ANTI-DIABETIC AND TOXICOLOGICAL STUDIES OF *GONGRONEMA LATIFOLIUM*

By

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LIST OF ABBREVIATIONS

ºC	Degree Celsius
%	Percent
µl	Microliter
AD	*Anno Domini* (After Christ)
ADA	American Diabetes Association
ALP	Alkaline Phosphatase
ALT	Alanin Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
b.w.	Body Weight
BGLs	Blood Glucose Levels
DC	Diabetic Control
DM	Diabetes Mellitus
DPP-4	Dipeptidyl Peptidase-4
FBG	Fasting Blood Glucose
FDA	Food and Drug Administration
GC-MS	Gas Chromatography – Mass Spectroscopy
GDM	Gestational Diabetes Mellitus
GL	*Gongronema latifolium* Benth.
GLES	*Gongronema latifolium* Ethanolic Soxhlet extract
GLP-1	Glucagon-Like Peptide 1
HDL	High-Density Lipoprotein
IDDM	Insulin-Dependent Diabetes Mellitus
LD_{50}	Lethal dose enough to kill 50% of a test sample
LDL	Low-Density Lipoprotein
mg	Milligram
min	Minute
mM	Mili Molar
NC	Normal Control
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organization for Economic Co-operation and Development
p.o.	per os (oral)
PC	Positive Control
$PPAR_{\gamma}$	Peroxisome Proliferator Activated nuclear Receptor
Rpm	Rounds Per Minute
SD	*Sprague-Dawley*
SEM	Standard Error of the Mean
SGLTs	Sodium–Glucose co-Transporters
STZ	Streptozotocin
TG	Treatment Group
TZD	Thiazolidones
VLDL	Very-Low-Density Lipoprotein
WAT	White Adopose Tissue
WHO	World Health Organization

KAJIAN ANTIDIABETIK DAN TOKSIKOLOGI EKSTRAK *GONGRONEMA LATIFOLIUM*

ABSTRAK

Gongronema latifolium Benth. (Apocyanaceae) (GL) mempunyai aktiviti menurunkan glukosa yang berpotensi untuk digunakan secara meluas. Kajian ini mengkaji kesan antidiabetik dan toksik GL. Etanol dan peralatan Soklet digunakan untuk mendapatkan ekstrak soklet etanolik GL (GLES) daripada daunnnya. Bagi mengkaji kesan antidiabetik, GLES diberikan secara oral kepada tikus – tikus jantan *Sprague-Dawley* (SD) yang normal dan diabetik aruhan STZ. Aras glukosa darah (BGLs), profil lipid serum, aras insulin dan pankreas dinilai selepas rawatan selama 14 hari. Bagi menilai ketoksikan GL, GLES diberikan secara oral pada dos 250, 500, dan 1000 mg/kg kepada tikus – tikus SD selama 90 hari berdasarkan garis panduan OECD. Indeks ketoksikan yang diukur, termasuk berat badan, parameter – parameter biokimia dan hematologi, dan berat organ – organ penting. Penilaian histopatologi bagi organ – organ penting yang terlibat dalam metabolisma xenobiotik dan perkumuhan – hati dan ginjal – juga dijalankan. GLES menurunkan BGLs tikus normal secara signifikan ($P < 0.05$) dalam ujian toleransi glukosa pada dos 2 g/kg berat badan, tetapi gagal merekodkan kesan yang sama pada tikus diabetik dalam rawatan akut selama 7 jam. Rawatan dengan 1 g/kg berat badan, dua kali sehari mengawal BGLs secara sederhana seawal hari kesepuluh. Selepas 14 hari rawatan, 1 g/kg dan 0.5 g/kg berat badan GLES menyebabkan peningkatan dalam purata luas kawasan Langerhans islet, masing – masing sebanyak 44% dan 50% dibandingkan dengan DC. Dengan menggunakan otot abdominal tikus, GLES dikesan bertindak sebagai penggalak insulin yang sederhana. Analisis GC-MS menunjukkan kehadiran Sitostenon, komponen fitosterol yang diketahui mampu menurunkan glukosa. Dalam kajian toksisiti, GLES tidak

menunjukkan sebarang kesan signifikan terhadap panel – panel fungsi ginjal dan hati, dan parameter hematologi yang diukur. Trigliserida serum, jumlah kolesterol dan lipoprotein ketumpatan rendah mencatatkan penurunan di dalam tikus jantan seiring dengan pengurangan dalam pengumpulan lemak badan retroperitoneal ($P < 0.05$). Terdapat percanggahan yang signifikan antara berat organ pada dos yang paling tinggi. Sebagai contoh, hati menunjukkan pembesaran secara signifikan pada kedua – dua jantina ($P < 0.05$). Walaubagaimanapun, kajian histopatologi tidak menunjukkan sebarang lesi patologi pada hati mahupun ginjal. Secara keseluruhannya, penggunaan GL dalam mengawal DM menunjukkan kesan yang setara dengan ubat antidiabetik oral konvensional dan ia mengandungi struktur penyembuhan terhadap pankreas, mungkin disebabkan oleh kehadiran Sitostenon dan komponen bukan fenolik yang lain. Di samping itu, kajian ini telah mengesahkan bahawa pengambilan GL secara jangka panjang adalah selamat, sama ada sebagai sayuran, herba mahupun untuk tujuan perubatan; tetapi tidak pada dos yang sangat tinggi melebihi tempoh masa tertentu.

ANTI-DIABETIC AND TOXICOLOGICAL STUDIES OF *GONGRONEMA LATIFOLIUM*

ABSTRACT

Gongronema latifolium Benth. (Apocyanaceae) (GL) possesses a considerable glucose lowering activity to be utilized on a large-scale. This study investigates the antidiabetic and toxic effects of GL. Ethanol and a Soxhlet apparatus were used to obtain GL ethanolic Soxhlet extract (GLES) from the leaves. To examine the antidiabetic effect, GLES was orally administered to male *Sprague-Dawley* (SD) normal and STZ-induced diabetic rats. Blood glucose levels (BGLs), serum lipid profile, insulin levels and the pancreas were evaluated after 14 days of treatment. To assess GL toxicity, GLES was administered orally at 250, 500 and 1000 mg/kg to SD rats for 90 days following OECD guidelines. Toxicity indices were measured, including body weight; biochemical, and hematological parameters; and weights of vital organs. Histopathological assessment of the key organs involved in xenobiotic metabolism and excretion – liver and kidneys - was conducted. GLES significantly ($P < 0.05$) decreased BGLs of normal rats in glucose tolerance testing at a dose of 2 g/kg b.w., but failed to do so in diabetic rats undergoing acute 7-h treatment. Treatment with 1 g/kg b.w. twice daily moderately controlled diabetic BGLs starting from day 10. After 14 days of treatment, 1 g/kg and 0.5 g/kg b.w. of GLES caused 44% and 50% respective increases in the average area of Langerhans islets compared to DC. Using isolated rat abdominal muscle, GLES was found to be a mild insulin-sensitizer. GC-MS analysis revealed the presence of known glucose-lowering phytosterol, Sitostenone. In the toxicity study, GLES did not exert a significant effect on measured liver and kidney function panels, and hematological parameters. Serum triglycerides, total cholesterol and low density lipoproteins were decreased in the male rats along with depletion in retroperitoneal body

fat depots ($P < 0.05$). Significant organ weight discrepancies were observed at the highest dose. For instance, the liver was significantly enlarged in both sexes ($P < 0.05$). However, histopathological studies did not show any pathological lesions in the liver or the kidneys. Overall, GL use in DM management was found to be comparable to a conventional oral antidiabetic drug and precipitate structural recovery in the pancreas, probably due to Sitostenone and other non-phenolic components. Furthermore, this work validated the safety of GL long-term use as a vegetable, spice and for medicinal purposes; but not at very high doses over an extended period of time.

CHAPTER ONE: INTRODUCTION

1.1 Diabetes overview

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by altered carbohydrate, lipid, and protein metabolism causing hyperglycemia, which results from insufficient insulin secretion, insulin action or both (Joseph and Jini, 2011; Mutalik *et al.*, 2003). Loss of control over blood glucose homeostasis is DM's main feature, which is attributed either to insulin deficiency or to insulin resistance— hence, the etiologic classification: Type I DM and Type II DM. The World Health Organization (WHO) projects that DM will become the 7th leading cause of death in the world in 2030. The state of chronic hyperglycemia and abnormal metabolism of nutrients which accompanies DM leads to severe consequences on one's wellbeing if not properly managed, such as retinal bleeding, nerve damage, renal failure, and immunity depression (Vivek *et al.*, 2010). Unfortunately, increasingly ageing populations worldwide, consumption of calorie-rich diets, obesity, and sedentary lifestyles have led to a tremendous increase in the number of diabetics in the past century (Rao and Subramanian, 2009).

1.1.1 Terminology

Diabetes as a term was used for the first time by the Greek doctor Aretaeous of Capadocia in the 2nd century AD. *Diabētēs* was derived from the verb *diabainein,* which is made up of the prefix *dia-*, "across/apart" and the word *bainein*, "to walk/stand". The word "diabetes" translates into "to pass through", which probably referred to one of the main manifestations of the disease, excessive urination; which is accompanied by failure to achieve satiety with eating, and recurring thirst (MacFarlance *et al.*, 1997).

"Mellitus" was added centuries later by the English physician John Rollo in the 18th century AD to reflect the glucose-containing nature of the urine excreted by those inflicted with the condition i.e., glycosuria. Hence, mellitus, which is a word of a Latin-Greek origin that denotes honey, served the purpose of distinguishing "diabetes mellitus" from other polyuric conditions (Rollo, 1797), mainly diabetes insipidus 2013).

1.1.2 Classification

There are two chronic types of DM: Type I, which is more sever, describes a condition in which insulin secretion is completely absent due to immune destruction of pancreatic β-cells (i.e., Insulin-Dependent Diabetes Mellitus: IDDM), whereas Type II describes a condition in which bodily cells (other than neurons) become irresponsive to insulin (i.e., Non-Insulin-Dependent Diabetes Mellitus: NIDDM). Gestational diabetes mellitus (GDM) is another common non-chronic class of DM that can occur during pregnancy. IDDM patients rely on insulin injections or pumps for treatment, which poses risks of ketosis and coma associated with overdosing (ADA, 2005). Patients of Type II DM make nearly 90% of the global diabetic population; and treatment for type II DM, a multifactorial disease, includes many different, expensive combinations of drugs whose use is restricted by their pharmacokinetic properties, secondary failure rates, and many accompanying side effects (Hammouda and Amer, 1966), which include weight gain, increased risk of hypoglycemia, and increased risk of mortality (Rang and Dale, 1999).

1.1.2.1 Insulin-Dependent Diabete Mellitus (IDDM)

IDDM, Type 1 DM, is a chronic disorder caused by immune destruction of β-cells in the pancreas, leading to insulin deficiency and hyperglycemia (Van Belle *et al.*, 2011). Chronic hyperglycemia results in long-term damage, dysfunction, and failure of several

tissues and organs, including the eyes, kidneys, nerves, heart, and blood vessels (Mellitus, 2005). IDDM has been linked to increased risk of microvascular and neurological complications (Control and Trial, 2005). IDDM incidence features an onset of weight drop, excessive thirst, urination, and polyphagia (Van Belle *et al.*, 2011). Management of IDDM necessitates regular insulin parenterals on daily basis, self-monitoring of BGLs, and the calorie values of foods (Loghmani, 2005). Treatment for IDDM is risky. Insulin injections are associated with increased risk of fatal hypoglycemia (Van den Berghe *et al.*, 2006) and although the use of insulin pumps can reduce the risk of treatment-elicited hypoglycemia (Boland *et al.*, 1999), the side effects of regular insulin intake, such as weight gain, and the daily dependence on insulin preparations is far from convenient.

1.1.2.2 Non-Insulin-Dependent Diabetes Mellitus (NIDDM)

NIDDM, Type 2 DM, is underlined by a spectrum of etiologies, ranging from predominant insulin resistance and relative impaired insulin secretion to predominant impaired insulin secretion coupled with relative insulin resistance (Mellitus, 2005). This makes treatment a kind of trial-and-error, particularly upon initiation (Genuth *et al.*, 2003). Impaired insulin action often underlies a deficiency in several molecules involved in insulin signaling pathways (ADA, 2014). NIDDM diabetic population represents over 90% of the world diabetic burden. Alarmingly, epidemiological data indicates that about 50% of those inflicted with NIDDM are actually undiagnosed (Rosenbloom *et al.*, 1999). The risk factors for developing NIDDM include rampant obesity, aging, lack of exercise, genetic predisposition and ethnicity (Loghmani, 2005). Genetic predisposition, obesity and a sedentary lifestyle stand out as the major factors associated with the onset of NIDDM in adults (Hauner and Scherbaum, 2002). The

United Kingdom Prospective Diabetes Study (UKPDS) described NIDDM as a progressive disorder that is initially manageable with one oral hypoglycemic drug, but that ultimately calls for the addition of other medications (DeFronzo, 1999). Management of this disorder later in life will require the administration of several hypoglycemic agents. However, available NIDDM drugs have been known to vary in their pharmacokinetic properties and cause a spectrum of side effects (Hammouda and Amer, 1966), including weight gain and hypoglycemia (Rang and Dale, 1999). Yet, medical practitioners advise the inclusion of complex drug regimens for the management of NIDDM to avoid its morbid complications.

1.1.2.3 Gestational Diabetes Mellitus (GDM)

GDM is a form of DM that may be experienced by some pregnant women. Women who are diagnosed with GDM usually presenting with elevated BGLs, serum insulin levels, exaggerated insulin spike upon food consumption, and blunted intravenous insulin responses (Carpenter and Coustan, 1982; WHO, 1980). The condition is commonly self-limiting. However, a significant adverse outcome may happen when GDM coincides with rampant obesity (Catalano *et al.*, 2012). A vigorous treatment regimen is necessary in such cases and evidence indicates marked risk reduction with such regimens (Langer *et al.*, 1994). If symptoms of GDM do not subside after child birth, which occurs in some scenarios, the diagnosis is changed to NIDDM and treatment is adjusted accordingly (ADA, 2005).

1.1.3 Classes of conventional hypoglycemic agents

DM medications are pharmacologically classified as inulinotropic agents (or secretogoges), insulin sensitizers, glucose absorption down-regulators, glucose

excretion inducers and hormone analogs. From a chemical perspective, as of 2014, ten classes of hypoglycemic agents have been employed for the management of elevated BGLs in DM patients. They include insulin analogs, pramlintide (amylin analog), biguanides (metformin), sulfonylureas, glinides, α-glucosidase inhibitors, thiazolidinediones, DPP-4 inhibitors, GLP1 Receptor agonists, and SGLT2 inhibitors. As evident from several surveys, Metformin has been the agent of choice for the treatment of NIDDM, followed by sulfonylureas, thiazolidinediones, DPP-inhibitors and α-glucosidases inhibitors as monotherapy or in combination (Alam *et al.*, 2014; Sharma *et al.*, 2016), whereas insulin replacement therapy has been the standard approach for the management of IDDM.

1.1.3.1 Insulin-replacement therapy

Insulin is a product of β-cells whose level increases in the blood following meals to ameliorate elevated BGLs. It is the agent of choice for the treatment of IDDM. However, the UK Prospective Diabetes Study Group propose that NIDDM patients ought to require the administration of insulin over the course of the disease (UKPDS, 1998). Synthetic human insulin analogues have been utilized for years, differing mainly in their onset of action. Recently, the patents of many known insulin analogs expired, and some are about to (insulin lispro, 2013; insulin glargine, 2014–2015; insulin aspart, 2017) (Rotenstein *et al.*, 2012), which should make way for pharmaceutical companies to develop biosimilar products freely in the near future. Owing to the lower cost of these alternatives, non-traditional insulin products have already spread in China, India, Pakistan, Thailand, Peru, and Mexico (Polimeni *et al.*, 2015). The main shortcoming of insulin preparations is the fact that insulin degrades in the GI tract, making its use as an injection an inconvenient necessity. This continues to put IDDM at high risk of adverse

reactions as the drug reaches the blood stream rapidly by subcutaneous injection (Nathan *et al.*, 2009).

1.1.3.2 Pramlintide

Pramlintide is an FDA approved synthetic analogue of amylin that is administered parenterally. The use of this hormonal analog started in 2005 as an adjunctive therapy with regular and rapid-acting insulins. Amylin is secreted by the pancreas after meals to regulate BGLs. It delays glucose absorption at the level of the intestine and suppresses glucagon. Concomitant administration of both pramlintide and insulin was reported to better control BGLs and body weight in NIDDM patients (Hollander *et al.*, 2003). However, like all new drugs, pramlintide is still relatively costly. Moreover, its safety has not yet been established (Nathan *et al.*, 2009). Studies have shown the use of pramlintide to cause adverse gastrointestinal effects (Riddle *et al.*, 2007).

1.1.3.3 Metformin

Metformin was derived from Guanidine, a secondary metabolite of a Europe-native herb called *Galega officinalis*. The use of this herb for the management of DM can be traced back to medieval Europe (Panzram, 1987). Metformin is considered a great example of a widely used medication derived from nature in 1957 (Luft *et al.*, 1978). It belongs to the biguanides family, of which metformin has been the only available member since the 1970s (Song, 2016). For years it was classified as an insulin sensitizer that lowered BGLs primarily by reducing hepatic glucose output and increasing cells' response to insulin. However, very recently the systemic effect of metformin was shown to be inferior to its main action on the distal part of the GI tract (Buse *et al.*, 2016). Although the mechanism of action of metformin has not been fully elucidated yet, it was reported

as the most prescribed drug for NIDDM in 2016 (Sharma *et al.*, 2016). It is available as a monotherapy and in combinations (Bailey, 1992). Metformin is also the drug-of-choice for diabetics with large waist circumferences and is preferred over sulfonylureas and insulin preparations in this patient category to avoid weight increments and hypoglycaemic events (Campbell *et al.*, 1996). It was also shown to enhance the lipid profile in NIDDM patients (DeFronzo *et al.*, 1991). Despite being considered safe and effective, metformin use is associated with gastrointestinal disturbance (Nathan *et al.*, 2009). Moreover, its use is not recommendable in a good proportion of NIDDM patients who suffer from renal complications (Kramer *et al.*, 2003).

1.1.3.4 Sulfonylureas

Sulfonylureas are hypoglycemic agents classified as insulin secretogogs. Examples include glibenclamide, also known as glyburide; tolbutamide; chlorpropamide; glipizide; gliclazide; and glimpepiride (Rosskamp, 1996), which is the most prescribed drug of this class (Sharma *et al.*, 2016). Intensive administration of a sulfornylurea in NIDDM patients is comparable to insulin intensive therapy in terms of decreasing the risk of nephropathy, retinopathy, and neuropathy (UKPDS, 1998). On the other hand, the side effects of progressive use include weight increment and potentially-fatal hypoglycemia (Nathan *et al.*, 2009). This drug class is also associated with adverse cardiovascular events (Evans *et al.*, 2006) due to lack of specificity because cardiac myocytes express the same sulphonylurea receptor as pancreatic β-cells.

1.1.3.5 Metiglinides

Metglinides (glinides) share the same mechanism of action with sulfonylureas. Examples include repaglinide and nateglinide. Both glinides and sulfonylureas block

potassium channels associated with the sulfonylurea receptor to increase serum insulin levels. However, a glinide binds to a different site as compared with a sulfonylurea (Malaisse, 2003). They are extremely effective agents that have been successful in managing DM in patients who are irresponsive to other treatments (Füchtenbusch *et al.*, 1999). They are safer than sulfonylureas (Damsbo *et al.*, 1999). However, glinides are considered a costly option. To make things worse, they necessitate multiple doses during the day because of their short half-lives (Nathan *et al.*, 2009). Furthermore, glinides practically cause the same unwanted body weight increment as sulfonylureas in NIDDM patients.

1.1.3.6 α-Glucosidase Inhibitors

Alpha-glucosidases are enzymes in the small intestine responsible for the breakdown of starch and sucrose. Examples include acarbose and miglitol. These antidiabetic agents target the gastrointestinal absorption of carbohydrates in the small intestine and result in prolonged absorption, which effectively blunts the postprandial spike in BGLs (Ron *et al.*, 2002). Alpha-glucosidase inhibitors do not cause fatal hypoglycemia or weight loss/gain (Nathan *et al.*, 2009). They are also advantages over other treatments because they facilitate the body to respond to food intake with lower levels of insulin (Rosskamp, 1996). The use of acarbose, in particular, is associated with a lower risk of cardiovascular disease and hypertension (Chiasson *et al.*, 2003). Unfortunately, at the beginning of treatment, this drug class causes flatulence and diarrhea as seen in those suffering from lactose (Swagerty Jr *et al.*, 2002). This embarrassing side effect leads a large number of diabetics to discontinue the medication.

1.1.3.7 Thiazolidinediones

Thiazolidinediones (TZDs), also referred to as glitazones, are synthetic hypoglycemic and antihyperglycemic agents that act both as insulin sensitizers and as insulin mimickers by induction of the peroxisome proliferator activated nuclear receptor ($PPAR_{\Upsilon}$) (Day, 1999). Two of the most common glitazones are rosiglitazone and pioglitazone. When used concomitantly with metformin, rosiglitazone achieves better glycemic control (Khan *et al.*, 2016). However, it may illicit adverse cardiovascular complications and more weight gain than some sulfonylureas (Kahn *et al.*, 2006). Moreover, a considerable number of health agencies have either totally banned or cautioned against the administration of pioglitazone in NIDDM patients following reports of medication-elicited bladder cancer (Sharma *et al.*, 2016).

1.1.3.8 Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

DDP-4 is an enzyme responsible for the rapid removal of incretins, insulinotropic peptides that are secreted to help ameliorate the rise in postprandial BGLs and reduce the rate of glucagon secretion. Consequently, DPP-4 inhibiting agents exert their hypoglycemic effect in an incretin-like mechanism (Takahashi *et al.*, 2015) by increasing GLP-1 levels, a natural incretin (Nauck, 2011). Examples include sitagliptin and vildagliptin. These medications were reported to decrease elevated BGLs as effectively as metformin (Migoya *et al.*, 2010) and lower HbA_{1c} by 0.5–1.0% without causing significant adverse effects or weight increment (Drucker and Nauck, 2006). Nevertheless, the long-term safety of DDP-4 inhibitors has not been established (Nathan *et al.*, 2009). They are classified as non-selective agents because the DDP-4 enzymes are expressed all around the body, including immune cells.

1.1.3.9 GLP-1 Receptor Agonists

Glucagon-Like Peptide 1 (GLP-1) is a product of intestinal cells that increases the level of circulating insulin. The use of a GLP-1 receptor agonist – named exendin-4 and exenatide – was officiated in 2005 by the FDA in combination with metformin, sulfonylureas and TZDs. Exenatide suppresses gastric motility and glucagon secretion (Nathan *et al.*, 2009). Reports linked its use to mild to moderate gastrointestinal side effects, including vomiting and diarrhea (DeFronzo *et al.*, 2005). However, as an adjuvant, metformin can help ameliorate these unwanted adverse effects (Thong *et al.*, 2015). Yet, alarmingly, a recent report linked exenatide to the incidence of pancreatitis in NIDDM patients (Monami *et al.*, 2014).

1.1.3.10 SGLT2 inhibitors

This is the newest class of the antihyperglycemic agents available. Inhibitors of Sodium–Glucose co-Transporters (SGLTs) (current example: Dapagliflozin) were approved for use as monotherapy and in combination with classical antidiabetic medications in 2014. These agents increase the execration of glucose in the urine and were shown to decrease BGLs remarkably, even when administered as monotherapy, without serious side effects (Chao and Henry, 2010). Nevertheless, this therapeutic approach might illicit unwanted urinary tract infections and general lack of energy because of the glucose shed with the urine.

1.1.4 Diagnosis

A person is said to suffer from DM when he/she has symptoms of diabetes (polyuria, polydipsia, and unexplained weight loss) and shows a plasma glucose level of 200 mg/dl (11.1 mmol/l) or above at any time of the day. The diagnosis can also be DM if a

patient had BGLs of 126 mg/dl (7.0 mmol/l) while fasting, which is defined as no caloric intake for at least 8 h (ADA, 2005).

1.1.5 Worldwide burden

In the year 2000, DM cases worldwide reached 171 million. This number was expected to double in thirty years, regardless of the prevalence of obesity, a major risk factor of DM, remaining stable or not (Sarah *et al.*, 2004). Yet, only ten years later, an estimated 285 million people worldwide had diabetes mellitus (Shaw *et al.*, 2010). This made DM one of the most immanent public health challenges to all nations. A few decades ago, NIDDM was relatively rare in developing countries, but recently, developing countries have suffered the major burden of diabetes mellitus (Chen *et al.*, 2012). Sadly, probably due to inferior health care systems, 80% of diabetes deaths occur in low- and middle-income countries (WHO). The problem is continuously aggravating as the number of diabetics in the countries of the Western Pacific Region is expected to double within the next twenty years (Whiting *et al.*, 2011).

1.1.6 Malaysian Burden

In the period between the years 1996-2006, the incidence of diabetes in Malaysia increased by 80%. Furthermore, the number of obese Malaysian adults, who are at high risk of developing DM, was estimated at 14% of the population in 2006, owing to an increased daily consumption of sugar and a sedentary life style (Institute for Public Health, Health Ministry of Malaysia). Unfortunately, with current trends, a proportional increase of 72.4% in the number of people with diabetes in Malaysia is projected to occur by the year 2035. Thus, it is estimated that about 3,299,000 Malaysian diabetics

will be in need of life-time medical care in just two decades (Guariguata *et al.*, 2014)—which, to say the least, would be crippling for all governmental health service sectors.

1.2 Clarifying ambiguous terminology: antidiabetic vs. antihyperglycemic and hypoglycemic

Drugs used in diabetic care are usually described as antidiabetic, antihyperglycemic, and/or hypoglycemic. These terms seem to be often used by researchers interchangeably. However, subtle differences between these words may render such practice, on occasions, incorrect. Hence, it seems only warranted that a brief description be provided herein for accuracy purposes.

As defined in Farlex Partner Medical Dictionary, the term "anti-diabetic" denotes an agent that reduces blood sugar. Though this might be the case for most antidiabetic drugs, it is not necessarily true for all. A more accurate definition may be found in Mosby's Medical Dictionary, in which an antidiabetic drug is rather defined as "any agent that prevents or relieves the symptoms of diabetes", whereas an antihyperglycemic agent is described as "a substance or therapy that counteracts high levels of glucose in the blood". Lastly, the term "hypoglycemic", according to the definition of The American Heritage® Medical Dictionary, may apply to any agent that lowers the concentration of glucose in the blood. However, a drug might be labeled as "hypoglycemic" to indicate that it can induce a state of hypoglycemia, whereby BGLs fall below the normal range (2015).

1.3 Trend to herbal therapy

Several studies carried out in the field of diabetes research have shown that traditional medicines could provide better glycemic control than currently used conventional drugs

(Rates, 2001; Roja and Rao, 2000). Hence, plants possessing hypoglycemic activity may provide a useful source of new oral antidiabetic compounds for the development of pharmaceutical entities or as simple dietary adjuncts to existing therapies (Ogundipe *et al.*, 2003). However, despite a lack of knowledge about most of the interactions between herbs and drugs, nearly 20% of patients taking prescription medication also take herbal and other dietary supplements (Kaufman et al., 2002; Gardiner et al., 2006). Though there are various approaches to reducing the ill effects of diabetes and its secondary complications, herbal medicines are still preferred due to less side effects and lower costs (Srivastava et al., 2012). The use of herbs from various corners of the world is being increasingly popularized in the modern world as adjuncts to conventional treatments for NIDDM, mainly by immigrants (Bailey et al., 1986).

1.4 *Gongronema latifolium* Overview

Gongronema latifolium Benth. (Apocyanaceae) (GL) is commonly grown in gardens in Calabar, Cross River State, Nigeria. Owing to its extensive presence in the tropical and the subtropical regions of Africa, GL has long been an integral part of the African traditional medicine. It has been used for a variety of medical conditions, such as hypertension, diabetes mellitus, malaria, mental, intestinal disorders (Ekong *et al.*, 2014; Ugochukwu *et al.*, 2003), intestinal worms, cough, dysentery, and dyspepsia (Iwu, 2014). GL extracts have anti-inflammatory (Morebise *et al.*, 2002), antifungal (Nwosu and Okafor, 1995), and moderate anti-laxative effects (Gamaniel and Akah, 1996). Overall, GL's beneficial properties have been well documented, with a focus on it antidiabetic use (Eze and Nwanguma, 2012). It has also been commercially utilized as a substitute for commercial hops in large-scale beer production operations (Adenuga *et al.*, 2010). Recently in the United States, it has been integrated into a DM Tea blend

claimed to maintain healthy BGLs (Akpaso *et al.*, 2011) by Neimeth Pharmaceuticals (Iwu, 2014). Early reports justified GL antidiabetic use based on its antioxidant potential (Ugochukwu and Babady, 2002) and glucose lowering effect (Ugochukwu and Babady, 2003). However, over a decade after these preliminary reports, GL use in the treatment of DM, its mechanisms of action and potential long-term toxicity have remained vaguely understood.

1.4.1 Morphological description

GL is a perennial edible herbaceous shrub, with milky or less often, clear latex and soft flexible stems (Akpaso *et al.*, 2011). Its flowers are mainly yellow (Edet *et al.*, 2009) and, as seen in **(Plate 1.1),** the leaves are simple, smooth, opposite or occasionally whorled, very rarely alternate, usually without obvious stipules; and the margins are nearly always entire (Bingtao *et al.*, 1977). It is a climbing plant with a soft and fibrous stem (Iwu, 2014).

1.4.2 Taxonomy

GL belongs to the kingdom Plantae and the Magnoliophyta phylum. It is of the Magnoliopsida class and the order of Gentianales. The family of GL is Apocyanaceae and it is of the *Gongronema* genus. The species is called *Gongronema latifolium*, and is commonly referred to as Bush Buck and Tafel Boom. Traditionally, GL has been called *Utasi* (Efiks, Ibibios and Quas tribes), *Utazi* (Igbos), *Arokeke* (Yorubas in Nigeria), as well as *Aborode, akam, nsurogya* (Ghana).

Plate 1.1: *Gongronema latifolium* Benth. (Apocyanaceae) courtesy of West African Plants.

1.4.3 Chemical composition

(Eneji *et al.*, 2011) have reported that GL extracts contained a variety of phytochemical compounds, including alkaloids, saponins, tannins, flavonoids, and glycosides. Furthermore, different part of *GL* contain some phytochemicals like B-sistosterol, lupenyl esters, pregnane ester, and essential oils (Ekundayo, 1980; Morebise *et al.*, 2002). It is believed that these phytochemicals in GL can influence cellular proteins with enzymatic activities (Eze and Nwanguma, 2012).

1.4.4 GL use in DM treatment

GL leaves, which are distinctly bitter (Eze and Nwanguma, 2012), were cooked as a vegetable soup and eaten as such for diabetes (Ogundipe *et al.*, 2003). In some African cultures it is utilized as a spice for pancreas-related ailments (Okafor, 1999). A handful of studies have shown that GL possesses a considerable antihyperglycemic effect in animal diabetic models and in human subjects (Ejike *et al.*, 2013). Yet, like most of the medicinal plants which have been identified in the recent decades, there is a lack of data on GL antidiabetic use, chronic and subchronic toxicity, dosing, and mechanisms of action. (Ugochukwu and Babady, 2003) argued that GL had an insulin-like activity. (Adebajo *et al.*, 2012) reported that it acted by increasing insulin release. Meanwhile, (Ogbu *et al.*, 2013) concluded that it attenuated blood glucose excursions partly by delaying gastric emptying.

1.5 Problem statement

The prevalence of DM is on the rise in South East Asia, particularly in Malaysia. Available DM drug classes vary in efficacy and method of administration. They often cause a spectrum of side effects, including weight gain, hypoglycemia and increased risk of mortality. Most are costly and have poor safety profiles. Natural alternatives like antidiabetic herbs can have similar capabilities and less adverse effects and/or cost. Several reports on GL have confirmed the validity of its traditional use in Africa in the treatment of DM. This suggests that GL might be beneficial and may make for a source of antidiabetic medication, or that it may work to commemorate the effects of antidiabetic drugs as an adjunct or a supplement. Hence, due to existing problems with currently available antidiabetic drugs, as well as a global trend towards herbal medicine, it was decided that it was of interest to look into GL further for potential therapeutic

agents. Studies have, in the recent years, managed to demonstrate its antioxidant potential and glucose lowering effect. However, GL use in the treatment of DM, its mechanisms of action and long-term toxicity remain vaguely understood. Hence, this study assessed GL blood glucose lowering activity, anti-diabetic mechanisms of action and sub-chronic toxicity using *in vitro* techniques and rat models.

1.6 Tested hypothesis

A soxhlet ethanolic extract of GL, GLES, retains a significant antihyperglycemic activity and produces structural recovery on the level of the pancreas. It can delay the transport of glucose via the intestinal wall and act as an insulin sensitizer. Furthermore, it has a good safety profile and does not cause systemic toxicity.

1.7 Study objectives

Main objective of the study:

- To investigate the antihyperglycemic/antidiabetic activity of Nigerian *Gongronema latifolium* ethanolic extract in normal and chemically-induced (by STZ) diabetic rats.

Secondary objectives:

1) To assess the effects of *Gongronema latfolium* ethanolic extract on pancreatic Langerhans islets.
3) To investigate the mechanism(s) of action of *Gongronema latifolium* extract by examining its effect on intestinal glucose absorption and glucose uptake by isolated body tissues.
4) To determine the safety of oral sub-chronic (90 days) exposure to *Gongronema latifolium* extract in *Sprauge-Dawely* rats of both sexes.

5) To make recommendations based on *in vivo*/*in vitro* antidiabetic and toxicity results to provide researchers and health care professionals with the safe and effective levels of the doses of GL ethanolic extract.

CHAPTER TWO: GENERAL METHODOLOGY

2.1 Plant material

Gongronema latifolium Benth (Apocynaceae) (GL), locally known as Utazi or Arokeke, was collected as whole plant from Yakkur, Cross River State, Nigeria at the following GPS coordinates (6° 08'17.35"N 8° 41'15.54"E elev 420 ft) under the supervision of the Department of Biochemistry at the University of Calabar. Authentication was carried on by Pastor Frank, a botanist in the Department of Botany, and voucher specimen (ERU/2011/718) was deposited at the same department. The leaves were plucked of the twigs, washed with tap water and dried in the shade. The dried leaves were ground into powder. The powder was placed into a properly packaged courier to arrive at the Department of Pharmacology, Universiti Sains Malaysia via courier within 7 days. Upon receipt of the powder, it was placed at 4 °C for further use. The plant name was verified by checking with www.theplantlist.org (website accessed on 16th of September 2015).

2.2 Preparation of plant extract

Upon receipt, 400 g of the powdered dried GL were extracted in ethanol using Soxhlet apparatus at 40-60 °C in ratio of 1:5 to 1:10 of material : solvent (w/v) for three days. Anti-pumping silicon granules were added each day before the apparatus was turned on. The extract was filtered and concentrated to about 1/10 of its original volume in a rotary evaporator at 40 °C. Thereafter, the concentrate was freeze-dried to obtain dried extract. The totally dry extract, referred to as GLES, amounted to a yield of ~ 12% and was stored at 4 °C until further use.

2.3 Experimental animals

Male and female *Sprague-Dawley* (SD) rats were used over the course of the present research. The animals weighed initially between 180 g - 220 g (6-7 weeks old) and were obtained from the Animal Research and Service Centre, Main Campus, Universiti Sains Malaysia (USM), Penang. The rats were housed pair-wise under standard environmental conditions (temperature, 25 ± 5 ºC; relative humidity, 50 ± 5% and 12 h light/dark cycle) throughout the period of the experiments. Prior to experimentation, the animals were acclimatized to laboratory conditions for one week in the Animal Transit Room at the School of Pharmaceutical Sciences, USM. The animals were allowed free access to standard rat pellets (Gold Coin Feedmills, Butterworth, Penang, Malaysia) and tap water *ad libitum*. The toxicity study was carried out according to OECD guidline 408 (Murbach *et al.*, 2014; OECD, 1998) and *US FDA Redbook 2000*, IV.C.4.a (90-day study) (FDA, 2003). Care and handling of study animals were performed according to the guidelines set by the WHO (World Health Organization, Geneva, Switzerland) with consideration to the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act 1998 XXVIII) regulating animal protection. The institutional Animal Ethics Committee at Universiti Sains Malaysia approved the research [Approval number: USM/Animal Ethics Approval/2013/(90)(509)].

2.4 Induction of diabetes

Diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) at a dose of (55 mg/kg) to rats fasted for 12 hours. The injection solution was freshly prepared by reconstituting STZ in cold normal saline. The diabetic condition of the animals was confirmed 72 hours after the STZ

injection through measurement of the fasting blood glucose level (FBG) using the One Touch Glucometer (Accu-Chek Performa, Roche Diagnostics, Mannheim, Germany) via a single puncture of the tail vein. Only diabetic rats with FBG within 15.0-20.0 mMol/L (270-360 mg/dl) were included in the study.

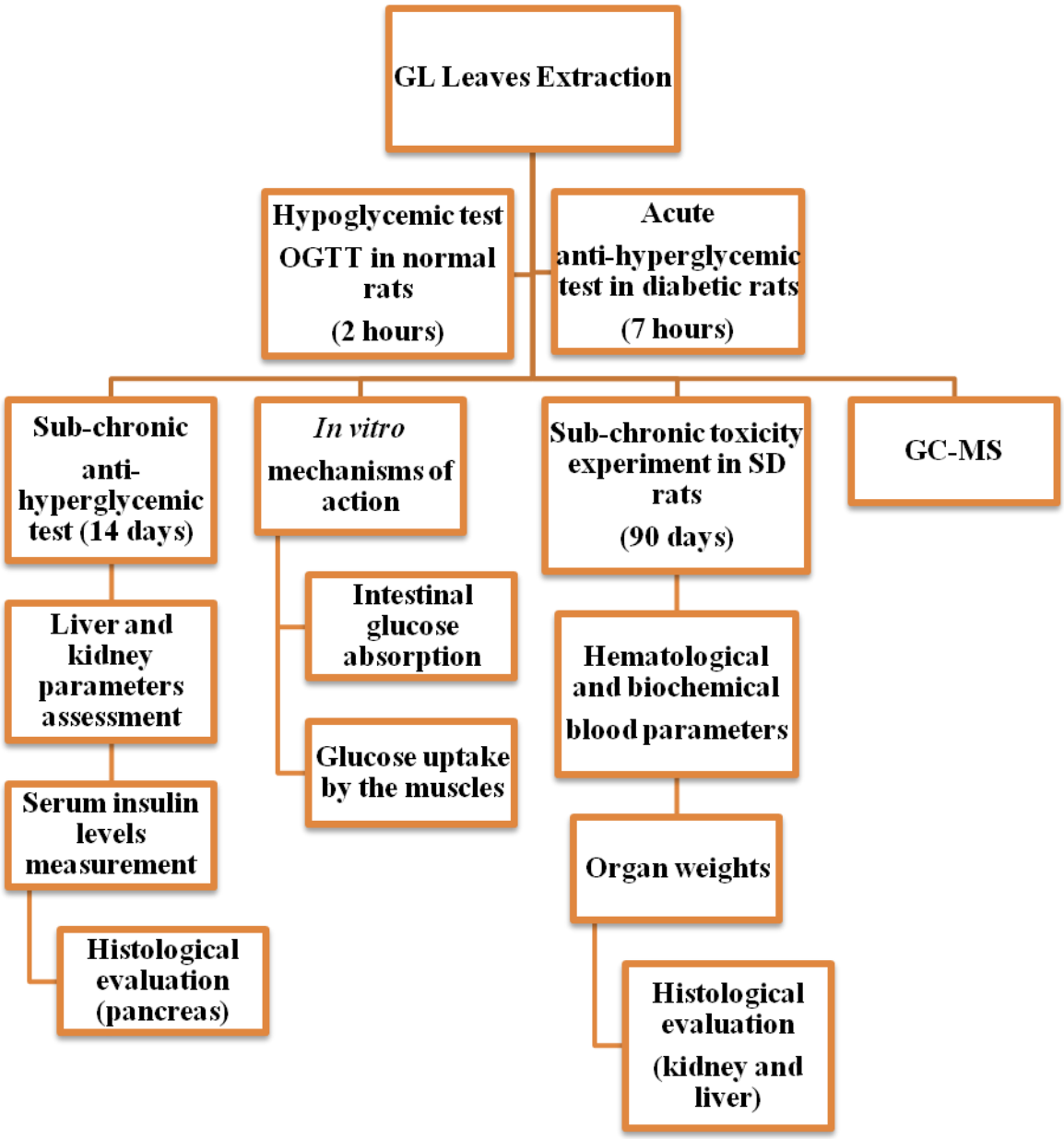
2.5 Preparation of metformin for oral dosing

Metformin 500 mg tablet (Glucophage®, Bristol-Myers Squibb, New York, USA) was grinded in a suitable amount of distilled water using a mortar and a pestle followed by ultra-sonication (UC-10 Ultrasonic Cleaner, Jeiotech, Seoul, Korea) for 15 min at 37 °C. The amount of water used and the desired concentration of the solution were calculated for an administration volume of 10 ml/kg b.w. in all of the treated rat groups.

2.6 Statistical analysis

Data obtained from the male and female treatment groups were compared separately. The statistical comparison aimed at determining whether the differences observed between the treatment groups and the control resulted from GL consumption. Results were expressed as the mean ± SEM. Graphs were drawn and data was typed into Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using version 21 of the IBM-SPSS statistical program (IBM Corp., Armonk, NY). Normal distribution of the data was confirmed using the relevant tool in the same software program. One-way ANOVA was used followed by Dunnett's Test as a Post Hoc Test for parametric multiple comparisons between the control and the treatment groups. Pre-treatment and post-treatment comparisons were performed using the paired *t*-test. Differences were considered significant when the P value was less than 0.05.

2.6 Flow chart of study protocols



CHAPTER THREE: ANTIDIABETIC STUDY OF *GONGRONEMA LATIFOLIUM*

3.1 Background

Diabetes Mellitus (DM) refers to a medical condition marked by hyperglycemia due to loss of control over blood glucose homeostasis. Diabetes Mellitus is a major cause of death worldwide. As projected in 2013 by the World Health Organization (WHO), DM is expected to become the 7th leading cause of death in 2030. There are two types of Diabetes: Type I, which is more severe, describes a condition in which insulin secretion is completely absent due to immune destruction of pancreatic β-cells (i.e., Insulin-Dependent Diabetes Mellitus: IDDM), whereas Type II describes a condition in which bodily cells (other than neurons) become irresponsive to insulin (i.e., Non-Insulin-Dependent Diabetes Mellitus: NIDDM). According to the Malaysian Ministry of Health, there were more than 3 million Malaysians with DM in 2011 (Amal *et al.*, 2011). It is very alarming considering the fact that 80% of diabetes deaths occur in low- and middle-income countries, and that the number of diabetics in the Western Pacific Region is expected to double within twenty years from now (WHO). Treatment for NIDDM, a multifactorial disease, includes many different, expensive combinations of drugs whose use is restricted by their pharmacokinetic properties, secondary failure rates, and many accompanying side effects (Hammouda and Amer, 1966), which include weight gain, increased risk of hypoglycemia, and increased risk of mortality (Rang and Dale, 1999). Thus, there is a grave need for natural drugs that have similar efficacy but do not have such adverse effects or cost.

Gongronema latifolium Benth (Apocynaceae) is a perennial edible shrub with a soft stem. Its leaves are usually simple, opposite or occasionally whorled with no clear

stipules (Bingtao *et al.*, 1977). It is widely used in the West African sub region for a number of medicinal and nutritional purposes (Akpaso *et al.*, 2011) and to treat a variety of ailments, such as hypertension, diabetes mellitus, malaria, mental and intestinal disorders (Ekong *et al.*, 2014; Ugochukwu *et al.*, 2003). Traditionally, the leaves of GL were cooked as a vegetable soup and eaten as such for diabetes (Ogundipe *et al.*, 2003) and in some African cultures it is utilized as a spice to support the pancreas (Okafor, 1999). Recently in the United States, it has been integrated into a DM Tea blend which is claimed to maintain healthy glycemia (Akpaso *et al.*, 2011). Previous reports (Ugochukwu *et al.*, 2003; Ugochukwu and Cobourne, 2003), justified its traditional use for DM based on its effect in ameliorating the oxidative stress which accompanies DM and which underlies many of the complications of diabetes. Although more recent reports (Akpaso *et al.*, 2011; Ejike *et al.*, 2013; Nnodim *et al.*, 2012) have confirmed that GL possesses antihyperglycemic activity, its use in the treatment of DM, as well as its mechanisms of action, remain vaguely understood. While (Ugochukwu and Babady, 2003) attributed an insulin-like activity to GL ethanolic extract, (Adebajo *et al.*, 2012) reported that the extract acted by increasing insulin release.

This study evaluates the effect of GL leaf-extract on BGLs upon acute and sub-chronic administration in diabetic rats. Furthermore, it examines the effect of sub-chronic administration of GL extract on serum lipid profile, kidney function indices and the insulin-secreting cells (Langerhans islets). It explores for the first time, the effect of GL extract on *in vitro* insulin and glucose transduction in isolated rat abdominal muscle and jejunum. Lastly, a profiling of the chemical composition of the extract is done with the use of Gas Chromatography-Mass Spectroscopy (GC-MS).