered orally, twice daily, for 6 days. The blood glucose levels were measured before and 6 days after treatment.

### 2.7.6. The effect of twice-daily oral administration of the two subfractions on the blood glucose levesl of STZ-induced diabetic rats

The diabetic rats were equally divided into four groups (n=6). The first group was administered with SF-1 (250 mg/kg). The second, third, and fourth, were treated with SF-2 (250 mg/kg), metformin (500 mg/kg) and saline (10 mL/kg), respectively. All treatments were administered orally, twice daily, for 6 days. The blood glucose levels were measured before and 6 days after treatment.

### 2.7.7. The effect of different doses of methanolic extract of *S. polyanthum* leaves on the blood glucose levels of STZ-induced diabetic rats

Diabetic rats were divided into six groups, with each group consisting of six rats (n=6). The groups were treated twice daily orally for six days with different doses of ME (125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg), metformin (500 mg/kg), which served as the positive control, and saline (10 mL/kg), which served as the negative control. The blood glucose levels were measured before treatment and on day 6.

## 2.8. Phytochemical screening of methanol extract, chloroform fraction, water fraction and *n*-hexane fraction of *S. polyanthum* leaves

### 2.8.1. Identification by chemical tests

#### 2.8.1.1.Identification of free reducing sugars

Half a gram of the sample (extract) was dissolved in distilled water and filtered. The filtrate was heated with Fehling solutions A (1 mL) and B (1 mL). A red precipitate of cuprous oxide indicated the presence of reducing sugars.

Fehling A: 7 g of $CuSO_4 . 7H_2O$ in 100 mL of distilled water.

Fehling B: 24 g of KOH + 34.6 g Na-K-tartrate in 100 mL of distilled water.

#### 2.8.1.2. Identification of tannins

Half a gram of the extract was stirred with 10 mL of distilled water and filtered. Four drops of 1% ferric chloride solution were added to 2 mL of the filtrate. A bluish-black, green or bluish-green precipitate indicated the presence of tannins.

#### 2.8.1.3. Identification of steroids (Liebermann-Burchard test)

Approximately 0.2 g of extract was mixed with 2 mL of acetic acid and cooled well on ice followed by the addition of concentrated $H_2SO_4$ carefully in fume chamber. The formation of violet, blue or bluish-green steroid ring indicates the presence of steroids.

#### 2.8.1.4. Identification of terpenoids

Approximately 0.1 g of the extract was dissolved in 10 mL ethanol. Then, 1mL of acetic anhydride was added, and this was followed by the addition of 3 drops of $H_2SO_4$. Pink or violet coloration indicated the presence of terpenoids.

#### 2.8.1.5. Identification of flavonoids (Ferric chloride test)

Half a gram of the extract was boiled with 3 mL of distilled water and filtered. To 2 mL of the filtrate, 5 drops of a 10% ferric chloride solution were added. A color changes to greenish-blue or violet indicated the presence of the phenolic hydroxyl group.

### 2.8.1.6. Identification of soluble starches

Approximately 0.1 g of the extract was boiled with 1 mL of 5% KOH, cooled and acidified with concentrated $H_2SO_4$. Yellow coloration was taken as a sign for the presence of soluble starches.

### 2.8.1.7. Identification of saponins

Approximately 0.2 g of the extract were dissolved in 5 mL of distilled water on a water bath until boiling. The solution was then cooled and filtered. Three mL of distilled water were added to the filtrate and the solution was shaken vigorously. Formation of froth indicated the presence of saponin.

### 2.8.1.8. Identification of glycosides

Half a gram of the extract was dissolved in 1 mL of distilled water. Aqueous NaOH 2M was added to the solution, and yellow coloration indicated the presence of glycosides.

### 2.8.2. Identification by gas chromatography-mass spectrometry (GC-MS)

ME, CF and SF-1 (4 mg respectively) were dissolved in 1 mL of the appropiate solvents. ME was dissolved in methanol, whereas CF and SF-1 were dissolved in chloroform. According to the literature, so far, no compound with anti-diabetic activity has been reported to be found in *Syzygium polyanthum*. Therefore the samples were tested using GC-MS and compared with the data-base. The compound squalene (SQ) was found in each of the samples. The compound was then purchased from Sigma as a refference compound.

Squalene (synonym: 2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene $[(CH_3)_2C[=CHCH_2CH_2C(CH_3)]_2=CHCH_2-]_2$; molecular weight 410.72, >98% liquid, was dilluted in methanol into 1 mg/mL concentration.

GC-MS analysis was conducted under the following conditions: column used: HP-5MS, initial oven temperature: 70°C (2 min), raised to 280°C at 20°C per min, final time: 20 min, total run time: 32.50 min, flow: 1.2 mL/min, splitless. One microliter of each of ME, CF and SF-1 (4 mg/mL respectively) solutions was injected into the GC apparatus. Mass spectrometry apparatus conditions: scan parameter for low mass 35.0 and high mass 650.0; MS Quad 150 °C; MS Source: 230 °C. The mass spectrum generated by GC-MS was analysed using the data-base of the National Institute of Standards and Technology (NIST 02). A compound was considered a match, when the match quality was more than 90% which indicated good similarity with the spectrum of squalene in the library. Confirmations were conducted with ion fractionation to identify squalene in ME, CF and SF-1.

### 2.9. The effect of different doses of squalene on the blood glucose level of STZ-induced diabetic rats

The diabetic rats were divided into six groups, with each group consisting of six rats (n=6). The groups were treated orally with different doses of squalene (SQ) (20 mg/kg, 40 mg/kg, 80 mg/kg and 160 mg/kg), metformin (500 mg/kg), which served as the positive control, and saline (10 mL/kg), which served as the negative control twice daily respectively for six days. The fasting blood glucose levels were measured on day 0 and six days after the first treatment.

## 2.10. Statistical analysis

The data was expressed as mean ± standard error of the mean (SEM). The differences between the groups were statistically analyzed by one way ANOVA at alpha value 0.05, followed by Dunnett's test as a post hoc test. The differences between two groups were analysed using the independent t-test. Pre-treatment and post-treatment comparisons were performed using the paired *t*-test. Pearson's correlation coefficient test was applied to assess the correlation between SQ and BGL. For statistical analysis, SPSS Version 21.0 (IBM®, Chicago, Illinois, USA) software package was used.

## 2.11. Results

### 2.11.1. Extracts, fractions and subfractions yields

One and a half kilogram of powdered *S. polyanthum* leaves were extracted and yielded: PEE (46.16 g), CE (74.47 g), ME (79.71 g) and WE (44.97 g). Furthermore, from 25 g of ME, CF (4.8 g), EAF (1.7 g), *n*-BF (5 g) and WF(7.8. g) were obtained. The yields from 10 g of CF were 6.3 g of SF-1 and 1.4 g of SF-2 (Table 2.1).

Table 2.1. The yields of *S. polyanthum* (Wight) leaf extraction.

| **Dried Leaves (1.5 kg)** | | | **ME (25 g)** | | | **CF (10 g)** | | |
|---|---|---|---|---|---|---|---|---|
| Extract | g | % | Fraction | g | % | Subfraction | g | % |
| PEE | 46.16 | 3.07 | CF | 4.8 | 19.2 | SF-1 | 6.3 | 63 |
| CE | 74.47 | 4.96 | EAF | 1.7 | 6.8 | SF-2 | 1.4 | 14 |
| ME | 79.71 | 5.31 | *n*-BF | 5 | 20 | | | |
| WE | 44.97 | 8.9 | WF | 7.8 | 31.2 | | | |

### 2.11.2. Hypoglycaemic effect of the four extracts in normal rats

Figure 2.4 shows that oral treatment with CE, ME and WE (1 g/kg), did not significantly alter the blood glucose level (BGL) of normal rats, as compared to the control group during a seven-hour observation. However, BGL in PEE ($P<0.05$) and glibenclamide ($P<0.01$)-treated rats were significantly reduced. PEE significantly reduced the BGL at the seventh hour after treatment, whereas glibenclamide did so from the first hour until the seventh hour after orally treatment.

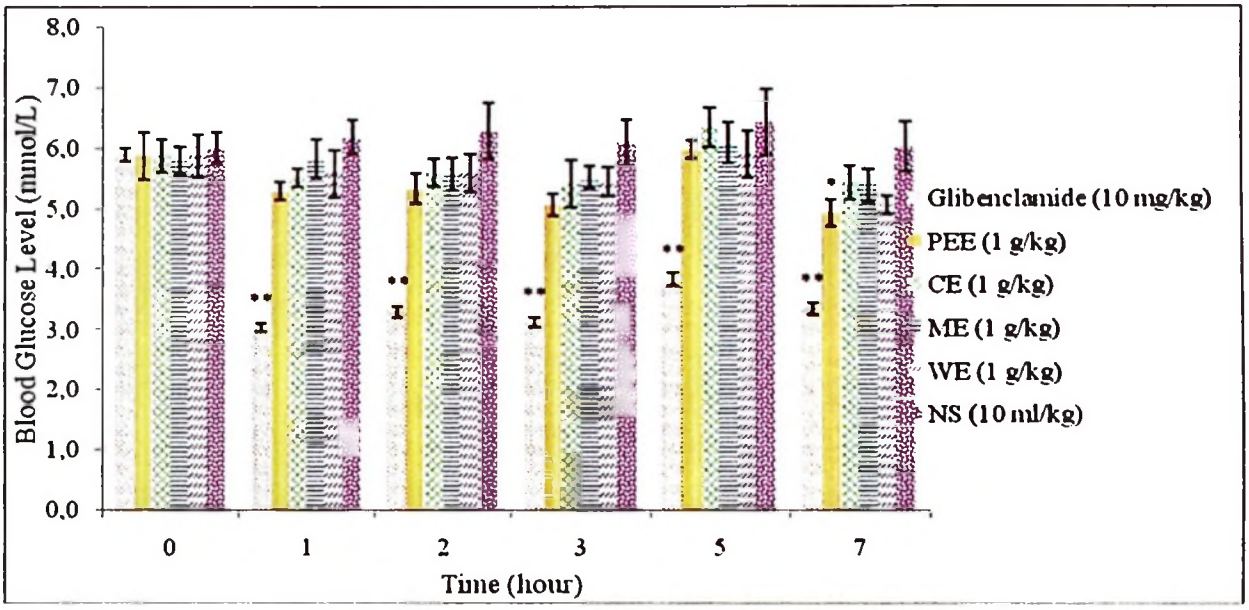


Figure 2.4. The effects of *S. polyanthum* leaf petroleum ether extract (PEE), chloroform extract (CE), methanol extract (ME) and water extract (WE) on the blood glucose levels of normal rats.
The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA followed by Dunnett's test as a post hoc test. (*$P$ < 0.05, **$P$<0.01)

### 2.11.3. Preliminary intra-peritoneal glucose tolerance test (IPGTT) in normal rats

Figure 2.5 shows that the rise in the blood glucose levels of the first set of glucose-loaded rats (500 mg/kg) was significantly different between the metformin-treated group and the control group within at time 45, 90 and 120 min ($P<0.05$) after glucose loading. Figure 2.6 shows that the rise in blood glucose levels in the second group of glucose-loaded rats (1000 mg/kg) was significantly different between metformin-treated and the control groups at the following points of time: 15, 90 and 120 min ($P<0.05$- $<0.001$). Figure 2.7 shows that the rise in blood glucose levels in the final set of glucose-loaded rats (1500 mg/kg) was significantly different between the metformin-receiving and the control groups at the 45 min observation point only ($P<0.01$).

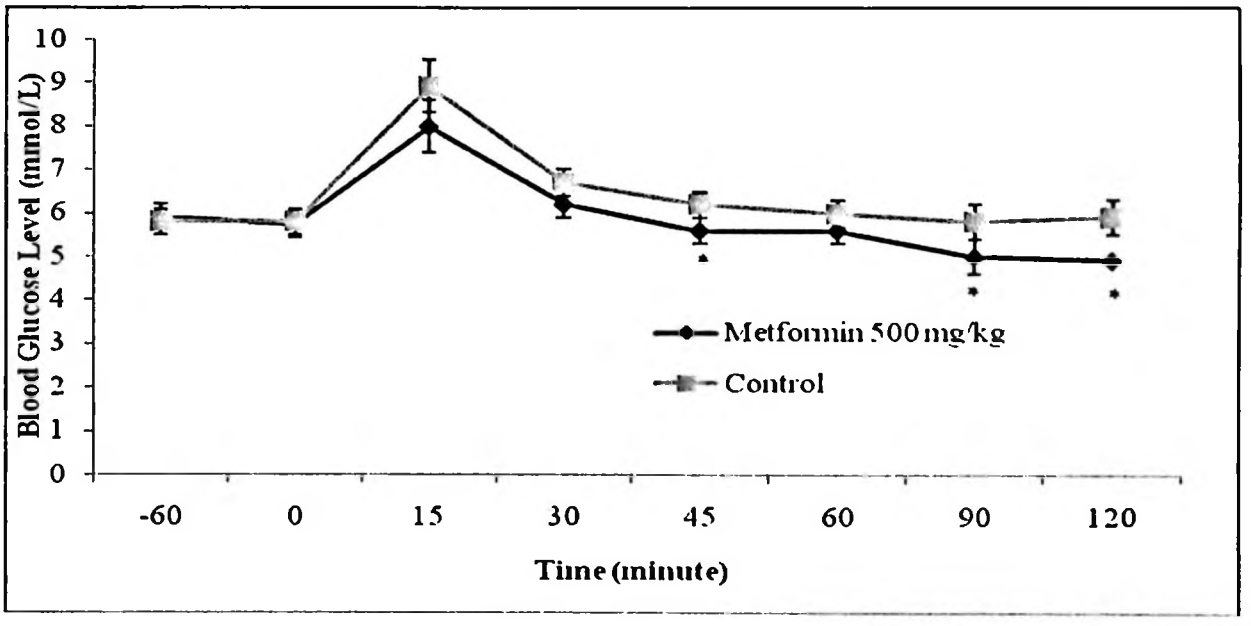


Figure 2.5. The effects of oral administration of metformin (500 mg/kg) on the blood glucose levels of rats loaded intra-peritoneally with glucose (500 mg/kg). The values are mean ± SEM (n=6). The mean differences between the two groups were analysed using the independent *t*-test (*$P<0.05$)

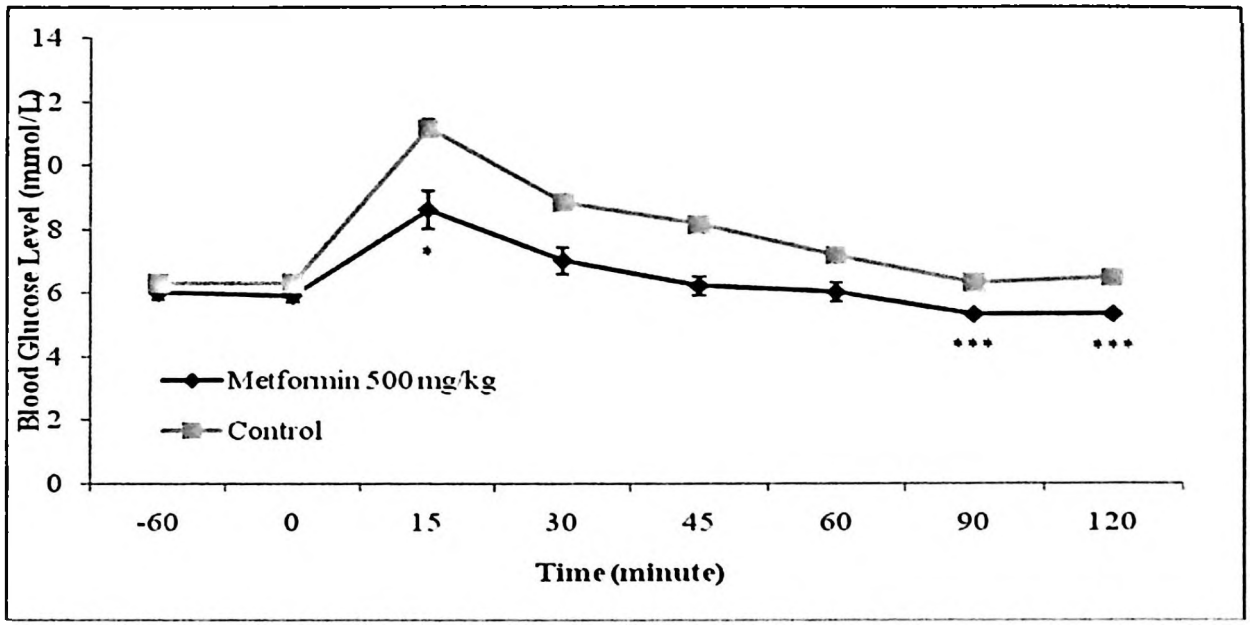


Figure 2.6. The effect of oral administration of metformin (500 mg/kg) on the blood glucose levels of rats loaded intra-peritoneally with glucose (1000 mg/kg).
The values are mean ± SEM (n=6); The mean differences between the two groups were analysed using the independent *t*-test (**P*<0.05, ****P*<0.001)

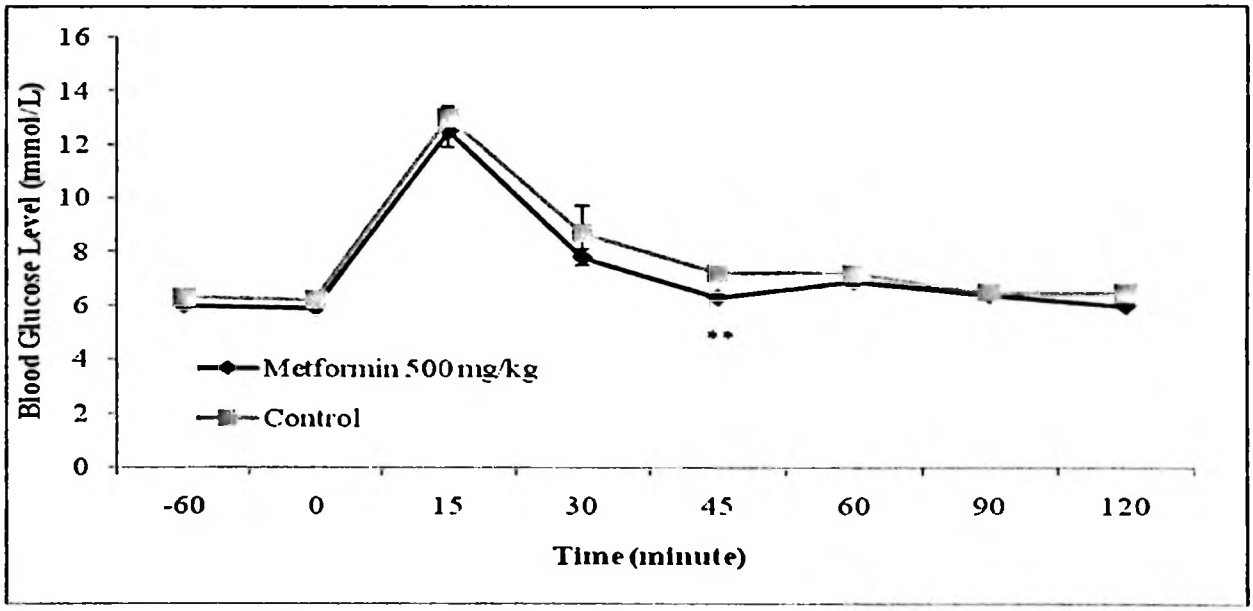


Figure 2.7. The effect of oral administration of metformin (500 mg/kg) on the blood glucose level of rats loaded intra-peritoneally with glucose (1500 mg/kg).
The values are mean ± SEM (n=6) ; The mean differences between the two groups were analysed using the independent *t*-test (***P*<0.01).

### 2.11.4. Intra-peritoneal glucose tolerance test (IPGTT) in normal rats

The increase in the blood glucose levels of the glucose-loaded normal rats (1g/kg) was not significantly altered by oral treatment with CE and ME (1 g/kg) within 120 min after glucose loading (Figure 2.8.). However, BGL in both WE- and PEE-treated groups were significantly altered 30 and 90 min after glucose loading ($P < 0.05$, respectively). Metformin significantly reduced BGL as observed after 90 min ($P<0.01$) and 120 min ($P<0.05$) in comparison with the control group.

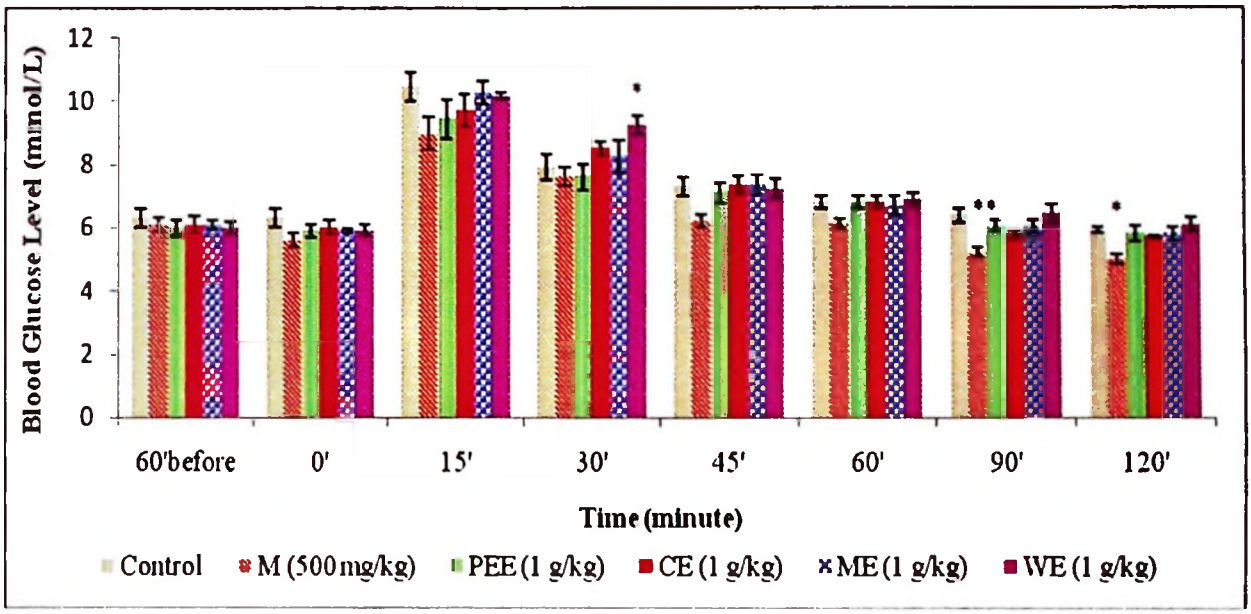


Figure 2.8. The effects of *S. polyanthum* leaf petroleum ether extract (PEE), chloroform extrac (CE), methanol extract (ME) and water extract (WE), and metformin (M) on the blood glucose levels following intra-peritoneal glucose loading (1 g/kg) in normal rats.
The values are expressed as mean ± SEM (n=6); *$P < 0.05$, **$P<0.01$ compared with the control group.

### 2.11.5. The effects of single-dose administration of the four extracts on the blood glucose levels of STZ-induced diabetic rats

The diabetic rats groups showed diabetic signs of high fasting BGL and weight loss 72 hours after STZ injection (Figure 2.9 and Figure 2.10). Diabetic rats with BGL above 11 mmol/L were included in the study. Figure 2.9 shows that there were significantly higher BGL ($P < 0.001$) in the diabetic rats (18.8 ± 1.6 mmol/L) 3 days after diabetes induction with streptozotocin compared to the normal control baseline BGL (4.9 ± 0.3 mmol/L).

Figure 2.10 shows that the body weight of the diabetic rats was significantly ($P < 0.05$) lower than that of the normal rats. Body weights were measured in fasted rats prior to and 72 hours after diabetes induction with STZ.

As shown in figure 2.11, the BGLs were not significantly affected by treatment with single-doses of PEE, CE and WE (1 g/kg) compared with the control group. On the other hand, ME- and metformin-treated groups showed significant BGL lowering effects. The BGLs were significantly reduced at the seventh hour after ME administration ($P < 0.01$), whereas metformin showed the significant effect of reducing BGL from the first hour ($P < 0.01$) until the seventh hour ($P < 0.001$) after treatment.

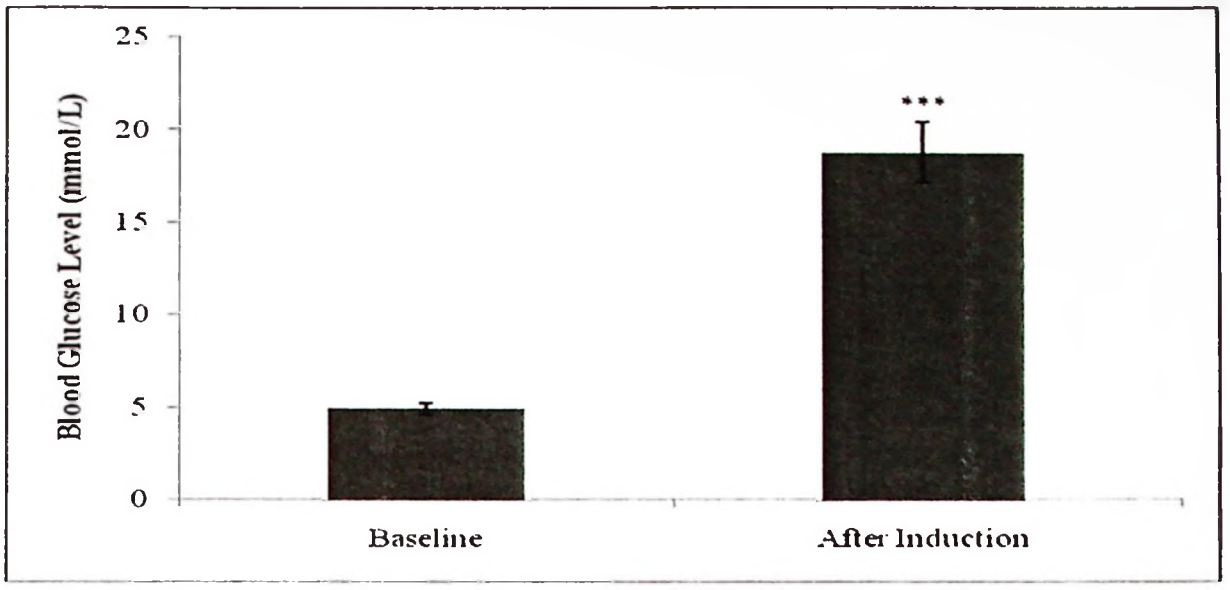


Figure 2.9. Blood glucose levels of Sprague-Dawley rats before (baseline) and 3 days after induction of diabetes with STZ.
The values are mean ± SEM (n=6). Pre-treatment (baseline) and post-treatment (after induction) comparisons were performed using the paired *t*-test.; ***$P < 0.001$

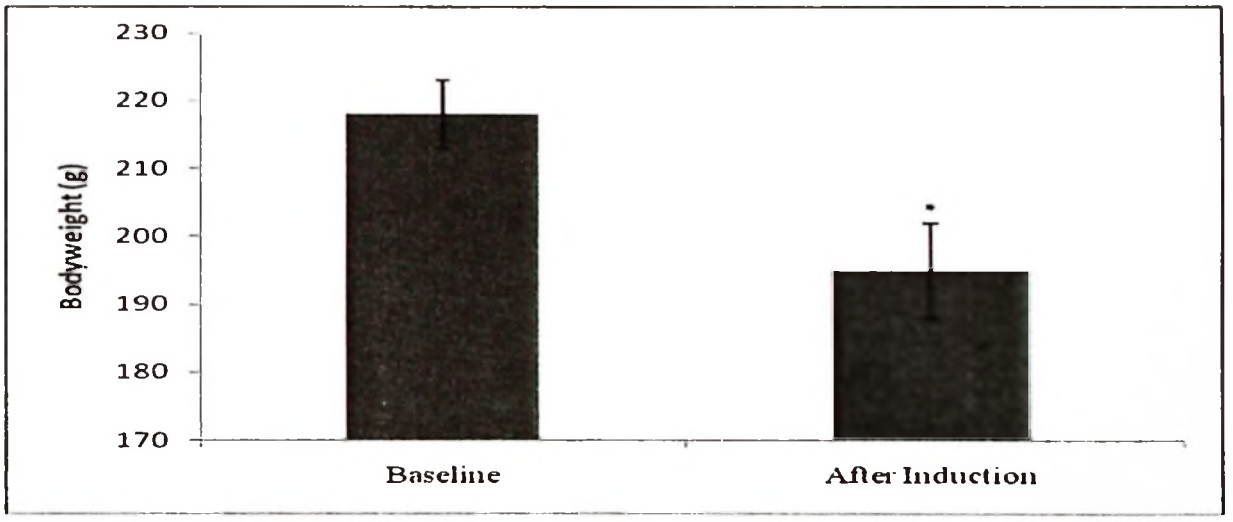


Figure 2.10. The body weights of Sprague-Dawley rats before (baseline) and 3 days after induction of diabetes with STZ.
The values were mean ± SEM (n=6). Pre-treatment (baseline) and post-treatment (after induction) comparisons were performed using the paired *t*-test.; *$P < 0.05$

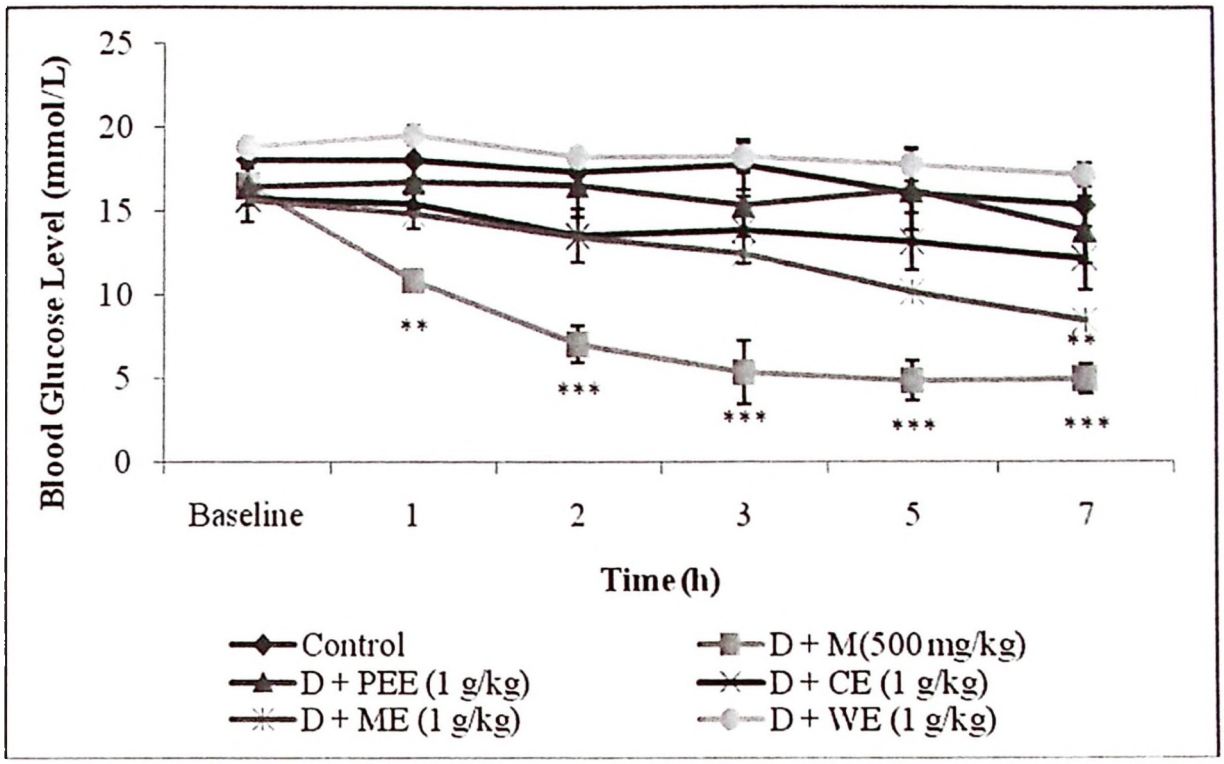


Figure 2.11. The effects of petroleum ether (PEE), chloroform (CE), methanol (ME), water (WE) extracts of *S. polyanthum* leaf and metformin (M) on the blood glucose levels of STZ-induced diabetic rats.

The values are expressed as mean ± SEM (n=6); $^{**}P < 0.01$, $^{***}P<0.001$ compared with the control group.

### 2.11.6. The effects of twice-daily oral administration of the four extracts on the blood glucose levels of STZ-induced diabetic rats

Figure 2.12 shows that the changes of BGLs were not significantly affected after twice-daily administration for 6 days with CE and WE (1 g/kg twice daily) in STZ-induced diabetic rats. On the other hand, after 6 days of treatment, BGLs of PEE-, ME- and metformin-treated group were significantly reduced ($P$<0.001).

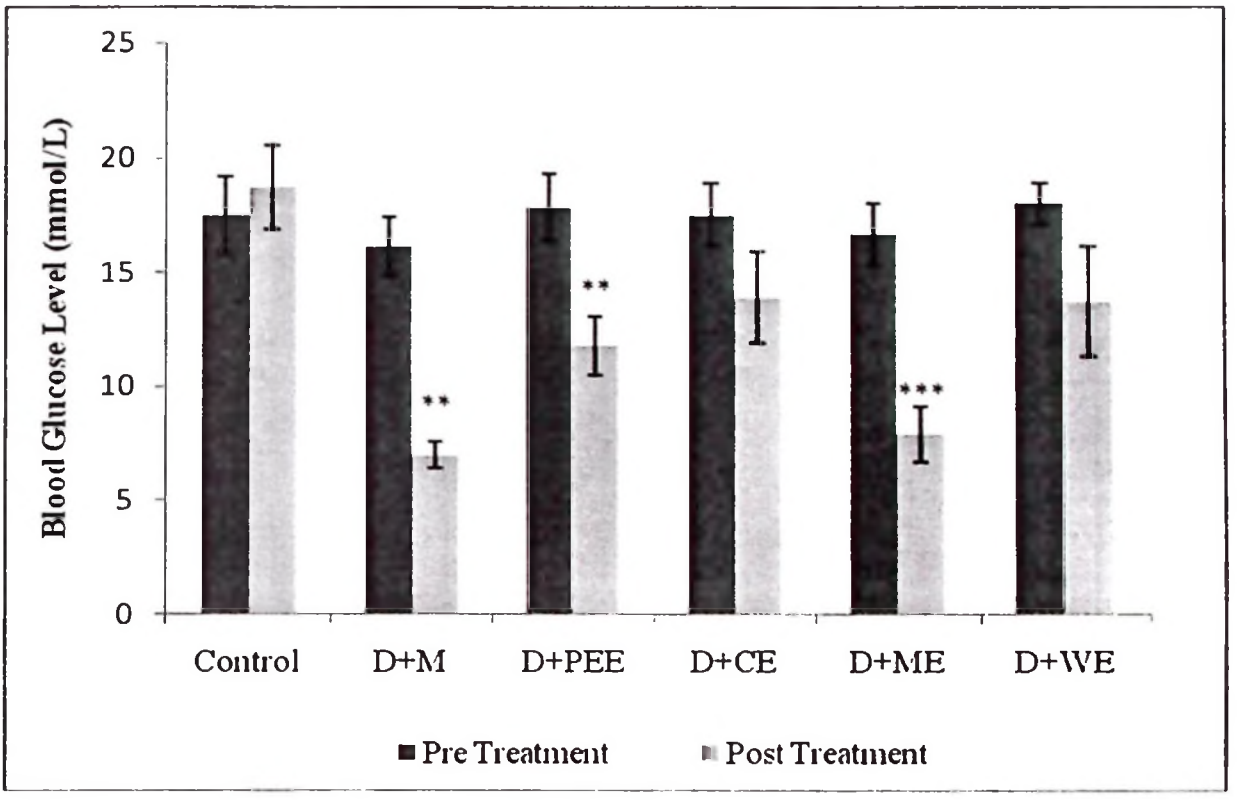


Figure 2.12. The effects of twice-daily oral administration of petroleum ether (PEE), chloroform (CE), methanol (ME) and water (WE) extracts of *S. polyanthum* leaf, 1 g/kg respectively and 500 mg/kg of metformin (M) for 6 days on the blood glucose level of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); Mean differences between pre-treatment and post-treatment BGL were analyzed using paired $t$-test, $*P < 0.05$, $***P<0.001$

### 2.11.7. The effects of twice-daily oral administration of the four fractions on the blood glucose levels of STZ-induced diabetic rats

Figure 2.13 shows that the changes of BGLs were not significantly affected after repeated oral administration for 6 days with EAF and *n*-BF in STZ-induced diabetic rats. On the other hand, the CF-, WF- and metformin-treated groups showed significant reductions in BGL ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) after 6 days of treatment.

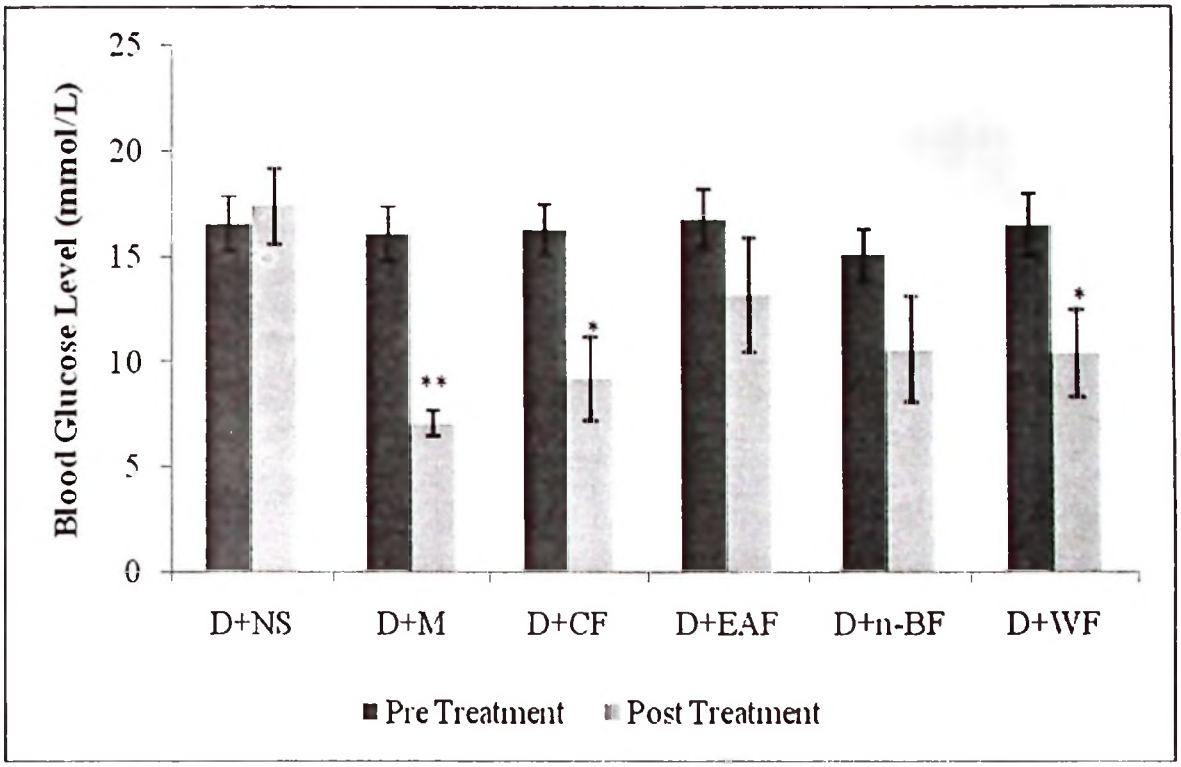


Figure 2.13. The effects of twice-daily oral administration of chloroform (CF), ethyl acetate (EAF), butanol (*n*-BF) and water (WF) fractions of *S. polyanthum* leaf extract, and metformin (M), administered at 500 mg/kg respectively, for 6 days on the blood glucose levels of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); The mean differences between pre-treatment and post-treatment BGL were analyzed using paired *t*-test, $*P < 0.05$; $**P < 0.01$

### 2.11.8. The effects of twice-daily oral administration of the two subfractions on the blood glucose levels of STZ-induced diabetic rats

Figure 2.14 shows that the changes of BGL were not significantly affected after twice-daily oral administration with SF-2 in STZ-induced diabetic rats. Meanwhile, BGL of SF-1- and metformin-treated groups were significantly decreased ($P < 0.01$).

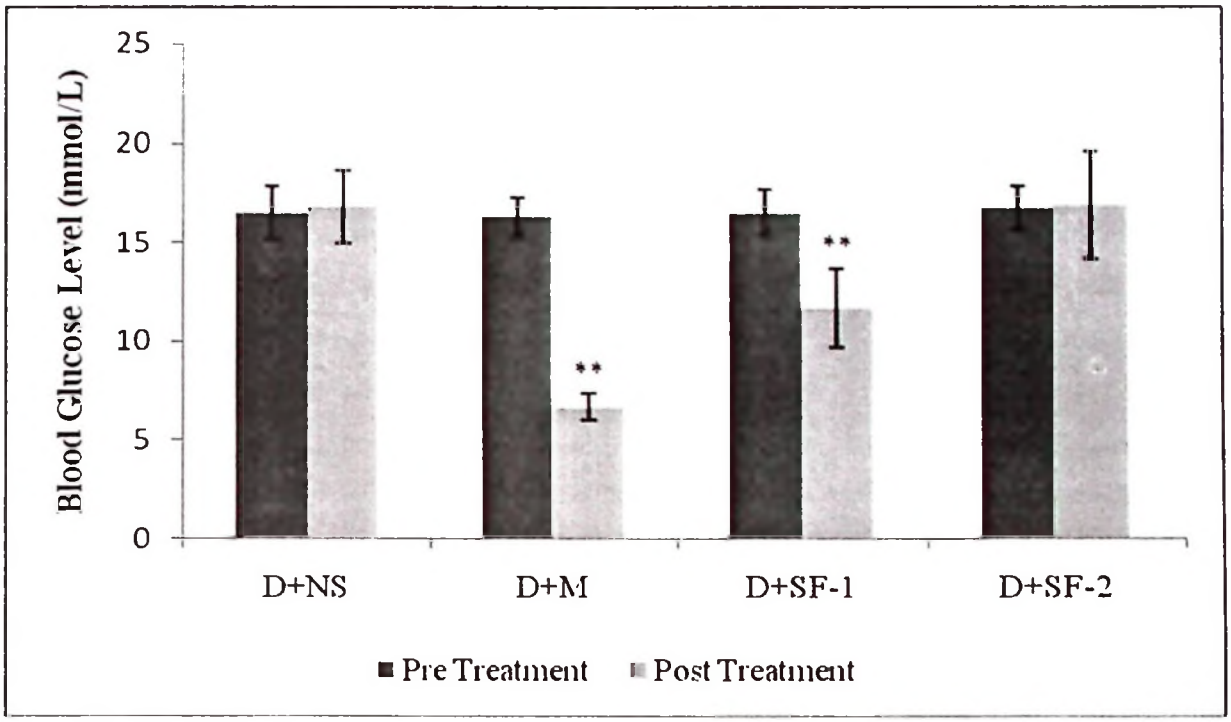


Figure 2.14. The effects of twice-daily oral administration of subfraction-1 (SF-1), subfraction-2 (SF-2) of *S. polyanthum* leaf extract (250 mg/kg, respectively), and 500 mg/kg of metformin (M) for 6 days on the blood glucose levels of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); The mean differences between pre-treatment and post-treatment BGL were analyzed using paired *t*-test, $^{**}P < 0.01$

### 2.11.9. The effect of different doses of the methanolic extract on the blood glucose levels of STZ-induced diabetic rats

Figure 2.15 shows that the decreases observed in fasting BGL after 6 days of twice-daily oral administration of ME 125 mg/kg were not significant. However, the doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg significantly (P< 0.05-0.001) and dose-dependently reduced the fasting BGL.

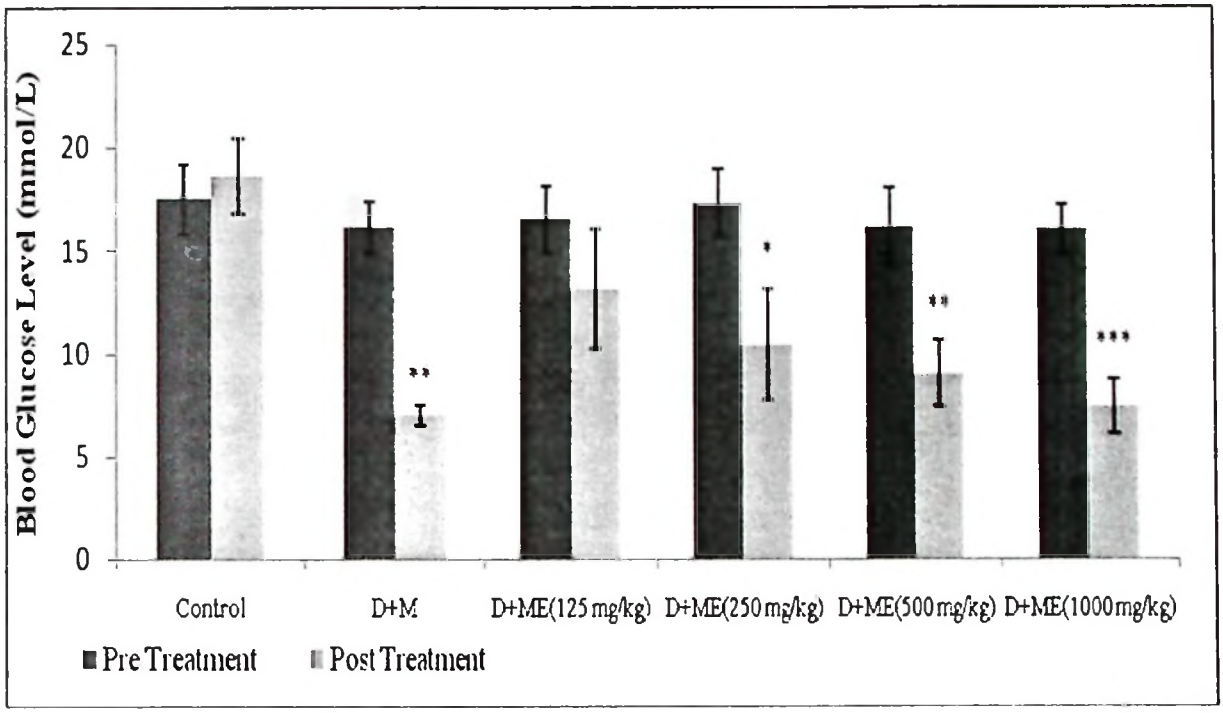


Figure 2.15. The effects of twice-daily oral administration of 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg of the methanolic extracts (ME) of *S. polyanthum* leaf and 500 mg/kg of metformin (M) on the blood glucose levels of STZ-induced diabetic rats.
The values were expressed as mean ± SEM (n=6); The mean differences between pre-treatment and post-treatment BGL were analyzed using paired *t*-test, $*P < 0.05$; $** P < 0.01$;$***P < 0.001$

### 2.11.10. Phytochemical study of *S. polyanthum* leaf methanol extract (ME), and its chloroform fraction (CF), water fraction (WF) and *n*-hexane fraction (SF-1)

#### 2.11.10.1. Identification by chemical tests

Table 2.2 shows the results of phytochemical screening of ME, CF, WF and SF-1. Qualitative assay of ME, CF and SF-1 showed the presence of tannins, steroids, terpenoids, flavonoids, saponins and glycosides. Whereas, WF showed the presence of sugars, tannins, flavonoids, saponins and glycosides.

Table 2.2. Phytochemical screening of ME, CF, WF and SF-1 of *Syzygium polyanthum* (Wight) leaf.

| No | Test | ME | CF | WF | SF-1 |
|---|---|---|---|---|---|
| 1 | Reducing sugars | (-) | (-) | (+) | (-) |
| 2 | Tannins | (+) | (+) | (+) | (+) |
| 3 | Steroids | (+) | (+) | (-) | (+) |
| 4 | Terpenoids | (+) | (+) | (-) | (+) |
| 5 | Flavonoids | (+) | (+) | (+) | (+) |
| 6 | Soluble starches | (-) | (-) | (-) | (-) |
| 7 | Saponins | (+) | (+) | (+) | (+) |
| 8 | Glycosides | (+) | (+) | (+) | (+) |

(+) = detected
(-) = not detected

**2.11.10.2. Identification by gas chromatography-mass spectrometry (GC-MS)**

ME, CF and SF-1 of *S. polyanthum* leaf were found to consist of many constituents that may have contributed to their medicinal activitities. Based on the matching quality with the library, one compound in ME, five compounds in CF and three compounds in SF-1 have been identified (Table 2.3). One compound was identified as squalene (SQ), and was consistently found in each of the samples. The percentages of SQ were based on the peak height in ME 0.11%, CF 0.04% and SF-1 0.26% (Table 2.4).

Table 2.3. Phytochemical components identified in the methanolic extract (ME), chloroform fraction (CF) and *n*-hexane subfraction (SF-1) with squalene serving as the standard by gas chromatogaphy-mass spectrometry (GC-MS).

| Samples | RT | Peak(%) | Compound(s) | MF |
|---|---|---|---|---|
| ME | 14.96 | 7.60 | Squalene | $C_{30}H_{50}$ |
| CF | 10.74 | 6.90 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ |
| | 11.49 | 2.03 | 9,12-Octadecadienoic acid, methyl ester | $C_{19}H_{34}O_2$ |
| | 14.97 | 8.92 | Squalene | $C_{30}H_{50}$ |
| | 18.37 | 4.73 | Vitamin E | $C_{29}H_{50}O_2$ |
| | 21.66 | 22.57 | Stigmasterol, 22,23-dihydro- | $C_{29}H_{50}O$ |
| SF-1 | 14.92 | 4.54 | Squalene | $C_{30}H_{50}$ |
| | 18.28 | 4.26 | Vitamin E | $C_{29}H_{50}O_2$ |
| | 21.50 | 33.37 | Stigmasterol, 22,23-dihydro- | $C_{29}H_{50}O$ |

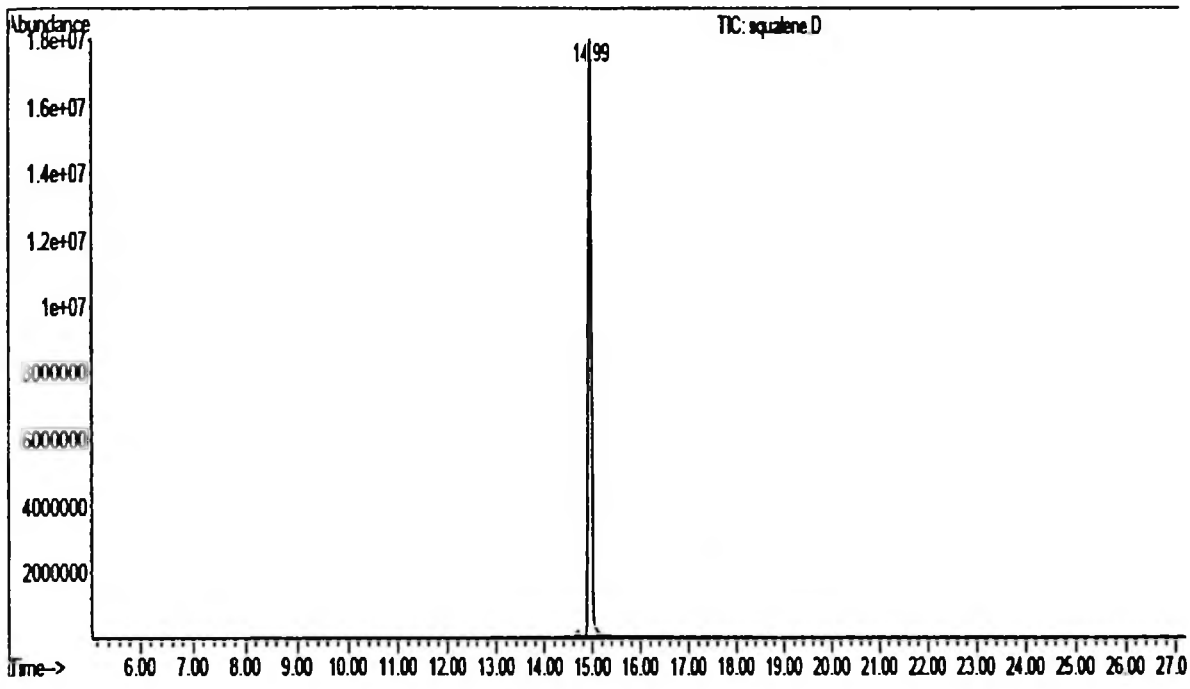


Figure 2.16. Gas chromatography-mass spectrometry (GC-MS) chromatogram of squalene as a standard compound

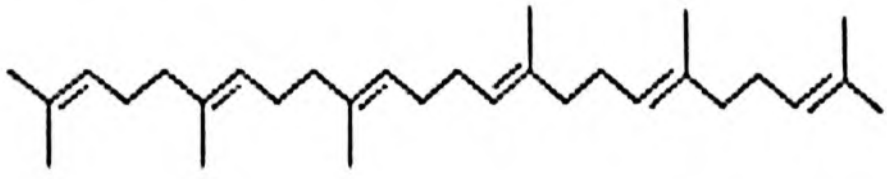

Figure 2.17. Chemical structure of squalene

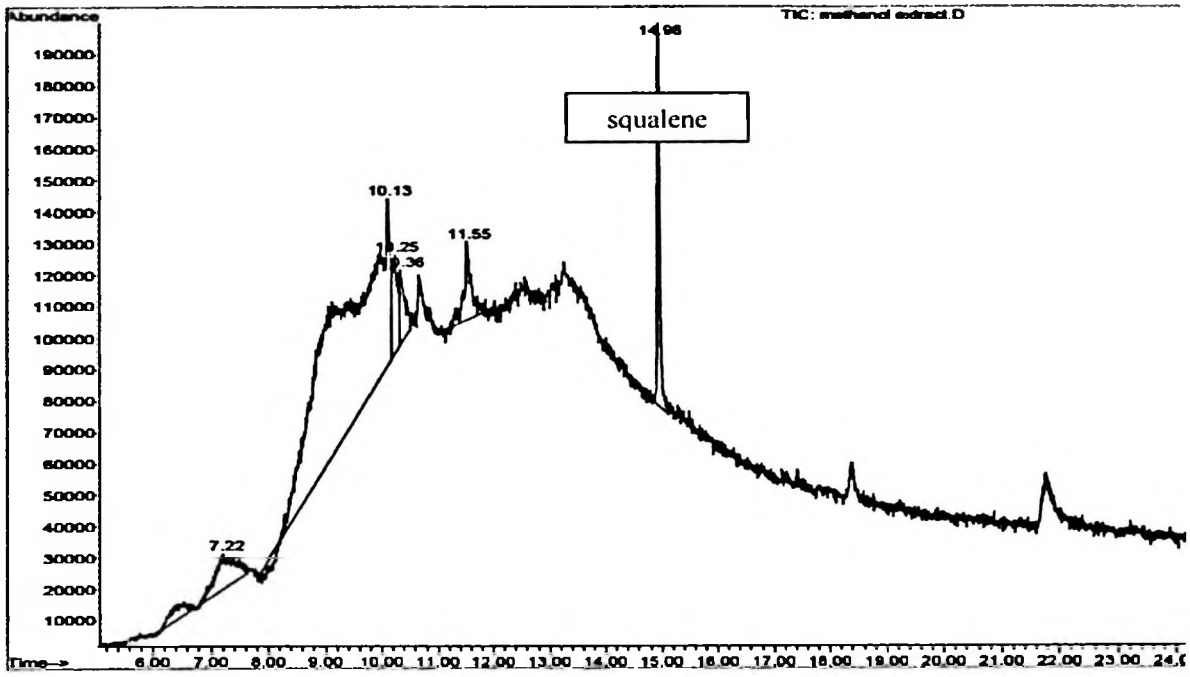


Figure 2.18. Gas chromatography-mass spectrometry (GC-MS) chromatogram of methanol extract (ME) of *S. polyanthum* leaf with squalene as the standard.

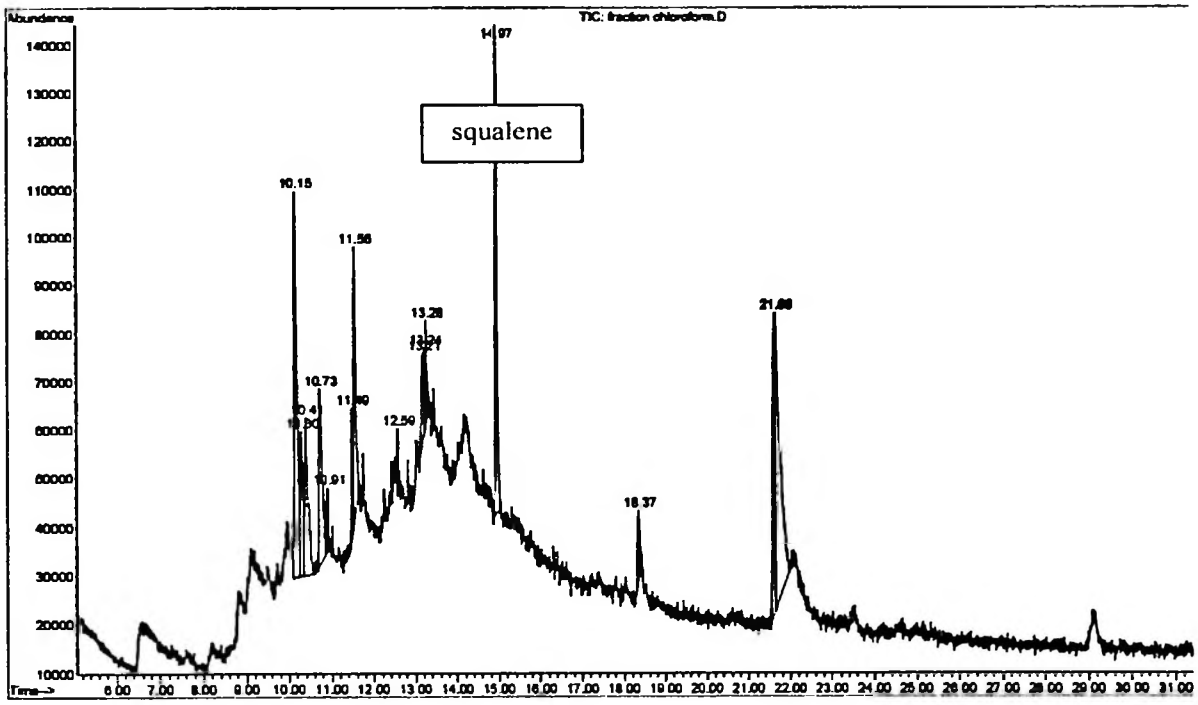


Figure 2.19. Gas chromatography-mass spectrometry (GC-MS) chromatogram of the chloroform fraction (CF) of *S. polyanthum* leaf extract with squalene as the standard.

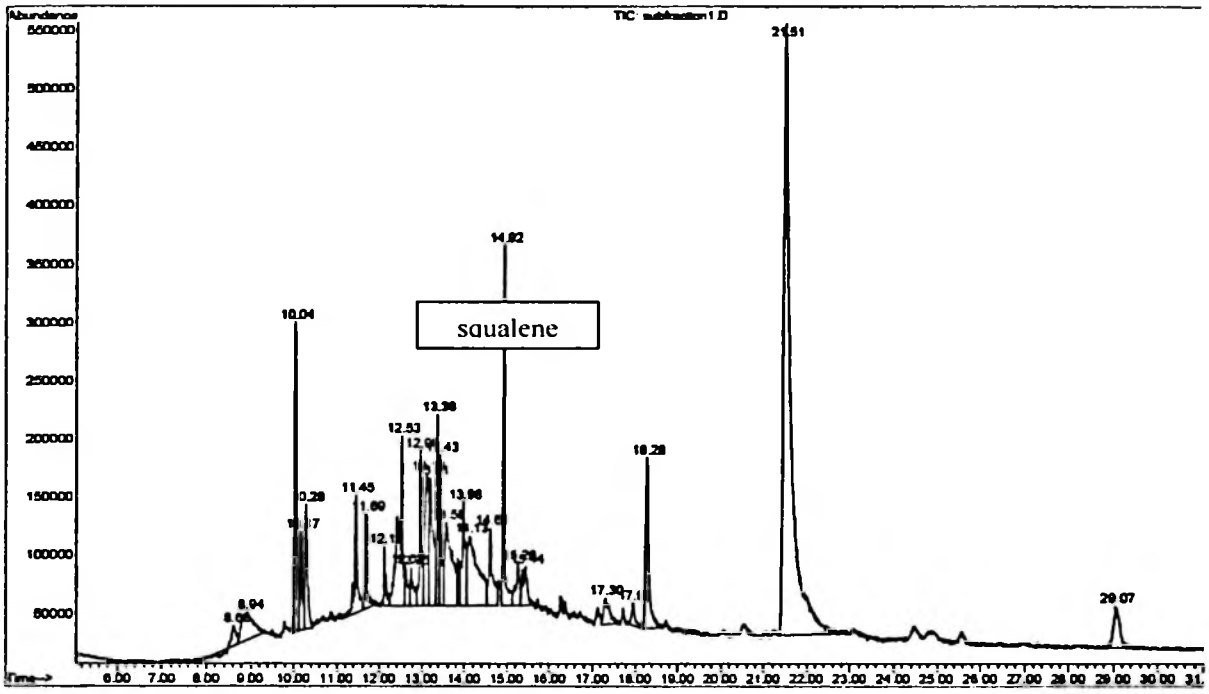


Figure 2.20. Gas chromatography-mass spectrometry (GC-MS) chromatogram of *n*-hexane fraction (SF-1) of *S. polyanthum* leaf extract, with squalene as the standard.

Table 2.4. The percentages of squalene in the methanol extract (ME), chloroform fraction (CF) and *n*-hexane fraction (SF-1).

| Sample | Amount (mg/mL) | Conc (µg/mL) | Percentage (%) |
|---|---|---|---|
| ME | 5 | 4.17 | 0.11 |
| CF | 10 | 5.59 | 0.04 |
| SF-1 | 5 | 13.19 | 0.26 |

**2.11.11. Anti-hyperglycaemic test of squalene (SQ) in STZ-induced diabetic rats**

Figure 2.21 shows that squalene (20 mg/kg) administered twice daily for six days had no significant effect on BGL of diabetic rats. However, higher doses of SQ, 40 mg/kg, 80 mg/kg and 160 mg/kg significantly reduced BGL compared to the pre-treatment levels ($P$<0.05-0.001). Metformin (500 mg/kg) also significantly ($P$<0.001) reduced BGL 6 days after treatment. At the present study, a significant ($P$<0.05) positive correlation between squalene use and lower BGL was observed in STZ-induced diabetic rats ($r^2$=0.976).

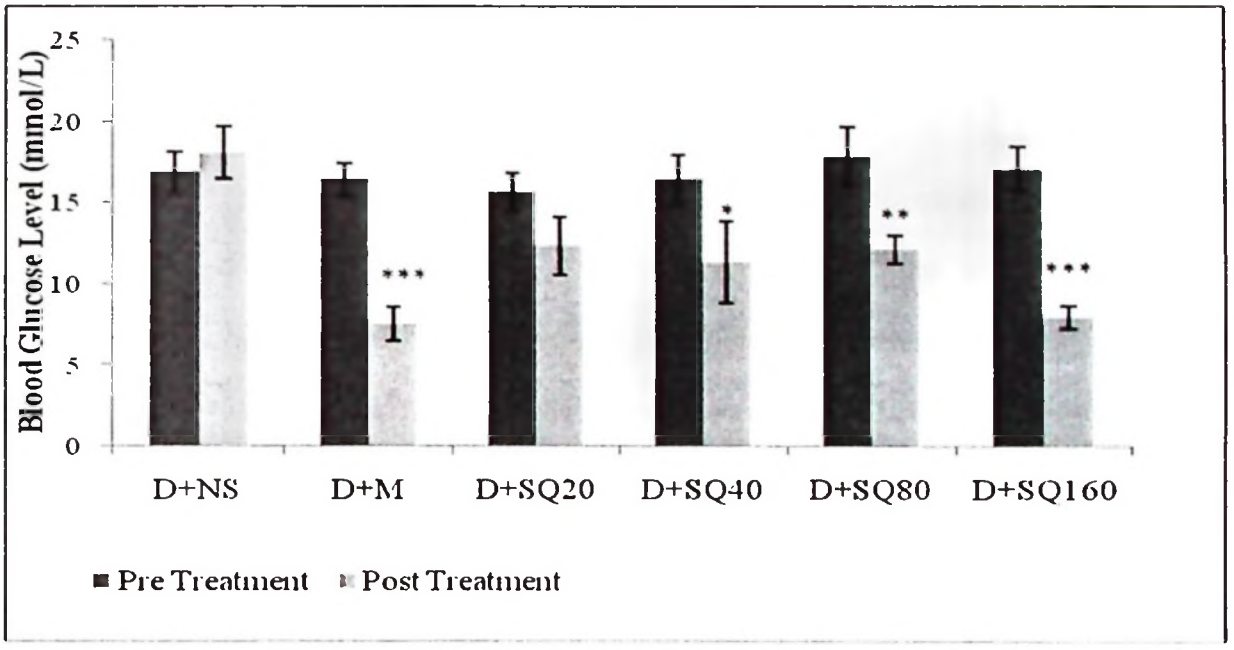


Figure 2.21. The effects of twice-daily oral administration of 20 mg/kg, 40 mg/kg, 80 mg/kg and 160 mg/kg of squalene (SQ), and 500 mg/kg of metformin (M) for six days on the blood glucose levels of streptozotocin-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); The mean differences between pre-treatment and post-treatment BGL were analyzed by the paired $t$-test, *$P$ < 0.05; ** $P$ < 0.01;***$P$ < 0.001

## 2.12. Discussion and conclusions

The present study was conducted in normal and diabetic rats to investigate the hypoglycaemic and anti-hyperglycaemic effects of *S.polyanthum* (Wight) leaf extracts. Preliminary studies in normal rats, the hypoglycaemic tests, used a sulphonylurea agent (glibenclamide) as the control, whereas the intra-peritoneal glucose tolerance tests (IPGTT) were conducted using a biguanide (metformin). The hypoglycaemic tests demonstrated that glibenclamide reduced the BGL from the first hour until the seventh hour after oral administration (Figure 2.4.). Glibenclamide is of the sulphonylurea class of the anti-diabetic agents. It act by increasing insulin secretion from the pancreatic β-cells. The cellular mechanism underlying its actions is by closing the ATP-sensitive potassium channels, depolarizing the β-cell plasma membranes, and increasing the intracellular calcium concentrations. Increased intracellular calcium results in increased insulin secretion (Nolte & Karam, 2004). These actions cause the effectiveness of glibenclamide in decreasing both fasting blood glucose levels, and postprandial hyperglycaemia (Chew & Leslie, 2006). Treatment with a single dose of CE, ME and WE did not significantly affect BGL of normal fasting rats. Even though PEE reduced fasting BGL significantly at the seventh hours, lowering them from 5.9 ± 0.38 mmol/L to 4.9 ± 0.22 mmol/L, the levels were not within the hypoglycaemic range. Hypoglycaemia is a condition characterized by blood glucose levels, falling below 3.8 mmol/L (Yakubovich & Gerstein, 2011). Glibenclamide, in the present study showed the ability to reduce BGL to be within the hypoglycaemic range (5.9 ± 0.11 mmol/L to 3.4 ± 0.10 mmol/L), which validated its activity as a hypoglycaemic agent. A previous study by

Anggadiredja (1998) reported that an ethanol-insoluble aqueous extract fraction of *S.polyanthum* leaves (0.7 g/kg) showed the ability to reduce BGL from 109.4 mg/dL (6.07 mmol/L) to 59.6 mg/dL (3.31 mmol/L) 3 hours after oral administration in normal mice.

Preliminary studies using IPGTT showed the effects of different doses of glucose. When the glucose load was low (500 mg/kg), the increase in BGL was not clearly visible. The higher the glucose load was, the higher the BGL (1000 mg/kg) rose. If the glucose load was not too high and the rise of BGL was also not too high, metformin would be able to inhibit the rise of BGL clearly. If the dose of glucose load was too much, the rise in BGL could become very high, too high for metformin to suppress it. The present study used a dose of 1000 mg/kg for glucose loading based on the results. The inhibition of the rise of BGL is used to determine whether an extract or a drug has an anti-hyperglycaemic activity (Kasmuri, 2006).

In IPGTT, metformin inhibited the rise of BGL significantly at the 90 and 120 min observation points. Conversely, WE showed a significant effect 30 min after glucose loading, adding to the increasing BGL. Water extracts usually containe glucose in the form of glycosides. Therefore, the administration of exogenous glucose in addition to glucose loading would increase BGL further. Metformin is of the biguanide class, and acts as an anti-hyperglycaemic, rather than a hypoglycaemic agent (Brunton & Parker, 2006; Kirpichnikov, McFarlane, & Sowers, 2002). Its blood glucose-lowering action does not depend on the presence of functioning

pancreatic β-cells (Brunton & Parker, 2006; Nolte & Karam, 2004). It does not stimulate insulin release from the pancreas, and generally does not cause hypoglycaemia, even at substatial doses. Metformin reduces glucose levels primarily by decreasing hepatic glucose production and increasing insulin sensitivity in the muscles and fat tissues. These actions are mediated, at least partly, by the activation of adenosine monophosphate-activated protein kinase (AMPK) (Brunton & Parker, 2006; Chew & Leslie, 2006; Nolte & Karam, 2004). Metformin activates an upstream primary kinase called LKB1, thereby resulting in the the phosphorylation of AMPK. Phosphorylated AMPK then results in cytosolic sequestering of a CREB transcription factor, named the transducer of regulated CREB activity 2 (TORC2). TORC2 and CREB within the nucleus then transcribe a transcriptional co-factor named the peroxisome proliferator-activated receptor-g co-activator 1a (PGC1a). PGC1a gene expresssion activates the transcriptional of glucose-6 phosphate and PEPCK thereby leading to a "slowing down" of the excessive basal rates of hepatic gluconeogenesis. Metformin improves fasting BGL by slowing down "excessive" basal hepatic gluconeogenesis, without significant changes in the insulin levels, which would have otherwise caused hypoglycaemia (Figure 2.22) (Cusi, Consoli, & Defronzo, 1996). Consequently, metformin can reduce the rise of BGL in IPGTT, but cannot decrease them below the normal level. Agents of the sort are therefore more appropriately termed "euglycemic" agents (Nolte & Karam, 2004).

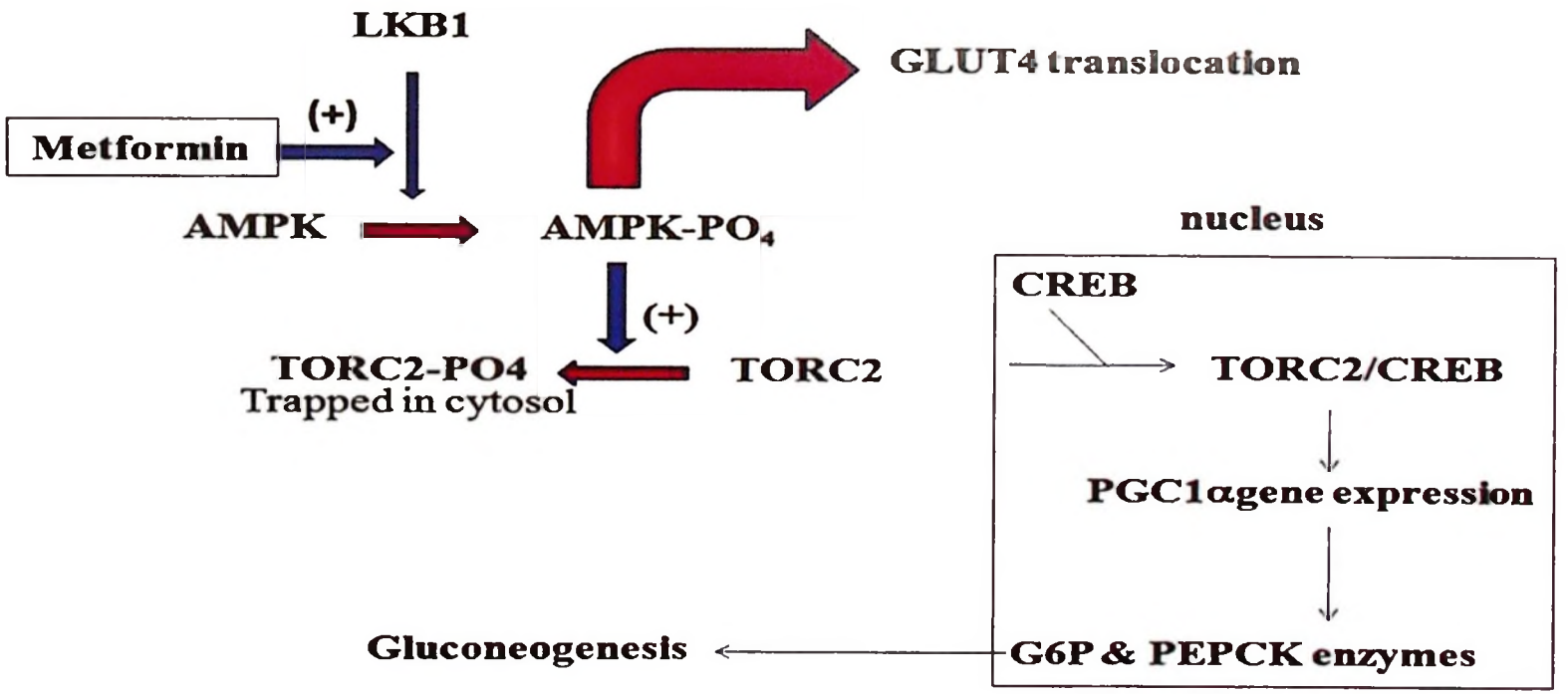


Figure 2.22. The role of metformin in hepatic gluconeogenesis (Adapted from "Pharmacologyweekly", n.d.) (AMPK: Adenosine monophospahte activated-protein kinase; LKB1: Liver kinase B1; GLUT4: Glucose transporter type 4; CREB: CyclicAMP-response element-binding; TORC2: Transducer of regulated CREB activity 2 ; PGC1a : Peroxisome proliferator-activated receptor-g co-activator 1a; G6P: Glucose-6 phosphate; PEPCK: Phosphoenolpyruvate carboxykinase).

Animals may exhibit the symptoms of diabetes with characteristics identical to humans (Srinivasan & Ramarao, 2012), such as hyperglycaemia, polydipsia and polyuria. Streptozotocin (STZ) is an antibiotic (Wu & Huan, 2008), synthesized using *Streptomycetes achromogenes* and used to induce both type 1 and type 2 diabetes mellitus (Szkudelski, 2001). STZ can cause pancreatic β-cell destruction accompanied by hyperglycaemia and blood insulin level reduction (West, Simon, & Morrison, 1996; Szkudelski, 2001).

The dose of STZ to induce diabetes varies between 40 to 70 mg/kg in rats. In adult rats, 60 mg/kg is the most common dose of STZ used to induce insulin dependent diabetes mellitus, as a single dose below 40 mg/kg may be ineffective (Brondum, Nilson & Aalkjaer, 2005; Federiuk et al., 2004). The present study used a dose of 55 mg/kg of STZ to induce hyperglycaemia in the rats. The diabetic rats showed polyuria, polydipsia, polyphagia (data not recorded but based on observations only) and also significant weight loss and hyperglycaemia. A rat was included in the study if the fasting BGL was above 11 mmol/L, as indicated by much literatures evidence on anti-diabetic studies reported (Kasmuri, 2006; Hassan, 2010). Moreover, the World Health Organisation (WHO) defines impaired fasting glucose (pre-diabetes) as fasting BGL between 6.1 to 6.9 mmol/L, and diabetes as BGL of 7.0 mmol/L and above (American Diabetes Association [ADA], 2013). To determine wether the included diabetic rats had stable hyperglycaemia and were not still at the stage of pre-diabetes, so that the effect of *S.polyanthum* extracts could be observed, the inclusion criteria for the present study was fasting BGL of 11 mmol/L or above. The present study showed 80 percent of the rats became diabetic after being injected STZ intra-peritoneally.

In STZ-induced diabetic rats, anti-hyperglycaemic tests with single-dose administration of the extracts demonstrated that metformin- and ME-treated group significantly reduced BGL, with metformin showing an earlier onset of action compared to ME. BGL were significantly reduced from the first hour until the seventh hour after oral administration of metformin, whereas, ME lowered BGL at the seventh hour after treatment (Figure 2.11). This finding showed that only the ME had an anti-hyperglycaemic activity in diabetic rats. Further studies were carried out by treating STZ-induced diabetic rats with a repeated dose daily for 6 days to observe the consistency of the single-dose administration effect previously. The repeated-dose study showed that metformin-, PEE- and ME-treated groups had significantly reduced BGL (Figure 2.12).

ME, the most active extract, was fractioned into four fractions and examined in STZ-induced diabetic rats with repeated doses for 6 days. The study demonstrated that both of the CF- and WF-treated groups had significantly reduced BGL, which were similar effects to metformin treatment (Figure 2.13). It was decided to fractionate CF further, which yielded two subfractions. Repeated dose administration for 6 days of the subfractions demonstrated that only SF-1 significanlty reduced BGL (Figure 2.14). The anti-hyperglycaemic activity results were consistent with the results of a previous investigation which reported that the ethanolic extract of *S.polyanthum* leaves reduced BGL of alloxan-induced diabetic mice (Studiawan & Santosa, 2005). Principally, alloxan and streptozotocin injections cause damage to β-cells in the pancreas which leads to an insulin-dependent type 1-like diabetes syndrome (Lenzen, 2008).

Repeated-dose administration of metformin and three doses of ME (250, 500 and 1000 mg/kg, respectively) for 6 days caused significant reductions in fasting BGL in STZ-induced diabetic rats (Figure 2.15). Different doses of ME reduced BGL dose-dependently. This finding is in line with the findings of Studiawan and Santosa (2005) who reported that two doses of the ethanolic extract of *S. polyanthum* leaves (2.62 and 5.24 mg/20 g) decreased BGL of alloxan-induce diabetic mice. Another study by Wahyono and Susanti (2008) reported that 30% and 70% ethanolic extracts of *S. polyanthum* leaves showed anti-hyperglycaemic activities in glucose-loaded rabbits. However, the present study was different from those previous studies because in the present study, the methanol extract was obtained by a serial extraction process. Administering different doses in the present study also showed that the minimum dose of ME that still possess anti-hyperglycaemic activity was 250 mg/kg.

The present study showed that ME, CF and SF-1 of *S. polyanthum* leaves contained tannins, steroids, terpenoids, flavonoids, saponins and glycosides, WF showed the presence of sugars, tannins, flavonoids, saponins and glycosides.These results are consistent with a previous study by Wahyono & Susanti (2008) which reported the anti-hyperglycaemic activities of the ethanolic extracts of *S. polyanthum* leaves in glucose-loaded rabbits and confirmed the presence of flavonoids using thin layer chromatogaphy. Another plant from the same family, *Syzygium cumini* was also reported to contain similar compounds such as flavonoids, tannins, glycosides and triterpenoids (Ayyanar & Subash-Babu, 2012).

Sharma et al. (2008) reported that a flavonoid-rich seed extract from *Eugenia jambolana* (EJ) had both hypoglycaemic and hypolipidemic effects. Flavonoids are

typical phenolic compounds and therefore act as potent metal chelators and free radical scavengers. The flavonoid isolated from *Ipomoea batatas* leaves decreased the blood glucose level and lipid parameters in alloxan-induced diabetic mice (Li et al., 2009). Oral administration of a flavonoid-rich fraction of the alcoholic extract of the root bark of *Morus alba* significantly reduced blood glucose levels, and also decrease lipid peroxidase activity levels (Singab et al., 2005). Some flavonoids have been reported to have anti-hyperglycemic activity through various mechanisms of action, such as inhibiting α-glucosidase activity (Jung, et al 2006) and increasing insulin levels (Sharma et al, 2008; Singab et al., 2005). Certain flavonoids e.g. quercetin, glycoside (Tapas, Sakarkar, & Kakde, 2008) and phytol, are also able to regenerate β-cells of the pancreas (Jananie, Priya, & Vijayalakshmi, 2011). Sharma, (2008) reported that a flavonoid-rich fraction of *Eugenia jambolana* seed, besides possessing hypoglycaemic effects, significantly decreased serum triglycerides and cholesterol levels in STZ-induced diabetic rats. The study demonstrated a dual regulator function for peroxisome proliferator-activated receptor (PPAR) α/γ which could be attributed to some polyphenolic constituents within the extract (Sharma et al., 2008). A similar pattern of PPARγ activation and anti-hyperlipidemic effects has been reported earlier in relation to flavonoids isolated from *Hawtorn* leaves (Fan et al., 2006). It has been also reported earlier that phenolic compounds from *Glycyrrhiza uralensis* roots (Kuroda et al., 2003) may activate the PPARy-signaling system (Kuroda et al., 2003; Shen et al., 2006). PPAR, a sub-family of the 48-member steroidal and nuclear receptor superfamily, are ligand-dependent transcription factors that control energy homeostasis by regulating carbohydrate and lipid metabolism (Shen et al., 2006). PPARγ are predominantly expressed in adipose tissues and involved in controlling insulin resistance, adipocyte differentiation and

lipid storage. One theory suggests that thiazolidiniones, agonists of the receptor PPAR$_{\gamma}$ mediate changes in adipocyte gene expression that cause fat metabolism changes, and alter the environment of the liver and the muscle cells, ultimately increasing insulin sensitivity in these tissues (Shu & Myers, 2004).

Matsuda (1998) studied oleanic acid glycosides' structure-activity relationships and concluded that the hypoglycaemic activity effect was by suppressing the transfer of glucose from the stomach to the small intestine, and by inhibiting glucose transport at the brush border of the small intestine. Syringin (a phenylpropanoid glycoside) from *Musa paradisiaca* extract normalizes hyperglycemia by increasing the plasma insulin level (Krishnan, Subramanian, & Subramanian, 2014).

An alkaloid-rich fraction from *Capparis decidua* showed anti-diabetic potential in streptozotocin-induced diabetic mice (Sharma et al., 2010). Another study reported that alkaloids (vindoline, vindolidine, vindolicine and vindolinine) induced relatively high glucose uptake in mouse β-TC6 pancreatic cell line and mouse myoblast (skeletal muscle) C2C12 cells. Improving glucose up take in pancreatic or muscle cells could reduce hyperglycaemia in type 2 diabetes (Tiong et al., 2013).

The administration of the tannin-rich fractions of *Ficus racemosa* for 30 days significantly reversed increased blood glucose, total cholesterol, triglyceride, and low density lipoprotein levels, and restored insulin and high density lipoprotein levels in the serum of a STZ-induced hypercholesterolemia-associated diabetic rat model (Velayutham, Sankaradoss, & Ahamed, 2012).

The GC-MS analyse of ME, CF, and SF-1 showed that all of the samples contained squalene (Table 2.3). Squalene, a triterpene is an isoprenoid compound (Spanova & Daum, 2011) which belongs to the terpenoid family and has a similar structure to beta-carotene (Reddy & Couvreur, 2009). It contains six isoprene units (Figure 2.17) and has been known to have several beneficial properties (Spanova & Daum, 2011) including an antioxidant activity (Ko, Weng & Chiou, 2002). A study conducted by Farvin et al. (2004) concluded that administration of squalene 2% along with the feeding for 45 days was able to prevent isoproterenol-induced myocardial infarctions in rats. The study suggested that the cardioprotective effect was due to the counteraction of free radicals by the antioxidant activity of squalene. Squalene has the ability to maintain the activities of anti-free radical enzyme and the levels of glutathione (GSH). The cardioprotective effect was also probably related to its hypolipidemic properties that are able to inhibit lipid accumulation (Farvin et al., 2006). Moreover, in animals, supplementation of the diet with squalene can reduce cholesterol and triglyceride levels. This contributes to the possibility of the usage of this compound in the prevention and treatment of hyperlipidemia (Kelly, 1999; Das, 2000: Aguilera et al., 2005). It is well known that obesity and hyperlipidemia are the important determinants of insulin sensitivity and the hyperglycaemic state (Kahn, 2003). Hyperglycaemia and hyperlipidemia are two important characteristics of diabetes mellitus, which is an endocrine-based disease (Kumar et al., 2012). Once hyperglycaemia exists, β-cells dysfunction is clearly present in the subjects inflicted with diabetes mellitus (Kahn, 2003). This shows an active compound that affects lipid metabolism may also have a beneficial effect on glucose homeostasis.

Squalene has been used as an emolient in the adjuvants of vaccine (Fox, 2009), and contributes to skin hydration (Huang, Lin, & Fang, 2009). It prevents breast cancer and tumors, and possess a cardioprotective, tumor protective (Kelly, 1999; Rao, Newmark, & Reddy, 1998), chemopreventive (Smith, 2000), and anti-hyperglycaemic activities.

Squalene has been implicated in several studies as a compound that contributed to the anti-hyperglycaemic activities of plants. Both of the studies of Baskar et al. (2011) and Jananie et al. (2011) reported that n-hexadecanoic acid, octadecanoic acid and squalene were present among the phytochemicals in *Mucuna pruriens* and *Cynodon dactylon*. These phytochemicals demonstrated an anti-hyperglycaemic activity in STZ-induced diabetic rats. The hypoglycaemic action of the fruit seeds' extract of *Syzygium cumini,* which contains triterpenes, also been experimentally verified (Evans, 2009). Several studies have been conducted to determine the anti-diabetic mechanisms of squalene derived from plants (Castellano et al., 2013; Perez & Vargas, 2002). Among the anti-diabetic mechanisms proposed are α-glucosidase inhibition, as demonstrated using squalene from *Lagerstroemia speciosa* leaves (Hou et al., 2009), and increased insulin sensitivity, as shown for the squalene from *Poria cocos* extract (Li et al., 2010).

The present study quantified squalene in ME (0.11%), CF(0.04%), and SF-1(0.26%). Although the contribution of squalene to an anti-hyperglycaemic activity has been reported in several studies, none of the earlier reports used an *in vivo* model. Therefore, the present study, investigated the anti-hyperglycaemic activity of squalene in STZ-induced diabetic rats. No reports provided evidence on the dose of

SQ as an anti-hyperglycaemic agent *in vivo*. A study by Strandberg et al., (1989) reported that a feeding of 1% squalene given to rats had an anti-hyperlipidemic activity. Based on this, attempts were made to find the anti-hyperglyaemic dose-response relationship of squalene. The doses of 10 mg/kg (data was not presented) and 20 mg kg were found to be not active in lowering BGL of STZ-induced diabetic rats. Hence, the present study included the dose of 40 mg/kg, 80 mg/kg and 160 mg/kg. The results showed that SQ given at 40 mg/kg, 80 mg/kg and 160 mg/kg dose-dependently reduced BGL of STZ-induced diabetic rats. There is a positive correlation of SQ and BGL in STZ-induced diabetic rats, as confirmed using Pearson's correlation coefficient test, which validates the anti-hyperglycaemic activity of squalene.

# CHAPTER THREE

# ANTI-DIABETIC MECHANISMS OF ACTION OF *SYZYGIUM POLYANTHUM* [WIGHT] LEAF EXTRACTS

## 3.1. Introduction

The usage of plant-based medicinal products has been known since ancient times (Mentreddy, 2007; Trojan-Rodrigues et al., 2012), and more than 1000 plants and their products (active natural principles and crude extracts) have been used to control diabetes in many cultures worldwide (Coman et al., 2012; Marles & Farnsworth, 1995; Trojan-Rodrigues et al., 2012). Unfortunately, the mechanisms of action of traditional anti-diabetic plants are under-explored, preventing them from being used in standard diabetes care (Coman et al., 2012).

According to the pharmacological approach, treatments for diabetes mellitus is dependent on the type of the diabetic condition (Daneman, 2006). The pharmacological strategy for type 1 diabetes is to administer a sufficient amount of exogenous insulin (Sesti, 2002). For type 2 diabetes, it is multifaceted, first, obese patients should change their lifestyles to reduce the body weight, increase excercise frequency to improve insulin sensitivity, and modify their diets. Reducing the body weight has been shown to have improved glycaemic control (McFarlane, 2009). Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (Wasada et al., 2004) are associated with obesity (especially abdominal or visceral obesity), and dyslipidemia with high triglycerides and/or low high-density lipoprotein levels (HDL) (American Diabetes Association[ADA], 2014). Thus, from a pharmacologically perspective, treatment comprises orally available agents that act to inhibit glucose absorption from the intestines (α-glucosidase inhibitors),

increasing the release of insulin from β-cells (sulfonylureas and meglitinides), or increase insulin sensitivity at the target tissues (thiazolidinediones and biguanides) (Brunton & Parker, 2006; Harvey, 2012; Shu & Myers, 2004). A novel category of anti-hyperglycaemic therapies include both incretin mimetic and dipeptidyl peptidase-IV (DPP-IV) inhibitors. Incretin mimetics are a new class of pharmacological agents with multiple anti-hyperglycaemic actions that mimic several of the actions of the incretin hormones that originate from the guts, such as the glucagon-like peptide (GLP)-1. Dipeptidyl peptidase-IV (DPP-IV) inhibitors suppress the degradation of many peptides, including GLP-1, thereby extending their bioactivity (Hinnen et al., 2006; Inzucchi & McGuire, 2008). Consequently, both a GLP-1 agonist and a DPP-IV inhibitor inhibit glucagon release which in turn increases insulin secretion, and decreases gastric emptying time and blood glucose levels. All of the mechanisms of actions described above could be investigated using *in vitro* and/or *in vivo* methods (Jung et al., 2006).

The present study shows a significant anti-hyperglycaemic effect of single-dose oral administration of *S. polyanthum* methanolic extracts. Furthermore, the chloroform fraction (CF) (500 mg/kg), the water fraction (WF) (500 mg/kg), the *n*-hexane subfraction-1 (SF-1) (250 mg/kg) and squalene (SQ) (160 mg/kg) also demonstrated the ability to significantly reduce the blood glucose levels (BGL) of STZ-induced diabetic rats.

The present study investigates the possible anti-diabetic mechanisms of action of *S. polyanthum* leaves using both *in vitro* and *in vivo* methods. Blood glucose levels, insulin levels, body weights and lipid profiles were determined to investigate the correlations between the reduction of BGL to insulin level, body weight and lipid profile alterations. The histopathology of the islets of Langerhans of the STZ-induced diabetic rats (Kasmuri et al., 2010; Wu & Huan, 2008), was investigated to find a correlation with the glycaemic response. The anti-diabetic mechanism related to the inhibition of α-glucosidase activity was evaluated using *in vitro* and *in vivo* methods. The averted jejunal sac method of Wilson and Wiseman (1954) was used to investigate the effects of extracts on glucose absorption in the intestine. Glucose uptake by the muscles, was examined using isolated abdominal and soleus muscles following the method of Gray and Flatt (1998).

**3.2. Objectives**

3.2.1. To evaluate the effects of twice-daily oral administration of *S. polyanthum* leaf extracts for 12 days on blood glucose, insulin, body weight, lipid profiles, and histological structure of the islets of Langerhans.

3.2.2. To evaulate the effect of *S. polyanthum* leaf extracts on:

3.2.2.1. α-glucosidase inhibition activity *in vitro.*

3.2.2.2. α-amylase inhibition activity *in vitro.*

3.2.2.3. glucose absorption in averted jejunal sac.

3.2.2.4. α-glucosidase inhibition activity *in vivo.*

3.2.2.5. glucose uptake by the abdominal muscle.

3.2.2.6. glucose uptake by the soleus muscle.

## 3.3. Materials and methods

### 3.3.1.Preparation of *S. polyanthum* extracts

The leaves of *S. polyanthum* were collected from Titi Kuning, Medan, Indonesia, and identified at the School of Biological Sciences, University of Sumatera Utara, Medan, Indonesia. The fresh leaves were dried at room temperature for one week. The dried material was milled using a ginder into powder. The extraction procedures incorporated the maceration technique using various solvents sequentially, similar to the description given in the previous chapter. First, *S. polyanthum* leaf powder was sequentially extracted with petroleum ether, chloroform and methanol. Secondly, the methanol extract (ME) was fractionated by liquid-liquid partition using chloroform (CF), ethyl acetate, *n*-butanol and water (WF). Thirdly, the chloroform fraction (CF) was further fractionated with *n*-hexane to obtain SF-1. The extracts, fractions and subfractions were evaporated under reduced pressure using a rotary evaporator. The concentrated extracts obtained were further dried in an oven. The dried extracts were kept in tight bottles and put in a freezer (-25 °C) until further analysis. ME, CF,WF and SF-1 were dissolved in saline (NaCl 0.9%) and tween 80 (5%) and used for experiments.

### 3.3.2. Animals

Male Sprague-Dawley (SD) rats (200-250 g) were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM), Penang. They were housed at room temperature (25 ± 3°C) with 12 h light/12 h dark cycles and fed with a commercial diet and water *ad libitum*.

### 3.3.3. Streptozotocin-induced diabetic rats

Diabetes was induced by intra-peritoneal injection of streptozotocin (STZ) (Sigma-Aldrich, USA) 55 mg/kg in 0.9% NaCl solution to 16 hrs fasted rats. The rats with blood glucose levels (BGL) of more than 11 mmol/L were considered diabetic and included in the study.

## 3.4. The effect of twice-daily oral administration of *S. polyanthum* (Wight) leaf extracts on the blood glucose levels, insulin levels, body weights, and lipid profiles of STZ-induced diabetic rats

### 3.4.1. Experimental set up

Streptozotocin (55 mg/kg) was injected intra-peritoneally. The rats with BGL above 11 mmol/L 3 days after STZ treatment were included in the study. The blood glucose levels and body weights of the diabetic rats were measured before and 12 days after twice-daily oral treatment with ME, CF, WF, SF-1, SQ, metformin and saline. The blood lipid profiles and insulin levels were determined at the end of 12-days treatment.

#### 3.4.1.1. Blood glucose level assay

The diabetic rats were fasted overnight before BGL assay. A drop of blood was obtained from the tail vein of each rat and BGL were assayed using Accu-Check Advantage Clinical Glucose Meter (Roche Diagnostics Co., IN, USA).

#### 3.4.1.2. Insulin measurement

The 12-days treated diabetic rats were anaesthetised with gaseous $CO_2$, and 3 mL blood were obtained from each of the rat via a cardiac puncture. The blood was

centrifuged at 3000 rpm for 10 min (Eppendorf, Centrifuge 5403). The plasma was collected and stored at -20°C until it was measured. The concentrations of insulin in the sera were assayed in triplicate for each of the samples using a commmercial ELISA kit for rat insulin (Crystal Chem Inc, Illinois, USA).

**The principles of the assay:**

1. **First reaction**

   Rat insulin in the sample was bound to the guinea pig anti-insulin antibody coated on the microplate well.

2. **Washing**

   The unbound material was removed by washing.

3. **Second Reaction**

   The horse radish peroxidase (POD)-conjugated anti-insulin antibody was then bound to the guinea pig anti-insulin antibody/rat insulin complex immobilized to the microplate well.

4. **Washing**

   Excess POD-conjugates were removed by wahing.

5. **Enzyme reaction**

   The bound POD conjugate in the microplate well were detected by the addition of a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution.

6. **Measuring the absorbance**

7. **Evaluating the results**

   Insulin concentration was determined via interpolation using a standard curve generated by plotting the absorbance values against the corresponding concentrations of the rat insulin standard.

The antibody-coated microplate was affixed to the frame

95 µL per well of the sample diluent were dispensed

5 µL per well of the sample (or working rat insulin standard) were pipetted

The microplate was incubated for 2 h at 4°C.

Each well was washed five times with the washing buffer*

100 µL per well of the anti-insulin enzyme conjugate solution were dispensed.

The microplate was incubated for 30 min at room temperature.

Each well was washed seven times with the washing buffer*.

100 µL per well of the enzyme substrate solution were dispensed.

The microplate was incubated for 40 min at room temperature while avoiding exposure to light.

The enzyme reaction was stopped by adding 100 µL of the enzyme reaction stop solution per well.

A450 and subtract 630 values were measured within 30 min.

Insulin concentrations were calculated using the standard curve.

Figure 3.1. Insulin assay procedure

### 3.4.1.3. Body weight measurements

The body weight of the rats were measured before and 12 days after the twice-daily oral treatment using an electronic balance (Navigator™, Ohaus Coorporation, Nanikon, Switzerland).

### 3.4.1.4. Lipid profiles measurement

Rat plasma samples were sent to Gibbles Pathology (Malaysia, Sdn Bhd) for lipid profiles determination. Sera lipid profiles were determined using an ADVIA 2400 Chemistry Analyzer (Siemens, Erlangen, Germany).

## 3.5. Histological assessment of the islets of Langerhans of diabetic rats after twice-daily oral administration of *S. polyanthum* (Wight) leaf extracts for 12 days

Histological assesments were conducted using the immunohistochemical method as described by Kasmuri (2006).

### 3.5.1. Experimental set up

Groups of STZ-induced diabetic rats, with BGL above 11 mMol/L, were treated orally with ME (1 g/kg), CF (500 mg/kg), WF (500 mg/kg), SF-1 (250 mg/kg), SQ (160 mg/kg), metformin (500 mg/kg) and saline (10 ml/kg), twice-daily for 12 days.

### 3.5.2.Specimen collection and preparation

The 12-days treated diabetic rats were sacrified with the carbogen gas (95% $O_2$ & 5% $O_2$) and their abdomen were cut opened. The pancreas was identified, isolated out and fixed in 10 % formalin for 48 hours. Processing of the fixed pancreatics

tissues was then undertaken as shown in the scheme in Table 3.1. The embedded pancreatic specimens were individually turned into blocks using liquid paraffin (58°C) in embedding rings. They were left to cool down before being removed from the embedding moulds (Kasmuri, 2006).

### 3.5.3.Specimen preparation

The pancreatic paraffin blocks were sectioned to obtain 5 μm-thick specimen using a microtome. The sections were mounted on glass slides and wetted with ethanol 70%. The mounted slides were heated on a hot plate (53°C) overnight before immunohistochemical staining method.

### 3.5.4. Immunohistochemical staining method

The mounted slides were stained using an immunohistochemical staining procedure (Table 3.2.). This method was used to detect insulin in viable β-cells. The presence of insulin was detected based on an antigen-antibody reaction. The tissues were deparaffinized with a series of solvents (xylene, xylene, absolute ethanol, 95% ethanol) for 3 min each before being washed with phosphate buffer saline (PBS) and distilled water for 5 min, respectively (Table 3.2). The tissues sections were incubated for 5 min in 3% $H_2O_2$ in methanol to quench the endogenous peroxides. The sections were then washed for 5 min in PBS. Excess PBS was wiped off using a tissues paper. The sections were blocked by incubation for 20 min in a diluted normal serum, and the excess of the serum was blotted from the sections using a tissue paper. Diluted primary antibody min (A guinea-pig polyclonal antibody of rat's insulin (DAKO, Glostrup, Denmark), 1:100 in PBS, was used to incubate the sections. After 30 min of incubation, the sections were washed with PBS for 5 min.

Excess PBS was wiped out. Then, the sections were incubated with a diluted biotinylated secondary antibody solution for 30 min. The slides were then washed for 5 min with PBS. This was followed by incubation with Vectastain® ABC kit (Vector laboratories, Burlingame, CA, USA) for 30 min. The slides were dipped in PBS for 5 min and incubated in a 3,3'-diaminobenzidine tetrahydrochloride (DAB) mixture (Zymed laboratories, San Fransisco, CA, USA) for 20 min at room temperature. Then, the slides were washed with distilled water and hematoxylin (Harris Hematoxylin), by dipping once in each to counterstaine the nuclei. The tissues were then dehydrated in 95% ethanol and absolute ethanol for 3 min, respectively. Insulin present in the tissues acted as an antigen, hence, it reacted with the antibody of rat's insulin and appeared as reddish brown in coloration.

### 3.5.5. Quantifying method

The assessment was done by direct microscopic examination and by a computerized image analyzer (Leica ®microsystemQwin plus, camera Olympus bx51m). The computerized image analyser was used in assessing the specimens which had been stained. Evaluation was made by examination of 10 islets per group (Kasmuri, 2006).

Table 3.1. Dehydration scheme of fixed pancreatic specimen.

| Step | Activity | Chemical/solvent | Time span (min) |
|---|---|---|---|
| 1 | Soak | 70% v/v ethanol | 30 |
| 2 | Soak | 80% v/v ethanol | 30 |
| 3 | Soak | 90% v/v ethanol | 30 |
| 4 | Soak | Absolute ethanol | 30 |
| 5 | Soak | Absolute ethanol | 30 |
| 6 | Soak | Xylene | 30 |
| 7 | Soak | Xylene | 30 |
| 8 | Soak | Paraffin at 58°C | 60 |
| 9 | Soak | Paraffin at 58°C | 60 |

Table 3.2. Imunohistochemical staining procedure.

| Step | Activity | Chemical/solvent | Time span (min) |
|---|---|---|---|
| 1 | Soak | Xylene | 3 |
| 2 | Soak | Xylene | 3 |
| 3 | Soak | Absolute ethanol | 3 |
| 4 | Soak | 95% v/v ethanol | 3 |
| 5 | Soak | Phosphate Buffer Saline (PBS) | 5 |
| 6 | Wash | Running tap water | 5 |
| 7 | Rinse | 3% Hydrogen peroxide in methanol | 5 |
| 8 | Soak | Phosphate Buffer Saline (PBS) | 5 |
| 9 | Soak | Diluted normal serum | 20 |
| 10 | Soak | Primary antibody (Guinea pig-polyclonal anti-insulin antibody) | 30 |
| 11 | Soak | Phosphate Buffer Saline (PBS) | 5 |
| 12 | Soak | Biotinylated Secondary Antibody solution | 30 |
| 13 | Soak | Phosphate Buffer Saline (PBS) | 5 |
| 14 | Soak | Vectastatin ABC reagent | 30 |
| 15 | Dip | Phosphate Buffer Saline (PBS) | 5 |
| 16 | Soak | 3,3-diamino benzidine tetrahydrochloride (DAB) | 20 |
| 17 | Soak | Distilled water | 1 dip |
| 18 | Soak | Hematoxyllin (Harris Hematoxyllin) | 1 dip |
| 19 | Soak | 95% Ethanol | 3 |
| 20 | Soak | Absolute Ethanol | 3 |
| 21 | Soak | Xylene | 3 |

| | | | |
|---|---|---|---|
| 22 | Soak | Xylene | 3 |

### 3.6. *In vitro* α-glucosidase inhibition assay

The *in vitro* α-glucosidase inhibition assay was conducted according to Kwon et al. (2008).

#### 3.6.1. Experimental set up

ME, CF, WF, SF-1, SQ and acarbose were prepared in several concentrations by serial dillution of the stock solutions of ME (1 mg/mL), CF (0.25 mg/mL),WF (2 mg/mL), SF-1 (0.25 mg/mL) and SQ (4 mg/mL). Fifty μL of each of the homogenized extract solutions and 100 μL of a 0.1 M phosphate buffer (pH 6.9) containing a yeast α-glucosidase solution (1.0 U/mL, source from *Saccharomyces cerevisiae*) were incubated in 96-well plates at 25°C for 10 min. After incubation, 50 μL of a 5 mM p-nitrophenyl-α-D-glucopyranoside solution in a 0.1 M phosphate buffer (pH 6.9) were added to each of the wells at time intervals. Subsequently, the reaction mixtures were incubated at 25°C for 5 min. Before and after incubation, absorbance as recorded at 405 nm by a micro-plate reader. The reading were compared to the control which had 50 μL of the buffer solution in place of the extract. α-glucosidase inhibitory activity was expressed as inhibition % and calculated as follow:

$$\% \text{ inhibition} = \frac{(\text{average A405 control} - \text{average A405extract})}{\text{average A405 control}} \times 100\%$$

Acarbose, which served as the positive control, was prepared in several concentrations: 1, 0.5, 0.25, 0.125 , 0.0625 and 0.03125 mg/mL. A procedure similar

to the above procedure was conducted to assess the α- glucosidase inhibition activity of acarbose.

The $IC_{50}$ values were calculated from the dose-response curves by interpolation using the linear regession analysis (Gao et al, 2013).

### 3.7. *In vitro* α-amylase inhibition assay

The *in vitro* α-amylase inhibition assay was conducted according to Kwon et al. (2008).

**Preparation of the solutions:**

1. Sodium chloride (6.7 mM): 117 mg sodium chloride was dissolved in 300 mL distilled water

2. Sodium phosphate (20 mM) buffer, pH 6.9: 1.4 g anhydrous monobasic sodium phosphate was dissolved in 250 mL of a 6.7 mM sodium chloride solution, and pH was adjusted to 6.9 using 1 M sodium hydroxide.

3. Starch (1% w/v): 1 g of starch was dissolved in 100 mL of a 20 mM sodium phosphate buffer (pH 6.9). Solubilization was facilitated by heating the starch solution directly on a heating/stirring plate. The solution was allowed to reach the boiling point and was maintained at this temperature for 15 min. Then the starch solution was cooled down to room temperature with contineous stirring.

4. Sodium hydroxide (2 M): 8 g of sodium hydroxide pelletes were dissolved in 100 mL of distilled water.

5. Dinitrosalicylic acid color reagent: It was prepared by dissolving 1 g of the 3,5-dinitrosalicylic acid in 50 mL of reagent-grade water, followed by the slow addition of 30 g of sodium potassium tartrate tetrahydrate and 20 mL of 2 N NaOH. Distilled

water was then added for a volume of 100 mL. The preparation was protected from $CO_2$ and stored for no longer than 2 weeks before being used.

6. α-amylase (0.5 mg/mL): 25 mg of α-amylase were dissolved in 50 mL of a sodium phosphate buffer (pH 6.9).

7. Serial dilution of of *S.polyanthum* ME, CF, WF and SF-1, SQ and acarbose were prepared from the respective initial concentration of 10 mg/mL, 8 mg/mL, 4 mg/mL, 0.25 mg/mL, 2 mg/mL and 4 mg/mL.

### 3.7.1. Experimental set up

500 µL of each sample (ME, CF, WF, SF-1 and SQ) and 500 µL of a 0.02 M sodium phosphate buffer (pH 6.9) were mixed with a 0.006 M NaCl solution containing porcine α-amylase (0.5 mg/mL) and incubated at 25°C for 10 min. After incubation, 500 µL of a 1 % starch solution in a 0.02 M phosphate buffer (pH 6.9) and 0.006 M NaCl were added. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1 mL of a 96 mM 3.5-dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and then cooled down to room temperature. The reaction mixtures were then diluted by adding 10.0 mL of distilled water, and the absorbance was measured at 540 nm using a spectrophotometer (Hitachi U-2800). The absorbance of the blank sample (containing buffer instead of the enzymatice solution) and the control (containing buffer in place of the sample extract) were recorded. Acarbose was used as the positive control.

The inhibition activity was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{average A540 control} - \text{average A540extract}}{\text{average A540 control}} \times 100\%$$

The $IC_{50}$ values were calculated from the dose-response curves by interpolation using the linear regession analysis (Gao et al, 2013).

### 3.8. Glucose absorption from averted jejunal sacs

Glucose absorption from the intestine was determined based on the method of Wilson & Wiseman (1954) as described by Hassan et al. (2010).

#### 3.8.1. Experimental set up

Male normal Sprague-Dawley rats, weighing 200-250 g, were sacrificed, and their abdominal walls dissected. The jejunum, identified as the segment located 20-25 cm away from the pylorus was cut and placed in a cold glucose-Ringer solution, aerated with carbogen (95% $O_2$ & 5% $CO_2$). The jejunum was inverted using a rod, and cut into 5 cm segments. One end of each segment was tied to become a sac, and the other end was tied loosely with a longer thread (20 cm). Each sac was filled with 1 mL of the Tyrode buffer solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.2 mM $NaH_2PO_2$ and 5.5 mM glucose) by slipping a blunt needle attached to a syringe through the loose end and tighten the knot as the needle was being withdrawn. Each sac was immersed into a test tube containing 15 mL of the Tyrode buffer solution mixed with 1 mg/mL of acarbose, phlorizin, ME, CF, WF, SF-1 and SQ, respectively. One end of the thread was allowed to hang over the opening of the tube. All of the tubes were then placed in a water bath (37°C) and incubated for 1 hour. The sacs were removed from the test tubes by pulling the thread ends using a forceps after the incubation period. Three mL of a peridochrome-

glucose reagent were pipetted into each test tube. Thirty µL of each test solution were added into a tube. The mixtures were incubated for 20 min in a water bath at 37°C. Glucose concentration was determined using a Stat Fax machine 1937 (Awareness Technology Inc., FL, USA).

Glucose absorption was calculated using the following formula: (Amount of glucose in Tyrode's solution (mg/dL) – Amount of glucose in the sample (mg/dL)/weight of the intestinal sac (g)

### 3.9. *In vivo* enzyme inhibition studies

Oral carbohydrate tolerance tests were conducted in both normal and diabetic rats.

#### 3.9.1. *In vivo* enzyme inhibition studies in normal rats

Seven groups of six rats were fasted for 16 hours and treated orally as follows:

Group I : Saline 10 mL/kg (normal control)

Group II : Acarbose 10 mg/kg (positive control)

Group III: ME 1 g/kg

Group IV: CF 500 mg/kg

Group V: WF 500 mg/kg

Group VI: SF-1 250 mg/kg

Groups VII: SQ 160 mg/kg

##### 3.9.1.1. Oral starch tolerance test in normal rats

The rats were treated orally with starch (3 g/kg) (R & M Chemicals, Essex, UK) ten min after administering the above treatments.

### 3.9.1.2. Oral sucrose tolerance test in normal rats

The rats were treated orally with sucrose (4 g/kg) (R & M Chemicals, Essex, UK), ten min after administering the treatments listed in section 3.9.1.

### 3.9.1.3. Oral glucose tolerance test in normal rats

The rats were treated orally with glucose 2 g/kg (R & M Chemicals, Essex, UK), ten min after receiving the treatments listed in section 3.9.1.

### 3.9.2. *In vivo* enzyme inhibition studies in STZ-induced diabetic rats

Seven groups of six STZ-induced diabetic rats were fasted for 16 hours and treated as follows:

Group I : Saline10 mL/kg (normal control)

Group II : Acarbose 10 mg/kg (positive control)

Group III: ME 1 g/kg

Group IV: CF 500 mg/kg

Group V: WF 500 mg/kg

Group VI: SF-1 250 mg/kg

Groups VII: SQ 160 mg/kg

### 3.9.2.1. Oral starch tolerance test in STZ-induced diabetic rats

The rats were treated with starch 3 g/kg (R & M Chemicals, Essex, UK) orally, ten min after giving the above treatments.

### 3.9.2.2. Oral sucrose tolerance test in STZ-induced diabetic rats

The rats were treated with sucrose 4 g/kg (R & M Chemicals, Essex, UK) orally, ten min after treatment administration as listed in section 3.9.2.

### 3.9.2.3. Oral glucose tolerance test in STZ-induced diabetic rats

The rats were treated with glucose 2 g/kg (R & M Chemicals, Essex, UK) orally, ten min after treatment administration as listed in section 3.9.2.

Blood specimens (one drop) were obtained by pricking the rat's tail vein, and BGL were measured using an Accu-Check Advantage Clinical Glucose Meter (Roche Diagnostics Co., IN, USA) at the following time points: 0 (before treatment), 30, 60 and 120 min after starch, sucrose and glucose administration, respectively. The area under the curve (AUC) was determined using the formula below:

AUC (mmol/hr) = 0.25(BGL0 + BGL30) +0.25(BGL30 + BGL60) + 0.5(30/2 (BGL60 + BGL120)

where BGL0, BGL30, BGL60 and BGL 120 represent BGL at 0, 30, 60 and 120 min.

## 3.10. Glucose uptake by isolated rat abdominal muscle

Glucose uptake by isolated rat abdominal muscles was measured afollowing the method of Gray and Flatt (1998), as described by Hassan et al. (2010).

### 3.10.1. Experimental set up

Male normal Sprague-Dawley rats (200-250 g) were sacrificed and skinned to expose their abdominal muscles. The abdominal muscles were then excised without the

*rectus abdominus* and were transferred into a Kreb's-Ringer bicarbonate buffer solution (KRB; 18 mMNaCl, 5 mMKCl, 2.0 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM$NaHCO_3$, and 1.28 mM $CaCl_2$) in the presence of 95% $O_2$ and 5% $CO_2$ at 37°C. The muscles were then cut into small squares weighing approximately between 90-150 mg. The small muscle squares were then put into 1.5 mL eppendorf tubes containing KRB, and the tubes were aerated for 10 min to allow the muscles to acclimatize the muscles. After acclimitization, the KRB solution was changed with a new KRB solution containing 11.1 mmol/L of glucose. The muscle segments were then aerated in the presence of the tested substances with or without the presence of insulin (1 IU/mL) for 5 min before being incubated for 30 min at 37°C in a water bath. The tested substances (ME, CF, WF, SF-1, SQ and metformin) were prepared at the concentration of 1 mg/mL. Three mL of the peridochrome-glucose reagent were pipetted into each of the test tubes before adding 30 µL of each test supernatant into the appropiate tubes. The mixtures were incubated for 20 min in a water bath at 37°C, and glucose uptake by the muscle segments was determined using a Stat Fax machine 1937 (Awareness Technology Inc., FL, USA).

Calculation: Glucose uptake= amount of glucose before incubation (mg/dL)-amount of glucose after incubation (mg/dL)/weight of the muscle segment (g)

### 3.11. Glucose uptake by isolated rat soleus muscle

Glucose uptake by the isolated rat soleus muscle was measured based on the method of Gray and Flatt (1998).

### 3.11.1. Experimental set up

Male normal Sprague-Dawley rats (200-250 g) were sacrificed and skinned to expose the soleus muscle. Then, a similar procedure to that shown in section 3.10.1. was conducted to assess glucose uptake by the isolated rat soleus muscle.

## 3.12. Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed using the one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey HSD as the *post hoc* tests. $P < 0.05$ was considered significant. The mean differences between the pre-treatment and post-treatment levels were analyzed using the paired *t*-test.

### 3.13. Resuls

#### 3.13.1.The effects of twice-daily oral administration for 12 days of *S. polyanthum* extracts (ME, CF, WF, SF-1) and squalene on the blood glucose levels, insulin levels, body weights and lipid profiles of STZ-induced diabetic rats

Figure 3.2. shows that the fasting BGL of all of the treated rats were significantly decreased compared to the pre-treatment levels on day 0. The blood glucose level lowering activity of ME-was similar to metformin-treated groups ($P$<0.001). CF-, SF- and SQ-treated group showed similar effects ($P$<0.01, $P$ <0.001, $P$<0.05, respectively).

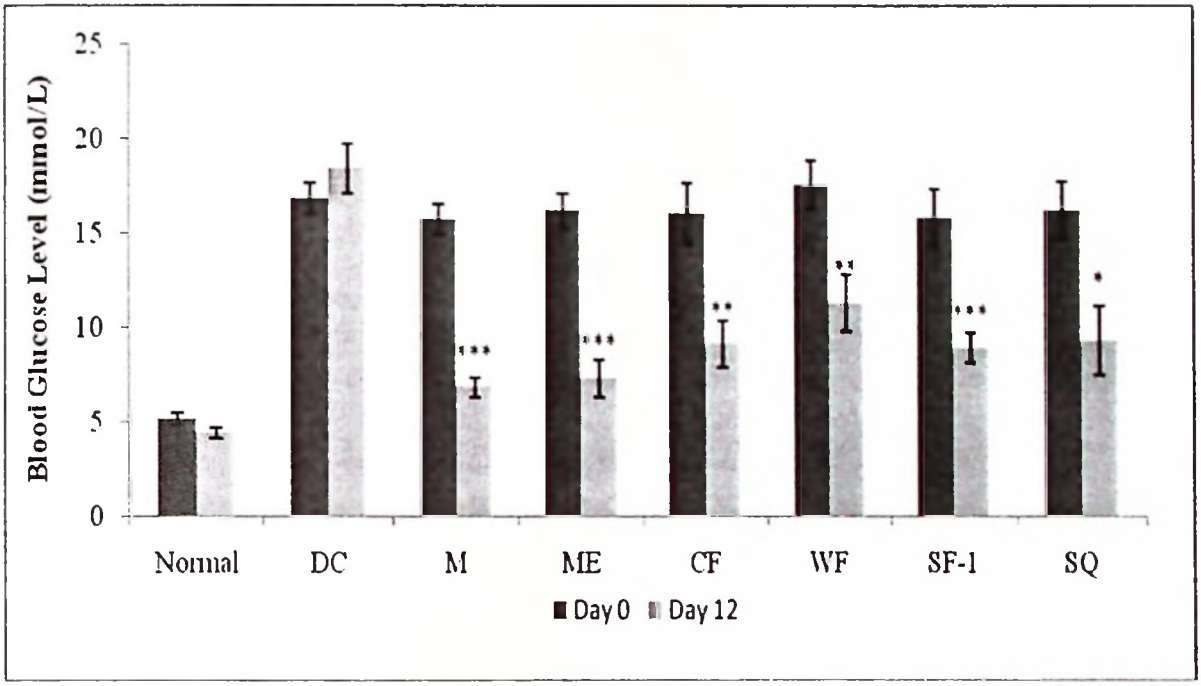


Figure 3.2. The effects of a 12-days twice-daily oral administration of *S. polyanthum* methanol extracts (ME) (1 g/kg), chloroform fractions (CF) (500 mg/kg), water fractions (WF) (500 mg/kg), *n*-hexane fractions (SF-1) (250 mg/kg), squalene (SQ) (160 mg/kg), metformin (M) (500 mg/kg) and saline (DC) (10 ml/kg) on the blood glucose levels of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); The mean differences between the pre-treatment (Day 0) and post-treatment (Day 12) levels were analyzed using paired *t*-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The insulin assay showed that only the insulin levels of the normal control were significantly different compared to those of the diabetic control after 12 days of treatment ($P$<0.001). The STZ-induced diabetic rats treatment groups (receiving ME, WF, SF-1 and squalene) showed higher levels than the diabetic control, but the results were insignificant (Figure 3.3).

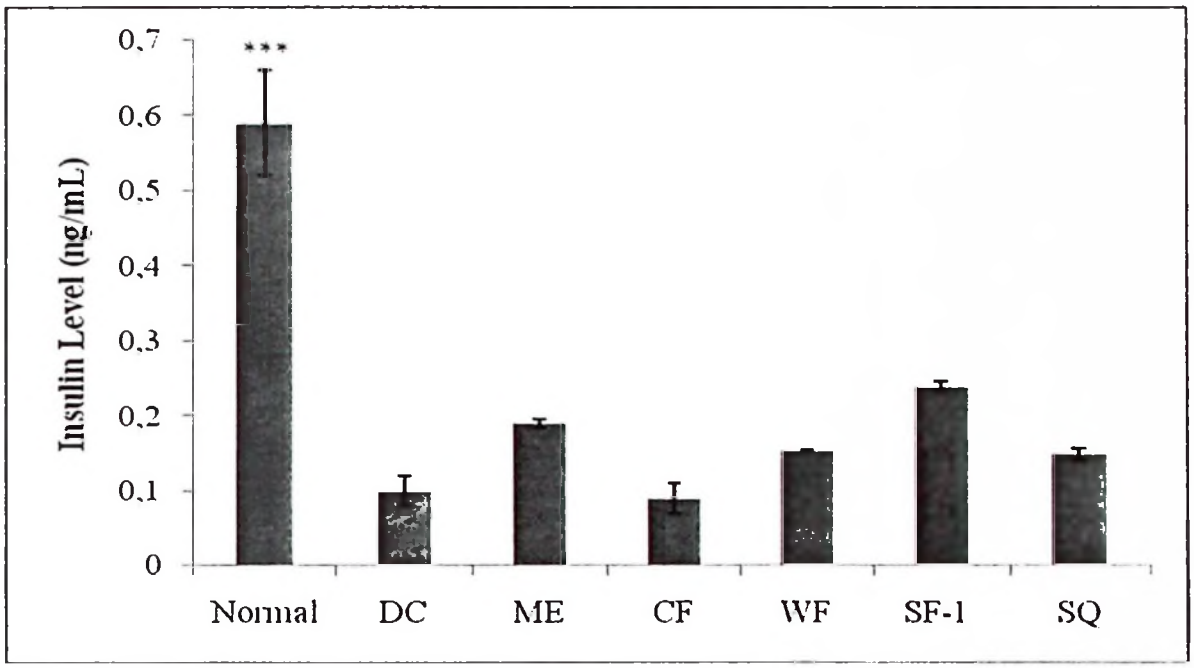


Figure 3.3. The effects of 12-days twice-daily oral administration of *S. polyanthum* methanol extracts (ME) (1 g/kg), chloroform fractions (CF) (500 mg/kg), water fractions (WF) (500 mg/kg), *n*-hexane fractions (SF-1) (250 mg/kg), squalene (SQ) (160 mg/kg), metformin (M) (500 mg/kg) and saline (DC) (10 ml/kg) on the insulin levels of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=3); ***$P$<0.001 compared with the diabetic control group (DC), as analyzed using Tukey HSD as a *post hoc* test.

The normal rats showed significant increase in body weight after the 12-days experimental period ($P<0.01$). Meanwhile, CF-, WF-, SF-1- and SQ-treated groups showed a tendency to having reduced body weights. However, only ME-, WF-, SF-1- and metformin-treated groups showed significant body weight reductions compared to the pre-treatment levels (day 0) ($P<0.05$, $P<0.001$, $P<0.05$, $P<0.05$, respectively) (Figure 3.4).

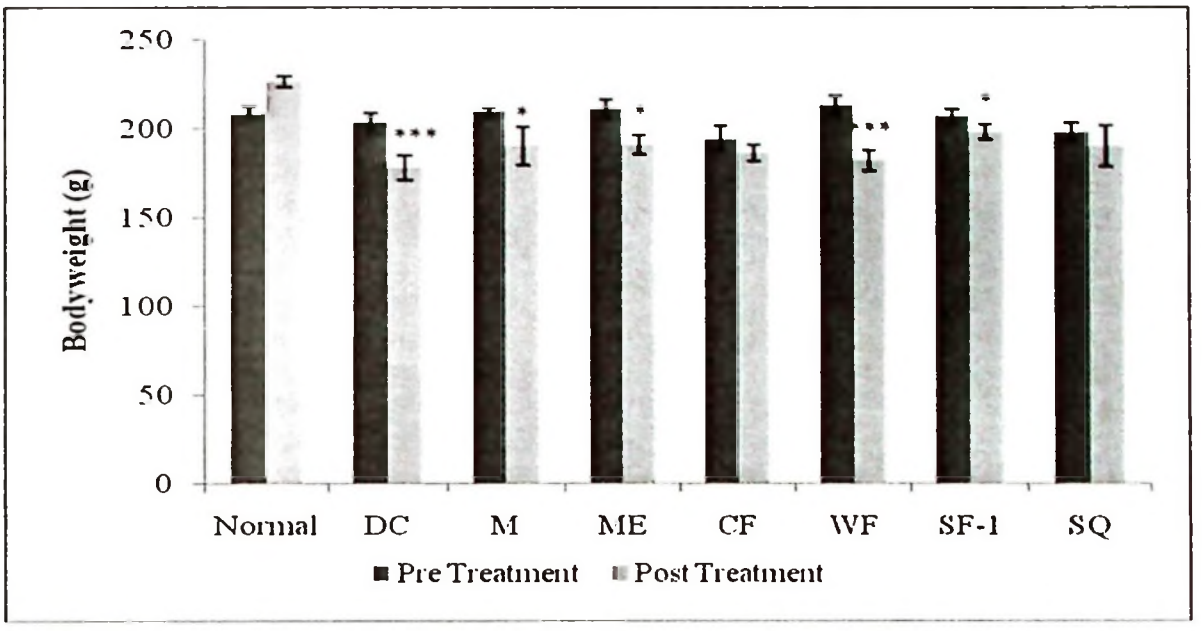


Figure 3.4. The effects of 12-days twice-daily oral administration of *S. polyanthum* methanol extract (ME) (1 g/kg), chloroform fractions (CF) (500 mg/kg), water fractions (WF) (500 mg/kg), *n*-hexane fractions (SF-1) (250 mg/kg), squalene (SQ) (160 mg/kg), metformin (M) (500 mg/kg) and saline (DC) (10 ml/kg) on the body weights of STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=6); The mean differences between pre-treatment (Day 0) and post-treatment (Day 12) levels were analyzed by the paired *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The serum levels of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL), and the ratioo of cholesterol/high-density lipoprotein levels (Chol/HDL) on ME-, CF-, WF-, SF-1- and SQ-treated groups were lower compared to the diabetic control. Significant reductions in TC levels were observed in SF-1- and SQ-treated groups, which was similar to the metformin-treated group ($P<0.05$). Both metformin- and SQ-treated groups showed significant reduction in LDL levels compared to the diabetic control group ($P<0.05$) (Table 3.3).

Table 3.3. The effects of 12-days twice-daily oral administration of *S. polyanthum* methanol extracts (ME) (1 g/kg), chloroform fractions (CF) (500 mg/kg), water fractions (WF) (500 mg/kg), *n*-hexane fractions (SF-1) (250 mg/kg), squalene (SQ) (160 mg/kg), metformin (M) (500 mg/kg) and saline (DC) (10 ml/kg) on the lipid profiles of STZ-induced diabetic rats.

| Group | Lipid Profiles (mmol/L) | | | | |
|---|---|---|---|---|---|
| | TC | TG | HDL | LDL | Chol/HDL |
| Normal | 1.68±0.16 | 0.39±0.02 | 1.20±0.10 | 0.48±0.07 | 1.57±0.06 |
| DC | 1.70±0.09 | 0.58±0.10 | 0.95±0.08 | 0.46±0.06 | 1.82±0.08 |
| M | 1.22±0.12* | 0.58±0.03 | 0.74±0.04 | 0.14±0.06*** | 1.65±0.07 |
| ME | 1.45±0.17 | 0.39±0.07 | 0.87±0.12 | 0.39±0.04 | 1.62±0.03 |
| CF | 1.43±0.10 | 0.58±0.09 | 0.82±0.10 | 0.43±0.07 | 1.79±0.09 |
| WF | 1.20±0.04 | 0.80±0.04 | 0.73±0.04 | 0.28±0.03 | 1.61±0.04 |
| SF-1 | 1.20±0.06* | 0.40±0.05 | 0.74±0.07 | 0.39±0.04 | 1.70±0.10 |
| SQ | 1.17±0.16* | 0.46±0.13 | 0.71±0.09 | 0.25±0.05* | 1.65±0.06 |

The values are expressed as mean ± SEM (n=6); * $P < 0.05$, ***$P<0.001$ compared with the diabetic control group (DC).

### 3.13.2. Histological assesment

Plate 3.1-2 shows the visualization of immunohistochemical staining with a 40x10 magnification power. The brown-colored area represents insulin in the viable β-cells. In the islets of Langerhans (iL) of the normal rat, clear and large brownish spots was seen (Plate 3.1). In the STZ-induced diabetic rats, the size of the brown coloration was reduced, even though the sizes of the areas seen were large (Plate 3.2). The average percentage of β-cells in the normal rats was 88%. The diabetic control showed a lower quantity (Figure 3.5). All of the extracts- and SQ-treated groups showed a tendency to have larger immunostained areas compared with that of the diabetic control group. Yet, only the normal rats showed a significantly different immunostained area size ($P<0.001$) compared to the diabetic control. This indicated that the treatments were ineffective in terms of increasing β-cell viability.

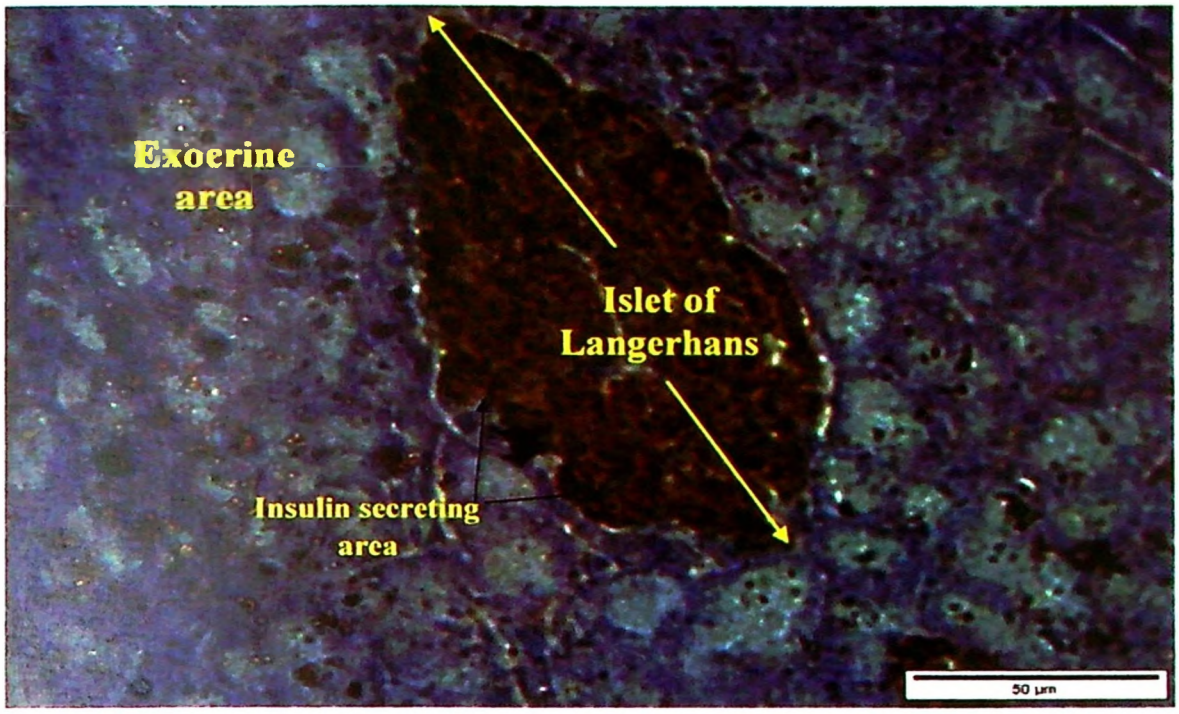


Plate 3.1. Histological appearance of an islet of Langerhans with immunohistochemical staining in a normal rat (40x10 magnification). The brown area represents the insulin-secreting area.

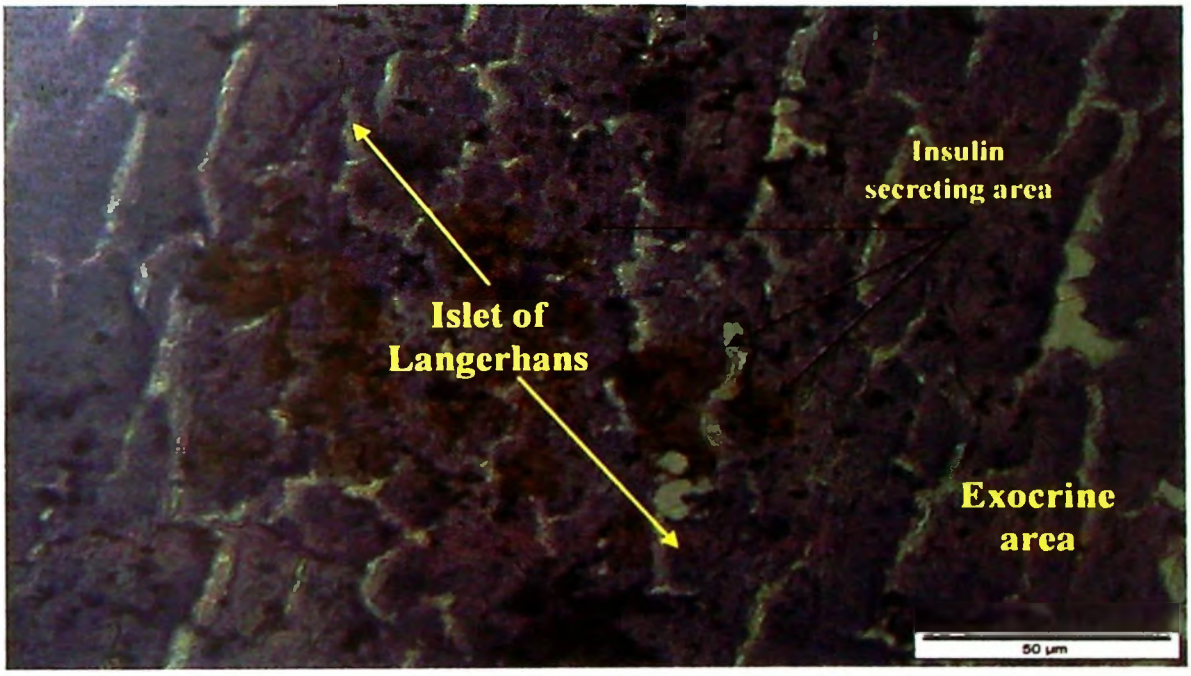


Plate 3.2. Histological appearance of an islet of Langerhans with immunohistochemical staining in a STZ-induced diabetic rat (40x10 magnification). The brown area represents the insulin-secreting area.

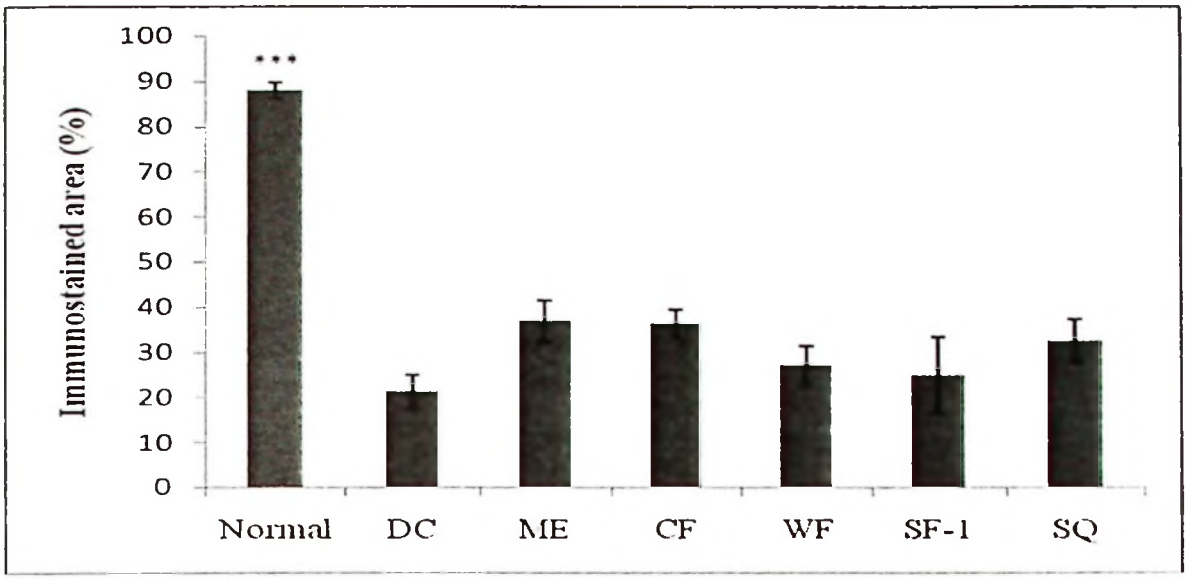


Figure 3.5. The effect of oral administration of *S. polyanthum* methanol extract (ME) (1 g/kg), chlorofrom fractions (CF) (500 mg/kg), water fractions (WF) (500 mg/kg), *n*-hexane fractions (SF-1) (250 mg/kg), squalene (SQ) (160 mg/kg) and saline (DC) (10 ml/kg) twice daily for 12 days on the percentage of the immunostained area of the islets of Langerhans in STZ-induced diabetic rats.
The values are expressed as mean ± SEM (n=10); The statistical analysis was done using one-way ANOVA, followed by Tukey HSD as a *post hoc* test (***$P$<0.01).

### 3.13.3. *In vitro* α-glucosidase inhibition activity

The *in vitro* α-glucosidase inhibition assay showed that ME, CF and SF-1 had the most potent α-glucosidase inhibiting activity (Table 3.4). Their $IC_{50}$ values were lower than that of acarbose. WF and SQ required higher concentrations, compared to acarbose, to inhibit the activity of α-glucosidase. Their $IC_{50}$ values were 0.74 ±0.07 and 1.33 ±0.05, respectively. The descending order of α-glucosidase inhibition activity was as follows: CF > ME > SF-1 > SQ > Acarbose > WF (Table 3.4).

Table 3.4. The $IC_{50}$ values of acarbose, methanol extract (ME), chloroform fraction (CF), water fraction (WF), *n*-hexane fraction (SF-1) and squalene (SQ) based on the α-glucosidase inhibition assay.

| Group | $IC_{50}$ (mg/mL) |
|---|---|
| Acarbose | 0.370 ± 0.210 |
| ME | 0.053 ± 0.004 |
| CF | 0.049 ± 0.002 |
| WF | 0.740 ± 0.070 |
| SF-1 | 0.100 ± 0.004 |
| SQ | 1.330 ± 0.050 |

Values are expressed as mean ± SD (n=3).

### 3.13.4. *In vitro* α-amylase inhibition activity

The *in vitro* α-amylase inhibition assay showed that SF-1 and SQ were the most potent inhibitors of α-amylase activity, with $IC_{50}$ values of 0.34 ±0.04 and 0.82 ±0.04, respectively (Table 3.5). The $IC_{50}$ values of ME and CF were 3.24 ±0.14 and 3.14 ±0.09, respectively, and they were higher than acarbose's 1.55 ±0.22 (Table 3.5). The descending order of α-amylase inhibition activities was as follows: SF-1 > SQ > Acarbose > WF > CF > ME (Table 3.5).

Table 3.5. The $IC_{50}$ values of acarbose, methanol extract (ME), chloroform fraction (CF), water fraction (WF), *n*-hexane fraction (SF-1) and squalene (SQ) based on the α-amylase inhibition assay.

| Group | $IC_{50}$ (mg/mL) |
|---|---|
| Acarbose | 1.55 ± 0.22 |
| ME | 3.24 ± 0.14 |
| CF | 3.14 ± 0.09 |
| WF | 1.57 ± 0.03 |
| SF-1 | 0.34 ± 0.04 |
| SQ | 0.82 ± 0.04 |

Values were expressed as mean ± SD (n=3).

### 3.13.5. Glucose absorption via averted jejunal sacs

Figure 3.6 shows the glucose absorption rate via averted jejunal sac. A lower glucose absorption value indicates a stronger inhibitory effect of intestinal glucose absorption exerted by the test substance. The present study showed that ME, CF, WF, SF-1 and SQ significantly inhibited glucose absorption via the jejunum in a similar manner to that of acarbose and phlorizin. CF, WF and SQ were the three most potent inhibitors of glucose absorption ($P$<0.001). Hence, the descending order of inhibition activity was as follows: SQ >WF > CF > Acarb = Phl = SF-1 > ME.

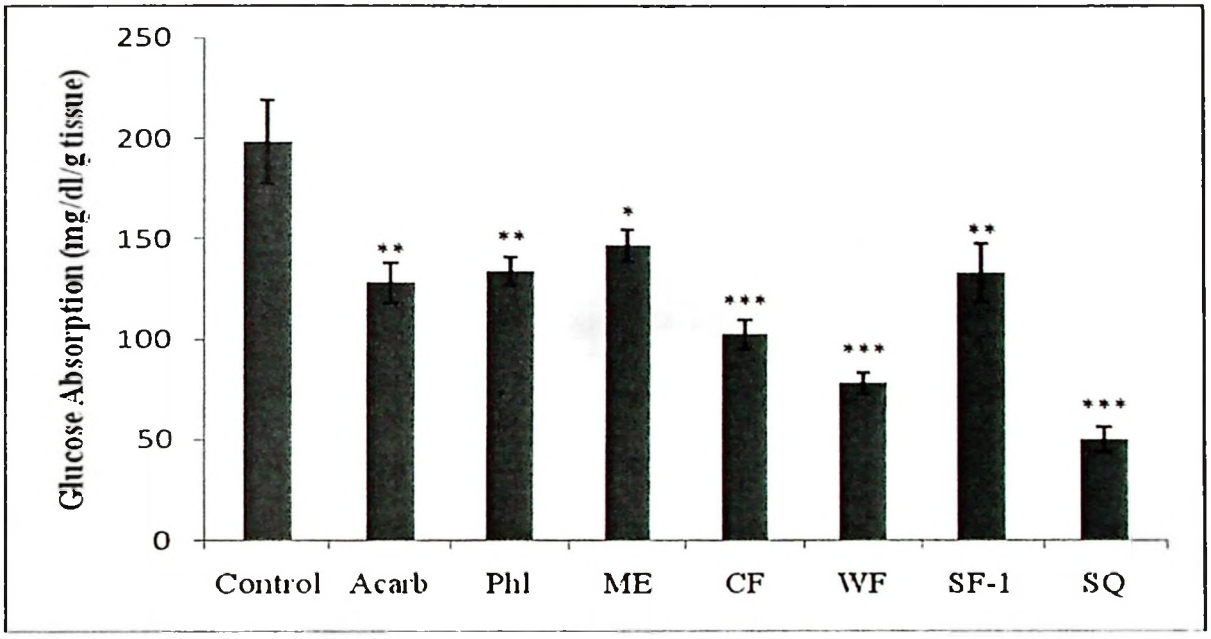


Figure 3.6. The effect of *S. polyanthum* methanol extract (ME), chloroform fraction (CF), water fraction (WF), and *n*-hexane fraction (SF-1), squalene (SQ), acarbose (Acarb) and phlorizin (Phl) on glucose absorption via averted rat jejunal sacs.
The values are expressed as mean ± SEM (n=5); statistical analysis was done using one-way ANOVA and followed by Dunnett's test as *post hoc* test (*$P$<0.05, **$P$<0.01, ***$P$<0.001)

### 3.13.6. Enzyme inhibition *in vivo*

Area Under the Curve (AUC) is the area under a plot of plasma concentration for a drug against time after drug administration (Katzung, 2004). AUC reflects the bioavailability of a drug. An increased AUC value means that more of a drug was absorbed. Conversely, a decreased AUC value means that a smaller amount of the drug was absorbed.

Starch is a polysaccharide, while sucrose is a disaccharide. After starch and sucrose loading, these carbohydrates should be converted by the carbohydrate digestive enzymes (α-glucosidase and α-amylase) into monosaccharides (glucose) to be absorbed. In the presence of an α-glucosidase inhibitor, the convertion of complex carbohydrates (starch) into disaccarides and monosaccharides (glucose) is inhibited. Therefore, the amount of glucose that can be absorbed is decreased. In the presence of an α-amylase inhibitor, the conversion of starch into disaccharides is inhibited.

In the oral starch, sucrose and glucose tolerance tests, oral administration of the extracts (ME, CF, WF, SF-1) and SQ as expected to inhibit the enzyme activity (if any), resulting in lower glucose absorption rates, and subsequently, lower AUC values.

### 3.13.6.1. Oral starch tolerance test in normal and STZ-induced diabetic rats

In non-diabetic rats, all of the groups treated with acarbose, ME, CF, WF, SF-1 and SQ showed significant decreases ($P<0.001$) in AUC after starch loading (Table 3.6). However, in STZ-induced diabetic rats, only the acarbose- and ME-treated groups had significant decreases in AUC after starch loading ($P<0.05$) (Table 3.6).

Table 3.6. Effect of acarbose (10 mg/kg), methanol extract (ME) (1 g/kg), chloroform fraction (CF) (500 mg/kg), water fraction (WF) (500 mg/kg), *n*-hexane fraction (SF-1) (250 mg/kg) and squalene (SQ) (160 mg/kg) on the Area Under the Curve (AUC) of the blood glucose levels of normal and STZ-induced diabetic rats after starch loading (3 g/kg).

| Group | AUC (mmol/L) | |
|---|---|---|
| | Normal | Diabetic |
| Control | 13.07 ± 0.30 | 39,43±2.00 |
| Acarbose | 10.03 ± 0.35*** | 28,73±1.90* |
| ME | 9.80 ± 0.14*** | 28,89±0.90* |
| CF | 10.68±0.49*** | 33,45±3.00 |
| WF | 10.34±0.49*** | 32,8±3.00 |
| SF-1 | 10.33±0.22*** | 35,59±3.00 |
| SQ | 10.1±0.44*** | 33,43±2.00 |

The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA and was followed by Dunnett's test as a *post hoc* test. (*$P<0.05$, ***$P<0.001$)

### 3.13.6.2. Oral sucrose tolerance test in normal and STZ-induced diabetic rats

In the oral sucrose tolerance test, acarbose- ($P<0.001$), ME-( $P<0.05$) and CF-($P<0.05$) treated groups showed significant decreases in AUC in the non-diabetic rats (Table 3.7). However, none of the extracts was able to reduce AUC in the STZ-induced diabetic rats. Only the acarbose-treated group showed a significant decrease in AUC after sucrose loading ($P<0.05$) (Table 3.7).

Table 3.7. Effects of acarbose (10 mg/kg), methanol extract (ME) (1 g/kg), chloroform fraction (CF) (500 mg/kg), water fraction (WF) (500 mg/kg), *n*-hexane fraction (SF-1) (250 mg/kg) and squalene (SQ) (160 mg/kg) on the Area Under the Curve (AUC) of the blood glucose levels of normal and STZ-induced diabetic rats after sucrose loading (4 g/kg).

| Group | AUC (mmol/L) | |
|---|---|---|
| | Normal | Diabetic |
| Control | 15.26 ± 0.90 | 42.58 ± 2.50 |
| Acarbose | 11.24 ± 0.24*** | 30.38 ± 1.90* |
| ME | 12.61 ± 0.76* | 35.78 ± 0.93 |
| CF | 12.74 ± 0.46* | 37.5 4± 3.09 |
| WF | 13.30 ± 0.69 | 40.11 ± 3.07 |
| SF-1 | 13.8 ± 0.30 | 37.79 ± 3.87 |
| SQ | 13.21 ± 0.30 | 38.89 ± 2.28 |

The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA and followed by Dunnett's test as a *post hoc* test. (*$P<0.05$, ***$P<0.001$)

### 3.13.6.3. Oral glucose tolerance test in normal and STZ-induced diabetic rats

In the oral glucose tolerance test, AUC levels of all treated groups (acarbose, ME, CF, WF, SF-1 and SQ) showed no significant difference ($P>0.05$) compared to the control groups in both normal and STZ-induced diabetic rats after glucose loading (Table 3.8).

Table 3.8. Effects of acarbose (10 mg/kg), methanol extract (ME) (1 g/kg), chloroform fraction (CF) (500 mg/kg), water fraction (WF) (500 mg/kg), *n*-hexane fraction (SF-1) (250 mg/kg) and squalene (SQ) (160 mg/kg) on the Area Under the Curve (AUC) of the blood glucose levels of normal and STZ-induced diabetic rats after glucose loading (2 g/kg).

| Group | AUC (mmol/L) | |
|---|---|---|
| | Normal | Diabetic |
| Control | 14.36 ± 1.40 | 50.38 ± 2.72 |
| Acarbose | 12.69 ± 0.90 | 44.24 ± 4.27 |
| ME | 12.18 ± 0.90 | 44.40 ± 4.54 |
| CF | 14.30 ± 0.40 | 45.17 ± 3.72 |
| WF | 13.17 ± 0.60 | 50.33 ± 2.75 |
| SF-1 | 12.98 ± 0.56 | 46.45 ± 0.69 |
| SQ | 12.18 ± 0.60 | 43.08 ± 4.17 |

The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA.

### 3.13.7. Glucose uptake by isolated abdominal muscle

Figure 3.7 shows that ME, CF and SF-1 caused significant increases in glucose uptake by the abdominal muscle, compared to the control, in a manner similar to metformin ($P$<0.01- 0.001) without the presence of insulin. In the presence of insulin, all of the substances (metformin, ME, CF, WF, SF-1 and SQ) increased glucose uptake more than the control ($P$<0.05-0.001) (Figure 3.8).

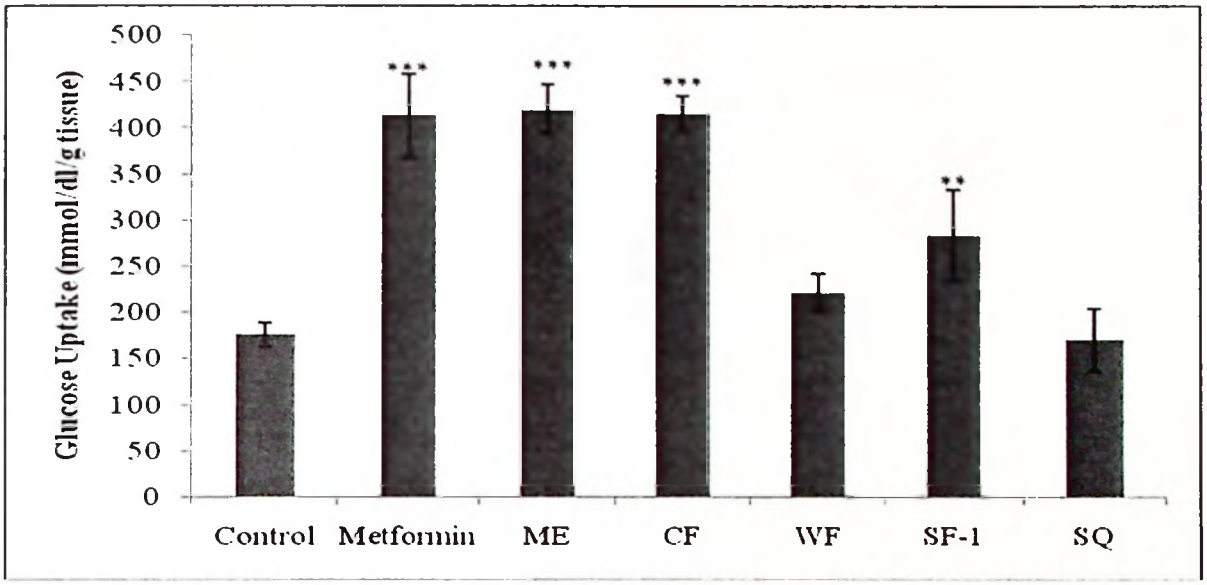


Figure 3.7. The effects of metformin, *S. polyanthum* methanol extract (ME), chloroform fraction (CF), water fraction (WF) and *n*-hexane fraction (SF-1) and squalene (SQ) on glucose uptake of abdominal muscle strips.
The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA followed by Dunnett's test as a *post hoc* test. (**$P$<0.01, ***$P$<0.001)

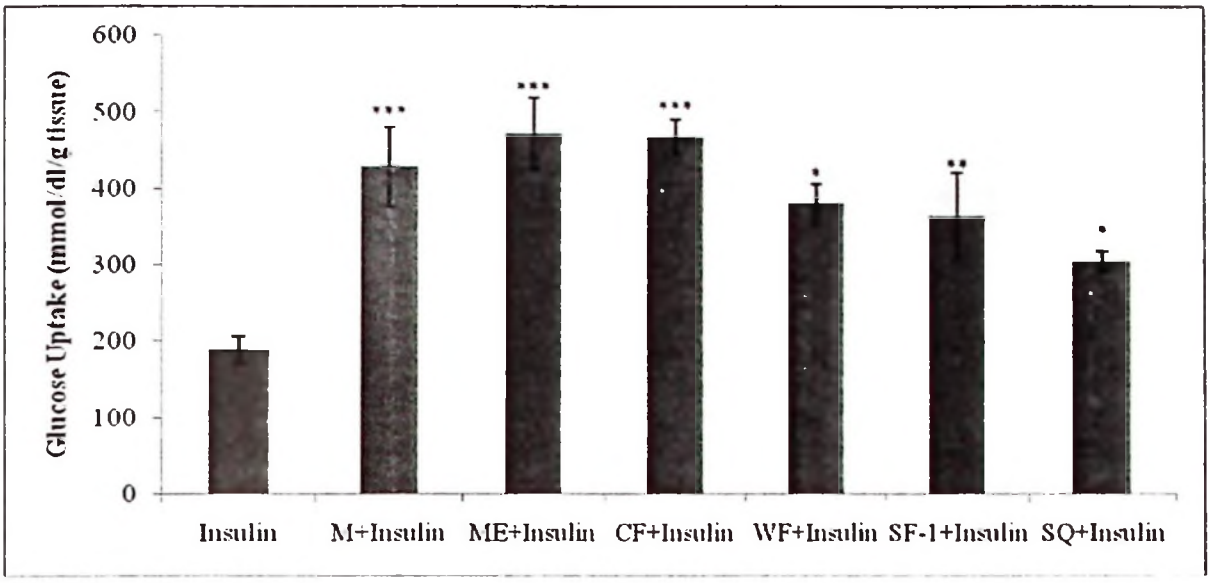


Figure 3.8. The effects of metformin (M), *S. polyanthum* methanol extract (ME), chloroform fraction (CF), water fraction (WF) and *n*-hexane fraction (SF-1), and squalene (SQ) on glucose uptake of abdominal muscle strips in the presence of insulin.
The values are expressed as mean ± SEM (n=6); statistical analysis was done using one-way ANOVA and was followed by Dunnett's test as a *post hoc* test. (**P*<0.05, ***P*<0.01, ****P*<0.001)

### 3.13.8. Glucose uptake by isolated soleus muscle

Metformin, ME, CF and SF-1 significantly increased glucose uptake by the soleus muscle (*P*<0.05) (Figure 3.9). Moreover, in the presence of insulin, metformin, CF, WF, SF-1 and SQ caused significant increases in glucose uptake by the soleus muscle (*P*<0.05-0.001) (Figure 3.10).

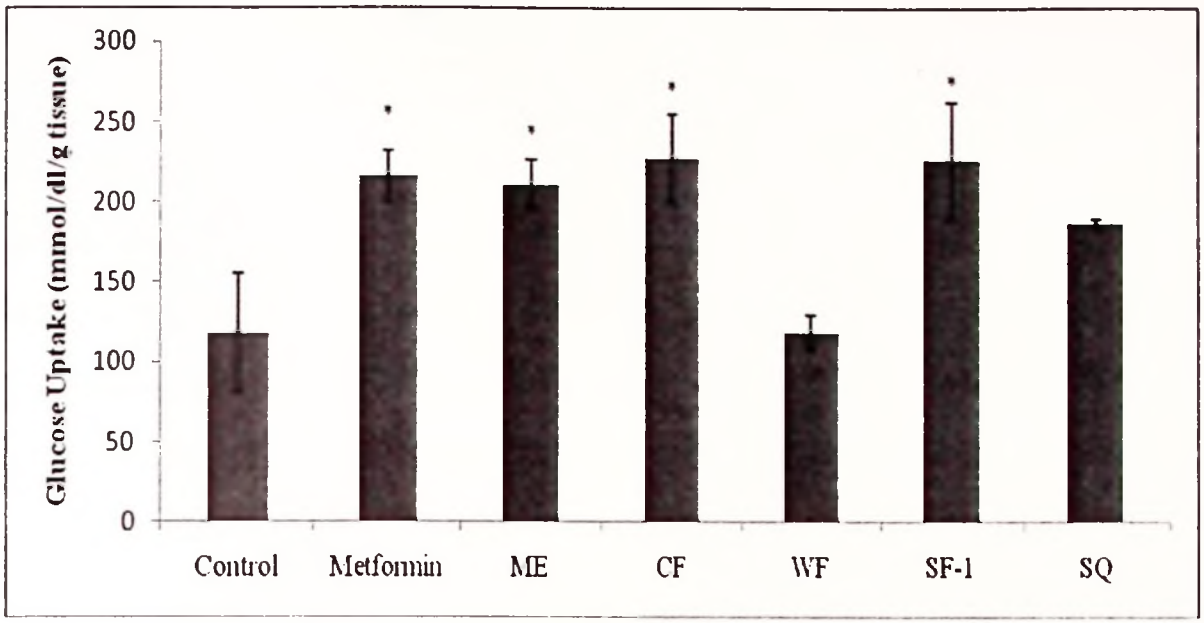


Figure 3.9. The effects of metformin, *S. polyanthum* methanol extract (ME), chloroform fraction (CF), water fraction (WF) and *n*-hexane fraction (SF-1), and squalene (SQ) on glucose uptake by soleus muscle strips.
The values are expressed as mean ± SEM (n=5); statistical analysis was done using one-way ANOVA followed by Dunnett's test as a *post hoc* test (**P*<0.05)

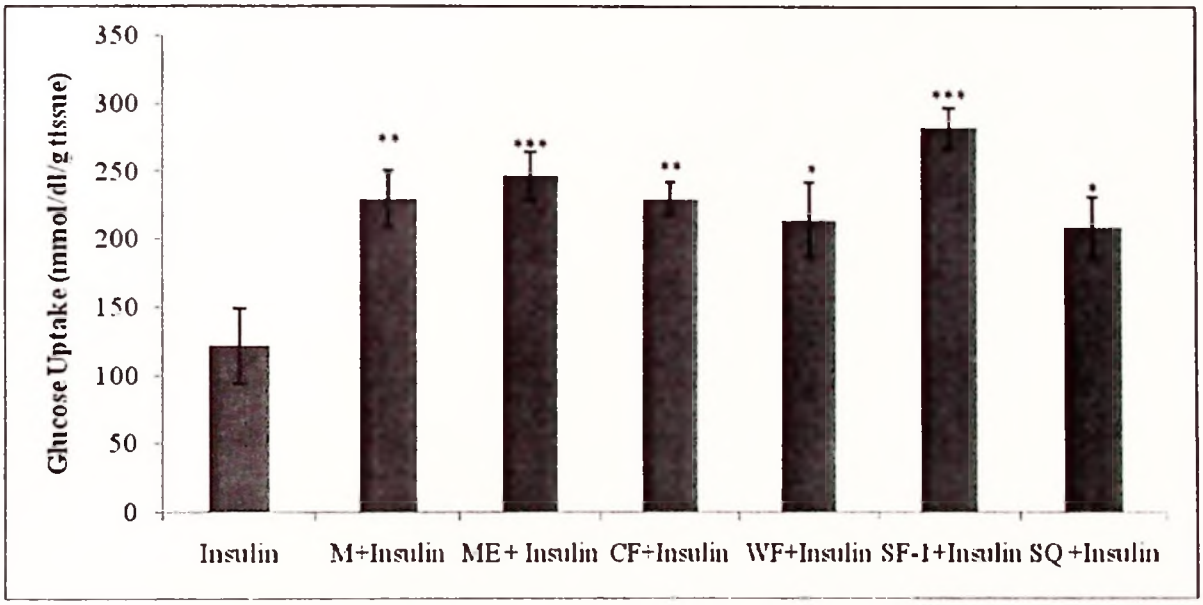


Figure 3.10. The effects of metformin, *S. polyanthum* methanol extract (ME), chloroform fraction (CF), water fraction (WF) and *n*-hexane fraction (SF-1), and squalene (SQ) on glucose uptake by soleus muscle strips in the presence of insulin.
The values are expressed as mean ± SEM (n=5); statistical analysis was done using one-way ANOVA followed by Dunnett's test as a *post hoc* test. (**P*<0.05, ***P*<0.01, ****P*<0.001)

### 3.14. Discussion and conclusions

As many as 80% of non-insulin-dependent diabetes mellitus (NIDDM) patients have been treated with oral anti-diabetic drugs (Krentz & Bailey, 2005) to control their disease (DeRuiter, 2003). The main goal of the pharmacological therapy (Rubin, 2005) of diabetes is to normalize BGL to reduce the risk of long-term complications (American Diabetes Association[ADA], 2014). This is achieved by treating with the oral anti-diabetic agents (Holstein & Egberts, 2003). Generally, these drugs act by inhibiting glucose absorption from the gut (α-glucosidase inhibitors e.g. acarbose, increasing insulin secretion by the β-cells (sulphonylureas and meglitinides) or increasing insulin sensitivity at the target tissues (thiazolidinediones and biguanides e.g. metformin) (Krentz & Bailey, 2005; Shu & Myers, 2004). Owing the great interest in the development of new drugs (Kecskemeti et al., 2002) to prevent the complications associated with diabetes mellitus, the scientific community has investigated both crude herbal extracts and isolated compounds of anti-diabetic activity and their possible mechanisms of action (Shukia et al., 2000) using different type of experimental techniques (Fröde & Medeiros, 2008; Patel et al., 2012). The previous chapters have shown the anti-hyperglycaemic activity of *S. polyanthum* in streptozotocin-induced diabetic rats, and its hypoglycaemic action in normal rats. Hence, in the present chapter, *in vitro* and *in vivo* assay were conducted to investigate the mechanisms of action of this plant to explain its pharmacological anti-diabetic activity.

The BGL of the STZ-induced diabetic rats treated with ME, CF, WF, and SF-1 of *S. polyanthum* leaves, SQ and metformin for 12 days were significantly decreased compared with the diabetic control. The descending order of the anti-hyperglycaemic

activity was as follows: M = ME (56%) > CF = SF-1 = SQ (43%) > WF (36%). These findings verified the previous ones in the 6-days treatment, whereby ME proved to be the most active agent. The initial investigation was carried out using repeated-dose administration for 6 days to evaluate the BGL lowering effects observed in the single-dose administration (7 hrs of observation). Furthermore, oral administration of ME, CF, WF, SF-1 and SQ was carried on twice-daily for 12 days to expand the aim of the study to include elucidating the mechanisms of action on the pancreatic pathway, and also the effect on the lipid profiles. None of the extract-, fraction-, subfraction- and SQ-treated groups showed a significant effect on the blood insulin levels compared with the control, which conformed to the histological findings, as none of the treatments effectively regenerate the islets of Langerhans. These results suggest that the mechanism of the anti-hyperglycaemic action of *S.polyanthum* is independent of insulin secretion.

Currently available oral anti-diabetic drugs, such as sulphonylureas and thiazolidinediones, often lead to weight gain, and cause obese conditions (Krentz & Bailey, 2005). Obesity is correlated to insulin resistance (Hirosumi et al., 2002). Insulin resistance is a condition whereby insulin-induced glucose uptake is impaired in the insulin-sensitive tissues (Ye, 2013), leading to hyperglycaemia. Weight reduction has been shown to improve glycaemic control due to improved insulin action (McFarlane, 2009; Schwartz & Kohl, 2010).

Weight loss increases the rate at which glucose is taken from the blood by muscle cells (glucose up-take) as a result of improved insulin sensitivity (Mathur & Conrad Stöppler, 2012). The present study also investigated the effects of ME, CF, WF, SF-1

and SQ on body weight. Interestingly, statistically significant weight losses were observed with the use of ME, WF, SF-1 and metformin from day 0 (pre-treatment) to day 12 (post-treatment) (Figure 3.4.). It is known that metformin is an insulin-sparing agent and does not increase the body weight (Nolte & Karam, 2004). This study showed that none of the extracts, fractions, SQ and metformin increased the body weights of the diabetic rats. The diabetic control group showed statistically significant reduction in the body weight after 12-days treatment with the fasting BGL maintained at the hyperglycaemic condition. The significant loss of body weight in the untreated diabetic rats may be due to the loss of muscle mass and adipose tissue, caused by an excessive breakdown of tissue proteins and fatty acids. Several studies have reported similar significant weight reductions in untreated diabetic rats (Rao et al., 1999; Sabu, Smitha, & Kuttan, 2002). In type 2 diabetes, the pancreas retains some β-cell function, but insulin secretion is insufficient to maintain glucose homeostasis. The β-cell mass may become gadually reduced (Harvey, 2012). The significance of the body weight reduction in the control diabetic group suggested a destruction of pancreatic β-cells, causing a disability to produce adequate insulin. Hence these results support the notion that the loss in body weight upon treatment with *S. polyanthum* ME, WF and SF-1can improve insulin sensitivity.

Diabetes is associated with hyperlipidemia (Rajasekaran et al., 2006), and STZ-induced diabetic rats have shown increased cholesterol and triglyceride plasma levels (Sachdewa & Khemani, 2003). The present study showed that the lipid profiles (TC, TG, LDL and Chol/LDL) of the ME-, CF-, SF-1-, SQ- and metformin-treated group were lower than those of the diabetic control after 12 days of treatment. However, only SF-1and SQ showed the ability to decrease the TC levels significantly as seen in

the metformin-treated group. The SQ-treated groups also showed a significant reduction in the LDL levels, which was similar to that observed with metformin use. The lipid profiles (TC, TG, HDL, LDL and Chol/LDL) of the normal rats were not significantly different compared to those of the diabetic control group. Similar finding were reported by Sriplang et al. (2007), who showed non-significant differences in TC, TG and HDL levels between the normal and the diabetic control groups upon 14 days of observation. The present study also found that the LDL levels in the SQ- and metformin-treated groups were significantly decreased compared to those in the diabetic control. Biguanides like metformin are associated with lowered lipid levels and a decreased in body weights (Shu & Myers, 2004). These findings additionally demonstrated the activities of *S. polyanthum* leaf extract/fractions and squalene in improving TC and LDL levels in STZ-induced diabetic rats.

In diabetes mellitus, controlling postprandial plasma glucose levels is critical from the beginning of treatment. Inhibiting some of the enzymes involved in the metabolism of carbohydrate, i.e., α-amylase and α-glucosidase, is one of the therapeutic approaches for delaying postprandial hyperglycaemia. α-Amylase, which is found in the saliva and the pancreatic juices, is involved in starch (polysaccharides) degradation and conversion into oligosaccharides and disaccharides. On the other hand, α-glucosidase, which is a collective term referring to the membran-bound enzymes in the small villi at the brush borders of the small intestines, is required for the breakdown of the α-linkages in oligosaccharides and disaccharides to be turned into absorbable monosaccharides (e.g., glucose) which results in postprandial hyperglycaemia (Soumyanath, 2005; Kumar et al., 2011).

Thus, this enzyme increases the bioavailability of glucose in the blood. Acarbose, a standard oral antidiabetic drug, reversibly binds to pancreatic α-amylase and the membrane-bound intestinal α-glucoside hydrolases, causing carbohydrate absorption and digestion to be delayed and prolonged, and resulting ameliorated postprandial hyperglycaemia (Munjal & Surendra, 2012).

The present *in vitro* study shows that ME, CF and SF-1 of *S. polyanthum* leaves, and SQ inhibited α-glucosidase in a fashion similar to that of the positive control, acarbose, with the inhibition order being as follows: CF > ME > SF-1 > SQ > Acarbose > WF. A similar finding has been reported on *S. cumini* by Shinde et al. (2008), who, based on similar results, concluded that a 70% ethanolic extract of the seeds had α-glucosidase inhibition activity. On the other hand, the *in vitro* α-amylase inhibition assay at the present study demonstrated that only SF-1 and SQ showed inhibition activities, with the inhibition order being as follows: SF-1 > SQ > Acarbose > WF > CF > ME. These findings suggest that the anti-hyperglycaemic effect of *S. polyanthum* leaves may at least be partially participated via inhibition of α-glucosidase and α-amylase activities.

The averted sac method of Wilson & Wiseman (1954) was used to investigate the effect of the extract/fractions on glucose absorption in the intestine. This technique has been declared to be reliable since 1954 (Carmona, 1998) to evaluate the inhibition of intestinal glucose absorption (Nistor et al., 2010). The present study used acarbose as an α-glucosidase inhibitor. Another agent that inhibits intestinal glucose absorption is phlorizin. This agent produce renal glycosuria and block

intestinal glucose absorption through the inhibition of the sodium-glucose symporters located in the proximal renal tubules and mucosa of the small intestines (Ehrenkranz et al., 2005). The intestinal part used in this study was the jejunal portion, which is the primary site of glucose absorption (Carmona, 1998). There are two aspects of intestinal glucose absorption, the classical and the diffusive absorption mechanisms. Classical active absorption is mediated by the Na+/glucose co-transporter, whereas the diffusive effect is mediated by the transient insertion of glucose transporter type 2 (GLUT2) into the apical membrane. This apical GLUT2 pathway of intestinal sugar absorption provides a major route at high sugar concentrations. The pathway is regulated by rapid trafficking of GLUT2 to the apical membrane, which is induced by glucose during the assimilation of a meal (Dyer et al, 2002; Kellet, 2008). ME, CF and SF-1 of *S. polyanthum*, and SQ inhibited glucose absorption from the intestine in a fashion similar to that of acarbose and phlorizin. The inhibition order of potency was as follows: SQ >WF > CF > Acarbose = Phlorizin = SF-1 > ME. This property may have partly contributed to the anti-hyperglycaemic activity of *S. polyanthum* leaves.

An inhibitory activity observed *in vitro* is uncertain to correspond to a similar *in vivo* activity (Subramanian, Asmawi, & Sadikun, 2008). Therefore *in vitro* data has to be confirmed by *in vivo* data. To confirm the enzyme inhibitory activity of *S. polyanthum* leaves, *in vivo* enzyme inhibition assays were performed by challenging normal and STZ-induced diabetic rats with starch, sucrose and glucose loadings. The present study showed that CF, WF, SF-1 and SQ inhibited the conversion of starch to glucose in the normal rats only and not in the diabetic ones. However, ME inhibited the conversion process in both, the normal and the diabetic rats. In the

sucrose challenge test, only ME and CF significantly reduced AUC values in the normal rats. None of the extracts, fractions, SQ or acarbose showed an inhibitory activity in the oral glucose tolerance test in the present study. These results suggested that ME and CF possessed both an α-glucosidase and an α-amylase inhibitory activity, whereas WF, SF-1 and SQ only retained the α-amylase inhibition activity. This may contribute to the overall anti-hyperglycaemic activity of *S. polyanthum*.

The glucose uptake by the muscles, was performed using rat isolated abdominal and soleus muscles prepared by Gray & Flatt (1998). The major effects of insulin on the muscles are stimulating the translocation of the insulin-responsive glucose transporter, GLUT4, from the intracellular vesicles to the cell surface; increasing amino acid uptake, stimulating the ribosomal protein synthesis machinery, and promoting the rate of glycogen synthase activity and the subsequent glycogen storage process, thus, decreasing the rate of glycogen breakdown (Dimitriadis et al., 2011; Shu & Myers, 2004). Metformin, a biguanide, is an insulin-sensitizing agent with potent anti-hyperglycaemic properties (Kirpichnikov et al., 2002) due to its effects on insulin receptors and glucose transporters (Ciaraldi et al., 2002). It reduces glucose absorption in the small intestines, increases glucose uptake into the cells and its utilization by the target tissues, decreases plasma free fatty acid concentrations and inhibits the gluconeogenesis process, thereby decreasing insulin resistance. Activationing AMP-dependent protein kinase (AMPPK) plays an important role in these processes (Grzybowska, Bober, & Olszewska, 2001; Shu & Myer, 2004). Since insulin resistance contributes greatly to the metabolic syndrome and causes type 2 diabetes mellitus, treatment with an insulin sensitizer may ameliorate the pathophysiological abnormalities of the metabolic syndrome (Kirpichnikov et al.,

2002). Biguanides activate AMPPK to block the breakdown of fatty acids, and inhibit hepatic gluconeogenesis and glycogenolysis (Musi et al., 2002; Shu & Myers, 2004). The effect of metformin on the peripheral insulin-sensitive tissues requires the presence of insulin for it to exert its full effect (Kirpichnikov et al., 2002). In the absence of insulin there is only a minor effect (Wiernsperger & Bailey, 1999). However, the direct effects of the drug on the glucose-transport have been shown to be unrelated as it enhanced glucose analogue transportation independently from insulin (Kasmuri, 2006; Klip & Leiter, 1990). Use of the isolated abdominal muscle model of the present study revealed that ME, CF, and SF-1 of *S. polyanthum* leaves enhanced glucose uptake by the muscle in the presence and absence of insulin in a fashion similar to metformin. Conversely, WF and SQ showed significant effects in the presence of insulin only. In the soleus muscle, ME, CF and SF-1 caused a significant increase of glucose uptake with and without insulin similar, which was comparable to metformin. However, WF and SQ showed significant effects in the presence of insulin only. In the presence of insulin, the extracts, fractions and SQ enhanced the sensitivity of insulin and increase the uptake of glucose, while in the absence of insulin, the actions were probably due to the activation of the glucose transporters that were directly responsible for the uptake of glucose. These findings support the previous study performed in the *in vivo* model of streptozotocin-induced diabetic rats. These mechanisms contributed to the anti-hyperglycaemic action of *S. polyanthum* leaves. The results on the mechanisms of action of *S. polyanthum* anti-diabetic activity in the present study are summarized in Table 3.9.

Table 3.9. Summary of the effects of *S. polyanthum* (Wight) leaf extract/fractions and squalene from the study

| Evaluation | ME | CF | WF | SF-1 | SQ |
|---|---|---|---|---|---|
| **Oral administration twice daily for 12 days** | | | | | |
| Fasting Blood Glucose Level | +++ | ++ | ++ | ++ | + |
| Insulin level | - | - | - | - | - |
| Body weight | + | - | ++ | + | - |
| **Lipid profiles** | | | | | |
| Total Cholesterol (TC) | - | - | - | + | + |
| Triglyceride (TG) | - | - | - | - | - |
| High-Density Lipoproteins (HDL) | - | - | - | - | - |
| Low-Density Lipoproteins (LDL) | - | - | - | - | + |
| Cholesterol/LDL | - | - | - | - | - |
| **Histological assesment** | | | | | |
| Insulin-secreting area | - | - | - | - | - |
| ***In vitro* enzyme inhibition assay** | | | | | |
| α- Glucosidase inhibition | ++++ | +++ | - | ++ | + |
| α-Amylase inhibition | - | - | - | ++ | + |
| **Glucose absorption from the intestine** | + | +++ | ++++ | ++ | ++++ |
| **OGTT (starch)** | | | | | |
| Normal rats | + | + | + | + | + |
| STZ-induced diabetic rats | + | - | - | - | - |
| **OGTT (sucrose)** | | | | | |
| Normal rats | + | + | - | - | - |
| STZ-induced diabetic rats | - | - | - | - | - |
| **OGTT (glucose)** | | | | | |
| Normal rats | - | - | - | - | - |
| STZ-induced diabetic rats | - | - | - | - | - |
| **Glucose uptake by the abdominal muscle** | | | | | |
| Without insulin | + | + | - | + | - |
| With insulin | + | + | + | + | + |
| **Glucose uptake by the soleus muscle** | | | | | |
| Without insulin | + | + | - | + | - |
| With insulin | + | + | + | + | + |

+ : significant effect

# CHAPTE FOUR

# GENERAL DISCUSSION AND CONCLUSION

Diabetes mellitus contributes to a major health-care burden worldwide and presents major challenges to patients, health-care systems and national economies. WHO estimates, that the worlds population will rise by 37% between 2000 and 2030, making Asia the main site of a rapidly emerging diabetes epidemic (Ramachandran, Wan Ma, & Snehalatha, 2010). These facts should be addressed by promoting education to prevent diabetes population from increasing in the community and to maintain blood glucose concentrations as close as possible to the normal level in both of the type 1 and the type 2 DM patients to decrease the incidence of microvascular and macrovascular complications. Nowadays, scientists are striving to find ideal blood glucose lowering agents from a variety of sources, including herbal remedies. Medicinal plants that are believed to have anti-diabetic activities, based on their uses in folk medicines, are being studied. In general, establishing pharmacological basis for the efficacy of medicinal plants is a constant challenge (Bhattaram et al., 2002).

*Syzygium polyanthum* (Wight) is easily found in Asia, commonly used as a natural flavor in Asian cuisines. It was been reported as one of the most commonly used herbs by the diabetic patients in Indonesia (Widyawati et al., 2012). Previous anti-diabetic studies on this plant were conducted using different solvents (ethanol, methanol and water) to extract the leaves and different experimental techniques (*in vitro* and *in vivo*) (Studiawan and Hadi, 2005; Kusnandar, 2006; Wahyono and Susanti, 2011). However, none of the previous works used the bioactivity-guided approach as conducted in the present study.

The initial aim of the present study was to verify the hypoglycaemic and anti-hyperglycaemic activities. Screening models were used, and subsequently, the study applied hypoglycaemic tests and IPGTT to screen the effects both in normal and STZ-induced diabetic rats. The preparation of the plant extracts was performed using selected solvents and based on their different polarities, starting from the crude extracts down to the subfractions. The study revealed that the non-polar (ME, CF) and the polar (WF) extracts of *S. polyanthum* leaves had anti-hyperglycaemic activities. The next task was to conduct similar tests on different doses of ME to determine whether or not that the effect was dose-related and to confirm the previous results. The study showed that the higher the doses of ME was, the higher the ability of ME to reduce BGL was. The minimum dose that produced an anti-hyperglycaemic activity was 250 mg/kg. It is important, however, to determine the optimum dose of pharmacological activity of medicinal plant. The required dose relates to the amount of the plant that needs to be administered. The amount of the dose is also correlated to the concentration and its effect, thus, it determines the possible response, whereby a drug may result sub-therapeutic, therapeutic or toxic effects (Eldawud et al., 2014).

Phytochemical screening showed the presence of tannins, flavonoids, saponins and glycosides in ME, CF, WF and SF-1. Some of these compounds are well known to have anti-diabetic properties. GC-MS revealed the presence of squalene in ME, CF and SF-1. There is so far no report on the role of squalene as an anti-hyperglycaemic agent. The previous studies had only identified the presence of squalene in other plant which had anti-diabetic activities. Thus, the present study was conducted to determine the effect of different doses of squalene in STZ-induced diabetic rats and it revealed its dose-dependent anti-hyperglycaemic activity.

The present study showed that ME had the greatest anti-hyperglycaemic activity, and it comprised, among other compounds, squalene. Squalene is a non-polar compound that was not supposed to be found in ME, yet, it was found there more compared with the non polar extracts (PEE and CE). However, the present study showed that PEE and CE had no anti-hyperglycaemic activity. Considering the relative amounts of squalene in ME, CF and SF-1, the findings from this present study infer that the anti-hyperglycaemic activity of *S. polyanthum* leaves could be mediated by synergistic molecular mechanisms or potentiation effects amongst the bioactive compounds in ME (involving polar compounds more than non-polar ones). Medical herbalists argue that, compared to the pharmacological effects observed with individual isolated compounds, better results are obtained with whole plant extracts and combinations of these extracts (Williamson, 2009). The 12-days treatments results in this current study supported this hypothesis by showing that ME exerted the greatest effect in decreasing BGL (56%), followed by CF (non-polar) =SF-1 (non-polar) =SQ (non-polar) (43%) and WF (polar) (36%). Furthermore, CF, SF-1, and SQ, which were of a non-polar nature and WF (polar) were shown to be less active than ME, which comprised of polar and non-polar components. These results indicate substantial synergism between the polar and the non-polar compunds found in *S. polyanthum* leaves, contributing to its anti-hyperglycaemic activity. To test this hypothesis, further research should be undertaken to further identify the polar bioactive compounds present in *S. polyanthum* leaf which may be related to ameliorating hyperglycaemia, as additional results from the present study served to assess the anti-hyperglycaemic activity of squalene, one of the bioactive non-polar compounds found in the most active extract (ME) and its fractions (CF and SF-1).

The body weight of the diabetic animals-treated with the extract/fractions (ME, CF, WF, SF-1) and squalene in the present study showed a tendency to decrease. Since obesity is correlated to insulin resistance, which is a major cause of hyperglycaemia, the reduction of body weight may probably be associated with the enhanced glycaemic control observed in the diabetic rats. The lipid profiles of the diabetic rats-treated with the extract/fractions (ME, CF, WF, SF-1) and squalene showed a tendency to decrease. TC significantly decreased in the SF-1- and SQ-treated groups, whereas LDL was significantly reduced in the SQ-treated groups. These results highlight the additional benefit of *S.polyanthum* (Wight) leaves in terms of lipid profile improvement.

To examine the possible mechanisms of the plant, the present study was performed using *in vitro* and *in vivo* models. The results show that each of ME, CF, WF and SF-1 has different effects, demonstrated by the different evaluation methods used in this study.

To examine whether the anti-hyperglycaemic effects of the extract/fractions and SQ were due to enhanced insulin secretion and/or improved function of the islets of Langerhans, a histological study was conducted. The results obtained using the *in vivo* models suggested that the extract/fractions and SQ did not significantly affect insulin secretion or Langerhans islet regeneration. Thus, it was concluded that the mechanism of action of S. *polyanthum* did not involve the pancreatic pathway.

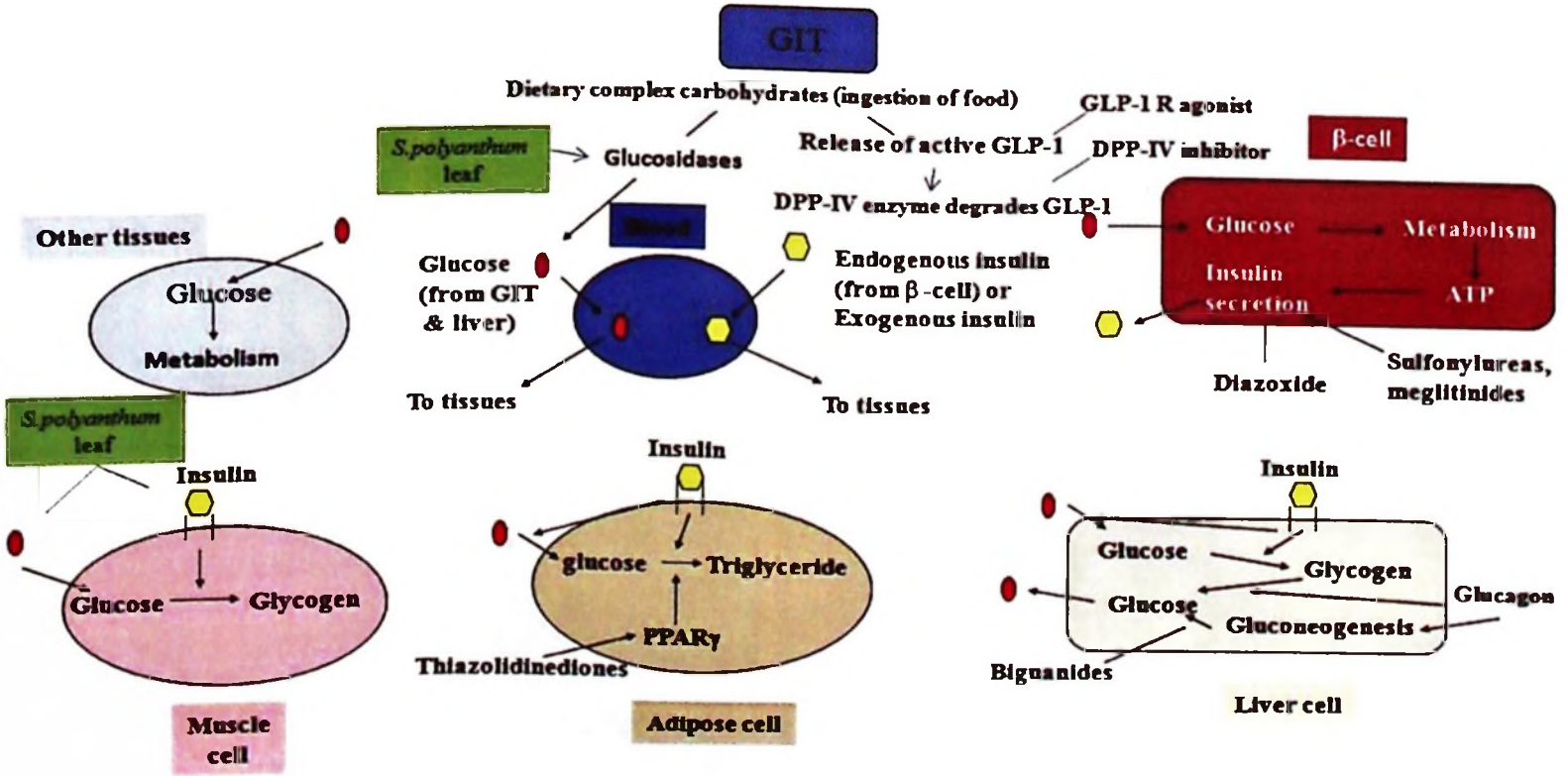


Figure 4.1. Mechanisms of action of *Syzygium polyanthum* (Wight) leaf extracts as anti-diabetic agent.
(GIT: Gastrointestinal tract, ATP: Adenosine triphosphates, PPARγ: Peroxisome proliferator-activated receptor γ; GLP-1R: Glucagon-like peptide-1 receptor; DPP-IV: dipeptidyl-peptidase-IV; : Glucose; : Insulin)

The *in vitro* α-glucosidase assay showed that ME, CF, SF-1 and SQ were able to inhibit α-glucosidase. Furthermore, the *in vitro* α-amylase assay showed that SF-1 and SQ were able to inhibit α-amylase as well. Based on the jejunal sac model, this study revealed that ME, CF, WF, SF-1 and SQ inhibited glucose absorption in a fashion similar to acarbose and phlorizin, the standard drugs used as positive controls. This effect suggested that the anti-diabetic activities of *S. polyanthum* and SQ were due to glucose absorption inhibition in the intestines (Figure 4.1).

Oral glucose tolerance tests involving the administration of starch, sucrose and glucose in normal and diabetic rats were conducted to confirm the observed enzymatic inhibition effects *in vivo*. They revealed that ME and CF were able to inhibit of α-glucosidase and α-amylase activities *in vivo*. Furthermore, ME, CF, WF, SF-1 and SQ showed the ability to increase glucose uptake in both the abdominal and the soleus muscles. The study suggested that in the presence of insulin, the tested substances enhanced the sensitivity of insulin to increase the uptake of glucose, whereas in the absence of insulin, their actions probably involved glucose transporter action, resulting in directly enhanced transport of glucose. Muscular tissues are the major site for insulin-stimulated glucose uptake, fundamentally due to the recruitment of GLUT4 to the plasma membranes (Michael et al., 2001). Investigating a regulatory effect exerted on the glucose transporters has been approached by scientists in two ways: First, by measuring the consequence of diabetic states on the expressions rates of the glucose transporter genes (surplus of glucose availability), and secondly, by measuring the effect of glucose availability and glucose deprivation in cell culture on glucose transporter gene expression rates (Klip et al.,1994).

In order to fully elucidate the mechanisms of action of a herbal extract, both studies above are required to confirm the involvement of GLUT4. The results of this study show that the anti-hyperglycaemic effect of *S.polyanthum* (Wight) leaf extract was exerted via the extra-pancreatic pathway.

In conclusion, this thesis successfully verified the anti-diabetic activity of *S. polyanthum* (Wight) leaf, and presented evidence to support the notion that the crude methanolic extract (ME) was the most active of *S. polyanthum* extracts. The anti-hyperglycaemic activity was found to be due to extra-pancreatic mechanisms, namely through the inhibition of α-glucosidase and α-amylase activities, the inhibition of glucose absorption from the intestines, and the elevation of glucose uptake by the muscles (Figure 4.1). The thesis has not only made novel findings, but it has also inspired a great impetus for subsequent studies. Nevertheless, the pharmacological role of the phytochemicals compounds present in *S. polyanthum* leaf extracts, especially the polar ones, needs to be further investigated. Other possible mechanisms of action, such as the inhibition of the dipeptidyl-peptidase-IV (DPP-IV) or glucagon-like peptide-1 receptor (GLP-1R) agonist, still need to be elucidated. Hopefully the data presented in this thesis can serve as further evidence to validate the traditional use of *S. polyanthum* as an anti-hyperglycaemic agent and this study may be used the basis for future studies for a better understanding of this herb's beneficial potential.

## REFERENCES


Adefegha, S. A., Oboh, G., Adefegha, O. M., Boligon, A. A., & Athayde, M. L. (2014). Antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of dietary clove (*Szyzgium aromaticum*) bud powder in a high-fat diet/streptozotocin-induced diabetes rat model. *Journal of the Science of Food and Agriculture*, *94*(13), 2726-2737.

Agoes, A. (2010). *Tanaman Obat Indonesia Buku 2*. Jakarta: Salemba Medika.

Aguilera, Y., Dorado, M. E., Prada, F. A., Martínez, J. J., Quesada, A., & Ruiz-Gutiérrez, V. (2005). The protective role of squalene in alcohol damage in the chick embryo retina. *Experimental Eye Care Research, 80*(4), 535-543.

Akhtar, M. S., Khan, M. A., & Malik, M. T. (2002). Hypoglycaemic activity of *Alpinia galanga* rhizome and its extracts in rabbits. *Fitoterapia, 73*(7–8), 623-628.

Alam, M. R., Rahman, A. B., Moniruzzaman, M., Kadir, M. F., Haque, M. A., Alvi, M. R. U. H., & Ratan, M. D. (2012). Evaluation of antidiabetic phytochemicals in *Syzygium cumini* (L.) Skeels (Family: Myrtaceae). *Journal of Applied Pharmaceutical Science*, *2*(10), 094-098.

Ali, R. B., Atangwho, I. J., Kuar, N., Mohamed, E. A., Mohamed, A. J., Asmawi, M. Z., & Mahmud, R. (2012). Hypoglycaemic and anti-hyperglycaemic study of *Phaleria macrocarpa* fruits pericarp. *Journal of Medicinal Plants Research, 6*(10), 1982-1990.

American Diabetes Association[ADA]. (2010). Standards of medical care in diabetes-2010. *Diabetes Care, 33*(Supplement 1), 11-61.

American Diabetes Association[ADA]. (2012). Diagnosis and classification of diabetes mellitus. *Diabetes Care, 35*(Suppl 1), 64-71.

American Diabetes Association[ADA]. (2013). Standards of medical care in diabetes-2013. *Diabetes Care, 36*(1), 11-15.

American Diabetes Association[ADA]. (2014). Standards of medical care in diabetes-2014. *Diabetes Care 37*(1), s16.

Anggadiredja, K. (1998). Studi aktivitas antidiabetes ekstrak air daun salam *(Syzygium polyanthum* (Wight) Walp., Myrtaceae*)* dan fraksinya pada mencit putih jantan galur ddY, (Master), ITB, Bandung. Retrieved from http://digilib.itb.ac.id

Anggorowati, R., Sukrasno, & Adnyana, I. K. (2004). Telaah absorpsi ekstrak daun salam [*Syzygium polyanthum* (Wight) Walp.] berefek hipoglisemik pada tikus. (Gaduate), ITB, Sekolah Farmasi ITB.

Antidiabetic. (n.d.) *Dorland's Medical Dictionary for Health Consumers*. (2007). Retrieved from http://medical-dictionary.thefreedictionary.com

Antihyperglycemic. (n.d.) *Dorland's Medical Dictionary for Health Consumers*. (2007). Retrieved from http://medical-dictionary.thefreedictionary.com

Arintawati, M. (2000). Identifikasi dan karakterisasi komponan aroma daun salam *Syzygium polyanthum* (Wight) Walp. Retrieved from http://repository.ipb.ac.id

Arumugam, B., Manaharan, T., Heng, C. K., Kuppusamy, U. R., & Palanisamy, U. D. (2014). Antioxidant and antiglycemic potentials of a standardized extract of *Syzygium malaccense*. *LWT-Food Science and Technology*, *59*(2), 707-712.

Ayyanar, M., & Subash-Babu, P. (2012). *Syzygium cumini* (L.) Skeels: A review of its phytochemical constituents and traditional uses. *Asian Pacific Journal of Tropical Biomedicine*, *2*(3), 240-246.

Bhaskar, A.; Nithya, V.; Vidhya, V.G. (2011). Phytochemical evaluation by GC-MS and antihyperglycemic activity of *Mucuna pruriens* on streptozotocin induced diabetes in rats. *Journal of Chemical and Pharmaceutical Research*, 3, 689–696.

Bauer, R. (1998). Quality criteria and standardization of phytopharmaceuticals: Can acceptable drug standards be achieved? *Drug Information Journal*, *32*(1), 101-110.

Bauer, R., & Tittle, G. (1996). Quality assessment of herbal preparations as a pre-condition of pharmacological and clinical studies. *Phytomedicine*, *2*(3), 193-198.

Bhattaram, V. A., Graefe, U., Kohlert, C., Veit, M., & Derendorf, H. (2002). Pharmacokinetics and bioavailability of herbal medicinal products. *Phytomedicine*, *9*, 1-33.

Brondum, E., Nilson, H.,& Aalkjaer. (2005). Functional abnormalities in isolated arteries from Goto-Kakizaki and streptozotocin-treated diabetic rat models. *Hormone and Metabolic Research, 37*(1), 56-60.

Brunton, L. L., & Parker, K. L. (2006). *Goodman & Gilman's: Manual of Pharmacology and Therapeutics* (11th ed.). United State of America. Mc Gaw Hill Medical.

Campbell, J. E., & Drucker, D. J. (2013). Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metabolism, 17*(6), 819-837.

Carmona, A. (1998). A simple in vitro perfusion system to measure intestinal nutrient uptake. *The Journal of Nutritional Biochemistry, 9*(1), 52-57.

Castellano, J. M., Guinda, A., Delgado, T., Rada, M., & Cayuela, J. A. (2013). Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. Diabetes, 62(6), 1791-1799.

Chew, S. L., & Leslie, D. (2006). *Clinical Endocrinology and Diabetes: An Illsutrated Colour Text*. China. Churchill Livingstone Elsevier.

Ciaraldi, T. P., Kong, A. P., Chu, N. V., Kim, D. D., Baxi, S., Loviscach, M., & Mudaliar, S. (2002). Regulation of glucose transport and insulin signaling by troglitazone or metformin in adipose tissue of type 2 diabetic subjects. *Diabetes, 51*(1), 30-36.

Coman, C., Rugina, O. D., & Socaciu, C. (2012). Plants and natural compounds with anti-diabetic action. *Notulae Botanicae Horti Agobotanici Cluj-Napoca, 40*(1).

Compton, C., Byrd, D., Garcia-Aguilar, J., Kurtzman, S., Olawaiye, A., & Washington, M. (2012). Exocrine and endocrine pancreas. In C. C. Compton, D. R. Byrd, J. Garcia-Aguilar, S. H. Kurtzman, A. Olawaiye, & M. K. Washington (Eds.), *AJCC Cancer Staging Atlas* (pp. 297-308): Springer New York.

Concannon, P., Rich, S. S., & Nepom, G. T. (2009). Genetics of type 1A diabetes. *New England Journal of Medicine, 360*(16), 1646-1654.

Cusi, K., Consoli, A., Defronzo, & R. A. (1996). Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *The Journal of Clinical Endocrinology & Metabolism, 81*(11), 4059-4067.

Daneman, D. (2006). Type 1 diabetes. *The Lancet, 367*(9513), 847-858.

Das, B. (2000). *The Science Behind Squalene: The Human Antioxidant*: Toronto Medical Pub., for the International Council for Bionutrient Research. Toronto, Ontario.

DeRuiter, J. (2003). Overview of the anti-diabetic agents. *Endocrine Pharmacotherapy Module*. Retrieved from http://www.auburn.edu.

Dey, L., Attele, A. S., & Yuan, C. (2003). Alternative therapies for type 2 diabetes. Textbook of complementary and alternative medicine, 267.

Diabetes Research Institute Foundation [DRIF]. (2014). What are islet cells? Retrieved from http://www.diabetesresearch.org/islet-cells.

Diabetes.co.uk. (2015. Blood sugar level ranges. Retrieved from http://www.diabetes.co.uk/diabetes_care/blood-sugar-level-ranges.html.

Dimitriadis, G., Mitrou, P., Lambadiari, V., Maratou, E., & Raptis, S. A. (2011). Insulin effects in muscle and adipose tissue. *Diabetes Research and Clinical Practice, 93*, 52-59.

Djoukeng, J. D., Abou-Mansour, E., Tabacchi, R., Tapondjou, A. L., Bouda, H., & Lontsi, D. (2005). Antibacterial triterpenes from *Syzygium guineense* (*Myrtaceae*). *Journal of Ethnopharmacology, 101*(1), 283-286.

Drucker, D. J., & Nauck, M. A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *The Lancet, 368*(9548), 1696-1705.

Drucker, D. J. (2006). The biology of incretin hormones. *Cell metabolism, 3*(3), 153-165.

Dyer, J., Wood, I. S., Palejwala, A., Ellis, A., & Shirazi-Beechey, S. P. (2002). Expression of monosaccharide transporters in intestine of diabetic humans. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 282*(2), G241-G248.

Ehrenkranz, J. R., Lewis, N. G., Ronald Kahn, C., & Roth, J. (2005). Phlorizin: a review. *Diabetes/ Metabolism Research and Reviews, 21*(1), 31-38.

Eldawud, R., Stueckle, T. A., Manivannan, S., Elbaz, H., Chen, M., Rojanasakul, Y., & Dinu, C. Z. (2014). Real-time analysis of the effects of toxic, therapeutic

and sub-therapeutic concentrations of digitoxin on lung cancer cells. *Biosensors and Bioelectronics, 59*(0), 192-199.

Elfahmi. (2006). Phytochemical and biosynthetic studies of lignans with a focus on Indonesian medicinal plants. (Doctor of Philosophy), University of Goningen.

Evans, W. C. (2009). *Trease and Evans Pharmacognosy* (16th ed.). China: Saunders Elsevier.

Fan, C., Yan, J., Qian, Y., Wo, X., & Gao, L. (2006). Regulation of lipoprotein lipase expression by effect of hawthorn flavonoids on peroxisome proliferator response element pathway. *Journal of Pharmacological Sciences*, *100*(1), 51-58.

Farvin, K. S., Anandan, R., Kumar, S. H. S., Shiny, K., Mathew, S., Sankar, T., & Nair, P. V. (2006). Cardioprotective effect of squalene on lipid profile in isoprenaline-induced myocardial infarction in rats. *Journal of Medicinal Food, 9*(4), 531-536.

Federiuk, I. F., Casey, H. M., Quinn, M. J., Wood, M. D., & Ward, W. K. (2004). Induction of type-1 diabetes mellitus in laboratory rats by use of alloxan: route of administration, pitfalls, and insulin treatment. *Comparative Medicine 54*, 252–257.

Fox, C. B. (2009). Squalene emulsions for parenteral vaccine and drug delivery. *Molecules, 14*(9), 3286-3312.

Fröde, T., & Medeiros, Y. (2008). Animal models to test drugs with potential anti-diabetic activity. *Journal of Ethnopharmacology, 115*(2), 173-183.

Golan, D. E. (2005). *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy*. United State of America. Lippincott William & Wilkins.

Gray, A. M., & Flatt, P. R. (1998). Antihyperglycemic actions of *Eucalyptus globulus* (Eucalyptus) are associated with pancreatic and extra-pancreatic effects in mice. *The Journal of Nutrition*, *128*(12), 2319-2323.

Grover, J. K., Yadav, S., & Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology*, *81*(1), 81-100.

Grzybowska, M., Bober, J., & Olszewska, M. (2010). Metformin-mechanisms of action and use for the treatment of type 2 diabetes mellitu]. *Postepy higieny i medycyny doswiadczalnej (Online)*, *65*, 277-285.

Handa, S. S., Khanuja, S. P. S., Longo, G., & Rakesh, D. D. (2008). *Extraction Technologies for Medicinal and Aromatic Plants*. Italy: ICS-UNIDO.

Harvey, R. A. (2012). *Lippincott's Illustrated Reviews* (5th ed.). China. Lippincott William & Wilkins.

Hassan, Z., Yam, M. F., Ahmad, M., & Yusof, A. P. M. (2010). Anti-diabetic properties and mechanism of action of *Gynura procumbens* water extract in streptozotocin-induced diabetic rats. *Molecules, 15*(12), 9008-9023.

Hinnen, D., Nielsen, L. L., Waninger, A., & Kushner, P. (2006). Incretin mimetics and DPP-IV inhibitors: new paradigms for the treatment of type 2 diabetes. *The Journal of the American Board of Family Medicine, 19*(6), 612-620.

Hirosumi, J., Tuncman, G., Chang, L., Görgün, C. Z., Uysal, K. T., Maeda, K., Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. *Nature, 420*(6913), 333-336.

Holstein, A., & Egberts, E. (2003). Risk of hypoglycaemia with oral anti-diabetic agents in patients with Type 2 diabetes. *Experimental and Clinical Endocrinology and Diabetes, 111*(7), 405-414.

Hou, W., Li, Y., Zhang, Q., Wei, X., Peng, A., Chen, L., & Wei, Y. (2009). Triterpene acids isolated from *Lagerstroemia speciosa* leaves as α-glucosidase inhibitors. *Phytotherapy Research, 23*(5), 614-618.

Huang, Z. R., Lin, Y. K., & Fang, J. Y. (2009). Biological and pharmacological activities of squalene and related compounds: potential uses in cosmetic dermatology. *Molecules*, *14*(1), 540-554.

Innes, J. A. (2009). *Davidson's Essential of Medicine:Diabetes Mellitus*. China: Churchill Livingstone Elsevier.

Inzucchi, S. E., & McGuire, D. K. (2008). New drugs for the treatment of diabetes part II: incretin-based therapy and beyond. *Circulation, 117*(4), 574-584.

Jananie, R.K., Priya, V., Vijayalakshmi, K. (2011). Phytoconstituents evaluation by GC-MS and anti-hyperglycemic activity of *Cynodon dactylon* on streptozotocin induced diabetes in rats. *Journal of Chemical and Pharmaceutical Research*, *3*(4):460-466.

Jung, M., Park, M., Lee, H. C., Kang, Y.-H., Kang, E. S., & Kim, S. K. (2006). Anti-diabetic agents from medicinal plants. *Current Medicinal Chemistry, 13*(10), 1203-1218.

Kahn, S. E. (2001). Clinical, review 135: The importance of β-cell failure in the development and progession of type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism, 86*(9), 4047-4058.

Kahn, S. E. (2003). The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. *Diabetologia, 46*(1), 3-19.

Kameswara Rao, B., & Appa Rao, C. (2001). Hypoglycemic and antihyperglycemic activity of *Syzygium alternifolium* (Wt.) Walp. seed extracts in normal and diabetic rats. *Phytomedicine, 8*(2), 88-93.

Kasmuri, A. R. (2006). Anti-diabetic properties of *Andrographis paniculata* and its possible mechanism of actions. (Doctor of Philosophy), Universiti Sains Malaysia, Malaysia.

Kasmuri, A. R., Ahmad, M., Sadikun, A., & Asmawi, M. Z. (2010). Assessment on functionality and viability of β-cells following repetitive dosage administration of ethanolic extracts of *Andrographis paniculata* on streptozotocin-induced diabetic rats. *International Medical Journal, 9*(1), 21-26.

Katsumata, K., Katsumata Jr., K., & Katsumata, Y. (1992). Protective effect of diltiazem hydrochloride on the occurrence of alloxan- or streptozotocin-induced diabetes in rats. *Hormone and Metabolic Research, 24*,508–510.

Katzung, B. G. (2004). *Basic and Clinical Pharmacology* (9th ed.).Singapore. The McGaw-Hill Companies.

Kecskemeti, V., Bagi, Z., Pacher, P., Posa, I., Kocsis, E., & Zs, K. M. (2002). New trends in the development of oral anti-diabetic drugs. *Current Medicinal Chemistry, 9*(1), 53-71.

Kellett, G. L., Brot-Laroche, E., Mace, O. J., & Leturque, A. (2008). Sugar absorption in the intestine: the role of GLUT2. *Annual Review of Nutrition, 28*, 35-54.

Kelly, G. S. (1999). Squalene and its potential clinical uses. *Alternative Medicine Review: A Journal of Clinical Therapeutic, 4*(1), 29-36.

Kirpichnikov, D., McFarlane, S. I., & Sowers, J. R. (2002). Metformin: an update. *Annals of Internal Medicine, 137*(1), 25-33.

Klip, A., & Leiter, L. A. (1990). Cellular mechanism of action of metformin. *Diabetes Care, 13*(6), 696-704.

Ko, T.-F., Weng, Y.-M., & Chiou, R. Y.-Y. (2002). Squalene content and antioxidant activity of *Terminalia catappa* leaves and seeds. *Journal of Agicultural and Food Chemistry, 50*(19), 5343-5348.

Kolb, H. (1987). Mouse models of insulin dependent diabetes: Low-dose streptozotocin-induced diabetes and non-obese diabetic (NOD) mice. *Diabetes/Metabolism Reviews, 3*(3), 751-778.

Krentz, A. J., & Bailey, C. J. (2005). Oral anti-diabetic agents. *Drugs, 65*(3), 385-411.

Krishnan, S. S. C., Subramanian, I. P., & Subramanian, S. P. (2014). Isolation, characterization of syringin, phenylpropanoid glycoside from *Musa paradisiaca* tepal extract and evaluation of its antidiabetic effect in streptozotocin-induced diabetic rats. *Biomedicine & Preventive Nutrition, 4*(2), 105-111.

Kumar, A., Ilavarasan, R., Jayachandran, T., Deecaraman, M., Aravindan, P., Padmanabhan, N., & Krishan, M. R. V. (2008). Anti-diabetic activity of *Syzygium cumini* and its isolated compound against streptozotocin-induced diabetic rats. *Journal of Medicinal Plants Research, 2*(9), 246-249.

Kumar, A., Lakshman, K., Jayaveera, K. N., Sheshadri Shekar, D., Narayan Swamy, V. B., Khan, S., & Velumurga, C. (2011). *In vitro* α-amylase inhibition and antioxidant activities of methanolic extract of *Amaranthus caudatus* Linn. *Oman Medical Journal, 26*(3), 166.

Kumar, B. A., Lakshman, K., Jayaveea, K. N., Shekar, D. S., Khan, S., Thippeswamy, B. S., & Veerapur, V. P. (2012). Antidiabetic, antihyperlipidemic and antioxidant activities of methanolic extract of *Amaranthus viridis* Linn in alloxan induced diabetic rats. *Experimental and Toxicologic Pathology, 64*(1), 75-79.

Kuo, Y. C., Yang, L. M., & Lin, L. C. (2004). Isolation and immunomodulatory effect of flavonoids from *Syzygium samarangense*. *Planta Medica, 70*(12), 1237-1239.

Kuroda, M., Mimaki, Y., Sashida, Y., Mae, T., Kishida, H., Nishiyama, T., Kitahara, M. (2003). Phenolics with PPAR-γ ligand-binding activity obtained from licorice (*Glycyrrhiza uralensis)* roots and ameliorative effects of glycyrin on

genetically diabetic KK-Ay mice. *Bioorganic & Medicinal Chemistry Letters, 13*(24), 4267-4272.

Kusuma, I. W., Kuspradini, H., Arung, E. T., Aryani, F., Min, Y. H., Kim, J. S., & Kim, Y. U. (2011). Biological activity and phytochemical analysis of three Indonesian medicinal plants, *Murraya koenigii*, *Syzygium polyanthum* and *Zingiber purpurea*. *Journal of Acupuncture and Meridian Studies*, *4*(1), 75-79.

Kwon, Y. I., Apostolidis, E., & Shetty, K. (2008). Inhibitory potential of wine and tea against α-amylase and α-glucosidase for management of hyperglycemia linked to type 2 diabetes. *Journal of Food Biochemistry*, *32*(1), 15-31.

Lee Wei, H. A. R., & Ismail, I. S. (2012). Antioxidant activity, total phenolics and total flavonoids of *Syzygium polyanthum* (Wight) Walp leaves. *International Journal of Medicinal and Aromatic Plants*, *2*(2), 219-228.

Lenzen, S. (2008). The mechanisms of alloxan-and streptozotocin-induced diabetes. *Diabetologia, 51*(2), 216-226.

Li, F., Li, Q., Gao, D., & Peng, Y. (2009). The optimal extraction parameters and anti-diabetic activity of flavonoids from *Ipomoea batatas* leaf. *African Journal of Traditional, Complementary and Alternative Medicines*, *6*(2).

Li, T. H., Hou, C. C., Chang, C. L. T., & Yang, W. C. (2010). Anti-hyperglycemic properties of crude extract and triterpenes from *Poria cocos*. *Evidence-Based Complementary and Alternative Medicine*, *2011*(2011).

Luo, J., Cheung, J., Yevich, E. M., Clark, J. P., Tsai, J., Lapresca, P., Hector, R. F. (1999). Novel terpenoid-type quinones isolated from *Pycnanthus angolensis* of potential utility in the treatment of type 2 diabetes. *Journal of Pharmacology and Experimental Therapeutics, 288*(2), 529-534.

Marles, R., & Farnsworth, N. (1995). Anti-diabetic plants and their active constituents. *Phytomedicine, 2*(2), 137-189.

Mathur, R., & Conrad Stöppler, M. (2012). Insulin resistance. *MedicineNet. com.* Retrieved from http://www. medicinenet. com/insulin_resistance/article. htm.

Matsuda, H., Li, Y., Murakami, T., Matsumura, N., Yamahara, J., & Yoshikawa, M. (1998). Antidiabetic principles of natural medicines. III. Structure-related inhibitory activity and action mode of oleanolic acid glycosides on hypoglycemic activity. *Chemical and Pharmaceutical Bulletin*, *46*(9), 1399-1403.

McFarlane, S. I. (2009). Anti-diabetic medications and weight gain: implications for the practicing physician. *Current Diabetes Reports, 9*(3), 249-254.

McGonigle, P., & Ruggeri, B. (2014). Animal models of human disease: Challenges in enabling translation. *Biochemical Pharmacology, 87*(1), 162-171.

Mentreddy, S. R. (2007). Medicinal plant species with potential anti-diabetic properties. *Journal of the Science of Food and Agiculture, 87*(5), 743-750.

Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., Spiegelman, B. M. (2001). Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proceedings of the National Academy of Sciences, 98*(7), 3820-3825.

Michel H. Porcher, 2011. Sorting *Syzygium* names. Multilingual multiscript plant name database. Retrieved from http://www.plantnames.unimelb.edu.au.

Miyazawa, M., & Hisama, M. (2003). Antimutagenic activity of phenylpropanoids from clove (*Syzygium aromaticum*). *Journal of Agricultural and Food Chemistry, 51*(22), 6413-6422.

Modak, M., Dixit, P., Londhe, J., Ghaskadbi, S., & Devasagayam, T. P. A. (2007). Indian herbs and herbal drugs used for the treatment of diabetes. *Journal of Clinical Biochemistry and Nutrition, 40*(3), 163.

Mu, Y.-M., Misra, A., Adam, J. M. F., Chan, S. P., Chow, F. C. C., Cunanan, E. C., & Tan, K. E. K. (2012). Managing diabetes in Asia: Overcoming obstacles and the role of DPP-IV inhibitors. *Diabetes Research and Clinical Practice, 95*(2), 179-188.

Munjal, Y. P., & Surendara, K. S. (2012). API text book of medicine, 9th ed. New Delhi India. Medical Publishers (P) Ltd.

Musi, N., Hirshman, M. F., Nygen, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Efendic, S. (2002). Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes, 51*(7), 2074-2081.

Mythili, M., Vyas, R., Akila, G., & Gunasekaran, S. (2004). Effect of streptozotocin on the ultrastructure of rat pancreatic islets. *Microscopy Research and Technique, 63*(5), 274-281.

Nadkarni, P., Chepurny, O. G., & Holz, G. G. (2014). Regulation of glucose homeostasis by GLP-1. *Progress in Molecular Biology and Translational Science, 121*, 23.

Nistor Baldea, L. A., Martineau, L. C., Benhaddou-Andaloussi, A., Arnason, J. T., Lévy, É., & Haddad, P. S. (2010). Inhibition of intestinal glucose absorption by anti-diabetic medicinal plants derived from the James Bay Cree traditional pharmacopeia. *Journal of Ethnopharmacology, 132*(2), 473-482.

Nonaka G., Aiko Y., Aritake K., and Nishioka I. (1992), Tannins and related compounds. CXIX. samarangenins A and B, novel proanthocyanidins with doubly bonded structures, from *Syzygium samarangense* and *S. aqueum*. *Chemical and Pharmaceutical Bulletin, 40*, 2671-2673.

Nolte, M. S., & Karam, J. H. (2004). *Pancreatic Hormones & Anti-diabetic Drugs.* In Pharmacology Katzung (9th ed.). Singapore: Mc Gaw Hill.

Offermanns, S., & Rosenthal, W. (2008). Encyclopedia of Molecular Pharmacology, Volume 1, 2nd ed. Springer Science & Business Media; p.116.

Ong, E. S. (2004). Extraction methods and chemical standardization of botanicals and herbal preparations. *Journal of Chromatogaphy B, 812*(1–2), 23-33.

Ong, K. W., Hsu, A., Song, L., Huang, D. J., & Tan, B. K. H. (2011). Polyphenols-rich *Vernonia amygdalina* shows anti-diabetic effects in streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology, 133*(2), 598-607.

Padee, P., Nualkaew, S., Talubmook, C., & Sakuljaitrong, S. (2010). Hypoglycemic effect of a leaf extract of *Pseuderanthemum palatiferum* (Nees) RadLk. in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology, 132*(2), 491-496.

Pandit, N. K. (2007). *Introduction to the Pharmaceutical Sciences*. Printed in The United States: Lippincott Williams & Wilkins.

Patel, D. K., Prasad, S. K., Kumar, R., & Hemalatha, S. (2012). An overview on anti-diabetic medicinal plants having insulin mimetic property. *Asian Pacific Journal of Tropical Biomedicine, 2*(4), 320-330.

Perez, G., & Vargas, S. (2002). Triterpenes from *Agarista mexicana* as potential antidiabetic agents. *Phytotherapy Research, 16*(1), 55-58.

Perumal, S., Mahmud, R., Piaru, S. P., Lee Wei, C., & Ramanathan, S. (2012). Potential antiradical activity and cytotoxicity assessment of *Ziziphus*

*mauritiana* and *Syzygium polyanthum*. *International Journal of Pharmacology, 8*(6), 535-541.

Pharmacologyweekly. (n.d.) How does metformin (glucophage) lower fasting glucose levels without causing hypoglycemia? Retrieved from http://www.pharmacologyweekly.com/articles/metformin-hypoglycemia-mechanism-diabetes

Puranik, N., Kammar, K. F., & Devi, S. (2010). Anti-diabetic activity of *Tinospora cordifolia* (Willd.) in streptozotocin diabetic rats; does it act like sulfonylureas. *Turkish Journal of Medical Sciences, 40*(2), 265-270.

Rajasekaran, S., Ravi, K., Sivagnanam, K., & Subramanian, S. (2006). Beneficial effects of *Aloe vera* leaf gel extract on lipid profile status in rats with streptozotocin diabetes. *Clinical and Experimental Pharmacology and Physiology, 33*(3), 232-237.

Ramachandran, A., Wan Ma, R. C., & Snehalatha, C. (2010). Diabetes in Asia. *The Lancet, 375*(9712), 408-418.

Rao, B. K., Kesavulu, M., Giri, R., & Rao, C. A. (1999). Anti-diabetic and hypolipidemic effects of *Momordica cymbalaria* Hook. fruit powder in alloxan-diabetic rats. *Journal of Ethnopharmacology, 67*(1), 103-109.

Rao, C. V., Newmark, H. L., & Reddy, B. S. (1998). Chemopreventive effect of squalene on colon cancer. *Carcinogenesis, 19*(2), 287-290.

Rasoanaivo, P., Wright, C. W., Willcox, M. L., & Gilbert, B. (2011). Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. *Malaria Journal, 10*(1), 1-4.

Reddy, L. H., & Couvreur, P. (2009). Squalene: A natural triterpene for use in disease management and therapy. *Advanced Drug Delivery Reviews, 61*(15), 1412-1426.

Rehab Ahmed, R. (2012). Effect of quercetin on the endocrine pancreas of the experimentally induced diabetes in male albino rats: A histological and immunohistochemical study. *Journal of Diabetes & Metabolism*.

Resurreccion-Magno, M., Hanshella, C., Villaseñor, I. M., Harada, N., & Monde, K. (2005). Antihyperglycaemic flavonoids from *Syzygium samarangense* (Blume) merr. and perry. *Phytotherapy Research, 19*(3), 246-251.

Riansari, A., & Suhardjono. (2008). Pengaruh pemberian ekstrak daun salam (*Eugenia polyantha*) terhadap kadar kolesterol total serum tikus jantan galur wistar hiperlipidemia. Universitas Diponegoro.

Rubin, R. R. (2005). Adherence to pharmacologic therapy in patients with type 2 diabetes mellitus. *The American Journal of Medicine, 118*(5), 27-34.

Sabu, M., Smitha, K., & Kuttan, R. (2002). Anti-diabetic activity of geen tea polyphenols and their role in reducing oxidative stress in experimental diabetes. *Journal of Ethnopharmacology, 83*(1), 109-116.

Sachdewa, A., & Khemani, L. (2003). Effect of *Hibiscus rosa sinensis* Linn. ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *Journal of Ethnopharmacology, 89*(1), 61-66.

Sacks, D. B. (2011). A1C versus glucose testing: a comparison. *Diabetes Care, 34*(2), 518-523.

Schwartz, S. S., & Kohl, B. A. (2010). Glycemic control and weight reduction without causing hypoglycaemia: the case for continued safe aggessive care of patients with type 2 diabetes mellitus and avoidance of therapeutic inertia. Paper presented at the Mayo Clinic Proceedings.

Seidemann, J. (2005). *World Spice Plants: Economic Usage, Botany, Taxonomy.* Printed in European Union: Springer-Verlag Berlin Heidelberg.

SenthilKumar, P., & Sudha, S. (2012). Evaluation of alpha-amylase and alpha-glucosidase inhibitory properties of selected seaweeds from gulf of Mannar. *International Research Journal of Pharmacy, 3*(8), 128-130.

Sesti, G. (2002). Apoptosis in the beta cells: cause or consequence of insulin secretion defect in diabetes? *Annals of Medicine, 34*(6), 444-450.

Sharma, B., Balomajumder, C., & Roy, P. (2008). Hypoglycemic and hypolipidemic effects of flavonoid rich extract from *Eugenia jambolana* seeds on streptozotocin induced diabetic rats. *Food and Chemical Toxicology, 46*(7), 2376-2383.

Sharma, U., Sahu, R., Roy, A., & Golwala, D. (2010). *In vivo* antidiabetic and antioxidant potential of *Stephania hernandifolia* in streptozotocin-induced-diabetic rats. *Journal of Young Pharmacists : JYP, 2*(3), 255–260.

Shen, P., Liu, M., Ng, T., Chan, Y., & Yong, E. (2006). Differential effects of isoflavones, from *Astragalus membranaceus* and *Pueraria thomsonii*, on the

activation of PPARα, PPARγ, and adipocyte differentiation in vitro. *The Journal of Nutrition, 136*(4), 899-905.

Shinde, J., Taldone, T., Barletta, M., Kunaparaju, N., Hu, B., Kumar, S., Zito, S. W. (2008). α-glucosidase inhibitory activity of *Syzygium cumini* (Linn.) Skeels seed kernel in vitro and in Goto–Kakizaki (GK) rats. *Carbohydrate Research, 343*(7), 1278-1281.

Shu, A. D., & Myers, M. G. (2004). Pharmacology of the endocrine pancreas. In D. E. Golan, A. H. Tashjian, E. J. Armstrong, J. M. Galanter, A. W. Armstrong, R. A. Arnaout, H. S. Rose, & . (Eds.), *Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy* (pp. 457-469). Baltimore: Lippincott Williams & Wilkins.

Shukia, R., Sharma, S., Puri, D., Prabhu, K., & Murthy, P. (2000). Medicinal plants for treatment of diabetes mellitus. *Indian Journal of Clinical Biochemistry, 15*(1), 169-177.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation, 106*(2), 171-176.

Singab, A. N. B., El-Beshbishy, H. A., Yonekawa, M., Nomura, T., & Fukai, T. (2005). Hypoglycemic effect of *Egyptian morus* alba root bark extract: effect on diabetes and lipid peroxidation of streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology, 100*(3), 333-338.

Singh, A. B., Chaturvedi, J., Narender, T., & Srivastava, A. K. (2008). Preliminary studies on the hypoglycemic effect of *Peganum harmala* L. seeds ethanol extract on normal and streptozotocin induced diabetic rats. *Indian Journal of Clinical Biochemistry, 23*(4), 391-393.

Smith, T. J. (2000). Squalene: potential chemopreventive agent. *Expert Opinion on Investigational Drugs, 9*(8), 1841-1848.

Soumyanath, A. (2005). *Traditional Medicines for Modern Times: Anti-diabetic Plants*. United States of America. CRC Press Taylor and Francis Group.

Spanova, M., & Daum, G. (2011). Squalene–biochemistry, molecular biology, process biotechnology, and applications. *European Journal of Lipid Science and Technology, 113*(11), 1299-1320.

Srinivasan, K., & Ramarao, P. (2007). Animal model in type 2 diabetes research: An overview. *Indian Journal of Medical Research, 125*(3), 451.

Sriplang, K., Adisakwattana, S., Rungsipipat, A., & Yibchok-Anun, S. (2007). Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology, 109*(3), 510-514.

Studiawan, H., & Santosa, M. H. (2005). Test pharmacological effect of ethanolic extract of *Eugenia polyantha* leaves as for decreasing glucose level activity on mice induced by alloxan. Retrieved from www.journal.unair.ac.id

Subramanian, R., Asmawi, M. Z., & Sadikun, A. (2008). *In vitro* alpha-glucosidase and alpha-amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta Biochimica Polonica, 55*(2), 391-398.

Suresh., K., Singh, R., Vasudeva, N., & Sharma, S. (2012). Acute and chronic animal models for the evaluation of anti-diabetic agents. *Cardiovascular Diabetology, 11*(9).

Swami, G., Nagpal, N., Rahar, S., Preeti, S., Porwal, A., Nagpal, M. A., & Kapoor, R. (2010). Effect of aqueous leaves extract of *Carissa carandas* Linn on blood glucose levels of normoglycemic and alloxan-induced diabetic wistar rats. *International Journal of Current Pharmaceutical Research,2*(3), 65-67

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in β-cells of the rat pancreas. *Physiological Research, 50*(6), 537-546.

Tapas, A. R., Sakarkar, D. M., & Kakde, R. B. (2008). Flavonoids as nutraceuticals: a review. *Tropical Journal of Pharmaceutical Research, 7*(3), 1089-1099.

Tian, L. W., Xu, M., Wang, D., Zhu, H. T., Yang, C. R., & Zhang, Y. J. (2011). Phenolic constituents from the leaves of *Syzygium forrestii* Merr. and Perry. *Biochemical Systematics and Ecology, 39*(2), 156-158.

Tiong, S. H., Looi, C. Y., Hazni, H., Arya, A., Paydar, M., Wong, W. F., & Awang, K. (2013). Antidiabetic and antioxidant properties of alkaloids from *Catharanthus roseus* (L.) G. Don. *Molecules, 18*(8), 9770-9784.

Toda, M., Kawabata, J., & Kasai, T. (2000). α-glucosidase inhibitors from clove (*Syzgium aromaticum*). *Bioscience,Biotechnology, and Biochemistry, 64*(2), 294-298.

Trojan-Rodrigues, M., Alves, T. L. S., Soares, G. L. G., & Ritter, M. R. (2012). Plants used as anti-diabetic in popular medicine in Rio Gande do Sul, southern Brazil. *Journal of Ethnopharmacology, 139*(1), 155-163.

Tushuizen, M. E., Bunck, M. C., Pouwels, P. J., Van Waesberghe, J. H. T., Diamant, M., & Heine, R. J. (2006). Incretin mimetics as a novel therapeutic option for hepatic steatosis. *Liver International, 26*(8), 1015-1017.

Upwar, N., Patel, R., Waseem, N., & Mahobia, N. K. (2011). Hypoglycemic effect of methanolic extract of *Berberis aristata* DC stem on normal and streptozotocin-induced diabetic rats. *International Journal of Pharmacy & Pharmaceutical Sciences, 3*(1), 222-224.

Useful Tropical Plants, (n.d.). *Syzygium polyanthum* images. Retrieved from http://tropical.theferns.info

Velayutham, R., Sankaradoss, N., & Ahamed, K. N. (2012). Protective effect of tannins from *Ficus racemosa* in hypercholesterolemia and diabetes induced vascular tissue damage in rats. *Asian Pacific Journal of Tropical Medicine, 5*(5), 367-373.

Wahyono D., & Susanti. Aktivitas hipoglikemik ekstrak etanolik daun salam *(Syzygium polyanthum* (Wight) Walp) dan pengaruhnya terhadap stimulasi parasimpatik pada kelinci jantan yang dibebani glukosa. Majalah Obat Tradisional, Vol 13, No 43, March 1st 2008

Wang, J.-l., Chinookoswong, N., Scully, S., Qi, M., & Shi, Z.-Q. (1999). Differential effects of leptin in regulation of tissue glucose utilization *in vivo*. *Endocrinology, 140*(5), 2117-2124.

Wasada, T., Kuroki, H., Katsumori, K., Arii, H., Sato, A., Aoki, K., & Hanai, G. (2004). Who are more insulin resistant, people with IFG or people with IGT? *Diabetologia, 47*(4), 759-760.

Wasito, H. (2011). *Obat Tradisional Kekayaan Indonesia* (1st ed.). Yogyakarta: Graha Ilmu.

West, E., Simon, O. R., & Morrison, E. Y. (1996). Streptozotocin alters pancreatic beta-cell responsiveness to glucose within six hours of injection into rats. *The West Indian Medical Journal, 45*(2), 60-62.

World Health Organization [WHO]. (2000). General guidelines for methodologies on research and evaluation of traditional medicine, *1*, 3-9.

World Health Organization [WHO]. (2015). Complications of Diabetes. Retrieved from http://www.who.int/diabetes/action_online/basics/en/index3.html

Widyawati, T., Purnawan, W. W., Yam, M. F., Asmawi, M. Z., & Ahmad, M. (2012). The use of medicinal herbs among diabetic patients in health community centre sering, Medan, Indonesia. Paper presented at the The 26th Malaysian Society of Pharmacology and Physiology (MSPP) Scientific Meeting, Penang, Malaysia.

Wiernsperger, N. F., & Bailey, C. J. (1999). The antihyperglycaemic effect of metformin. *Drugs, 58*(1), 31-39.

Wilson, T. H., & Wiseman, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *The Journal of Physiology, 123*(1), 116-125.

Wisetmuen, E., Pannangpetch, P., Kongyingyoes, B., Kukongviriyapan, U., Yutanawiboonchai, W., & Itharat, A. (2013). Insulin secretion enhancing activity of roselle calyx extract in normal and streptozotocin-induced diabetic rats. *Pharmacognosy Research, 5*(2), 65.

Wu, K. K., & Huan, Y. (2008). Streptozotocin-induced diabetic models in mice and rats. *Current Protocol in Pharmacology, 40*, 5.47.41-45.47.14.

Yakubovich, N., & Gerstein, H. C. (2011). Serious cardiovascular outcomes in diabetes: The role of hypoglycemia. *Circulation, 123*(3), 342-348.

Ye, J. (2013). Mechanisms of insulin resistance in obesity. *Frontiers of Medicine, 7*(1), 14-24.

Yoo, C. B., Han, K. T., Cho, K. S., Ha, J., Park, H. J., Nam, J. H., & Lee, K. T. (2005). Eugenol isolated from the essential oil of *Eugenia caryophyllata* induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. *Cancer Letters, 225*(1), 41-52.

Zou, J., Mi, Y., Chen, W., Liu, Q., Wang, J., Lou, L., & Zhao, W. (2006). Alkyl phloroglucinol derivatives from *Syzygium levinei* and their differentiation-inducing activity. *Planta Medica, 72*(6), 533-538.

## LIST OF PUBLICATIONS & WORKSHOP

### 1.0. PUBLICATIONS ON SEMINAR (POSTER SESSION)

1.1.**Tri Widyawati,** Willy Winardy Purnawan, Mun Fei Yam, Mohd. Zaini Asmawi, Mariam Ahmad. The Use of Medicinal Herbs among Diabetic Patients Health Community Centre Sering, Medan, Indonesia. 26th Annual Scientific Meeting Malaysian Pharmacology and Physiology Society *(*MSPP*)*, 19-20 May 2012, Equitorial Hotel Penang.

1.2.**Tri Widyawati,** Nor Adlin Md Yusoff, Yam Mun Fei, Bassel Hindi, Mohd. Zaini Asmawi and Mariam Ahmad. Anti-hyperglycaemic effects of *Syzygium polyanthum* leaves extracts and fractions in streptozotocin-induced diabetic rats. International Conference on Natural Product 2014, 18-19 March 2014, Putrajaya Malaysia.

1.3.**Tri Widyawati,** Nor Adlin Md Yusoff, Mohd. Zaini Asmawi and Mariam Ahmad. Anti-hyperglycaemic effects of methanol extract of *Syzygium polyanthum* leaves in streptozotocin-induced diabetic rats. 5th International Conference on Natural Products for Health and Beauty *(*NATPRO*5)*, 6-8 May 2014, Moevenpick Resort & Spa Karon Beach Phuket, Thailand.

### 2.0. PUBLICATIONS ON INTERNATIONAL JOURNAL

2.1. **Tri Widyawati,** Nor Adlin Md Yusoff, Mohd. Zaini Asmawi and Mariam Ahmad. Meeting Report: Anti-hyperglycaemic effects of methanol extract of *Syzygium polyanthum* leaves in streptozotocin-induced diabetic rats. *Nutrients.* 2014, *6*, 4145; ISSN 2072-6643. Available at www.mdpi.com/journal/nutrients

2.2. **Tri Widyawati,** Willy Winardi Purnawan, Item Justin Atangwho, Nor Adlin Yusoff, Mariam Ahmad and Mohd. Zaini Asmawi. Anti-diabetic activity of *Syzygium polyanthum* (Wight) leaf extract, the most commonly used herb among diabetic patients in Medan, North Sumatera, Indonesia. *International Journal of Pharmaceutical Sciences and Research.* 2015,6 (4), 1698-1704;
E-ISSN: 0975-8232; P-ISSN: 2320-5148. Available at www.ijpsr.com

2.3. **Tri Widyawati,** Nor Adlin Yusoff, Mohd. Zaini Asmawi and Mariam Ahmad. Anti-hyperglycaemic effects of methanol extract of *Syzygium polyanthum* leaves in streptozotocin-induced diabetic rats. *Nutrients.* 2015, 7, 7764-7780. Available at www.mdpi.com/journal/nutrients

## 3.0. WORKSHOP

3.1. Workshop on The Use of Animals in Science "Ethical & Practical Considerations'', 14-15 September 2011, Penang, School of Pharmaceutical Sciences, Universiti Sains Malaysia.