PHYTOCHEMICAL CHARACTERISATION AND EFFECTS OF THE NATIVE PLANT *Tabernanthe iboga* IN MODELS OF DIABETES

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Abstract

Preparations of the root bark of Gabonese plants, *T. iboga* and *G. tessmannii* have long been used in Central and West African traditional medicine for the treatment of diabetes. In this thesis phytochemical characterisation of *T. iboga* aqueous extract revealed the presence of 23 unknown alkaloids and 7 already known alkaloids, including ibogaine, the main indole alkaloid found in *T. iboga*. For the first time, phenolic compounds have been identified in significant quantity in *T. Iboga*, the most prevalent being 3-O-CQA. The therapeutic potential of these constituents in diabetes remains to be determined. The aqueous extracts of these Gabonese plants for the potential to improve insulin release was examined. *T. iboga* (1 μg/ml) and *G. tessmannii* (70 μg/ml) demonstrated insulinotropic effects in isolated rat pancreatic islets at non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM, for *T. iboga* and 2.8 to 16.7 mM, for *G. tessmannii*), similar to the insulin secretagogue, tolbutamide (200 μM). An additive effect on glucose-induced insulin release was observed for both plant aqueous extracts in the presence of tolbutamide (200 μM). The mechanism of action of the insulinotropic effect of *T. iboga* (1 μg/ml) was explored through the use of a σ2 receptor antagonist, SM-21 (1 and 10 μM) which significantly blocked *T. iboga* (1 μg/ml) insulin potentiation at non-stimulatory and stimulatory glucose concentrations (2.8 and 11 mM) indicating these effects were seen to be partly through σ2 receptors. *T. iboga* (50, 100 and 200 mg/kg) when administered daily over 28 days demonstrated no observable toxicity and exerted hypoglycaemic activity on glucose tolerance in healthy rats. Feeding a 10% fructose solution over 2 weeks combined with a low intraperitoneal dose of streptozotocin (STZ, 40 mg/kg) to rats led to the development of a T2D compared with T1D rats over 4 weeks. *T. iboga* at the lowest daily dose of 50 mg/kg significantly improved hyperglycaemia after 3 and 4 weeks of treatment compared with glibenclamide. After 2 and 4 weeks daily administration of *T. Iboga* (50 and 200 mg/kg) glucose tolerance over a 2 hour fasted glucose load was improved compared with T2D rats treated with vehicle alone. Feeding a high fat diet (HFD) to mice for 10 weeks produced manifestations of metabolic syndrome and T2D, as compared to mice fed a low-fat diet (LFD). Supplementation of HFD with *T. iboga* aqueous extract at ibogaine doses of 0.83 (low) and 2.07 (high) mg/kg/day did not improve these HFD-induced metabolic effects except for a reduction of plasma MCP-1 in the low dose group, indicative of an anti-inflammatory effect. Overall, *T. iboga* and *G. tessmannii* aqueous extracts have demonstrated insulin potentiation, hypoglycaemic and anti-hyperglycaemic activities. However, further investigations are needed to validate their safe use for the management of DM in Gabon.

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Publications:

Papers

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- Bading-Taika, B., Akinyeke, T., Magana, A., Choi, J., Ouanesisouk, M., Samson Torres, E.R., Lione, L.A., Maier, C.S., Bobe, G., Raber, J., Miranda, C.L. and Stevens, J.F. 2018. Phytochemical characterisation of *Tabernanthe iboga* root bark and its effects on dysfunctional metabolism and cognitive performance in high-fat-fed C57BL/6J mice. *J. Food Bioact.; 3: 11-123.*

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- Pharmacy retreat: 'Effect of *Tabernanthe iboga* (Bail) aqueous extract on insulin secretion in rat islets'. Annual Rising Lectures, Salishan Resort, 7760 Hwy 101 North in Gleneden Beach, north of Depot Bay. Friday, March 24th to Saturday, March 25th 2017.

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Abbreviations list

Abbreviation	**Definition**
σ2	Sigma-2 receptor
$[M^+H^+]$	Non-charged compound
%	Per cent
ADP/ATP	Adenosine diphosphastes/adenosine triphosphates ratio
AGE	Advanced glycation end
Akt	Also called protein kinase B
ALP	Alkaline phosphatase
ALS	Alloxan susceptible
ALS/Lt	Alloxan susceptible (the substrain maintained at the Jackson Laboratory)
ALT	Alanine aminotransferase
ALX	Alloxan
AMP	Adenosine monophosphate
APCI	Atmospheric pressure chemical ionisation
AS160	Akt substrate of 160 kDA
ASCVD	Atherosclerotic cardiovascular disease
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
b.w.	Body weight
BB	BioBreeding
BSA	Bovine Serum Albumin

C57BL /6J	Diet induced obese mouse model
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CBD	Common bile duct
CGA	Chlorogenic acid
CI	Chemical ionisation
cps	Count per second
CQA	Caffeoylquinic acid
db/bd	Mouse model of obesity
DDP4	Dipeptidylpeptidase-4
DIO	Diet-induced obesity
DM	Diabetes Mellitus
DMSO	Dimethylsulfoxide
e-	Electron
EI	Electron ionisation
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endethelial Nitric Oxide synthase
Epac2	Exchange proteins activated directly by cAMP 2
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
eV	Electron Volt
FBS	Fat Body Syndrome (macrosomia)
FCS	Fetal Calf Serum

FI	Field ionisation
FT-OT	Fourier transform orbitrap
FTICR	Fourier transform ion cyclotron resonance
g	grams
G. tessmannii	*Guibourtia tessmannii*
GC	Gas chromatography
GCK	Glucokinase
GDM	Gestational Diabetes Mellitus
GLP-1	Glucagon-like peptide-1
Glucose-6-P	Glucose-6-phosphate
GLUT-1	Glucose transporter-1
GSK-3	Glycogen synthase kinase 3
GTG	Gold Thioglucose
H_2O	water
HCL	Hydrochloric acid
HDL	High density lipoprotein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HED	Human equivalent dose
HIP	Human islet polypeptide
HIV	Human immunodeficiency virus
HLPC	High performance liquid chromatography
i.p.	intraperitoneal

IDA	Information-dependent acquisition
IDF	International Diabetes Federation
IR	Insulin Receptor
IR*	Infrared spectroscopy
IRS	Insulin receptor substrate
IT	Ion trap
JCR/LA-cp	James C Russel
k	kilogram
K^+	Potassium ion
K^+-$_{ATP}$	ATP- dependent potassium channel
KK	Kuo Kondo
KK/A_y	Yellow KK obese
L^{-1}	Per liter
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoprotein
LEW.1AR1/Ztm-iddm	Mouse induced diabetic model
m/z	Mass-to-charge ratio
M16	Mouse induced diabetic model
MALDI	Matrix-assisted laser desorption/ ionisation
MEM-NEAA	Minima Essential Medium
MetS	Metabolic Syndrome
mg	milligrams

$mgmL^{-1}$	Milligram per milliliter
MGO	Methylglyoxal
min	minutes
ml	Milliliter
mm	millimeter
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
N/A	Not available
NA^{+}/Ca^{2+}	Potassium/calcium pump
nM	Nano molar
nM	Nanomolar
NMR	Nuclear magnetic resonance spectroscopy
NO	Nitric Oxide
NOD	Non-obese diabetic
NONcNZO10	Obese mouse model
NZO	New Zealand obese
ob/ob	Obese mouce
OCT1	Organic transporter 1
OLETF	Otuska Long Evans Tokushima Fatty
OT	orbitrap
pH	Potential of Hydrogen

PI3K	Phosphatidylinositol 3'-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol 4,5-triphosphate
PKA	Protein Kinase A
PKB	Protein kinase B
pM	picomolar
PPAR	Peroxisome proliferator activated receptor
PPAR-g	Peroxisome proliferator activated receptor gama
ppm	Parts per million
PTP-1B	Protein-Tyrosine Phosphatase 1B (endothelial)
Q	quadrupole
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (culture medium)
SEM	Standard error of the mean
SGLT2	Sodium glucose co-transporter 2
SH2	Src-Homology-2 domain
SHR/N-cp	Spontaneously hypertensive rat /NIH-corpulent
SM-21	3-alpha-tropanyl-2-(-4chorophenoxy)-butyrate
SMCs	Smooth muscle cells
ß	Beta
std	Standard deviation
STZ	Streptozotocin
SUR1	Sulphonylurea receptor 1

SUs	Sulphonylureas
T. iboga	*Tabernanthe iboga*
T1D	Type 1 Diabetes
T1DM	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes
T2DM	Type 2 Diabetes Mellitus
TLC	Thin-layer chromatography
TOF	Time-of-flight
TSOD	Tsumara Suzuki obese diabetes
UCP1	Uncoupling protein 1
UV	Ultra-violet spectrophotometry
V	Volt
v/v	Volume/volume
VMH	Ventromedial hypothalamus
WHO	World Health Organisation
XIC	Extracting Ion Current
ZDF	Zucker diabetic fatty
µg	Micrograms
µL	microliter
µM	micromolar
µmol	micromole
Gli	Glibenclamide
NOAEL	No observed adverse effect level

MCP-1	Monocyte chemoattractant protein 1
LD_{50}	Lethal Dose that kills 50 % of a population
LD_{100}	Lethal Dose that kills 100% of a population
p.o.	Per os
CO_2	Carbon dioxide
TC	Total Cholesterol
TG	Triglycerides
NFBG	Non Fasting Blood Glucose
BGL	Blood Glucose Levels
Hba1c	Haemoglobin glycated
FBG	Fasting Blood Glucose
HOMA-IR	Homeostasis model assessment of insulin resistance
HOMA-ß	Homeostasis model assessment of beta cell function
W	Weight
FI	Fluid Intake
DV	Dose Volume
NC	Normal Control
OGTT	Oral Glucose Tolerance Test
ITT	Insulin Tolerance Test
FR10	Fructose (10%)
NMDA	N-Methyl-D-Aspartate
kDa	Kilo Daltons
5HT	5-Hydroxytryptamine (serotonin)

Chapter 1: General introduction

1.1. Diabetes Mellitus and Metabolic syndrome definition

Diabetes mellitus (DM) is a complex metabolic disorder characterised by constant elevated blood glucose levels (hyperglycaemia), caused by an insufficient or blunted insulin secretion and / or reduced tissue sensitivity to insulin. The disease has become a global issue and can now be considered a pandemic (Motala and Ramaiya, 2010).

Depending on the form of the insulin insufficiency, the disease can be categorised into two main types. Type 1 diabetes mellitus (T1DM) is caused by a profound reduction in the number of functional pancreatic β-cells, destroyed in many by autoimmune mechanisms leading to hyperinsulinemia and hyperglycaemia; while Type 2 diabetes mellitus (T2DM) results in impaired insulin secretion and its subsequent action, often referred to as insulin resistance, leading to hyperglycaemia. T2DM is the most prevalent form with over 85 % of the total DM prevalence (Forouhi and Wareham, 2018). Other types such as Gestational Diabetes Mellitus (GDM) also exist (Motala and Ramaiya, 2010). The cause of diabetes are multifactorial and certain conditions including metabolic syndrome, increase diabetes development especially T2DM.

Metabolic syndrome (MetS), is a group of different metabolic abnormalities which represents a clinical challenge, especially in developed countries. MetS has been considered as a concept rather than a diagnostic and is now defined by the association of physiological, biochemical, clinical and metabolic factors that are directly involved in the risk for developing atherosclerotic cardiovascular disease (ASCVD) and T2DM (Grundy *et al.,* 2004; Kaur, 2014). The international Diabetes Federation (IDF) estimates that more than 25% of world's adult population has MetS, although this estimate varies with age, ethnicity, and gender of the population studied (Nolan *et al.,* 2017). Unfortunately, no specific data are available for Southern countries including Sub-Saharan Africans, eastern Mediterranean and Middle East population (Kaur, 2014). MetS pathophysiology is defined by a state of low grade inflammation as a result of complex inter-related genetic and environmental factors (Jeppesen *et al.,* 2006; Kaur, 2014). Insulin resistance and/or impaired glucose tolerance, abdominal obesity (or visceral adiposity), atherogenic dyslipidemia, endothelial dysfunction, elevated blood pressure, pro-inflammatory state, and prothombotic state are components that constitute the syndrome. Adults having MetS are at increased risk for developing T2DM and

cardiovascular disease independently from other common risk factors including age, sex, serum cholesterol levels, and smoking (Grundy *et al.,* 2004; Kaur, 2014).

1.2. Prevalence and incidence of diabetes

More than 415 million people worldwide have been diagnosed with diabetes in 2015 and the International Diabetes Federation (IDF) has forecasted an increase up to 642 million by 2040 (Khanam *et al.,* 2017), mainly because of the increase in obesity. In African communities, more than 19 million people have diabetes and these figures are estimated to rise up to 41 million by 2035 (Peer *et al.,* 2014).

Commonly, diabetes is caused by a defected pancreas which fails to produce insulin, or to supply enough efficient insulin to allow glucose from the blood to be used by body cells (Motala and Ramaiya, 2010). T1DM is an auto-immune disease and occurs when the body immune system recognises pancreatic β-cells that produce insulin and destroys them. The destruction of the β-cells leads to an absolute insulin deficiency. This type of diabetes accounts for 5-10% of diabetics and people diagnosed with it needs insulin therapy for survival. T2DM, however, is the most common type of diabetes (90-95% of diabetics) and is defined by insulin resistance and/ or altered insulin secretion and the rise of glucose production (Motala and Ramaiya, 2010; Tripathi and Srivasta, 2006). This form is largely responsible for the high mortality and morbidity rates caused by diabetes due to its related complications (Levitt, 2008; Sobngwi *et al,* 2002).

1.3. Complications associated with diabetes

As a metabolic disease, diabetes mellitus with the hyperglycaemia state, triggers alterations of the metabolism of carbohydrates, fat, proteins within the body, leading to major chronic complications (Adeghate *et al.,* 2006). These complications are mostly the cause of diabetes high morbidity and mortality rates. Diabetes complications are subdivided into vascular and non-vascular complications. The vascular complications concern microvascular complications (small blood vessels damaged) such as nephropathy, neuropathy and retinopathy, and macrovascular complications (large blood vessels damaged) such as coronary artery disease, peripheral vascular disease and cerebrovascular disease (Laddha and

Kulkarni, 2019). In arteries, at early stage of the disease, intracellular hyperglycaemia induces alterations in blood flow and vasodilator availability such as nitric oxide (NO), leading to endothelial dysfunction and cellular damage (Alexandru *et al.,* 2016). The non-vascular complications include disorders such as gastroparesis, sexual dysfunction and skin troubles (Tripathi and Srivasta, 2006; Alexandru *et al.,* 2016). These complications combined with high blood glucose concentrations (> 11.1 mM or > 200 mg/dl) were for many years the main features of diabetes diagnosis. Furthermore, diabetes complications including chronic kidney disease, coronary artery disease (CAD), foot and leg ulcers or eye damage (Trikkalinou *et al.,* 2017), dramatically alter diabetics quality of life and contribute to the heavy economic burden of this disease. The association of increasing diabetes prevalence and the rise of diabetes complication rates is leading to a huge rise of financial and societal burden on healthcare systems, especially for southern countries which sanitary structures are not well developed (Sobngwi *et al*, 2002; Saleh *et al.,* 2014).

Nowadays, because of the steady rise of diabetes-related complications and the economic and social impact of diabetes, extensive investigations have been done in order to understand the pathogenesis of this disease. Now, it is fairly understood that diabetes is the result of an impairment of insulin function, and the permanent hyperglycaemia state is a major cause of the related complications (Brownlee, 2005; Llorente and Malphurs, 2007).

The causative mechanisms of diabetes complications are complex and not fully understood. Thus, the pathophysiology of diabetes, the hyperglycaemia role in the genesis of diabetes complications, insulin secretion, insulin signalling and roles are important notions to understand.

In addition, elevated levels of the highly reactive glucose and fructose metabolites, methylglyoxal (MGO) in diabetes (plasma levels > 800 nM) increases oxidative stress accelerating advanced glycation end (AGE) products resulting in pancreatic cell cytotoxicity exacerbating hyperglycaemia, further MGO production and complications. A recent review of the literature demonstrates a clear correlation between MGO and the diabetes complications of insulin resistance, neuropathies, vascular and gastrointestinal dysfunction and neuropathic pain (Shamsaldeen *et al.,* 2016). Monitoring changes in metabolic biomarkers, understanding the actions of MGO and other markers (e.g. adropin, oxidised LDL, Shamsaldeen *et al.,* 2018) should highlight new therapeutic targets for treating diabetes complications.

1.4. Insulin secretion (Insulin signalling and action)

The major role of insulin is to manage glucose homeostasis by stimulating glucose uptake into muscle and adipocyte cells while decreasing glucose generated from the liver via glucogenesis and glycogenolysis (Rains and Jain, 2011). The secretion of insulin is a complex process occurring in pancreatic ß-cells and involving Ca^{2+} outflow and inflow as reviewed by Herchuelz and Pachera, 2018. As shown in Figure 1.1, under physiological conditions, rise of blood sugar levels triggers glucose rapid metabolism via glucose transporter GLUT-1 into pancreatic ß-cells, causing a fall in ADP/ATP ratio (which generates ATP) and the closure of K^{+}-$_{ATP}$ dependent channels. This closure induces subsequent opening of the voltage-gated Ca^{2+} channels (T type) and the rise of intracellular Ca^{2+} which triggers the exocytosis of insulin-containing granules (Gustavsson *et al.,* 2008; Ashcroft and Rorsman, 2012). Moreover, other pathways can control Ca^{2+} intracellular concentrations such as plasma membrane NA^{+}/Ca^{2+} exchanger and Ca^{2+} channels (P Type) (Herchuelz and Pachera, 2018). In parallel, a Ca^{2+} independent pathway is also capable to induce insulin granules exocytosis via the protein kinase A (PKA)-dependent and Epac2-dependent mechanisms involving glucagon-like peptide-1 (GLP-1) (Ashcroft and Rorsman, 2012).

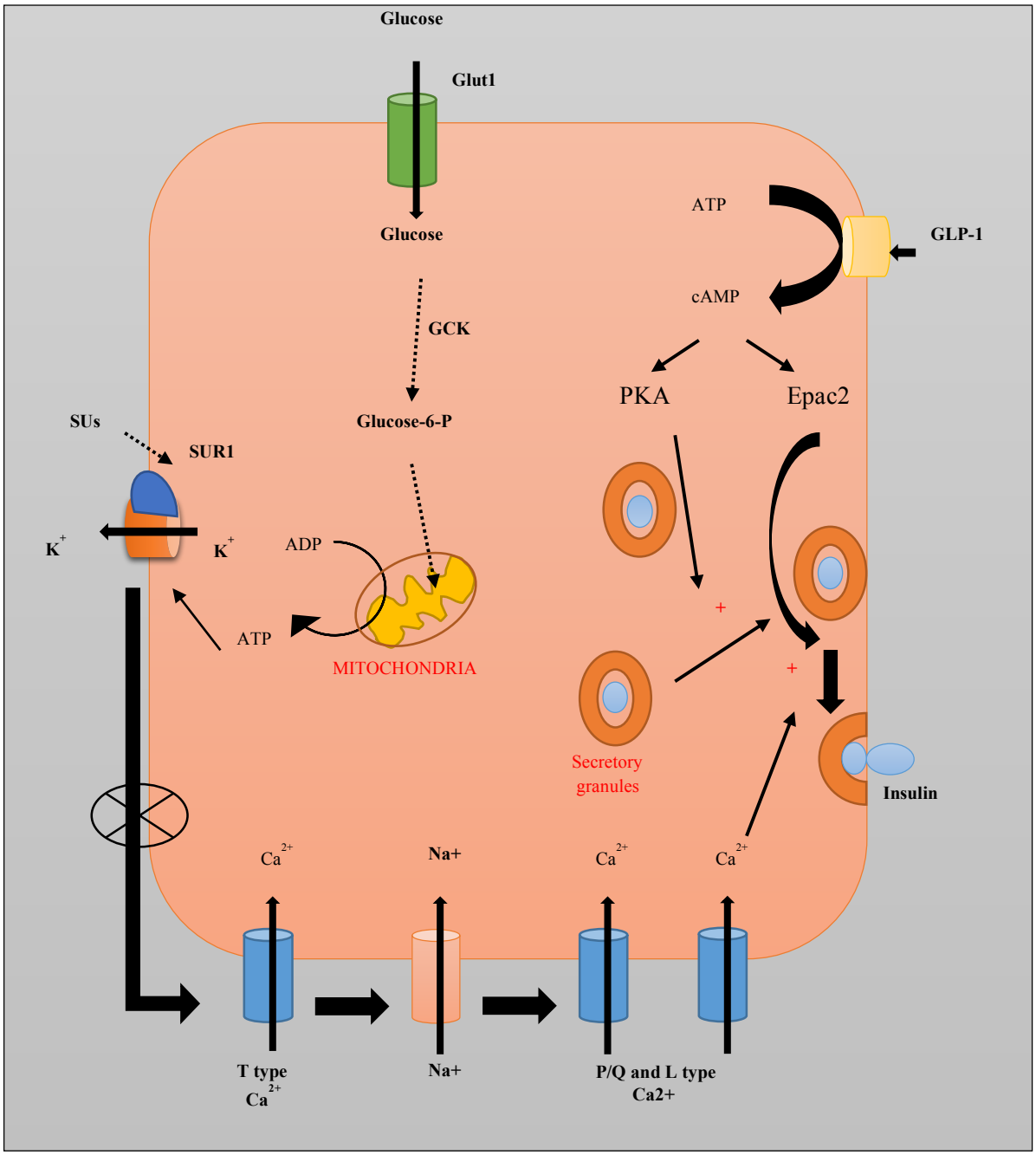


Figure 1.1. Insulin release pathways in pancreatic ß-cell, via transporter GLUT-1 as glucose uptake triggers K^+ channels closure thus, Ca^{2+} influx and insulin secretion, while glucagon-like peptide-1 (GLP-1) triggers PKA and Epac2 which stimulate insulin release (Adapted from Ashcroft and Rorsman, 2012).

Similarly, the action of insulin in muscle cells and adipocytes involves a succession of complex signalling events as shown in Figure 1.2. As soon as the hormone binds to its receptor, insulin receptor (IR), which is a tyrosine kinase-coupled receptor, it promotes IR auto phosphorylation at certain tyrosine residues which induces the phosphorylation of multiple tyrosine residues of insulin-receptor-substrates (IRS-1 to 4) and other intracellular protein substrates. The phosphorylated IRS binds to phosphatidylinositol 3'-kinase (PI3k)/protein kinase B (PKB) at its Src-Homology-2 (SH2) domain, which phosphorylates membrane phospholipids and phosphatidylinositol 4,5-biphosphate (PIP2) on the third position to generate phosphatidylinositol 4,5-triphosphate (PIP3). PIP3 recruits' protein kinase B/ Akt (PKB/ Akt) leading to its domain AS160 phosphorylation which stimulates GLUT-4 translocation to the membrane, glycogen synthesis by phosphorylation of GSK-3, and lipogenesis by up-regulating synthesis of the fatty acid synthase gene (Choi and Kim, 2010; Rains and Jain, 2011). In parallel, IRs expressed in the endothelial cells are also involved in the release of nitric oxide (NO) and the regulation of vascular tone via vasodilation. The phosphorylation of PKB/ Akt protein after IRS-1 binding by insulin also activate endothelial nitric oxide synthase (eNOS) which induces NO synthesis, an important vasodilator (Tripathi and Srivasta, 2006), as shown in Figure 1.2 below. Decrease of NO levels may lead to insulin resistance seen in T2D and diabetes complications including endothelial dysfunction (Alexandru *et al.,* 2016).

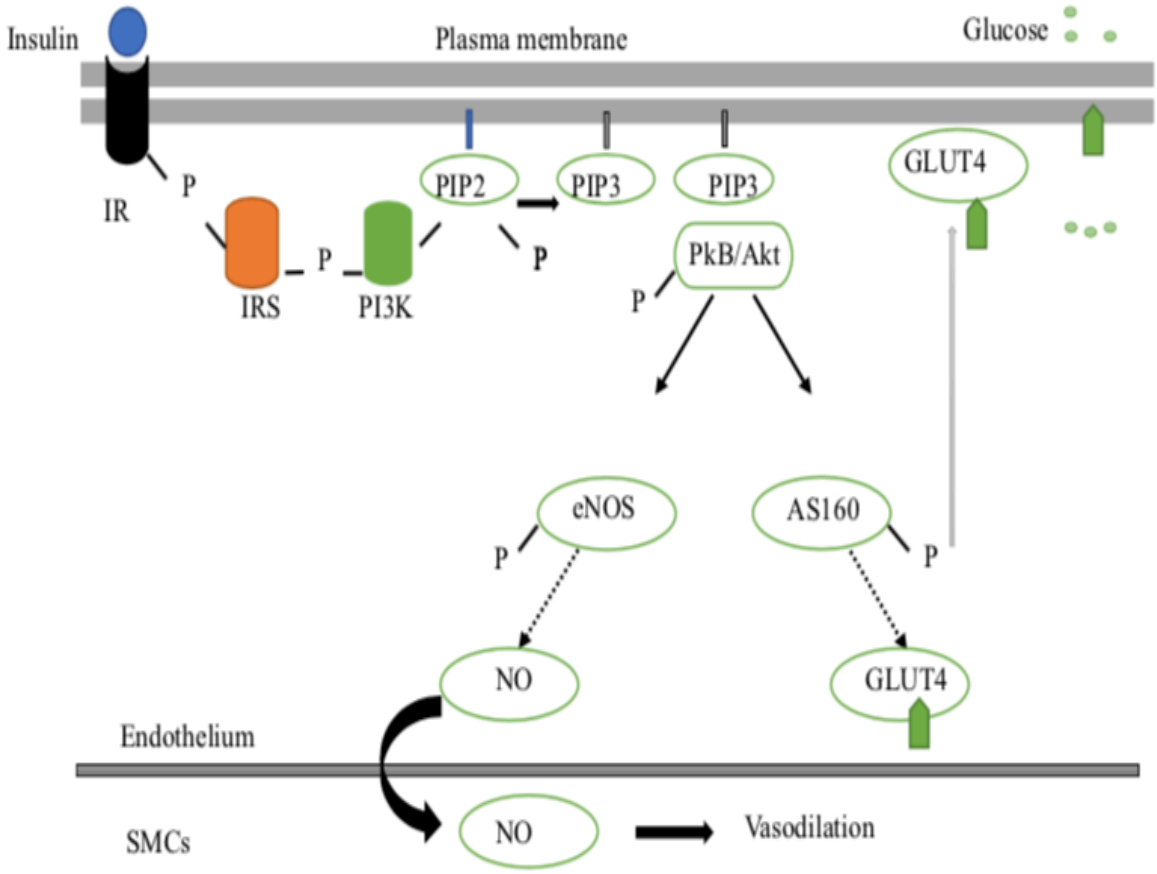


Figure 1.2. Insulin signalling cascades in muscle cells and adipocytes, after binding of insulin receptor (IR), insulin triggers IR auto phosphorylation, then phosphorylation of insulin receptor substrate which induces phosphorylation of phosphatidylinositol 3-kinase (PI3K) which phosphorylates membrane phosphatidylinositol 4,5-biphosphate (PIP2) at 3rd position (PIP3). PIP3 phosphorylates protein kinase B/ Akt which phosphorylates its AS160 domain, these triggers GLUT-4 translocation to the plasma membrane. Also, PKB/Akt phosphorylation induces endothelial nitric oxide synthase (eNOS) phosphorylation which leads to NO synthesis in smooth muscle cells (SMCs) (Adapted from Choi and Kim 2010).

The secretion and action of insulin are part of integrated mechanisms, responsible for carbohydrate uptake, from nutrient consumption, and storage at key sites after its conversion into lipids. However, this ingenious ability to avoid caloric scarcity has been altered drastically recently. Evidences strongly suggest that ectopic lipid metabolites accumulation such as diacylglycerols and/or ceramides, maybe one major cause of liver and muscle insulin resistance, pathology that has been previously associated with diabetes complications (Samuel and Shulman, 2012). Indeed, insulin resistance which is characterized by lowered tissues sensitivity to the hormone, induce higher insulin quantity production to overcome insulin reduced sensitivity and to achieve insulin-mediated biological activity (Kahn *et al.,* 2006). This impairment is well compensated for in healthy individuals but plays an important part in the development of T2D. Moreover, in T2D patients it contributes largely to the development of diabetes-related complications as mentioned previously (Giacco and Brownlee, 2010).

1.5. Diabetes management (conventional treatments)

DM is characterized by constant high blood glucose, thus, the control of blood sugars levels and diabetes-related complication risks such as high blood pressure and cardiovascular diseases, are the first targeted lines to improve diabetes outcomes. T1D patients who have an absolute insulin deficiency require insulin therapy to survive thus insulin or its analogs is administered daily to replace the endogenous hormone (Tripathi and Srivasta, 2006). While, T2D patients are generally prescribed oral anti-diabetic agents and/or exogenous insulin to help in the management of hyperglycaemia when lifestyle modifications are not sufficient (Packer and Castro, 2015; Tahrani *et al.,* 2011). Several pharmacological drugs with different mechanisms of action are available to complement lifestyle changes outcomes in the treatment of T2DM. However, most of these drugs have several limitations (Table 1.1) and cannot maintain initial glycaemia improvements as β-cells continue to degenerate during the course of the disease. Furthermore, several side-effects (Table 1.1) such as hypoglycemia, weight gain, potential cardiovascular risks and gastrointestinal disturbances, have been reported for these treatments (Tahrani *et al.,* 2011; Li *et al.,* 2018; Hsia *et al.,* 2017).

Table 1.1. Summary of anti-diabetic drugs and action

	Examples	**Mechanism (s) of action**	**Route**	**Dosing**	**Advantages**	**Disadvantages**
Insulin **(1922)**	Rapid acting (lispro, aspart), Short acting (actrapid, humulin S), intermediate acting (insulatard, humulin I), long acting (glargine, detemir)	Activate insulin receptor, diminution hepatic glucose production, increase peripheral glucose utilisation and decrease lipolysis	Subcutaneous injection	Once to four times a day	Sustained glycemic improvements	Weight gain, hypoglycemia, fluid retention and self-monitoring of blood glucose needed
Sulphonylureas (1946)	Glibenclamide, Glipizide, Tolbutamide	Increase insulin secretion by binding to SUR1, leading to membrane depolarization and Ca^{2+} influx which triggers insulin secretion	Oral	Once or twice a day	Long-term effects, low cost	Hypoglycemia, weight gain, self-monitoring of blood glucose needed and careful dose titration
Biguanide (1957)	Metformin	Inhibits hepatic glucose release, rises insulin sensitivity in muscle and interferes with lactate and glucose metabolism in the gut	Oral	Once or twice a day	Long-term effects, low risk of hypoglycemia, decrease myocardial infarction risk in	Possible link to lactic acidosis, gastrointestinal disorders, renal function altered

					experimental study, low cost	
α-glucosidase inhibitors (1995)	Acarbose, miglitol	Decrease carbohydrate digestion in the gut	Oral	Three times a day	Long-term effects, low cost	Gastrointestinal side-effects
Meglitinides (1997)	Nateglinide, Repaglinide	Bind to SUR1 on a different site than sulfonylureas leading to a more rapid insulin response	Oral	With each meal	Short acting	Few data for long-term safety, weight gain, hypoglycemia, blood glucose self-monitoring
Thiazolidine-diones (1997)	Pioglitazone, rosiglitazone	Peroxisome-proliferator-activated-receptor-γ agonists act in adipose tissue to increase adipogenesis and decrease free fatty acid release, also increase insulin sensitivity in muscle and liver	Oral	Once a day		Oedema and potential risks of heart failure, long-term safety not established, weight gain and fractures
Glucagon like-peptide-1 (2005)	Exenatide, liraglutide	Bind to GLP-1 receptor resulting in increased glucose-dependent insulin release and glucagon inhibition, delayed gastric emptying and appetite.	Subcutaneous injection	Once or twice a day	Low risk of hypoglycemia, weight loss, possible effect on β-cell decline	Long-term safety not known, gastrointestinal side-effects, might stimulate renal failure

Amylin analogue (2005)	Pramlintide S	Soluble analogue of human amylin which lowers postprandial sugar by inducing satiety sensation, suppressing postprandial glucagon secretion and delaying gastric emptying	Subcutaneous injection	Three times a day	Weight loss	Long term safety unknown, hypoglycemia, used associated with insulin only
Dipeptidyl-peptidase-4 inhibitors (DDP-4) (2006)	Saxagliptin, Sitagliptin	Rise of endogenous incretin levels (GLP1 and GIP) by inhibiting incretins degradation	Oral	Once a day	Low risk of hypoglycemia, possible effect on β-cell survival	Unknown long-term safety, association with pancreatitis
Sodium Glucose cotransporter 2 inhibitors (SGLT2) (2013)	Chapagliflozin, Empagliflozin	Reduced renal tubular glucose reabsorption, producing a reduction in blood glucose independently of insulin	Oral	Once/ twice a day	Low risk of hypoglycaemia, possible improvement of blood pressure	Genital infections, increase urinary frequency, rarely ortho static hypotension, pancreatitis

Metformin remains the first-line glucose-lowering treatment prescribed to newly diagnosed T2D patients. Indeed, this biguanide acts through enhancement of hepatic insulin sensitivity, increases glucose uptake by peripheral cells, reduces hepatic gluconeogenesis and contributes to weigh loss (Refuerzo *et al.,* 2015).

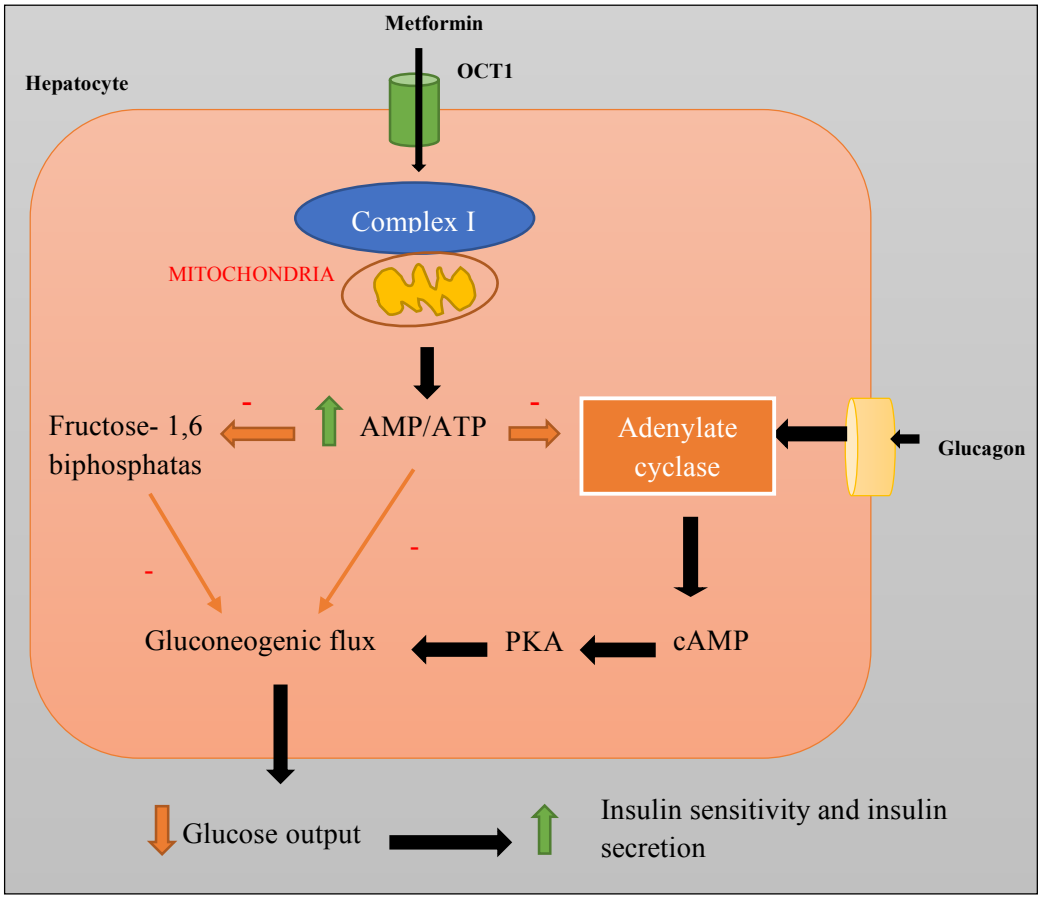


Figure 1.3. Understanding of how current anti-diabetic drugs increase insulin sensitivity and insulin secretion- Metformin case). Metformin uptake through OCT1 (organic cation transporter 1) in hepatocyte exerts mild and specific inhibition of the respiratory chain complex 1, resulting in decreased ATP levels and increase of AMP. ATP drop blocks the gluconeogenesis and AMP accumulation inhibits gluconeogenesis through allosteric regulation of fructose-1,6- biphosphatase and the reduction of glucagon-stimulated adenylate cyclase activity. Chronic high glucose is avoided and insulin secretion increased (Adapted from Ashcroft and Rorsman, 2012; Viollet and Foretz, 2013; Yang *et al.,* 2017).

All these processes would improve insulin resistance, which contributes to diabetes complications. However, metformin can also promote gastrointestinal adverse effects such as cramps, abdominal bloating, nausea, vomiting and diarrhea (Sharrat *et al.,* 2015; Mahmood *et al.,* 2013). Furthermore, for patients considered being "at risk", metformin can be harmful and may cause acute heart failure, shock and lactic acidosis (Packer and Castro, 2015; Scheen and Paquot, 2013). In addition, recent toxicity studies, have exhibited potential genotoxicity of chronic treatment of metformin (114.4 µg/ml or 572 µg/ml in CHO-K1 cells for 24 hr), while others suggested that metformin may act as a mild embryo-toxic agent since it exhibited cytotoxicity at 800 µg/ml in embryonic stem cells (D3 cells), drawing cautious to pregnant women that need metformin for treatment (Zhou *et al.,* 2018).

Sulfonylureas such as tolbutamide are generally prescribed as a second-line therapy in patients with T2D. Although, these drugs can be used as alternative treatment to metformin for patients facing intolerance to biguanides or who are overweight. Sulfonylureas can also be prescribed in combination with metformin for more efficient glycemic control. Sulfonylureas act by binding specific ß-cells K^+ channels receptors (SUR 1), inducing insulin secretion using glucose similar pathway (Tripathi and Srivasta, 2006). The major adverse effects of sulfonylureas are hypoglycaemia and weight gain (Packer and Castro, 2015; Tripathi and Srivasta, 2006).

Another class of drugs named thiazolidinedione have been prescribed as alternative second-line or third-line treatment for T2D patients. Although these drugs improve insulin resistance (detrimental for diabetic complications) by targeting specific receptor gamma and have great efficacy in combination with other anti-diabetic agents, the side effects reported, such as heart failure, oedema, anaemia and weight gain, have decreased their popularity (Packer and Castro, 2015; Tripathi and Srivasta, 2006).

Recently, newer therapies have been developed to improve glycemic control such as sodium glucose co-transporter 2 (SGLT2) inhibitors and incretin hormones such as GLP-1 receptor agonists, for example exenatide, liraglutide, lixisenatide, dulaglutide which increase insulin secretion (Table 1.1), reduce glucagon release and slow gastric emptying. Dipeptidylpeptidase-4 (DDP4) inhibitors such as sitagliptin, linagliptin, saxagliptin, and vildagliptin, have been reported to have a lower risk of hypoglycemia and do not lead to weight gain, although long-term safety is unknown. Newer, long lasting insulin formulations have also been developed to allow a better flexibility to patients but not avoiding the daily

injection disagreement. Moreover, novel insulin delivery routes are tested such as inhaled insulin with hypoglycemia and throat pain as major side effects reported (Packer and Castro, 2015).

Unfortunately, despite the presence and abundance of these well investigated anti-diabetic agents, diabetes and its devastating complications including sight loss, foot complications, infections and amputations, remain an important health problem in industrialized and economically developing countries (Kim *et al.,* 2006). Chronic hyperglycaemia in itself certainly plays a major precipitating and directly causative role in diabetes complications, but additional factors are likely to contribute because even with good glucose homeostasis in well-controlled diabetic, complications still occur and are resistant to resolution (Shamsaldeen *et al.,* 2016). These oral anti diabetic drugs decrease only relatively the risk of complications but cannot prevent them, and mostly increase the risk of hypoglycemia (King *et al.,* 1999). The expense of drugs and recommended diets in cities and rural regions, along with the social burden imposed by everyday treatment practices and culture pressures are the main factors of biomedical diabetes management failure in Africa (Levitt, 2008). Hence, a large number of African communities are still relying on traditional remedies as first line treatment for the management of diabetes mellitus and its related complications.

1.6. Medicinal plants widely used for diabetes

The efficacy and safety limitations of currently available conventional oral anti-diabetic drugs combined with the rise of the disease incidence and its related complications globally, have promoted alternative therapeutic research with the aim of managing diabetes more safely and efficiently (Pandey *et al.,* 2011). Indeed, the World Health Organisation (WHO) have listed more than 21, 000 plants used for medicinal purposes, supporting extensive investigations to find potential therapeutic agents among anti-diabetic-claimed plants (Jayaprasad *et al.,* 2011). Moreover, medicinal plants are ready, available and show few side effects which represent great advantages and prospect for the discovery of new anti-diabetic drugs (Arumugam *et al.,* 2013; Patel *et al.,* 2012). Besides, a large number of medicinal plants are accessible as there are domesticated in garden or are wild plants in forests near habitations, especially in African communities (Bading Taika *et al.,* 2018). In Africa, plant based medicine is widely used to treat numerous of ailments including diabetes without any scientific supportive data on the mechanism of action and the safe range of doses for long

term use. In countries such as Gabon where traditional medicine is used and relied on by more than half of population due to reduced cost of traditional medicines and the easy access of plants (Bading Taika *et al.,* 2018).

Numerous medicinal plants have been studied for their potential ability to restore pancreatic tissue functions by either increasing insulin release, inhibiting glucose intestinal absorption, or insulin-dependent metabolism processes (Kuete and Efferth, 2010). The most studied plants with hypoglycemic activity include *Momordica charantia* (bitter melon), *Trigonella foenum graecum* (fenugreek), *Gymnema sylvestre* (gurmar), *Panax quinquefolius* (ginseng), *Opuntia streptacanthia* (nopal cactus), *Aloe barbadenis* (Aloe), *Vaccinium mytillus* (bilberry), *Silybum marianum* (milk thistle), *Allium sativum* (garlic), *Allium cepa* (onions) and *Pterocarpus marsupium* (Vijayasar) (Upendra Rao *et al.,* 2010). Common culinary herbs and spices based extracts from cinnamon, cloves and bay leaf have also demonstrated insulin-like or insulin-potentiating *in vitro* action by improving glucose metabolism. Now, more than 200 isolated phytochemicals are known to be hypoglycemic, although caution has been made that it can be due to metabolic or hepatic toxicity (Broadhurst *et al.,* 2000).

Nowadays, it is well accepted that people around the world have used medicinal plants to treat diabetes safely and with good success for long time. Indeed, botanical products can improve glucose metabolism and the overall condition of persons with diabetes not only by direct hypoglycaemic effects but also by improving lipid metabolism, antioxidant status, and capillary function (Patel *et al.,* 2012). According to several phytochemical analyses, most of cited medicinal plants contain natural bioactive substances exhibiting anti-diabetic effects such as glycosides, alkaloids, terpenoids, flavonoids, carotenoids (Patel *et al.,* 2012). However, few of these lead compounds have been further developed into clinically useful phytomedicines which would have direct benefits on patients. In this context, it is important to mention that metformin, a biguanide, is a derivate of an active natural product, galegine a guanidine isolated from the plant *Galega officinalis* L. used in ancient times to relieve the intense urination in diabetic people (Andrade-Cetto and Heinrich, 2005).

1.7. Plants used in Gabon to treat diabetes

Gabon is a little country with 1.8 million population covered by 80% of forest. The national prevalence of diabetes is more than 9%. Thus, it is the third country in sub-Saharan Africa the most affected by diabetes mellitus (Bading Taika *et al.,* 2018).

There is a rise of diabetes features in Gabon that is caused by many factors including urbanisation, changes in lifestyle with fewer physical activities and more high fat food consumed, obesity and recently, more diagnosed diabetics. Commonly in Gabon, diabetes is fairly well managed in urban regions. However, in rural areas the poor availability of treatments and difficult access to medicines as well as their expensive cost, helped with the culture and religious beliefs, diabetic patients tend to relying more on traditional practitioners and medicinal plants for the management of this disease (Bading Taika *et al.,* 2018).

Indeed, plant based traditional medicine is widespread in Gabon and it is used to treat numerous ailments including renal insufficiency, HIV and diabetes. In Gabon, more than 1600 medicinal plants have been reported with beneficial biological activities including anti-diabetic property such as *Irvingia gabonensis* (Odika or wild mango), *Pseudospondias longifolia*, *Antrocaryon klaineanum* and *Tabernanthe iboga* (Bading Taika *et al.,* 2018). We recently published an ethnobotanical literature review identifying 69 medicinal plants, currently used in Gabon, that have been reported to have *in vivo or in vitro* hypoglycaemic activities, reducing blood glucose or stimulating insulin secretion in human or animal models (Bading Taika *et al.,* 2018). Active substances were defined in only 12 out of 69 plants, which included *Anacardium occidentale, Sclerocarya birrea, Ageratum conyzoides, Ceiba pentandra, Kalanchoe crenata, Bridelia ndellensis, Tabernanthe iboga (T. iboga), Irvingia gabonensis, Bersama engleriana and Morinda lucida* (Bading Taika *et al.,* 2018). These plants have revealed natural compounds that have shown anti-diabetic potential such as alkaloids, terpenoids, flavonoids, phenolics (Jung *et al.,* 2006). Also, other constituents frequently involved in anti-diabetic activities have been mentioned including carotenoids, peptidoglycan, and steroids (Akinmoladun *et al.,* 2014). These compounds can justify the use of these plants in traditional medicine for the management of diabetes (Bading Taika *et al.,* 2018). For example, the root bark of *T. iboga* and its preparations are used in Gabonese traditional medicine combating diabetes in Central and West Africa, but their active anti-diabetic constituents are not fully identified or characterised.

Since ancient time, treatments for numerous diseases including diabetes have been provided by African traditional healers through plants such as *T. iboga* in Cameroon and Gabon (Eyong, 2007). Traditional "doctors" use the air-dried root barks, stem barks or other plant parts in different recipes obtained usually via maceration (plants macerated in water) or decoction (boiled plants) to form the remedy given to patients (Bading Taika *et al.,* 2018).

However, as mentioned above, only 69 plants have been investigated to date, while more plants are claimed to be anti-diabetic and are currently used by population but have limited or no available scientific evidence on their anti-diabetic mechanism of action such as *T. iboga* and *G. tessmannii.*

T. iboga also called "iboga" or the "sacred wood" has been investigated for several biological activities such as anti-addiction, anti- cancer and anti-diabetic (Souza *et al.,* 2011). *T. iboga* belongs to the Apocynaceae family and grows in tropical forest of central Africa, including in Cameroon and Gabon (Eyong, 2007; Souza *et al,* 2011). *T. iboga* is a shrub of about 5 to 10 m height with white flowers and orange-yellow fruit as shown in Figure 1.4 below (Sanner, 2007).

(a) Shrub (b) Flower (c) Fruit

Figure 1.4. *T. iboga* in natural environment (a), flower (b) and fruit (c) (Bayissi Bading-Taïka, February 2014, Arboretum of Sibang, Libreville/Gabon)

In vitro experiments have shown potentiation of insulin secretion from isolated pancreatic rat ß-cells, at very low concentrations of *T. iboga* aqueous extract (1ug/ml) and in presence of stimulatory glucose concentration (11.1 mM), involving K^+-$_{ATP}$ channels (evidenced by diazoxide which prevented the closure of K^+-$_{ATP}$ channels and suppressed *T. iboga* effect) and extracellular Ca^{2+} (evidenced in presence of cobalt, an inorganic Ca^{2+} channel blocker, which strongly inhibited the insulinotropic effect of *T. iboga*) (Souza *et al.,* 2011). In this study, σ2 receptors were hypothesized to be involved in the insulin-secretion action of the plant (Souza *et al.,* 2011).

Previous phytochemical analysis of *T. iboga* aqueous extract of the roots has shown an association of indole alkaloids ibogaine (80 %), ibogaline (15 %) and up to 5 % of ibogamine (Mačiulaitis *et al.,* 2008) but whether any of these exhibit active anti-diabetic constituents has not been determined to date.

Similarly, *G. tessmannii* Harms Leonard, also known as 'Bilinga', has been investigated for its antioxidant activity (Nyangono *et al.,* 2013), and anti-hypertensive property (Madingou *et al.,* 2012). It has also been reported as medicinal plant used for the treatment of diabetes complications (Bading-Taika *et al.,* 2018), cardiovascular diseases such as arterial hypertension (Madingou *et al.,* 2012), cancer, and sexual troubles (Watcho *et al.,* 2013), supporting the use of this plant by Gabonese population for the treatment of diabetes complications, although no studies on its anti-diabetic effect were reported up to now. *G. tessmannii* is one of the largest tree occurring in forests in Cameroon and Gabon (growing up

to 30-280 meters) as shown in Figure 1.5. It belongs to the Leguminoseae family, and is mainly found in tropical Africa and tropical America (Madingou *et al,* 2012; Mbaveng *et al,* 2011).

(a) Trunk (b) Foliage

Figure 1.5. *G. tessmannii* in natural environment (Bayissi Bading-Taïka, February 2014, Arboretum of Sibang, Libreville/Gabon)

Phytochemical analyses of *G. tessmannii* aqueous extracts have revealed the presence of sterols, alkaloids, polyphenols, reductor compounds, saponosides and terpenes which are bioactive compounds also cited for anti-diabetic activity (Madingou *et al.,* 2012). Numerous studies of aqueous and hydro-ethanolic extracts of *G. tessmannii* at 200 mg/kg have revealed antioxidant and anti-hyperlipidemic activities in tritonised hyperlipidemic rats (Nyangono Beyegue *et al.,* 2012). Moreover, *in vitro* antioxidant activity of aqueous and hydro-ethanolic extracts of *G. tessmannii* was also demonstrated, which supports previous *in vivo* findings (Nyangono Beyegue *et al.,* 2013). It has been shown that alkaloids and flavonoids have antioxidant activity, which is beneficial on human nutrition and health care. Alkaloids may act through peroxidation inhibition and flavonoids are responsible of lipid peroxidation inhibition (avoid cell injury) by scavenging free radical, linking metal ions and/or inhibiting enzymes involved in oxidation systems (Nyangono Beyegue *et al.,* 2013). Recently, the study of biosafety of *G. tessmannii* aqueous extract has determined acute and sub-acute toxicities values. Large safety range of doses have been determined with an oral LD_{50} (lethal dose killing 50% of treated population) above 5000 mg/kg which place this plant in a Class 5 drug, recognised as a low toxic product (Koumba Madingou *et al.,* 2016). This result was

supported by intraperitoneal administration to Swiss mice, LD_{50} estimated at 328.78 mg/kg which is considered as low toxicity. The weak bioavailability of the active compounds that cause toxicity, a poor gastrointestinal absorption, or rapid metabolism to non-toxic metabolites might explain this low toxicity (Koumba Madingou *et al.,* 2016). Furthermore, sub-chronic exposition of aqueous extract of *G. tessmannii* (150-3000 mg/kg b.w.) to Wistar rats for 28 days, did not alter animal behaviour, food and water intake, nor vital organs and body weights, which could indicate side effects of the administered plant extract (Koumba Madingou *et al.,* 2016).

However, the lack of scientific evidence of anti-diabetic effects of Gabonese medicinal plants claimed to be anti-diabetic, endangers the health care systems already weakened by the heavy burden of this life time disease in this community. Indeed, as a large number of Gabonese population rely on traditional medicine for the management of diabetes, there is a warrant need of evidence either to prevent or promote the use of certain Gabonese medicinal plants as alternative treatment for the management of diabetes in this country.

1.8. Animal models of diabetes

Although medicinal plants have been long used for the treatment of diabetes around the world, few of them have received scientific validation and been tested in humans. (Bading Taika *et al.,* 2018; Fröde and Medeiros, 2008). Thus, the effectiveness of anti-diabetic medicinal plants is largely not supported by applied medical research (Fröde and Medeiros, 2008). In order to be able to evaluate *in vitro* and *in vivo* anti-diabetic efficacy, their side effects and mechanism of action of plants and plant-derived extracts, animal models have been used (Eddouks *et al.,* 2012). Diabetes is a complex metabolic disorder and is hard to model *in vitro.* Animal models of diabetes are therefore very important for the study of the disease pathogenesis and can be used for the study of diabetic complications that are seen clinically, including glucose homeostasis, insulin sensitivity, vascular, gastrointestinal and/ or urogenital dysfunction and neuropathic pain.

Over 130 years ago, the surgical removal of the pancreas in a dog and the consequent polyuria, polydipsia and diabetes, highlighted the role that the pancreas plays in diabetes (Von Mering and Minkowski, 1889). Due to the complexity of diabetes features in human, not one single animal model can be representative to a precise type of diabetes in man. Thus,

many different animal models have been used for T1D and T2D (Eddouks *et al.,* 2012). The majority of published experiments in ethno-pharmacology have used T1D or T2D models as presented on Table 1.2.

The most frequently used animal models are rodents, which are cheaper to maintain than larger animals and generally exhibit more rapidly the onset of their diabetic condition (Eddouks *et al.,* 2012; Fröde and Medeiros, 2008).

The induction of diabetes in animal models can be done by pharmacologic, surgical or genetic manipulations in a large number of animal species. Although, most experiments are carried out on small rodents, few studies are still conducted in larger animal such as dogs (Eddouks *et al.,* 2012; Fröde and Medeiros, 2008). To date, there is no evidence that a plant material can be use as substitute for insulin, though, a large number of plant products have been reported to mimic or improve endogenous insulin effects.

Streptozotocin (STZ) and alloxan are the most frequently used chemicals to induce diabetes that are selectively toxic to the ß-cells in the islet of pancreatic islets (Lenzen, 2008). Both drugs show their diabetogenic action when they are administered intravenously, intraperitoneally or subcutaneously. The dose required of these drugs for inducing diabetes mainly depends on the animal species and route of administration. According to the dose administered of these agents, features similar to either T1DM, T2DM or glucose intolerance can be induced (Eddouks *et al.,* 2012). In rodents, systemic administration of chemicals that are selectively toxic to the ß-cells of pancreatic islets such as STZ (Schein *et al.,* 1974) and ALX (Lenzen, 2008) induce a rapid and sustained diabetic state of hyperglycaemia and insulin deficiency. For many researchers, STZ is the agent of choice due to its more reproducible induction of diabetes through greater stability than ALX at physiological pH (Lenzen, 2008).

There are a wide variety of genetic models of T2D but these are expensive and not easy to maintain. Non-genetic models are widely available, such as nicotinamide-STZ model and high fat fed-STZ models but these can lead to permanent ß-cell damage (akin to T1D) and require special dietary formulations. Back in the 1950s it was recognised that diets high in fructose can rapidly induce hyperglycaemia, hyperinsulinemia and impaired glucose tolerance (Sleder *et al.,* 1980). Recent studies have combined fructose feeding in drinking water (10%) over 2 weeks with a lower dose (40 mg/kg i.p.) of STZ and demonstrated a stable hyperglycaemia, polydypsia, oral glucose tolerance, insulin resistance, hyperlipidaemia

and sensitivity to anti-diabetic drugs metformin and glibenclamide (Wilson and Islam, 2012). These STZ-induced rat models of diabetes are well-established at the University of Hertfordshire and are highly reproducible and lead to moderate severity. Within hours following STZ injection, animals show typical symptoms of diabetes, such as polydipsia, polyuria, glycosuria, dyslipidaemia and hyperglycaemia.

Transgenic or highly inbred animal strains have also been developed that spontaneously or conditionally develop diabetes (eg. ob/ob, Zucker), although the use of these can be restricted by their cost and the fact that diabetes is highly genetically determined unlike heterogeneity seen in humans (Rees and Alcolado, 2005). The most commonly used inbred models in research are the non-obese diabetic mouse (NOD) and the bio breeding (BB) rat (Rees and Acolado, 2005). Both of these models spontaneously develop diabetes but there are drawbacks in that the NOD model demonstrates a large gender difference with females showing an increased propensity of developing diabetes and the BB rat requires administration of exogenous insulin to survive (Rees and Alcolado, 2005). There are numerous models of T2D due to the complexity and heterogeneity of the human disease. They include ob/ob and db/db mice, which have been shown to be leptin deficient and leptin resistant respectively (Rees and Acolado, 2005) and the Zucker (fa/fa) rat (also leptin resistant). Transgenic models have been developed for the study of specific elements of the disease but the use of these is economically restrictive and has other drawbacks especially when wanting to look at a disease from a non-biased viewpoint. Advantages and disadvantages of different categories of T2 diabetic animal models are listed on Table 1.3.

Table 1.2: Classification of type 2 and type 1 diabetes models in animals (Rees and Alcolado, 2005)

Type 1	**Spontaneous syndromes**	**Experimentally induced**
	- BB rat - NOD mouse - LEW.1AR1/Ztm-iddm rat	- Chemically with ALX or STZ or surgically by near-total pancreatectomy
Type 2	Obese	Non Obese
1. Spontaneous or genetically derived diabetic animals	- ob/ob mouse, db/db mouse - KK mouse, KK/A_y mouse - NZO mouse - NONcNZO10 mouse - TSOD mouse, M16 mouse - Zucker fatty rat, ZDF rat - SHR/N-cp rat, JCR/LA-cp rat	- Cohen diabetic rat, GK rat; Torri rat Non-obese C57BL/6; (Akita) mutant mouse, ALS/Lt mouse

2. Diet-induced diabetic animals	- OLETF rat - Obese rhesus monkey - Sand rat, C57/BL 6J mouse, Spiny mouse - GTG-treated obese mice	- Low-dose ALX or STZ adult rats, mice; Neonatal STZ rat; Non-genetic rat models (Fructose-induced-STZ model type 2 model)
3. Chemically induced diabetic animals	- VMH lesioned dietary obese diabetic rat - ß$_3$ receptor knockout mouse - Uncoupling protein (UCP1) knock-out mouse	
4. Surgically diabetic animals		- Partial pancreatectomized animals

		(dog, primate, pig, and rat)
5. Transgenic/knock-out diabetic animals		- Transgenic or knockout mice involving genes of insulin, insulin receptor, and its components of downstream; Insulin signalling (IRS-1, IRS-2, GLUT-4, PTP-1B …); PPAR-g tissue-specific knockout mouse; Glucokinase or GLUT-2 knockout mice; Human islet amyloid polypeptide (HIP) over expressed rat

DIO: Diet-induced obesity; KK: Kuo Kondo; KK/A_y : yellow KK obese; VMH: ventromedial hypothalamus; ZDF: Zucker diabetic fatty; NZO: New Zealand obese; TSOD: Tsumara Suzuki obese diabetes; SHR/N-cp: spontaneously hypertensive rat/NIH-corpulent; JCR: James C Russel; OLETF: Otuska Long Evans Tokushima fatty; GTG: gold thioglucose; ALX: alloxan; STZ: streptozotocin; GLUT: glucose transporter; IRS: insulin receptor substrate; GK: Goto-Kakizaki; PPAR: peroxisome proliferator activated receptor; PTP: phosphotyrosine phosphatase; ALS: alloxan sensitive.

Table 1.3: Advantages and disadvantages of various categories of type 2 diabetic animal models (adapted from Eddooks *et al*., 2012)

Model category	**Advantages**	**Disadvantages**
Spontaneous diabetic animals	- T2D development is spontaneous involving genetic factors, and features resembling human T2D - Most inbred animal models are homogenous and in controlled environment, that allows easy genetic dissection - Variability of results is low and require small sample size	- Highly inbred, homogenous and in majority monogenic. Inheritance and development is genetically determined, unlike heterogeneity of humans - Limited availability and very expensive. High mortality due to ketosis in animals with brittle pancreas (db/db, ZDF rat P. obeus, and more), and it requires insulin in later stage for survival - Require sophisticated maintenance
Diet/nutrition induced diabetics	- Develop diabetes with obesity due to over nutrition like diabesity syndrome in humans - Toxicity of chemicals on other vital organs can be avoided	- Most of them require long dietary treatment - No direct hyperglycaemia develops upon dietary treatment in normal animals and hence unsuitable for screening antidiabetic agents on circulating glucose parameters

Chemical induced diabetic animals	- Selective loss of pancreatic ß-cells (ALX/STZ) leaving alpha and delta cells undamaged -Residual insulin secretion help animals to live long without insulin treatment - Cheaper, easier to develop and maintain comparing to other models	- Hyperglycaemia develops by cytotoxicity action on the ß-cells, leading to insulin deficiency rather than insulin resistance - Diabetes induced by chemicals is less stable and reversible due to spontaneous regeneration of ß-cells. Care is thus required to assess ß-cell function in log-term experiments - Chemically induced toxicity on other organs along with its action on ß-cells - High variability on hyperglycaemia development
Surgical diabetic animals	- Avoid cytotoxicity effects of chemicals inducing diabetes on other organs - Due to reduced ß-cell mass, resembles to human T2D	- Requires technical skills and post-operative procedure - Some digestive problem may occur due to excision of exocrine portion leading to the deficiency of amylase - Dissection of alpha cells (secreting glucagon) along with ß-cells leading to the counter regulatory response to hypoglycaemia - High mortality
Transgenic/ knock out diabetic animals	- In vivo effect of single gene or mutation on diabetes can be investigated - Study of complex genetics of T2D is easier	- Highly sophisticated and costly for maintenance and production - Expensive for regular screening experiments

1.9. Pharmacological approaches

In vitro experiments are usually designed using known drugs mechanism of action already used for the management of diabetes including inhibitory effects against activity on PPAR gamma receptors which activation increases glucose metabolism (thiazolidinediones-like effect), insulin release from pancreatic ß-cells (sulfonylureas), glucose uptake increase in muscle or liver, increase expression of glucose transporter GLUT4, which enhance glucose uptake into muscles and adipose tissues, DPP-IV inhibitors, or aldose reductase inhibitors like the drug epalrestat (Ezuruike and Prieto, 2014). This methodology supports an easier identification of potential medicinal plants with therapeutic value having identified natural compounds (chapter 2). Although, this experimental approach excludes extracts that might act on unknown targets with unknown mechanisms. The investigation of mixture of plants would not be appropriate using this approach only as numerous active metabolites and targets may be involved (chapter 2).

In vivo experiments need safe and effective drug dosing. The common perception of scaling of dose based on the body weight (mg/kg) is not the right approach on its own. Therefore, extrapolation of dose from animals to humans or *vice versa* needs consideration of body surface area, pharmacokinetics, and physiological time to increase experiments or preclinical trial safety (Nair and Jacob, 2016). Four different methods to assess the initial dose have been reported in the literature, including dose by factor, similar drug, pharmacokinetically guided, and comparative approaches (Nair and Jacob, 2016). In chapter 4 and 5, the dose by factor approach was used, using the same guideline values to back calculate the human dose to animal dose for preclinical toxicological studies. This empirical method use the No Observable Adverse Effect Level (NOAEL) of drug from preclinical toxicological studies to estimates human equivalent dose (HED). The dose selection is based on minimum risk of toxicity (Nair and Jacob, 2016).

1.10. Aims and objectives

The use of specific plants for the treatment of diabetes is very common in Gabon. However, the lack of scientific evidence on claimed anti-diabetic plants has raised safety and health concerns in the country. Only 69 plants used for the treatment of diabetes in Gabon have been documented in the literature, with little preliminary data. Most of these plants investigations

to date revealed poor general design and unrealistic doses unveiling the huge work to be done before obtaining plant-based medicines from the Gabon plants used (Bading Taika *et al.,* 2018).

To date, there is insufficient scientific information regarding the appropriate range of doses for *T. iboga* and *G. tessmannii* extracts as remedy for diabetes. Scientific Research is therefore needed to provide an insight into acute and long-term effects of aqueous extracts of *T. iboga and G. tessmannii* for the development of standardized extracts of these plant species for preventing or ameliorating diabetes in humans.

Overall aims of this study:

1. Perform a phytochemical characterization of *T. iboga* root bark to identify known and unknown novel compounds and determine if any (based upon literature) are insulinotropic agents.

2. Investigate the aqueous extracts from *T. iboga* and *G. tessmannii* plant material and to determine the effects on glucose stimulated insulin release from isolated rat pancreatic islets and the involvement of σ2 receptors. These experiments would determine whether concentrations of *T. iboga* and *G. tessmannii* aqueous extracts can potentiate insulin release from rat pancreatic islets *in vitro* before evaluating *in vivo* efficacy in diabetes disease models.

3. Investigate effects of the aqueous extract of *T. iboga* on animal models of diabetes. Specifically, the *in vivo* effects of the extract on naïve rats, as well as rat and mouse T2D. Effects on glucose tolerance, insulin tolerance and metabolic syndrome (including disease tissue biomarkers) were determined.

Phytochemical isolation and characterization of active *T. iboga* antidiabetic constituent compound(s) could lead to a patent and investment to designing and synthesising analogues that could be used in the controlled medical treatment of diabetes in Gabon in the future. Further this project will be the first to determine the *in vitro* and *in vivo* activity of *T. iboga* in models of diabetes and whether aqueous extract from widely used *G. tessmannii* plant stem bark has anti-diabetic potential.

The objectives of this study were investigated as the following:

1. To identify the Phytochemical characterization of *T. iboga* plant root bark using LC-MS/MS and metabolomics (Chapter 2)
2. To develop a methodology for isolation of rat pancreatic islets (Chapter 3)
3. To investigate the effects of low and high glucose concentrations on insulin secretion from isolated rat pancreatic islets (Chapter 3)
4. To investigate the concentration dependent effects of *T. iboga* and *G. tessmannii* aqueous extracts on low and high glucose induced insulin secretion from the isolated rat pancreatic islets (Chapter 3)
5. To investigate the effect of *T. iboga* and *G. tessmannii* on insulin release from isolated rat pancreatic islets in the presence of Tolbutamide (Chapter 3)
6. To investigate the effect of *T. iboga* on insulin release from isolated rat pancreatic islets in the presence of σ2 receptor antagonist SM-21 (Chapter 3).
7. To evaluate *T. iboga* toxicity and optimal dose regimen *in vivo* in rats (Chapter 4)
8. To investigate the in vivo anti-hyperglycemic and hypoglycemic effect of *T. iboga* in naïve control rats (Chapter 4)
9. To investigate the *in vivo* anti-diabetic effect of *T. iboga* in Fructose-induced STZ type diabetic animal model (Chapter 4)
10. To investigate *T. iboga* for the improvement of dysfunctional metabolism in mouse DIO models of metabolic disease (Chapter 5)

Chapter 2: Phytochemical characterisation of *T. iboga* plant root barks by LC-MS/MS and metabolomics

2.1. Introduction

Plants and plant-derived substances have become of great interest due to their wide range of pharmacological activities. Phytochemicals as referred to the biologically active compounds found in plants are great resource of drugs for traditional medicine, alternative modern medicine, food supplements, folk medicines and chemical patterns for synthetic drugs (Ncube *et al.,* 2008; Ingle *et al.,* 2017). It has been estimated that more than 74% of pharmacologically active plant-derived compounds were identified following ethno medicinal reports of the plants' use. A large number of interesting outcomes have been reported with the use of mixtures of natural products for the treatment of many diseases especially the synergistic effects and poly-pharmacological use of plant extracts (Ncube *et al.,* 2008; Panchal and Tiwari, 2017). Phytochemistry describes the abundance of secondary metabolites occurring in the plants. Nowadays, it is well known that plants are the reservoirs of natural chemical compounds with structurally diverse bioactive molecules (Ingle *et al.,* 2017).

The first step to the development of pharmaceuticals commences with the sample preparation, separation and then detection (Moein *et al.,* 2017). Sample preparation involves suitable extraction methods which can allow a better identification of bioactive principles, followed by in-depth biological assays, dosage formulations and clinical studies to evaluate safety, efficacy, and pharmacokinetic profile of the new drugs derived from plant extracts (Ncube *et al.,* 2008; Moein *et al.,* 2017). Numerous analytical techniques have been developed to identify unknown compounds involving extraction, isolation of compounds and qualitative determination of elements with spectrometry, MS (mass spectrometry), IR* (infrared spectroscopy), NMR (nuclear magnetic resonance spectroscopy), UV (ultra-violet spectrophotometry), chromatography, HPLC (high performance liquid chromatography), GC (gas chromatography), TLC (thin-layer chromatography), electrophoresis and fluorescence for the most cited in the literature (Milman, 2011; Ingle *et al.,* 2017).

Extraction

Extraction is the critical first step in the analysis of medicinal plants, as it is essential to extract the wanted chemicals from the plant materials for further separation and characterisation. Extraction is a technique of separation of compounds, usually solid, based on their solubility. Depending on the phases involved, it can be either liquid-solid or liquid-liquid, the latter being the most commonly used in organic laboratory (Hoffman and Stroobant, 2007). The common procedures include pre-washing, drying of whole plant or plant parts or freeze drying, grinding to obtain uniform sample and then reconstitution for further analysis. Precautious actions are required to avoid loss, destruction or deterioration of potential active principles during extraction process. Usually, when the plant was selected on the basis of traditional uses, the extract is prepared according to the traditional practitioner method in order to obtain a product as close as possible to the one used traditionally (Sasidharan *et al.,* 2011). The solvents for the extraction are selected according to the nature of potential bioactive compounds being targeted. Different solvents are available to extract the bioactive compounds from natural products. Hydrophilic compounds are usually extracted using polar solvents such as methanol, ethanol or ethyl acetate. Lipophilic components are usually extracted using less polar solvents such as DMSO (dimethylsulfoxide), hexane or heptane. The targeted compounds may be non-polar to polar and even thermally sensitive, thus the appropriate method of extraction must be assessed. Besides, since the final product obtained will contain traces of residual solvent, the solvent chosen should be non-toxic for bioassay. Generally, traditional practitioners use water extracts to prepare their remedies although organic plant extracts have been reported to carry more active molecules than water extracts (Ncube *et al.,* 2008).

Numerous methods of extraction are available such as sonication, heating under reflux and Soxhlet extraction. Alternatively, plant extracts are also obtained by maceration, decoction, infusion (Bading Taika *et al.,* 2018) or percolation of fresh green plants or dried powdered plant materials in water or organic solvents (Sasidharan *et al.,* 2011). Modern extraction techniques are also available including solid-phase micro-extraction, supercritical-fluid extraction, pressurised-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated methods, which present many advantages including automation of these methods with gain of time, less organic solvent use and sample degradation, fewer steps before chromatographic analysis with the elimination of additional sample clean-up and concentration, improvement in extraction efficiency, selectivity, kinetics

of extraction (Gupta *et al.,* 2012). Plant extracts usually appear as a mixture of various type of phytochemicals, which represent a real challenge for their separation, identification and characterisation processes. As mentioned previously, multiple separation and identification techniques of bioactive compounds in a sample are available (Sasidharan *et al.,* 2011), although in this project we will focus on mass spectrometry technique.

Mass spectrometry (MS)

Among the numerous methods of detection, mass spectrometry (MS) is one of the most powerful analytical techniques for the identification of unknown compounds, quantification of known compounds and to elucidate structure and chemical properties of molecules. Using MS spectrum, the molecular weight of sample can be determined (Ingle *et al.,* 2017). This method is used for the structural elucidation of organic compounds, for peptide identification and for monitoring the presence of previously characterised compounds in mixtures with high specificity by defining, at the same time, both molecular weight and a diagnostic fragment of the molecule.

MS has been referred to as the smallest scale in the world as this is a micro-analytical method used to selectively detect and determine the amount (~200 Da or less), the elemental composition and aspects of the molecular structure of a given component. MS has the capacity for direct determination of the nominal mass of an analyte, to produce and detect fragments of the molecule that match with distinct groups of atoms of various elements that disclose structure characteristics (Grebe and Singh, 2011; Kang, 2012). Mass spectrometers provide mass spectra which allow the exploitation of information about the analyte. A mass spectrometer produces ions from a specimen, screen the resulting mixture of ions according to their mass-to-charge (*m/z*) ratios, and delivers signals which are measures of relative abundance of each occurring ionic species. The principle of MS is to obtain ions from a specimen, to separate them according to their *m/z* ratios (it can be assimilated to the mass as most of the ion has usually a single charge), and to detect ions depending on their abundance. A mass spectrum of the molecule is then produced as a plot of ion abundance versus mass-to-charge (*m/z*) ratio. In mass spectra, the most intense peak or base peak is arbitrarily assigned the highest relative abundance of 100% and abundances of all other peaks are given as percentage of the base peak abundance ions obtained in MS provide information on the nature and structure of their precursor/ formal molecule (Hoffmann and Stroobant, 2007). A

typical mass spectrometry apparatus has four components as shown in Figure 2.1, which are all under vacuum (low pressure) to allow ions produced to reach the detector without unwanted collisions.

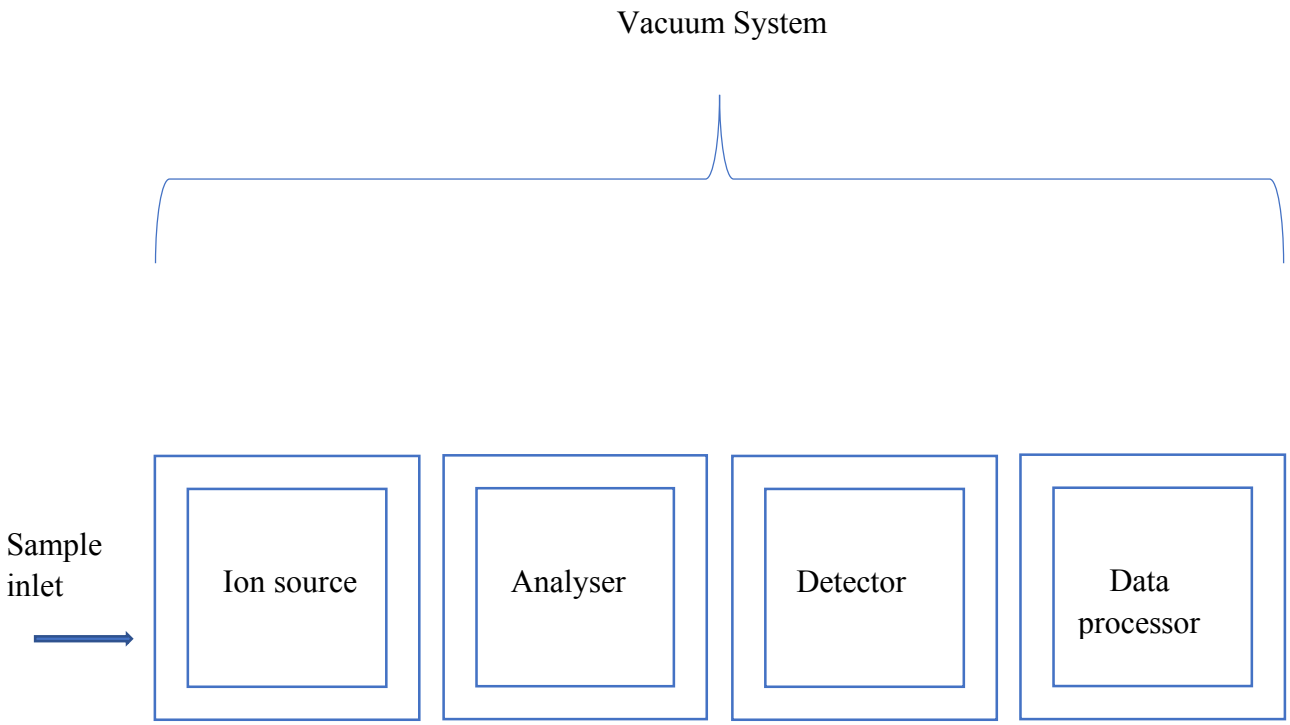
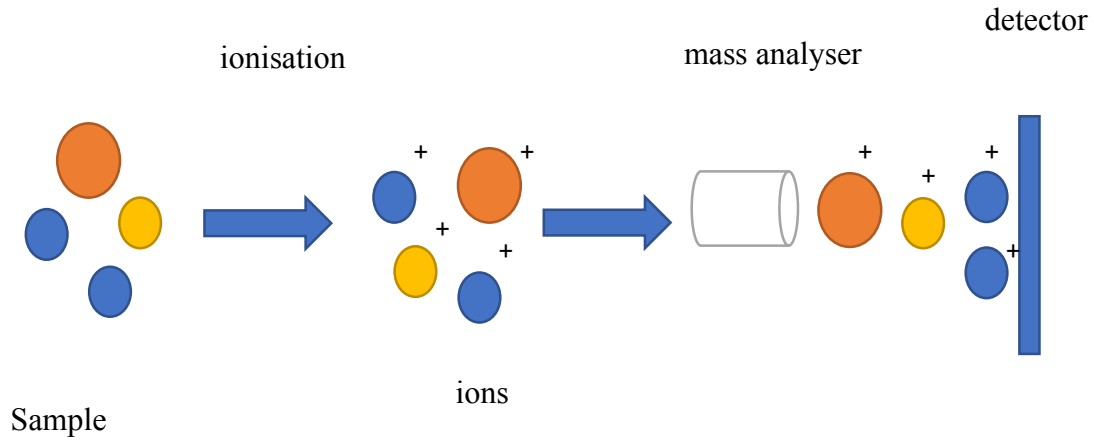


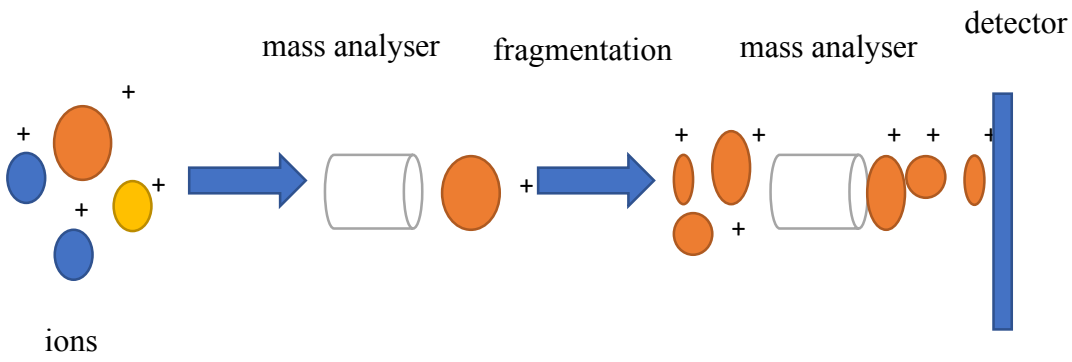
Figure 2.1. Basic diagram for a mass spectrometer comprising an ion source, an analyser, a detector and a data processor.

a



Simplified scheme of MS

b



Simplified scheme of MS-MS

Figure 2.2. Simplified scheme of mass spectrometry (MS) (a) and tandem mass spectrometry (MS-MS) (b). In MS-MS, after first analyser, analytes are fragmented and fragments analysed in second analyser

Sample inlet may be coupled to a liquid chromatography system (LC-MS), gas chromatography system (GC-MS) or capillary electrophoresis system (Hoffmann and Stroobant, 2007). Samples are ionised prior to analysis in the mass spectrometer. Only ions can be detected in MS and any particles that are not ionic can move freely from one point to another without being detected (Kang, 2012).

Ionisation is a process whereby an electrically neutral atom or molecule becomes electrically charged either by losing or gaining one or more of its extra nuclear electrons. All ions (positively or negatively charged atoms, group of atoms or molecules) can be analysed by mass spectrometer, although most of apparatus are used to investigate positive ions as there are more abundant in most ions sources than negative ions (Kang, 2012).

Multiple ionisation techniques are described in the literature for mass spectrometry, with some very energetic, causing extensive fragmentations and others softer, producing ions of the molecular species. Electron ionisation (EI), chemical ionisation (CI) and field ionisation (FI) are the most cited for gas-phase ionisation of volatile and thermally stable compounds. However, numerous compounds are not thermally stable and non-volatile and must be directly extracted from the condensed to the gas phase (Hoffmann and Stroobant, 2007). Matrix-assisted laser desorption/ ionisation (MALDI) is the most cited for non-volatile and thermally labile compounds, while, electrospray ionisation (ESI) is the preferred strategy of ionisation for sample in liquid form. Two types of ions sources exist: liquid-phase ion source and solid-state ion sources. In the first one, the analyte is in a solution which is introduced by nebulisation, as droplets into the mass spectrometer through vacuum pumping stages. Sources including electrospray, atmospheric pressure chemical ionisation and atmospheric pressure photoionisation correspond to this type. While, in solid-state sources, the analyte is in a non-volatile deposit. Sources are obtained through methods involving the introduction of a matrix (solid or viscous fluid) which is irradiated by energetic particles or photons that absorb ions close to the deposit surface. The resulted ions can then be extracted by an electric field and focus towards the analyser. Sources such as matrix-assisted laser desorption, secondary ion mass spectrometry, plasma desorption and field desorption use this system to produce ions (Hoffmann and Stroobant, 2007).

EI source is widely used in organic MS as it induces extensive fragmentations, however, the molecular ion is not always observed. Although, these fragmentations are useful as it provides structural information necessary to elucidate unknown analyte molecules. In this

technique, electron beam is produced by a heated filament of rhenium or tungsten wire at a temperature over 1000 k for electrons (e-) emission. The generated e- are accelerated to 70 eV resulting in an e- beam. Volatile sample or sample in a gaseous phase containing neutral molecules is introduced to the ion source in a perpendicular direction to the e- beam. The impact of e- on the analyte leads to loss of e-, producing cation M^+ or gain of e-, producing anion M^-.

CI, in another hand, produces ions with little excess energy which yield to a spectrum with less fragmentations and the molecular ions are generally observed. In this technique, the reagent gas is ionised due to e- impact. When analyte molecules are injected, they undergo many collisions with the reagent gas. In the cloud formed by reagent gas ions, there is a reaction leading to adduct ions formation, which are excellent proton donors for analyte. Both EI and CI sources are complementary (Hoffmann and Stroobant, 2007).

MALDI and ESI are soft ionisation techniques which lead to minimum fragmentation of the sample. The first one uses matrix (a metal plate where sample is spotted on and dried) which absorbs the laser light energy causing a small part of the target substrate to vaporise. As the process of forming analyte ions is not clear, it is believed that matrix protonates neutral analyte molecules after absorbing laser light energy. The second one, the analyte solution is introduced from a syringe pump or as an eluent flow from LC, through the electrospray needle that has a high potential difference applied to it, which forces the spraying of charged droplets. Solvent evaporation occurs as droplet moves towards counter electrode cone with droplets shrinking until it reaches the point that surface tension can no longer sustain the charge (the Rayleigh limit) causing droplets break which leads to smaller droplets. This process is repeated until all solvent is evaporated and charge passed on to analyte. The charged analyte molecules can have single or multiple charges (Hoffmann and Stroobant, 2007).

Among the wide variety of possible ionisation, proton transfer is the most common one. Usually, when analyte molecules M are introduced in the ionisation plasma, the reagent gas ions GH+ can easily transfer a proton to the molecules M and produce protonated molecular ions MH+. Almost all neutral substances are able to yield positive ions, whereas negative ions require the presence of acidic groups or electronegative elements to produce them. This specificity allows some selectivity for their detection in mixtures. Negative ions can be produced by capture of thermal electrons by the analyte molecule or by ion-molecule

reactions between analyte and ions present in the reagent plasma. Additionally, ion pair production is observed with a large variety of electron energies above 15 eV, process which principally leads to negative ion production under conventional EI conditions (Hoffmann and Stroobant, 2007)

Mass analyser

Once ions have been produced, they need to be separated according to their mass-to-charge ratio (*m/z*) by a mass analyser. Several types of mass analysers have been developed related to the sources and the principle of separation such as Quadrupole (Q) using *m/z* (trajectory stability), Ion trap (IT), Fourier transform ion cyclotron resonance (FTICR) and Fourier transform orbitrap (FT-OT), the three using m/z (resonance frequency) and Time-of-flight (TOF) using velocity (flight time). They all use static or dynamic electric and magnetic fields alone or combined and can be divided into two main classes. The first includes scanning analysers which transmit the ions of different masses successively along a time scale. They can be magnetic sector apparatus with a flight tube in the magnetic field, allowing only the ions of a given mass-to-charge ratio to go through at a given time, or quadrupole instruments. The second includes the dispersive magnetic analyser, the TOF mass analyser and the trapped-ion mass analyser which allow the simultaneous transmission of all ions. Mass analysers can be combined in sequence in order to enhance the strengths of the apparatus and allow a large number of experiments to be performed. For instance, triple quadrupole and hybrid apparatus including quadrupole TOF instruments or the ion-trap triple quadrupole instruments allow the decomposition of an ion selected in the first analyser and generate multiple fragments (Hoffmann and Stroobant, 2007). Once ions pass through the mass analyser, there are detected and transformed into a signal by a detector capable to generate an electric current that is proportional to their abundance. A computer registers the data given by mass spectrometer and stores spectra in the library (Hoffmann and Stroobant, 2007).

In this study, we used Liquid Chromatography combined with tandem Mass spectrometry (LC-MS/MS) with reference to known compound libraries and computer matching. This method is rapid, simple and sensitive and has several advantages including small sample volume, simple sample preparation, good chromatographic resolution, specific and sensitive mass spectrometric conditions. LC-MS/MS consists on the fragmentation of charged ions and the detection of the resulting fragments. It has thus, much higher selectivity and sensitivity

than LC-MS and makes it possible to elucidate metabolite structures. LC-MS is a hyphenated technique, combining the physicochemical separation power of liquid chromatography HPLC, and the mass analysis with the detection power of mass spectrometry. HPLC is very useful in this technique as it allows to remove the interferences from the sample that would impact the ionisation (Maheswari *et al.,* 2013). Usually, the sample is ionised in the source of ionisation- electrospray ionisation or atmospheric pressure chemical ionisation (ESI or APCI), passes into the first mass filter, then into the collision cell, followed by a 2nd mass filter, finally to reach the detector (Figure 2.2). In ESI, the solvent-analyte flow from the LC passes into the source through a positively charged, very narrow capillary, and gets nebulised as microscopic, positively charged solvent-analyte droplets. These droplets fly towards the negatively-charged faceplate, with solvent evaporating on the way, until they disintegrate in a Coulomb explosion, when the repulsive charge of their ionised components exceeds their surface tension. The individual ionised analyte molecules then pass through the faceplate entry hole into the mass spectrometer. In APCI, the solvent-analyte stream from the LC is vaporised by heated nebuliser gas and the polar components of the solvent (s) vapour are ionised by a high-current discharge of a Corona needle. The solvent molecules subsequently transfer their charge to ionisable analyte molecules, which pass through the faceplate entry hole into the mass spectrometer (Grebe and Singh, 2011). This apparatus allows the user to perform, within the instrument's sensitivity and mass resolution multiple reaction monitoring (MRM) (mode of separation) which allows highly specific detection of an analyte with m/Q selected in Q1 (first mass filter) that is known to fragment specifically into the product ion selected in Q3 (2nd mass filter before detection) (Kang, 2012).

The raw data processing was enabled by use of Progenesis QI software (NonLinear Dynamics, UK). Progenesis QI is a new generation of bioinformatics vehicle targeting small molecule discovery analysis for metabolomics. This novel software platform enable to perform chromatographic alignment, peak-picking, and mining of metabolomics data to quantify and then identify subsequent molecular interactions between groups of samples. The software utilises a MetaScope search engine for metabolite identification, search parameters to be defined by user to probe both home and open-accessed available databases. The interface is easy to use with the possibility to combine information for metabolite identification such as accurate mass, retention time, collision cross section and the experimental or theoretical fragment ions (Arapitsas and Mattivi, 2018). These properties can improve the confidence of metabolites identification while decreasing the number of false

positives. This expensive tool is the fastest, more powerful and the most stable on the market to date, giving consistent high quality results, and were provided by Mass spectrometry centre of Oregon State University.

We report outcomes of analytical methodologies applied on *T. iboga* extracts in order to measure ibogaine concentrations from the plant extract for further experiments and also for the identification of other known or unknown compounds, never reported before for this plant.

2.2. Materials and Methods

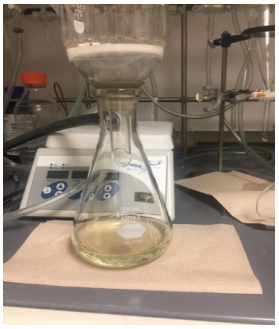
2.2.1. Plant material and preparation of *T. iboga* extract for quantitative and qualitative analysis

Root bark of *T. iboga* was provided by the Institute of Pharmacopoeia and Traditional Medicine (IPHAMETRA) in Libreville, Gabon voucher no 20358 deposited at the National Herbarium of Gabon. The barks of *T. iboga* were collected in Gabonese forest (at Libreville) during harvest time by botanists from IPHAMETRA and brought to Oregon (USA) in air-free plastic bags to avoid humidification. *T. iboga* extract for analytical chemistry study was prepared according to the method of Sadoon *et al.,* (2014) for optimal extraction result as traditional method do use analytical methods. Briefly, root barks chips were ground using a coffee grinder to obtain a fine powder. The powder obtained (50 g) was macerated in distilled water with magnetic stirring for 24h at room temperature. The slurry was filtered and the residue rinsed twice with distilled water. The water extract was adjusted to pH 9 with ammonium hydroxide and then extracted five times with 25 mL of chloroform in the fume hood. Anhydrous sodium sulphate (50 mg) was added to the combined chloroform extracts and the chloroform layer was filtered before drying under vacuum using a rotary evaporator. 4 g of dry residue was obtained and the different steps are shown in Figure 2.3 below. The residue (*T. iboga* organic extract) was characterised by LC-MS/MS, using the facilities of the Mass Spectrometry Centre at Oregon State University, and used for preparing the iboga-fortified diet for experiments of chapter 5.

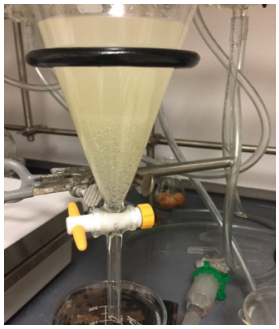
(a) *T. iboga* dried stem barks

(b) Grounded powder

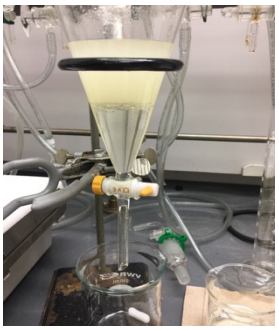
(c) Maceration in distilled water



(d) Filtration



(e) Chloroform extraction



(f) Chloroform layer separation

(g) Drying by rotavapor

Figure 2.3. Steps of the preparation of *T. iboga* extract for chemical characterisation (a) T. iboga dried stem barks, (b) Ground powder, (c) Maceration in distilled water, (d) Filtration, (e) Chloroform extraction, (f) Chloroform layer separation, (g) Drying by rotavapor (Linus Pauling Centre, February 2017, Bayissi Bading-Taïka)

2.2.2. Measurement of ibogaine concentration in *T. iboga* extract by liquid chromatography tandem mass spectrometry (LC-MS/MS)

A Shimadzu Prominence HPLC system (Shimadzu, Colombia, MD USA) was used for chromatography of the *T. iboga* extract. Chromatographic separation of ibogaine was achieved on an Agilent Poroshell 120 EC-C8 column (2.1 x 50 mm, 2.7 µm) eluted with solvent A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The column effluent was introduced into the electrospray ionisation source of an applied Biosystems 4000 QTRAP (Figure 2.4) hybrid linear ion trap-triple quadrupole instrument (Sciex, Concord, ON, Canada) operated at a source temperature of 400^{o}C with a needle

voltage of 5 kV in the positive ion mode. Ibogaine was detected using multiple reaction monitoring (MRM), using mass to charge ratio (*m/z*) 311.4 > 174.1 as the qualifier transition and m/z 311.4 > 122.1 as the quantifier transition. The concentration of ibogaine in the bark extract was determined by external calibration method using a standard of ibogaine obtained from Cambridge Chemicals (Woburn, Massachussetts, USA).

Figure 2.4. 4000 Q Trap hybrid (linear ion-trap triple quadrupole instrument (Mass Spectrometry Center, Oregon, April 2017, Bayissi Bading-Taïka)

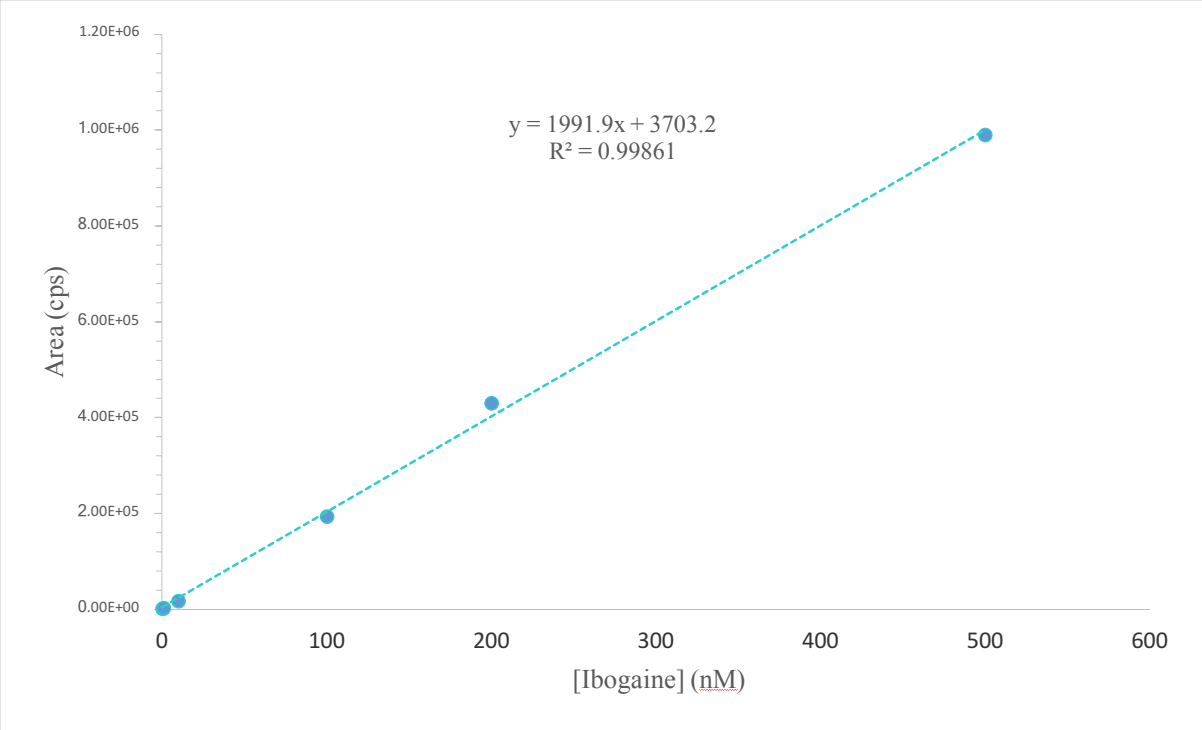


Figure 2.5. Standard curve of pure ibogaine

2.2.3. LC-MS detection of other phytochemicals in *T. iboga* root bark.

Pieces of iboga root bark were ground to a fine powder by using a coffee grinder. Ten milligrams of bark powder were extracted with 10 mL of 70 % aqueous methanol (v/v) by sonication for 30 min at 25 ºC. After sonication, the extract was centrifuged at 15,000 rpm for 10 min. Other phytochemicals from *T. iboga* were investigated by chromatographic separation, conducted using a Shimadzu Nexera UPLC system equipped with an Inertsil Phenyl-3 column (150 × 4.6 mm, 5 µm). The injection volume was 10 µl. Mobile phase A was water with 0.1 % formic acid, and mobile phase B was methanol with 0.1% formic acid. The gradient started with 5% B and was held for 1 min at 5% B, followed by a 10-min linear gradient from 5% to 30 % B. The gradient was increased linearly to 100% B at 20 min, held for 10 min at 100% B and, finally, stepped back to 5% B to equilibrate the column. The flow rate was 0.4 mL min^{-1}, with the auto-sampler set at 10 ºC, and the column temperature was maintained at 50 ºC. The column effluent was introduced into a QToF mass spectrometer (AB Sciex Triple TOF 5600) equipped with a TurboSpray electrospray ionization source operated in the negative and positive ionization modes. The instrument was operated in the

information-dependent acquisition (IDA) mode using a collision energy of 35 V. For analysis of phenolic compounds, compound identification was based on accurate mass, isotopic similarity, retention time and MS/MS spectral comparison with authentic compounds. A targeted analysis was performed for 1,3-di-*O*-caffeoylquinic acid, 1,5-di-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, caffeic acid, catechin, dihydrocaffeic acid, dihydroferulic acid, dihydroisoferulic acid, epicatechin, epigallocatechin, ferulic acid, isoferulic acid, kaempferol, naringin, quercetin and rutin. An external calibration curve was prepared for all precursor ions (Calibration points: 0.05, 0.1, 0.5, 1.0, 5.0 mg L^{-1}, R values > 0. 995).

2.2.4. Qualitative analysis of *T. iboga* extract (untargeted compounds)

For qualitative (untargeted) analysis, LC-MS/MS data were acquired on the AB Sciex Triple TOF 5600 system using the same chromatographic separation. In order to detect alkaloids as protonated molecular species, MS acquisition was performed in the positive ionisation mode. Raw data were processed by using Progenesis QI software (NonLinear Dynamics, Newcastle upon Tyne, UK). Progenesis QI is a bioinformatics tool for small molecule discovery and analysis. Over 6,300 raw mass spectra were collected. Molecules were assigned by extensive querying and comparison of molecular features, namely accurate mass-to-charge ration (*m/z*), MS/MS fragmentation patter, and isotopic similarity, against Metlin^{TM} MS/MS, HMDB (Wishart *et al.*, 2018), KEGG and KnapSack data bases. In the Progenesis QI workflow, we considered a confidence score higher than 50 sufficient for assignment of constituents in *T. iboga* extract. A score higher than 50 for a putative compound is reached when the deviation of the accurate mass from the extract mass is lower than 5 ppm combined with an isotopic pattern similarly > 90%.

2.3. Results

2.3.1. Estimation of ibogaine and total alkaloid concentration in *T. iboga* extract

Figure 2.6 below, shows the LC-MS/MS chromatograms of the ibogaine standard and ibogaine content in the bark aqueous extract of *T. iboga.* Different solvents (acid, alcohol and water) were used for extraction (Table 2.1 and 2.3) and allowed the determination of ibogaine concentration in the samples (Table 2.2). The analyte peaks of ibogaine detection from samples of acid/water extract diluted 10^5 and 10^4 times with acetonitrile are recorded on Table 2.1.

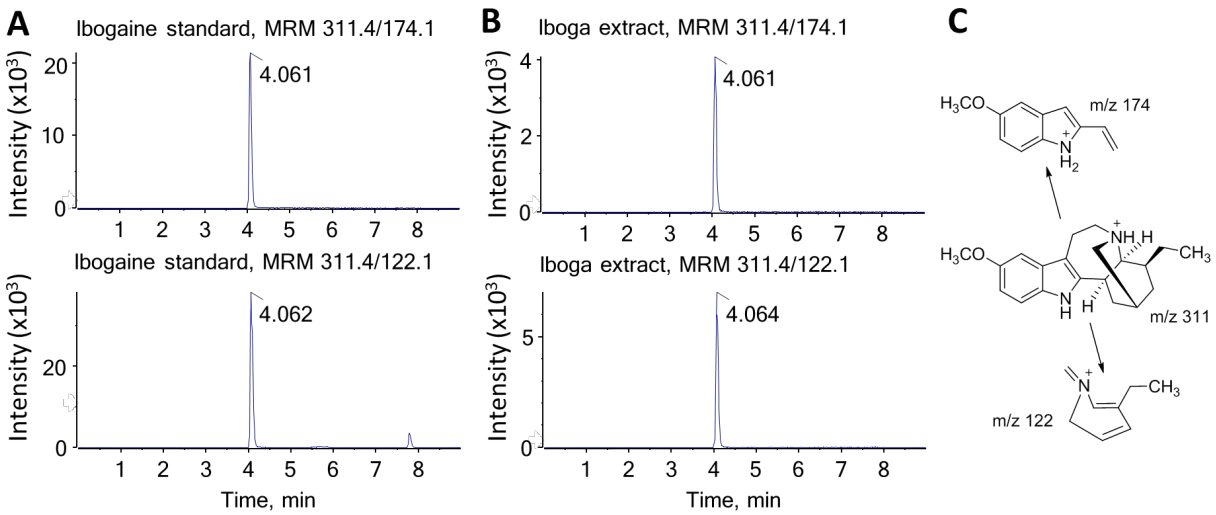


Figure 2.6. LC-MS/MS chromatograms of ibogaine. (**A**) 10 nM ibogaine standard. (**B**) ibogaine in root bark extracts. (**C**) Multiple reaction monitoring (MRM) transitions: *m/z* 311.4 > 174.1 (qualifier MRM), *m/z* 311.4 > 122.1 (quantifier MRM)

Plant extract was dissolved in 1ml of chloroform and then diluted 10^5 times with acetonitrile: water ratio 1:1.

- Sample 1 (S1) contained 21 x 10^5 (nM) of ibogaine in 1 ml solution, thus 21 µmol of ibogaine in 100 mg of plant powder.

This 2.1 µmol x 310 µg/ µmol (MW of pure ibogaine) gave 651 µg of ibogaine in S1.

- Sample 2 (S2) contained 20.5 x 10^5 (nM) of ibogaine in 1 ml solution, thus 2 µmol of ibogaine in 100 mg of plant powder.

This 2 µmol x 310 µg/ µmol gave 620 µg of ibogaine

- Sample 3 (S3) contained 26.5 x 10^5 (nM) in 1 ml, thus 2.6 µmol of ibogaine in 100 mg of plant powder.

This 2.6 µmol x 310 µg/ µmol gave 806 µg of ibogaine.

Ibogaine detection from ethanol and water extracts is reported in table 5. Dilution factor was x10^5 (with acetonitrile: water = 1:1, v/v). Plant extract was dissolved in 1ml chloroform and then diluted 10^5 times. We did not consider extraction efficiency thus, 312 x 10^5 (nM) in 1 ml solution gave 31.2 µmol of ibogaine in 500 mg of plant powder. Then, 31.2 µmol x 310 µg / µmol (MW of ibogaine) gave 9.67 mg of ibogaine in 500 mg of plant powder extracted with H_2O.

Table 2.1. Ibogaine content in *T. iboga* extracts (HCL/H_2O extracts) (*m/z* 311.4 >174.1 and *m/z* 311.4 >122.1)

Samples Name	**Analyte Peak Area (counts)**	**Analyte Peak Height (cps)**
std 100 pM	3.17 x 10^2	1.46 x 10^2
std 100 pM	1.81 x 10^2	1.02 x 10^2
std 1nM	1.83 x 10^3	5.41 x 10^2
std 1nM	1.15 x 10^3	3.74 x 10^2
std 10 nM	1.87 x 10^4	5.51 x 10^3
std 10 nM	9.89 x 10^3	2.84 x 10^3
std 100 nM	1.77 x 10^5	4.74 x 10^4
std 100 nM	9.70 x 10^4	2.68 x 10^4
std 200 nM	3.54 x 10^5	9.01 x 10^4

std 200 nM	1.94×10^5	4.82×10^4
std 500 nM	7.66×10^5	1.84×10^5
std 500 nM	4.20×10^5	1.02×10^5
std 1 µM	1.39×10^6	3.21×10^5
std 1 µM	7.43×10^5	1.67×10^5
HCL/H_2O (diluted x10^5)	5.67×10^5	1.31×10^5
HCL/H_2O (diluted x10^5)	3.01×10^5	7.09×10^4
s1 10^5	2.99×10^4	8.05×10^3
s1 10^5	1.61×10^4	4.28×10^3
s2 10^5	2.93×10^4	8.33×10^3
s2 10^5	1.55×10^4	4.02×10^3
s3 10^5	3.79×10^4	9.73×10^3
s3 10^5	2.00×10^4	5.10×10^3
s1 10^4	2.84×10^5	7.05×10^4
s1 10^4	1.52×10^5	3.72×10^4
s2 10^4	2.79×10^5	7.02×10^4
s2 10^4	1.50×10^5	3.55×10^4
s3 10^4	3.43×10^5	8.63×10^4
s3 10^4	1.90×10^5	4.59×10^4

Extraction of 500 mg of iboga powder using acid/water solvent lead a better yield of ibogaine content (9.67 mg/ml for 500 mg of iboga powder) than extraction with ethanol/water (2.20

mg/ml for 500 mg of iboga powder) or with water only (0.7 mg/ml for 100 mg of iboga powder, which would take to 3.5 mg/ml for 500 mg of iboga powder considering optimal yield). The powdered root bark of iboga (100 mg) contained 1.93 % ibogaine (w:w).

Table 2.2. Ibogaine concentrations in *T. iboga* extracts (HCL/H_2O extracts)

Samples (diluted x10^5)	**Area (cps)**	**Concentration (nM)**
s1	2.99 x 10^4	20.9
s2	2.93 x 10^4	20.5
s3	3.79 x 10^4	26.5

Table 2.3. Ibogaine content in *T. iboga* extracts (EtOH/ H_2O, H_2O extracts) (*m/z* 311.4>174.1 and *m/z* 311.4>122.1)

Sample Name	**Analyte Peak Area (counts)**	**Analyte Peak Height (cps)**
std 100 pM	1.49 x 10^3	3.82 x 10^2
std 1 nM	3.09 x 10^3	9.36 x 10^2
std 10 nM	1.83 x 10^4	5.05 x 10^3
std 100 nM	1.94 x 10^5	4.73 x 10^4
std 200 nM	4.31 x 10^5	9.12 x 10^4
std 500 nM	9.90 x 10^5	2.19 x 10^5
std 1 µM	1.61 x 10^6	3.40 x 10^5
EtOH H_2O (diluted x10^5)	1.46 x 10^5	3.22 x 10^4
EtOH H_2O (diluted x10^4)	1.10 x 10^6	2.36 x 10^5

H_2O (diluted x10^5)	6.25 x 10^5	1.37 x 10^5
H_2O (diluted x10^4)	3.83 x 10^6	7.55 x 10^5

Table 2.4. Ibogaine concentrations from different extracts

Samples	**Ibogaine concentration (mg/mL)**
EtOH/H_2O (diluted x 10^5 - 500 mg of plant)	2.20
HCL/H_2O (diluted x 10^5 - 500 mg of plant)	9.67
H_2O (diluted x 10^5 - 100 mg of plant)	0.7
H_2O (diluted x 10^6 - 50 g of plant)	8.39

2.3.2 Analysis of *T. iboga* extract by mass spectrometry (MS)

Analysis of over 6,300 mass spectra recorded in the positive ionisation mode revealed 38 compound identifications with high confidence using Progenesis QI software (score > 50, Table 2.5). Among them,30 compounds are alkaloids previously reported in plants (Afendi *et al.,* 2012; Kanehisa *et al.,* 2017; Smith *et al.,* 2005) (MetlinTM, HMDB, KEGG or KnapSack, table), with ibogaine as the major alkaloid (Figure 2.6 A-C).

Further analysis of 70 % aqueous methanol extract by negative ion mass spectrometry using several standards, showed the presence of five phenolic compounds, including 3-O-caffeoylquinic acid (Table 2.5, Figure 2.8).

The calculated amount of the 3-O-caffeoylquinic acid was 0.97 mg/g in 100 mg of plant root barks.

Table 2.5. Phytochemicals detected in *T. iboga* root barks. Compounds detected in the positive electrospray ionization (ESI) mode were assigned using Progenesis QI (score>50). For compounds detected by negative ESI-MS, identity was assigned based on comparison of *m/z*, retention time and MS/MS spectra comparison with authentic standards ([1]Afendi *et al.,* 2012; [2]Bartlett *et al.,* 1957; [3]Harborne and Baxter, 1999; [4]Taylor, 1968).

Alkaloid	**m/z**	**R.T (min)**	**Detected Adducts**	**Formula**	**Composition %[a]**
Alkaloids, positive ESI					
Ibogaine[1-3]	311.2115	21.63	$[M+H]^+$	$C_{20}H_{26}N_2O$	24.60
Iboxygaine[4]	327.2063	21.46	$[M+H]^+$	$C_{20}H_{26}N_2O_2$	11.00
Ibogaline[4]	341.2220	21.39	$[M+H]^+$	$C_{21}H_{28}N_2O_2$	10.77
Alloibogaine	325.1908	22.01	$[M+H]^+$	$C_{20}H_{24}N_2O_2$	8.21
Catharanthine[4]	337.1912	22.77	$[M+H]^+$	$C_{21}H_{24}N_2O_2$	5.69
Ibogamine[1-3]	281.2010	21.58	$[M+H]^+$	$C_{19}H_{24}N_2$	3.37
Noribogaine	297.1958	20.66	$[M+H]^+$	$C_{19}H_{24}N_2O$	2.76
Voacangine[1-3]	369.2167	21.82	$[M+H]^+$	$C_{22}H_{28}N_2O_3$	1.87
Yohimbine	355.2013	19.89	$[M+H]^+$	$C_{21}H_{26}N_2O_3$	1.81
Hydroxy-ibogamine (isomer of noribogaine)	297.1958	21.41	$[M+H]^+$	$C_{19}H_{24}N_2O$	1.79
Quinidine	325.1912	18.64	$[M+H]^+$	$C_{20}H_{24}N_2O_2$	1.01
Coronaridine[1-3]	339.2054	22.86	$[M+H]^+$	$C_{21}H_{26}N_2O_2$	0.97
11-O-demethyl-17-O-deacetylvindoline	383.1965	18.18	$[M+H-H_2O]^+$	$C_{22}H_{28}N_2O_5$	0.86
Affinisine	309.1959	20.47	$[M+H]^+$	$C_{20}H_{24}N_2O$	0.84
17-O-acetylnorajmaline	355.2015	23.68	$[M+H]^+$	$C_{21}H_{26}N_2O_3$	0.55
Affinine	325.1908	23.55	$[M+H]^+$	$C_{20}H_{24}N_2O_2$	0.43
Rhazidigenine N-oxide	315.2063	19.49	$[M+H]^+$	$C_{19}H_{26}N_2O_2$	0.40
1,2-Dihydrovomilenine	353.1858	23.95	$[M+H]^+$	$C_{21}H_{24}N_2O_3$	0.23
11-Hydroxyyohimbine	371.1958	20.03	$[M+H]^+$	$C_{21}H_{26}N_2O_4$	0.20
16-Methoxytabersonine	367.2010	22.43	$[M+H]^+$	$C_{22}H_{26}N_2O_3$	0.19
N^4-demethylechitamine	371.1959	21.72	$[M+H]^+$	$C_{21}H_{26}N_2O_4$	0.19

Quinine-N-Oxide	341.1855	19.42	$[M+H]^+$	$C_{20}H_{24}N_2O_3$	0.18
Iboluteine	327.2052	23.21	$[M+H]^+$	$C_{20}H_{26}N_2O_2$	0.08
Strictosidine aglycone	369.1808	22.68	$[M+H]^+$	$C_{21}H_{24}N_2O_4$	0.08
Aspidodasycarpine	353.1859	23.54	$[M+H-H_2O]^+$	$C_{21}H_{26}N_2O_4$	0.05
Reserpic acid	401.2069	20.48	$[M+H]^+$	$C_{22}H_{28}N_2O_5$	0.03
Ajmaline	327.2061	29.40	$[M+H]^+$	$C_{20}H_{26}N_2O_2$	0.02
Jerantinine B	399.1909	19.11	$[M+H]^+$	$C_{22}H_{26}N_2O_5$	0.02
Vinervine	321.1596	24.46	$[M+H-H_2O]^+$	$C_{20}H_{22}N_2O_3$	0.01
Ellipticine	247.1222	25.46	$[M+H]^+$	$C_{17}H_{14}N_2$	0.01
Unknown alkaloids (48)[b]	N/A	N/A	$[M+H]^+$	N/A	21.77

Polyphenols and other compounds, positive ESI					
4-Guanidinobutanoic acid	146.0918	6.13	$[M+H]^+$	$C_5H_{11}N_3O_2$	N/A
Betaine	118.0859	4.52	$[M+H]^+$	$C_5H_{11}NO_2$	N/A
Catechin	291.0859	13.76	$[M+H]^+$, $M+Na]^+$	$C_{15}H_{14}O_6$	N/A
Choline	104.1071	6.48	$[M+H]^+$	$C_5H_{13}NO$	N/A
Epicatechin	291.0861	15.97	$[M+H]^+$, $[M+Na]^+$	$C_{15}H_{14}O_6$	N/A
Glutamate	148.0599	4.59	$[M+H]^+$	$C_5H_9NO_4$	N/A
Indoleacetaldehyde	177.1015	10.27	[M+H]	$C_{10}H_9NO$	N/A
Phosphocholine	184.0728	5.29	$[M+H]^+$	$C_5H_{14}NO_4P$	N/A

Phenolic compounds, negative ESI					
5-O-Caffeoylquinic acid	353.0461	11.86	$[M-H]^-$	$C_{16}H_{18}O_9$	<LOQ[c]
1,3-Di-O-caffeoylquinic acid	515.1217	N/A	$[M-H]^-$	$C_{25}H_{24}O_{12}$	<LOD[d]

1,5-Di-O-caffeoylquinic acid	515.1210	N/A	$[M-H]^-$	$C_{25}H_{24}O_{12}$	<LOD
3,4-Di-O-caffeoylquinic acid	515.1207	N/A	$[M-H]^-$	$C_{25}H_{24}O_{12}$	<LOD
3,5-Di-O-caffeoylquinic acid	515.1208	N/A	$[M-H]^-$	$C_{25}H_{24}O_{12}$	<LOD
3-O-Caffeoylquinic acid	353.0409	15.28	$[M-H]^-$	$C_{16}H_{18}O_9$	0.97±.015 mg/g bark
4,5-Di-O-caffeoylquinic acid	515.1210	N/A	$[M-H]^-$	$C_{25}H_{24}O_{12}$	<LOD
4-O-Caffeoylquinic acid	353.0508	N/A	$[M-H]^-$	$C_{16}H_{18}O_9$	<LOD
Caffeic acid	179.0346	N/A	$[M-H]^-$	$C_9H_8O_4$	<LOD
Catechin	289.0729	N/A	$[M-H]^-$	$C_{15}H_{14}O_6$	<LOD
Dihydrocaffeic acid	181.0497	N/A	$[M-H]^-$	$C_9H_{10}O_4$	<LOD
Dihydroferulic acid	195.0651	N/A	$[M-H]^-$	$C_{27}H_{30}O_{16}$	<LOD
Epicatechin	289.0707	N/A	$[M-H]^-$	$C_{15}H_{14}O_6$	<LOD
Epigallocatechin	305.0668	N/A	$[M-H]^-$	$C_{15}H_{14}O_7$	<LOD
Ferulic acid	193.0497	19.6	$[M-H]^-$	$C_{10}H_{10}O_4$	<LOQ
Iso Ferulic acid	193.0499	N/A	$[M-H]^-$	$C_{10}H_{10}O_4$	<LOD
Kaempferol	285.0405	21.95	$[M-H]^-$	$C_{15}H_{10}O_6$	<LOQ
Naringin	579.1718	N/A	$[M-H]^-$	$C_{27}H_{32}O_{14}$	<LOD
Quercetin	301.0345	21.08	$[M-H]^-$	$C_{15}H_{10}O_7$	<LOQ
Rutin	609.1502	19.01	$[M-H]^-$	$C_{27}H_{30}O_{16}$	<LOQ

[a] Assuming comparable detector responses for detected alkaloids, all which contain two nitrogen atoms indicative of indole alkaloids.

[b] Compounds with a mass defect ranging from 0.1939 to 0.2100.

[c] LOQ Calibration quantification limit evaluated as S/N ratio 10:1; LOQ > 3.3 μg L^{-1}

[d] LOD- Calibration detection limit evaluated as S/N ratio 3:1; LOD > 1.0 μg L^{-1}

<LOD not detected; <LOQ detected below LOQ

2.3.3 Mass defect analysis

Alkaloids are suitable for LC-MS analysis in positive ionisation due to nitrogen presence in their structure. Filtering of data based on mass defect is a useful post-acquisition data processing technique for distinguishing metabolites and chemical components in traditional medicines (Xie *et al.,* 2012), and allowing the classification of chromatographic peaks in chemical families on the mass acquired in high resolution. To screen *T. iboga* alkaloids, we used as reference the mass defect range obtained from the monoisotopic neutral mass of previously reported alkaloids in *T. iboga* (Afendi *et al.,* 2012; Barlett *et al.,* 1958; Harborne *et al.,* 1999). The absolute mass defect or mass excess of alkaloids varied from 0.1939 to 0.2100. Major alkaloids reported in KnapSack database (Afendi *et al.,* 2012) for *T. iboga* possess a mass defect in the range of 0.201 to 0.212 (Harborne, 1999). In this experiment, we achieved 5 ppm or better as accurate mass, thus, we used a screening range from 0.1900 to 0.2190 (Figure 2.7).

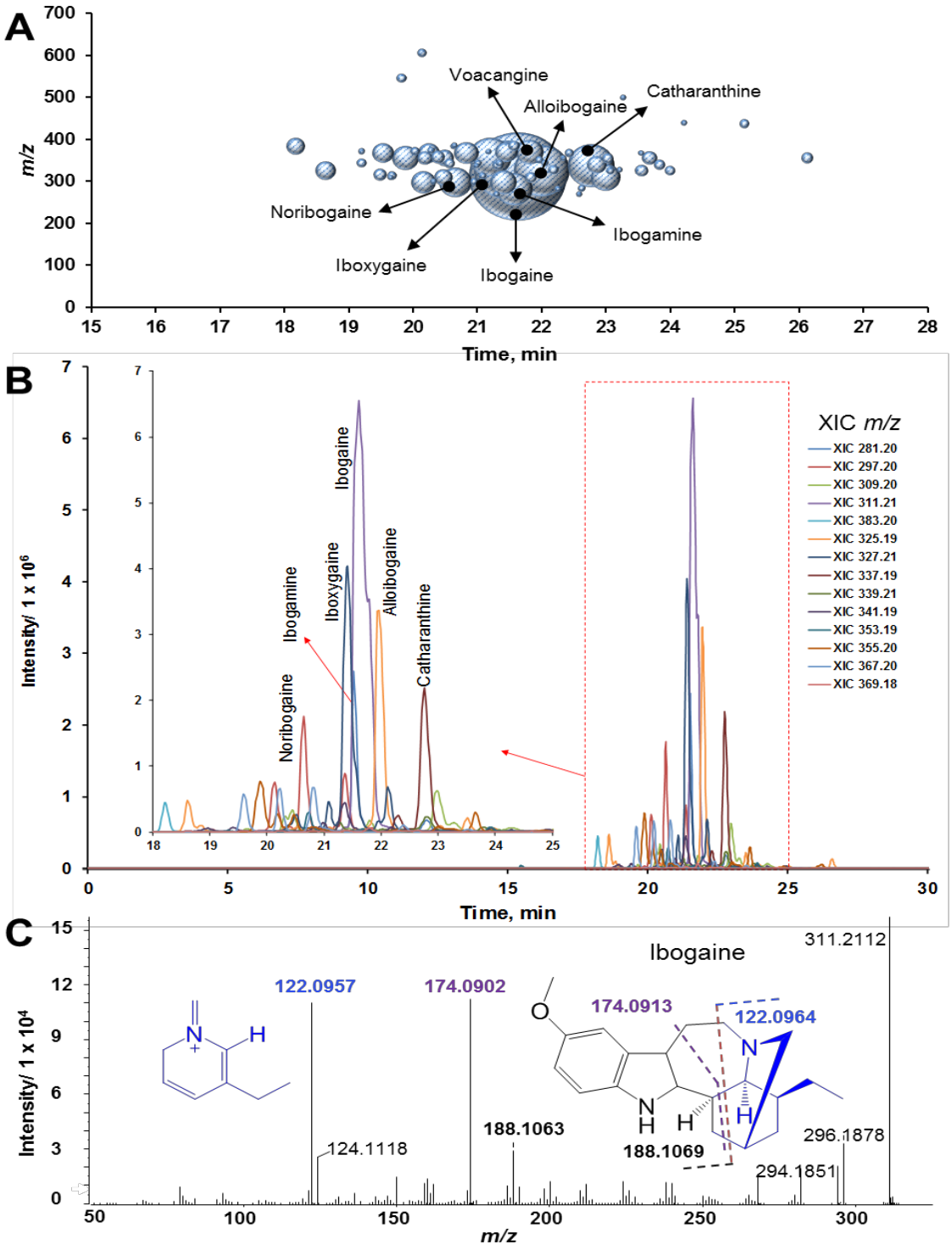


Figure 2.7. LC-HRMS/MS analysis of *T. iboga* extract in positive ionisation mode (1 mgmL^{-1} of dry mass re-suspended in 70% aqueous ethanol). **A**. Molecular features detected in *T. iboga* extract with a mass defect in a range from 0.1900 to 0.2190. The size of the circles corresponds to the magnitude of the chromatographic peak area of a particular feature; the largest circle represents ibogaine. **B**. [M^+H^+] extracted ion chromatograms (XIC) of indole alkaloids. **C**. Fragmentation spectrum and proposed dissociation of ibogaine.

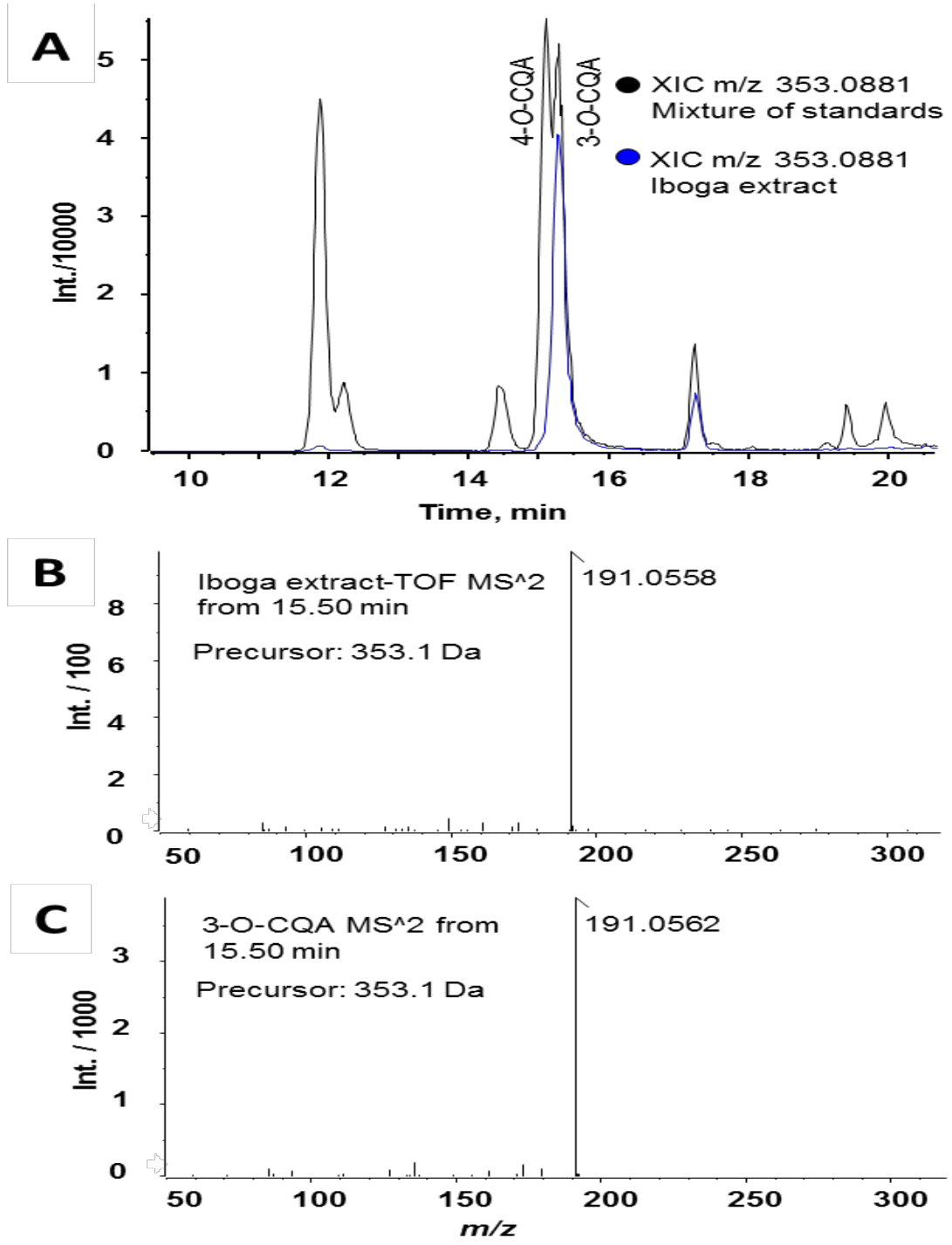


Figure 2.8. LC-HRMS/MS analysis of *T. iboga* root bark extract. **A**. extracted ion chromatogram $[M-H]^+$ *m/z* 353.09, 10 µL injection. Black line- 1 mg L^{-1} of standards 3-*O*-caffeyolquinic acid (3-*O*-CQA) and 4-*O*-caffeylquinic acid (4-*O*-CQA). Blue line- 1 mg mL^{-1} of bark extract, both re-suspended in 70% aqueous methanol. **B**. Fragmentation spectrum of 3-*O*-CQA detected in *T. iboga* extract. **C**. Fragmentation spectrum of 3-*O*-CQA standard (1mg L^{-1}).

2.4. Discussion

T. iboga is a plant belonging to the Apocynaceae family (Bading-Taika *et al.,* 2018), rich in indole alkaloids which are low molecular weight compounds containing nitrogen (Dewick, 2009). We extracted alkaloids from the powdered *T. iboga* root barks using extraction method as close as possible to the method used by traditional practitioners in Gabon. Therefore, water extraction which was not the most efficient method (ibogaine content of 1.93% vs 7.2% reported by Mazoyer *et al.,* 2013) was selected for further experiments in our project. We estimated the total alkaloid content of the bark to be 7.8% (Table 2.2). This number is higher than the 5-6% indole alkaloids in the root barks previously reported by Delourne-Houdé, 1948; Marion, 1952 and Dewick, 2002. However, this number is lower than the alkaloid content of powdered *T. iboga* root (7.2% ibogaine, 0.6% ibogamine) previously reported by Mazoyer *et al.* (2013). This discrepancy may be related to the techniques used for the extraction. Organic extraction was performed in that study, and it is the more used for complex molecules such as ibogaine (Mazoyer *et al.,* 2013; Dewick, 2009).

The chromatographic peak area of ibogaine comprised 24.6% of the total area of chromatographic peaks assigned to known and unknown alkaloids based on their common mass defect. Data are in accord with those reported by Kontrimaviciute *et al.,* 2006. Only seven alkaloids have been previously reported for *T. iboga* (Table 2.5) which are ibogaine, iboxygaine, ibogaline, alloibogaine, catharanthine, ibogamine and noribogaine (and its isomer hydroxy-ibogamine (Taylor, 1968; Alper, 2001; Gaignault and Delourme-Houdé, 1977; Bowen, 2001), all of which we identified by the plant metabolomics approach at the Mass Spectrometry Centre of Oregon State University (OSU, Table 2.5). Moreover, we tentatively (fingerprinting using libraries and data comparison) assigned 23 other alkaloids not previously reported for *T. iboga* but reported for other plants species including plants belonging to Apocynaceae family (Table 2.5), such as *Voacanga africana, Ervatamia coronaria* Stapf*,* and *Vinca roseus* Linn (Taylor, 1968). Unfortunately, as plant metabolome constitute heterogeneous group of molecules with complex physicochemical properties and wide fluctuation in concentration ranges, it is currently impossible to extract and analyse all metabolites of plant in single analysis. The currently characterised plant metabolites represent only a small fraction of the whole metabolome (Tugizimana *et al.,* 2013).

To the best of our knowledge, numerous of these ibogaine congeners possess biological properties such as voacangine, that is a small molecule largely occurring in *Voacanga africana* was reported to inhibits angiogenesis *in vitro* and *in vivo* (Kim *et al.,* 2012).

Yohimbine from *Rauwolfia* root bark may induce impulsivity and reinstate seeking of various drugs through κ opioids receptors (Funk *et al.,* 2019; Stavrinides *et al.,* 2015). Hydroxyl-ibogamine and coronaridine showed anti-addictive and anti-tumour properties (Arias *et al.,* 2011; Arias *et al.,* 2017). Quinidine has been identified as an effective agent for both atrial and ventricular arrhythmias in patient with Brugada syndrome (Halperin *et al.,* 2018; Yang *et al.,* 2009; Stavrinides *et al.,* 2015). Affine and affinisine might be chemotherapeutic for melanoma cells (Rosales *et al.,* 2019; Yang *et al.,* 2010). The 1,2-hihydrovomilenine, ajmaline, reserpic acid and strictosidine aglycone from *Rauwolfia species* might also be anti-arrhythmic (Von Schumann *et al.,* 2002; Stavrinides *et al.,* 2015). Ellipticine quinone has shown anti-proliferative activity in cell lines (Nishiyama *et al.,* 2017). Aspidocarpine from *Aspidosperma cuspa* (Kunth) Blake has shown anti-nociceptive activity that may be mediated through opioid receptors (Pérez *et al.,* 2012). Jerantinine B isolated from *Tabernaemontana corymbosa* has shown potential as chemotherapeutic agent (Qazzaz *et al.,* 2016), and ellipticine exhibited anticancer properties due to its size, shape and planarity (Zsila and Samsonov, 2018). Anti-addictive, anticancer and anti-HIV properties have been widely reported for *T. iboga* (Bading Taika *et al.,* 2018; Souza *et al.,* 2011).

For the first time, phenolic compounds are cited for *T. iboga* in chemistry analytical reports. The amount of 3-O-caffeoylquinic acid (3-CQA) in *T. iboga* root bark, extracted with 70% of aqueous methanol, was 0.97 mg/g of powdered root barks. 3-O-CQA, also called chlorogenic acid (CGA) is a bioactive natural phenolic compound found in food. CGA is formed by esterification of trans-cinnamic acids such as caffeic, coumaric and feluric acid, with (-)-quinic acid. It contains a group major of isomers including 3-CQA, 4-O-caffeoylquinic acid (4-CQA), and 5-O-caffeoylquinic acid (5-CQA) (Bagdas *et al.,* 2015). Several pharmacological properties of CGA have been reported including antioxidant, free radical scavenging, anti-inflammatory, radio protective, anti-tumour, anti ulcerogenic, and analgesic activities (Bagdas *et al.,* 2015; Jabeur *et al.,* 2016).

Thus here, we revealed known and unknown compounds of *T. iboga* aqueous extract including alkaloids and chlorogenic acid that may support anti-diabetic potential activity investigated in rat pancreatic islets in the next chapter.

Chapter 3: *In vitro* investigations into the effects of aqueous extracts from *T. iboga* and *G. tessmannii* on glucose-stimulated insulin release from isolated rat pancreatic islets

3.1. Introduction

T. iboga or "iboga" belonging the Apocynaceae family is one of the most widely used medicinal plants in Gabon due to religious beliefs. It is believed to help contact with ancestors and develop mind in Gabonese religious ritual such as 'Bwiti', when large amounts are chewed and consumed as doses from 100 mg to 1g of ibogaine cause trance-like visual and auditory hallucinations (Mazoyer *et al.,* 2013). However, small amounts are ingested to offset hunger and fatigue (Souza *et al.,* 2011; Chèze *et al.,* 2008). *T. iboga* is used traditionally for numerous medicinal purposes including drugs addiction, HIV1, psychological troubles, and as spasmolytic (Bading-Taika *et al.,* 2018). Ethnobotanical surveys conducted in the province of Estuaire (Gabon) show that many healers use *T. iboga* for the treatment of many diseases, including diabetes (Tjeck *et al.,* 2017).

Previous *in vitro* studies of *T. iboga* aqueous extract have demonstrated its sulfonylureas-like action on insulin release. Indeed, *T. iboga* aqueous extracts (1 µg/mL) potentiated the stimulation of insulin secretion at glucose stimulatory concentrations (11.1 and 16.7 mM) *in vitro* via the closure of K^+-$_{ATP}$ channels and the increase of Ca^{2+} influx in rat pancreatic ß-cells which triggered glucose-induced insulin release, in the same way of tolbutamide (Souza *et al.,* 2011).

G. tessmannii is another widely used medicinal plant for the treatment of diabetes complications in Gabon and has been largely linked to antioxidant activity (Bading Taïka *et al.,* 2018; Nyangono Beyegue *et al.,* 2012). Several phytochemical bioactive compounds have been identified, sterols, alkaloids, flavonoids, tannins, polyphenols, sugars and saponides (Koumba Madingou *et al.,* 2012; Nyangono Beyegue *et al.,* 2012) and *G. tessmannii* has been shown to restore functional status of liver (hepatic AST and ALT levels) and decrease glucose levels supporting its anti-diabetic potential (hypoglycaemic effect) of the plant extract (Koumba Madingou *et al.,* 2016). Few studies on pharmacological activities of *G. tessmannii* have been carried out, except for antioxidant activity (Nyangono Beyegue *et al.,* 2012).

In chapter 2, we identified novel phytochemical constituents with anti-diabetic potential in *T. iboga*. The main constituent is ibogaine which pharmacological activity is mediated by numerous classes of neurological receptors and transporters, including the σ2, κ and μ opioid receptors, $5HT_2$ and $5HT_3$ receptors, α, ß4 nicotinic receptors, and N-methyl-D-aspartic acid (NMDA) ion channel (Paškulin *et al.,* 2006). However, few studies of *in vitro* anti-diabetic property and mechanism of action have been reported for *T. iboga* and ibogaine. Despite the *in vitro* anti-diabetic efficacy data with *T. iboga* and ibogaine no *in vivo* efficacy in animal models or patients have yet been reported. Moreover, numerous safety studies raised concerns including neurotoxicity and cardiotoxicity which have been observed in animal and human studies using ibogaine (Mačiulatis *et al.,* 2008).

σ2 receptors and insulin release

Two major subclasses of σ receptors have been documented, σ 1 and σ 2 receptors are different on their pharmacological profile, function and molecular size; σ1 receptors are 25 kDa whereas σ2 receptors are 18-21.5 kDa in size (Bowen, 2001).

σ receptors are widely distributed throughout the brain, endocrine, immune and reproductive tissues, including ß pancreatic islets, kidney and liver organs (Bowen, 2001). Furthermore, both subtypes of σ receptors are found to be highly expressed in tumour cell lines derived from numerous tissues including neuroblastomas, glioma, melanoma, carcinoma cell lines of breast, prostate, lungs, and pancreas (Bowen, 2001; Kashiwagi *et al.,* 2009). No endogenous functional ligand for σ receptors has been convincingly identified, however, certain steroids may be endogenous ligands for σ receptors such as progesterone (Rybczynska *et al.,* 2009). The functions of σ1 receptors in the brain have been involved in the modulation of the synthesis and release of dopamine and acetylcholine (Sambo *et al.,* 2018) as well as the modulation of NMDA-type glutamatergic receptor electrophysiology (Gonzalez-Alvear and Werling, 1995) and modulation of NMDA-stimulated neurotransmitter release (Zamanillo *et al.,* 2013). There is no evidence supporting *T. iboga* constituents as σ1 ligands, although σ1 modulation could explain, in part, some of the central actions of *T. iboga* (addiction, fatigue). However, less is known about the function of σ2 receptors in the brain. Chronic activation of σ2 receptors leads to morphological changes and cell death by apoptosis (Bowen, 2001). It is

well established that Ca^{2+} plays a role in cytotoxicity and that alterations in cell Ca^{2+} levels induce apoptosis in various cell types (Bowen, 2001). σ receptors ligands such as CB-64D, produce two types of increases in cell (cytosolic) Ca^{2+} concentration ($[Ca^{2+}]i$). Mediation of the effect on $[Ca^{2+}]i$ by σ2 receptors was demonstrated by the high activity of the selective σ2 receptors against CB-64D (Bowen, 2001), which induced an immediate, dose-dependent, and transient rise in $[Ca^{2+}]i$ (reversible action). As this transient rise in $[Ca^{2+}]i$ occurred in the absence of extracellular Ca^{2+} (pre-treatment by thapsigargin), it has been shown that σ2 receptors stimulate a transient release of Ca^{2+} from endoplasmic reticulum stores. However, prolonged exposure of cells to σ2 receptors ligands lead to a latent and sustainable rise in $[Ca^{2+}]i$, not altered by removal of extracellular Ca^{2+} or thapsigargin treatment, indicating the release of Ca^{2+} from mitochondrial stores or from other Ca^{2+} stores as Golgi apparatus (Bowen, 2001). Moreover, recent study has shown that under diabetes conditions, high blood glucose levels and decreased insulin levels (STZ-induced T1D in mouse), Ca^{2+} homeostasis was disrupted and intracellular Ca^{2+} levels altered, which may induce cell death through depletion of Ca^{2+} from endoplasmic reticulum (ER) and ER stress (Ahn *et al.,* 2015).

Ibogaine alkaloids have been found to induce a rise in $[Ca^{2+}]i$ by selective interaction with σ2 receptors and induced morphological changes and apoptosis in neuroblastoma and glioma cells (Bowen, 2001). Ibogaine and related iboga alkaloids including 10-t-Butoxy-ibogamine act as σ2 receptor agonists to gate Ca^{2+} ions from cytoplasmic reticulum towards cytosol (Bowen, 2001). However, it has been shown that in the absence of extracellular Ca^{2+}, iboga alkaloids failed to induce intracellular Ca^{2+} release (Souza *et al.,* 2011), in contrast with previous findings and expectations (Bowen, 2001). Although numerous σ2 receptor agonists have been documented, there are few selective pharmacological antagonists for this subtype. SM-21 (3 alpha-tropanyl-2-(4-chorophenoxy)-butyrate has been reported to possess high preferential affinity in mice and rat brain (nearly 10-fold preferential affinity compared to σ1 receptor) for σ2 receptors, and acts as a potent and selective σ2 receptor antagonist (Ghelardini and Bartoloni, 2000; Matsumoto and Mack, 2001; Matsumoto *et al.,* 2007).

In this chapter, we have investigated the effects of two plants aqueous extracts, *T. iboga* and *G. tessmannii*, on glucose stimulated insulin secretion from isolated rat pancreatic islets, in order to assess their potential therapeutic actions and elucidate the anti-diabetic mechanism of action *in vitro* via σ2 receptors prior to further investigations *in vivo* (chapters 4 and 5). Furthermore, we aimed to develop an assay which will provide viable islets, as pancreatic cells are extremely vulnerable to oxidative stress even in healthy conditions due to their lack

of strong antioxidant system (Miceli *et al.,* 2018). Hyperglycaemia (exposure to high glucose > 16.7 mM) and other metabolic or mechanical alterations of pancreatic cells may lead to increase of oxidative stress, thus enhanced NO production, leading to cellular damage and ultimately resulting to cell death and its associated complications (Adela *et al.,* 2015; Miceli *et al.,* 2018). We investigated whether the insulin potentiating concentrations of aqueous extracts of *T. iboga* and *G. tessmannii* and low and high glucose exposure induces changes in NO production in isolated rat pancreatic islets.

3.2. Materials and methods

3.2.1. Plant material and preparation of *T. iboga* and *G. tessmannii* aqueous extracts

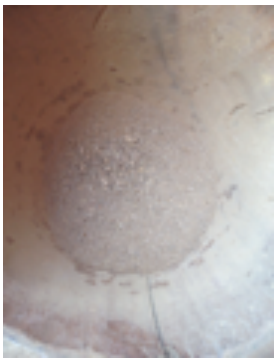
The plants were prepared according to the traditionally recommended method for oral administration, in chemistry laboratory at the University of Hertfordshire. The barks of *T. iboga* and *G. tessmannii* were collected in Gabonese forest (at Libreville) during harvest time by botanists from IPHAMETRA, authenticated, a voucher specimens have been identified and deposited at the National Herbarium of Gabon and brought to London in air-free plastic bags to avoid humidification.

In short, *G. tessmannii* stem barks were collected from Mandji forest by botanists, authenticated at the National Herbarium of Gabon (voucher no 16067), grounded into fine pounder using a traditional grounder at IPHAMETRA institute. The aqueous extract was obtained after maceration of the dried barks fine powder (100 g) in distilled water (500 mL) for 96 hours and under magnetic stirrer (500 rpm) and at room temperature at the University of Hertfordshire chemistry laboratory. The aqueous extract was filtered with Whatman paper number 1 and freeze-dried in a lyophilisator.

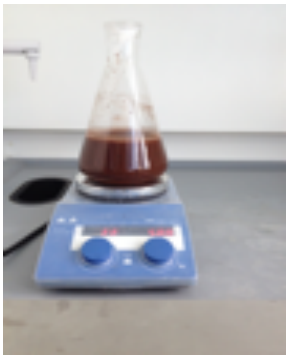
Similarly, *T. iboga* root barks were collected by botanist from IPHAMETRA at Lambaréné forest where the plant grows near the sand and have bigger roots. The plant was authenticated at the National Herbarium of Gabon (voucher no 20358), the root barks were grounded in a traditional big grounder to obtain a fine powder. The powder of the root barks (75 g) was macerated for 24 hours first in n-hexane (500 mL), under magnetic stirrer and at room temperature at the University of Hertfordshire chemistry laboratory. The solid material

obtained was then macerated with distilled water (500 mL) at room temperature and under magnetic stirrer for another 24 hours. The macerate obtained was filtered using cotton and Whatman number 1 filter paper placed in a Buchner funnel, and freeze-dried in a lyophilisator. Both powders were stored at room temperature in a desiccator to avoid humidity as shown in Figure 3.1 below.

(a) air-dried root barks



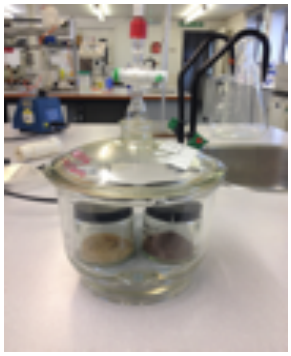
(b) grounded fine powder



(c) maceration under magnetic stirrer

(d) filtration

(e) freeze-drying



(f) aqueous extract in dessicator

Figure 3.1. T. iboga: from the root barks to the aqueous extract powder

(a) air-dried root barks; (b) grounded fine powder; (c) maceration under magnetic stirrer; (d) filtration; (e) freeze-drying; (f) aqueous extract stored in desiccator. Iphone 7+ camera (Bayissi Bading-Taïka, 2014)

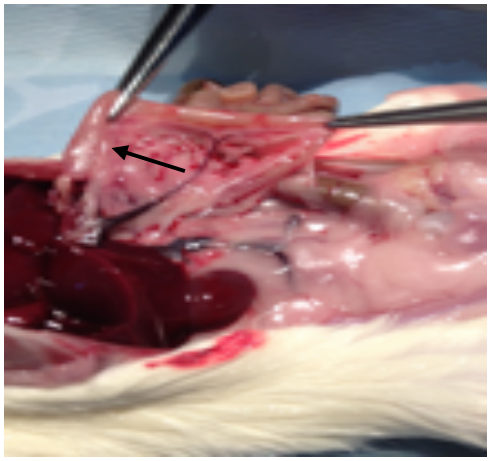
3.2.2. Animals and environment

Male Wistar rats were used in this chapter (source Charles River) with baseline body weights 350 g (10 weeks of age). Animals were housed in the University of Hertfordshire Biological Services Unit in pairs in standard cages (Techniplast 2000P) with sawdust (Datesand grade 7 substrate) and shredded paper wool bedding with water and food (5LF2 10% protein LabDiet) freely available. The housing room was maintained at a constant temperature of 22 ± 2 ^{o}C, under a 12h light dark cycle (lights on: 07:00 to 19:00 h). All studies were approved by the Institutional Animal Welfare and Ethnics review committee and conducted in accordance with guidelines established by the Animals (Scientific Procedures) Act, 1986 and European directive 2010/63/EU.

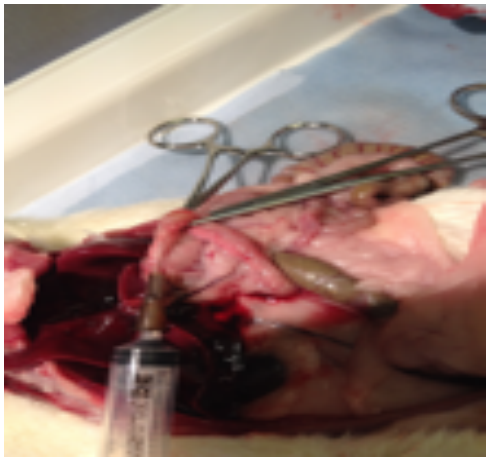
3.2.3. Isolation and preparation of rat pancreatic islets

Two methods were tested for the isolation of rat pancreatic islets. The first method was using histopaque gradient for pancreatic islets separation according to Carter *et al.,* 2009 method and the second was using Ficoll gradient to separate islets according to the method of Szot *et al.,* 2007.

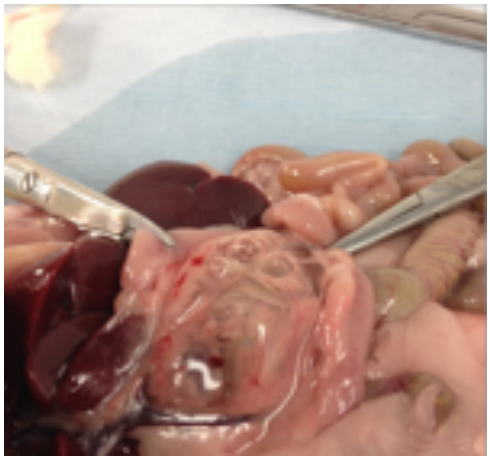
Carter *et al.*, 2009 have given a well detailed protocol for pancreatic islets isolation from rats. Briefly, after opening the rat abdomen cavity in a V-shape, the common bile duct (CBD) was clamped off near the small intestine. At the junction of the cystic duct and the gall bladder, the CBD was cannulated with 10 mL syringe filled with 8 mL of collagenase, and a 27-30-gauge needle (an insulin needle can be used as well), 1.4 mg/mL of collagenase solution was injected into the CBD as shown in Figure 3.2 below.



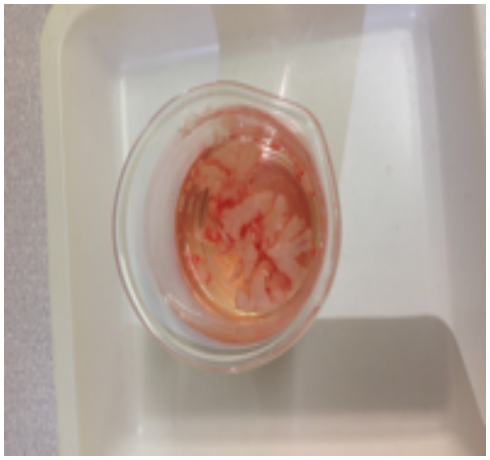
(a) CBD



(b) Cannulation



(c) Inflated pancreas



(d) Extracted pancreas

Figure 3.2. Islets isolation procedure (a) Common Bile Duct (CBD) clamped off; (b) Cannulation; (c) Inflated pancreas; (d) Extracted pancreas in collagenase, Bayissi Bading-Taïka, February 2015

Pancreas was removed from rodent and placed in a beaker containing 2 mL of collagenase. The separated pancreas was incubated for 20 min in a 37^{o} C water bath to digest the pancreatic cells. After digestion in the water bath, the tube was shaken by hand for few seconds to complete tissue separation. After this step, the tube containing the digested pancreas was rapidly placed in ice to slow the digestive process. The isolated islets were obtained from extracted pancreas following six centrifugations. First four centrifugations for

2 min at 1,200 rpm followed by a centrifugation at 1,500 rpm for 4 min to spin down the islets and a last one at 1,200 rpm for 3 min to obtain isolated islets. This step allows the separation of islets according to their density with a mix of histopaque 1077 solution and histopaque 1119 solution to have the histopaque 1100 solution (1.100 g/mL) which enhance islets purity. Islets were transferred to a Petri dish containing culture medium composed of RPMI 1640 + L-Glutamide, 10% FBS and Penicillin/ Streptomicin for cell culture possibilities or incubation. The cells were picked up under light microscope (Letz laborlux 11) at 10X magnitude using low-retention pipette and placed in a 6 wells plate for insulin secretion studies. For counting purposes the cells were frozen.

The second method followed Szot *et al.,* 2007 protocol with Ficoll gradient. The distension of pancreas followed the same procedure as in Carter *et al.,* 2009 protocol. However, there is only 3 steps of centrifugation at 1,300 rpm to spin down the cells. The pancreatic cells separation was performed quickly with 7 mL of Ficoll density 1.108, and 3 layers of 2 mL of Ficoll density 1.096, 1.069 then 1.037 in a 15-mL conical. After a centrifugation at 1,800 rpm for 15 min, islets were picked up from the second layer using an eye dropper as shown in Figure 3.3 below.

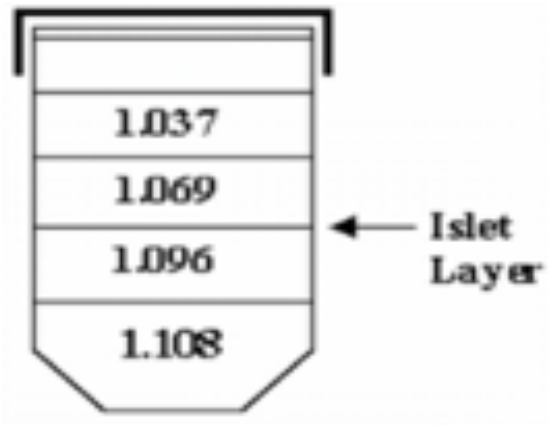


Figure 3.3. Islet layer with Ficoll gradient (Szot *et al.,* 2007)

The collected islets were re-suspended in 20 mL RPMI-1640 (containing 10% FCS and penicillin and streptomycin, HEPES, MEM-NEAA) and mix gently. A 100 µL was transferred for counting to a 35 mm Grid-plate containing 1 mL of media and 1 mL dithizone. Another method of counting was under light microscope with a Petri dish divided into four

parts. Cells were counted in the first section and an extrapolation was done for the three other sections. The number of islets counted is mean ± SEM (standard error of the mean) of 30 experiments (cells counting).

3.2.4. Insulin secretion experiments

The insulin secretion experiments followed the modified protocol of Bardy *et al.,* 2013 and Souza *et al.,* 2011. In brief, isolated islets were counted under light microscope 10 X magnification or with inverted microscope, separated and placed in glass tubes (10 islets per tube) containing a bicarbonate buffer solution containing (in mM): 115 Nacl, 5 Kcl, 24 $NaHCo_3$, 2.56 $CaCl_2$, 1.3 $MgCl_2$, 2.8 glucose, gassed with 95 % O_2 and 5% CO_2 and supplemented with 1 g/L BSA and kept at room temperature.

First, the islets were pre-incubated in 1 mL of continuously gassed (95% O_2 and 5% CO_2) bicarbonate buffer solution for 45 to 60 min at 37^{o}C. Following the pre-incubation time, the medium bicarbonate buffer solution was removed from each tube and replaced by fresh bicarbonate buffer without glucose (0 mM) or with increasing glucose concentrations (2.8 mM, 11.1 mM, 16.7 mM and 25 mM), various drugs or aqueous extracts for 90 minutes, gassed with 95 % O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin experiments performed, to evaluate the possible mechanisms of action underlying the action of the plant extracts, the effects of the extracts were studied in the presence of insulin secretagogue, as tolbutamide (an inhibitor of ß-cell K^{+}-$_{ATP}$ channels) and SM-21 (a σ2 receptor antagonist).

Measurement of insulin

ELISA or enzyme-linked immunosorbent assay, is a technique used to detect the presence of an antibody or antigen in samples. Sandwich ELISA measures the amount of antigen between two layers of antibodies (capture and detection antibody). The antigen to be measured must contain at least two antigen sites of binding to antibody, since at least two antibodies act in sandwich. Either monoclonal or polyclonal antibodies can be use as the capture and detection antibodies in Sandwich ELISA systems. A polyclonal is often used as the capture antibody to

pull down as much as of the antigen possible. In Sandwich ELISA, the sample does not need to be purified before analysis and the assay can be very sensitive (Büyükköroglu *et al.,* 2018).

3.2.5. Measurement of the effects of *T. iboga* (0.001 to 100 µg/ml) on glucose-induced insulin secretion from isolated rat pancreatic islets

Following insulin secretion experiments in presence of no glucose (0 mM) or with increasing glucose concentrations (2.8 mM, 11.1 mM, 16.7 mM and 25 mM), the effect of *T. iboga* aqueous extracts on glucose-induced insulin release was evaluated. As described above, after the pre-incubation period, the medium bicarbonate buffer solution was replaced by fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 11.1 mM, 16.7 mM and 25 mM without or with *T. iboga* extracts (0.001, 0.01, 0.1, 1, 10 and 100 µg/mL), for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin ELISA was performed.

3.2.6. Measurement of the effect of *T. iboga* (1 µg/mL) on glucose-induced insulin secretion in presence of Tolbutamide (200 µM) from isolated rat pancreatic islets

As described above, after the pre-incubation period, the medium bicarbonate buffer solution was replaced by fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 1.11 mM, 16.7 mM or 25 mM without or with *T. iboga* extracts (1 µg/mL) and with or without tolbutamide (200 µM) for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin ELISA was performed.

3.2.7. Measurement of the effects of *T. iboga* (1 µg/mL) on glucose-induced insulin secretion in presence of SM-21 concentrations (1, 10 µM) from isolated rat pancreatic islets

As described above, after the pre-incubation period, the medium bicarbonate buffer solution was replaced by fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 11.1 mM, 16.7 mM or 25 mM without or with *T. iboga* extracts (1 µg/mL) and with or without the σ receptor antagonist, SM-21 (1, 10 µM) for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin ELISA was performed.

3.2.8. Measurement of the effects of *G. tessmannii* (1 µg/mL to 10 mg/mL) concentrations on glucose-induced insulin secretion from isolated rat pancreatic islets

The effects of *G. tessmannii* aqueous extracts at 1, 10, 30, 50, 70 100 µg/mL and 1, 10 mg/mL on glucose-induced insulin release was evaluated following the same protocol as described above. Concentrations were selected according to previous results by Madingou *et al.*, 2011. As described above, after the pre-incubation period, the medium bicarbonate buffer solution was replaced by fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 11.1 mM, 16.7 mM or 25 mM without or with *G. tessmannii* extracts (1, 10, 30, 50, 70, 100 µg/mL and 1, 10 mg/mL), for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin ELISA was performed.

3.2.9. Measurement of the effects of *G. tessmannii* (70 µg/mL) on glucose-induced insulin secretion in presence of Tolbutamide (200 µM) from isolated rat pancreatic islets

As described above, after the pre-incubation period, the medium bicarbonate buffer solution was replaced by fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 11.1 mM, 16.7 mM or 25 mM without or with *G. tessmannii* extracts (70 µg/mL) and with or without tolbutamide (200 µM) for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. At the end of incubation periods, aliquots of supernatant were collected and stored at -20^{o}C until the insulin ELISA was performed.

3.2.10. Measurement of the effects of *T. iboga* (1 µg/mL) and *G. tessmannii* (70 µg/mL) on NO production at low and high glucose concentrations from rat pancreatic islets.

Since ibogaine has shown neurologic cytotoxic effects (Bowen et al., 2001), and is the major constituent of *T. iboga*, a Griess assay was performed to determine levels of nitrates as a marker of oxidative stress following exposure of isolated rat pancreatic islets to low and high glucose concentrations and 1 µg/mL of *T. iboga* and 70 µg/mL of *G. tessmannii*. After incubation of isolated rat pancreatic islets with fresh bicarbonate buffer containing glucose at 0 mM, 2.8 mM, 11.1 mM, 16.7 mM or 25 mM with/without *G. tessmannii* extract (70 µg/mL) and with/without *T. iboga* (1 µg/mL) for 90 minutes gassed with 95% O_2 and 5% CO_2 at 37^{o}C. Aliquots of 100 µL of supernatant were removed for Griess reaction assay.

Griess assay reaction is a colorimetric assay that has been extensively used because of its simplicity since it was first described in 1879, to measure the levels of Nitrite (NO_2^-) in biological samples. Nitric oxide (NO), a gaseous free radical secreted by endothelium, is a major modulator of endothelial function (Adela *et al.,* 2015). NO is a highly reactive molecule with a short half-life in vivo (few seconds), thus, levels of more stable metabolites, nitrite (NO_{2-}) and nitrate (NO_{3-}) have been used to indirectly measure NO in samples. NO concentrations are based on the enzymatic conversion of NO_{3-} to NO_{2-} by nitrate reductase. The reaction is followed by colorimetric detection of NO_{2-} as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotisation reaction in which acidified NO_{2-} produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm (Parameter^{T}).

Briefly, a series of nitrite concentrations as standards were prepared from 65 µM to 1.01 µM to stay within the working range of the Griess reagent modified (0.43-65 µM nitrite). In a 96 wells plate, 100 µL of the standards and aliquot samples were added into their respective wells followed by 100 µL Griess reagent modified into each well. After 15 min incubation at room temperature, the absorbance at 540 nm was measured using a plate reader, and absorbance of each sample was recorded to assess the amount of NO produced.

3.2.11. Data analysis

Data were analysed using GraphPad Prism 7.0 software to determine the level of significance. All data are expressed as means ± standard error mean (SEM). The level of probability (*p*) was considered statistically significant when values were less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****). One- way analysis of variance (ANOVA) was performed to examine the effect of a single variable on more than two groups (effect of increasing glucose concentrations on insulin release) followed by Tukey's tests. Two-way ANOVA were performed to examine the effect of two independent variables with Dunnett or Tukey's post-hoc tests.

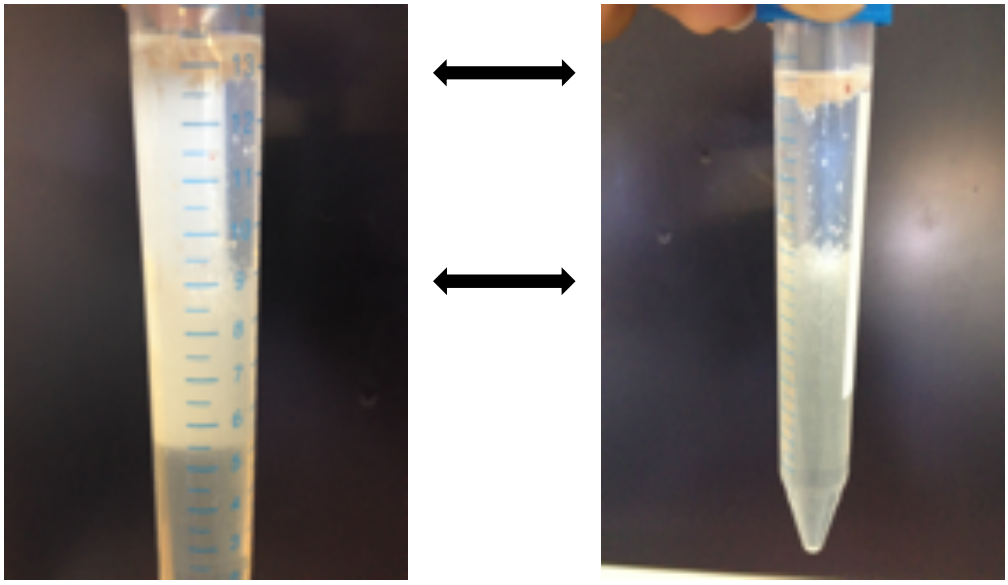
3.3. Results

3.3.1. *T. iboga* and *G. tessmannii* aqueous extracts yield

The aqueous extraction of *G. tessmannii* (100 g) and *T. iboga* (75 g) gave a yield of 12.49 g (12.5 %) and 2.11 g (3 %), respectively of aqueous extract.

3.3.2. Isolation of rat pancreatic islets

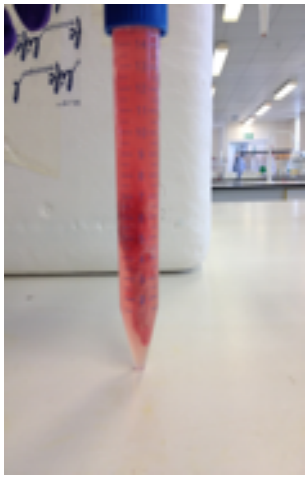
The pancreatic islets were separated by gradient (graduation 9) from other acinar and pancreatic tissues (graduation 13).



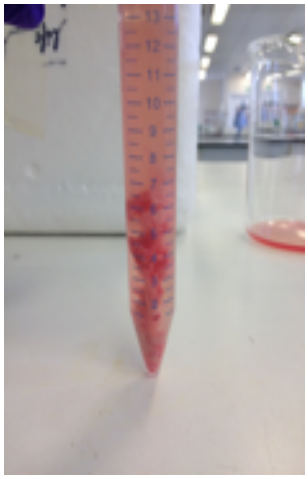
(a) Islets layer by Ficoll gradient (b) Islets layer by Ficoll gradient

Figure 3.4. Islets layer (graduation 9) by Ficoll gradient, other pancreatic and acinar tissues at graduation 13, UH Laboratory, Bayissi Bading-Taïka, 2015

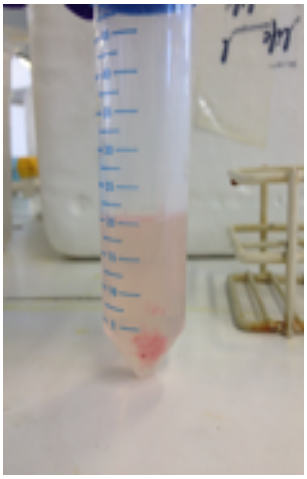
Islets were mainly found as clusters Figure 3.5 a, e, however, some isolated ones were localised Figure 3.5 b, c, d, f with variable sizes. Cells have a normal appearance with ß granules inside and some were surrounded with contaminating exocrine tissue Figure 3.5 a, c, e.



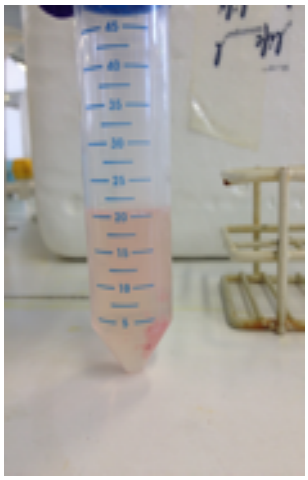
(a) Digested pancreas



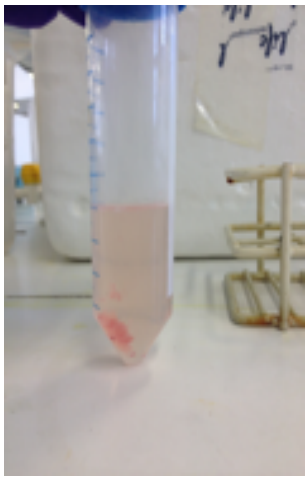
(b) Islets and other tissues spin down



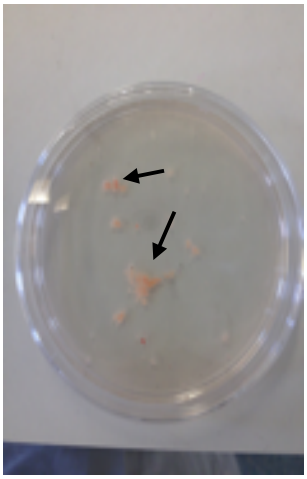
(c) Islets cells separation



(d) Islets further separation



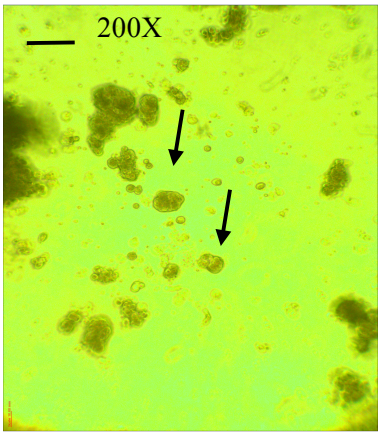
(e) Islets isolated



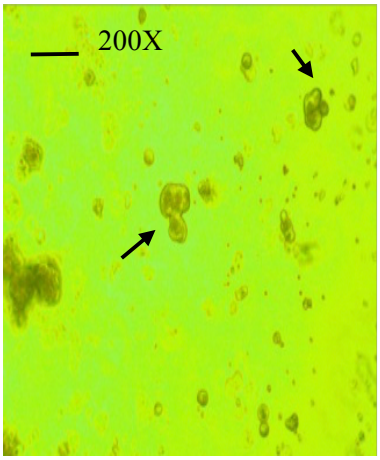
(f) Collected isolated islets

Figure 3.5. Rat pancreatic islets centrifugation steps using Histopaque gradient, University of Herfordshire Bayissi Bading-Taïka, 2015

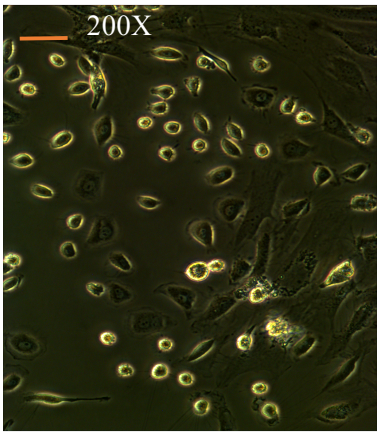
The number of islets per pancreas was approximately SEM 1887 ± 428 (n=30) and a visual inspection under a light microscope shown spherical and golden-brown cells with varying diameter from 50 µm to 600 µm.



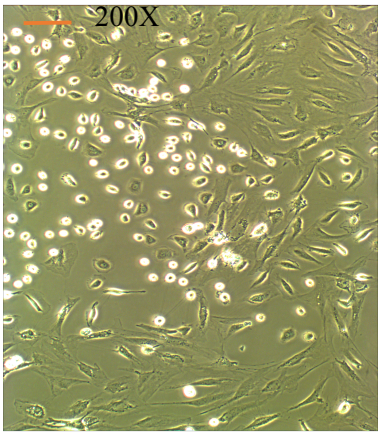
(a) Isolated islets



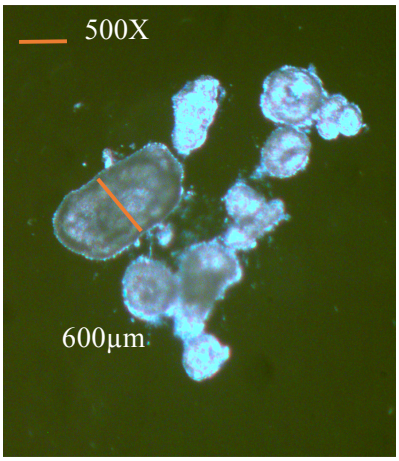
(b) Cluster of islets



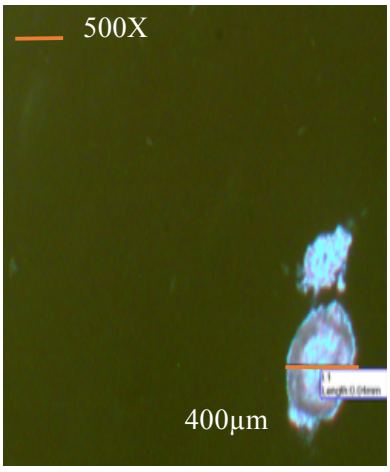
(c) Isolated islets



(d) Isolated islets



(e) Isolated islets



(f) Isolated islets

Figure 3.6. Isolated rat pancreatic islets (200X). Light microscope (a-b), Inverted microscope (c-d), Isolated islets diameter (X500) (e-f)

3.3.3. Effect of increasing glucose concentration on insulin release from isolated rat pancreatic islets

Before each experiment, the effect of glucose was tested on rat isolated islets. The Figure 3.7 below shows the effect of glucose concentrations on insulin release after a static incubation of 90 min. Increasing glucose concentrations (0 mM, 2.8 mM, 11.1 mM, 16.7 mM and 25 mM) were added to the medium of each tube containing isolated pancreatic rat islets (~10) and insulin secretion was measured. The data shows a concentration dependent increase of insulin release from 11.1 mM of glucose. Insulin secretion was not induced in medium with no glucose (0 mM) (0.406 ng/mL/10 islets), however, a slight increase, non-significant, was shown at glucose concentration of 2.8 mM (0.928 ng/mL/10 islets). There is a significant concentration-dependent increase of insulin release from 11.1 mM glucose (3 ng/mL/10 islets) to 25 mM glucose (7 ng/mL/10 islets) compared to non-stimulatory glucose concentrations (0 mM and 2.8 mM). The isolated pancreatic cells were secreting insulin in a concentration-dependent manner in the presence of increasing glucose concentrations, which confirmed that freshly isolated rat pancreatic islets were insulin secreting cells.

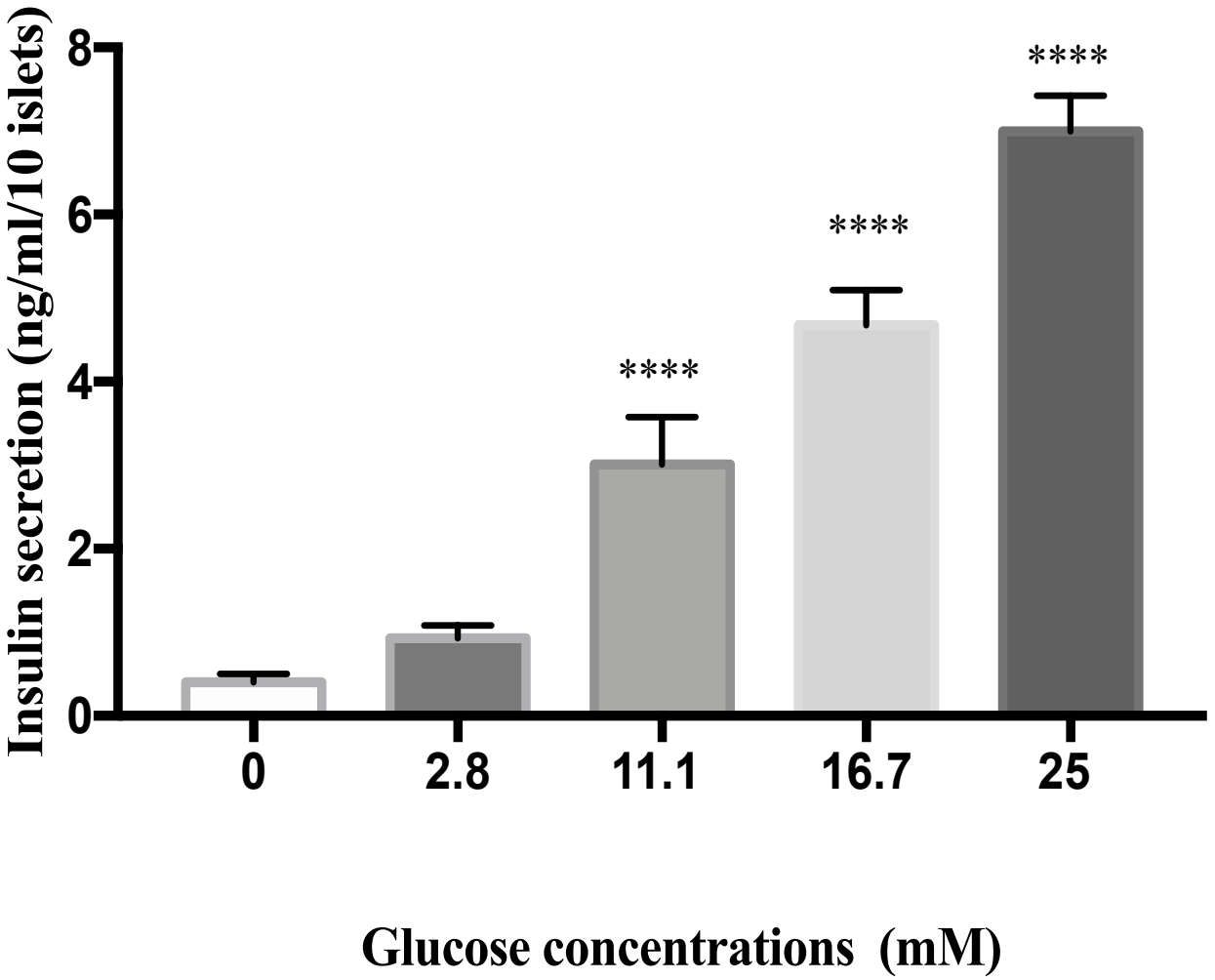


Figure 3.7. The effect of increasing glucose concentrations (0, 2.8, 11.1, 16.7 and 25 mM) on insulin release from isolated rat pancreatic islets. Data is presented as mean ± SEM of 21 independent experiments. Data was analysed by one-way ANOVA and Tukey's post-hoc test. Statistical significance is presented as **** $p < 0.001$ compared 0 mM (no glucose) group.

3.3.4. Concentration dependent effects of *T. iboga* (0.001 to 100 µg/mL) aqueous extract on low and high glucose (0 to 25 mM) induced insulin secretion from isolated rat pancreatic islets

To screen the effect of *T. iboga* aqueous extract on insulin secretion, the freshly isolated islets were tested with various concentrations (0.001, 0.01, 0.1, 1, 10, 100 µg/mL) of the plant extract, in the presence of increasing glucose concentrations (0, 2.8, 11.1, 16.7 and 25 mM), over a 90-min static incubation. The data shows that in the absence of glucose (0 mM) *T. iboga* did not influence insulin release. The stimulatory effect of the extract was then compared in presence of non-stimulatory (Figure 3.8 a) and stimulatory glucose concentrations (Figure 3.8 b, c, and d). Glucose-induced insulin secretion was significantly increased in the presence of *T. iboga* extract (1 µg/mL) at 2.8 mM (Figure 3.8 a) compared to control (no extract, no glucose) by more than 9-fold (0.39 vs 3.63 ng/mL/10 islets, $p < 0.001$) and more than 3-fold compared to control at same concentration (0.92 vs 3.63 ng/mL/10 islets, $p < 0.001$). At 11.1 mM glucose (Figure 3.8 b), a substantial increase of insulin secretion was observed at 1 µg/mL of *T. iboga* (7.31 ng/mL/10 islets, $p < 0.0001$) compared to the control (no extract, no glucose) and at the same glucose concentration (no extract) (3 ng/mL/10 islets, $p < 0.001$). However, an inhibition on insulin potentiation at 1 µg/mL of *T. iboga* was observed at 16.7 mM glucose compared to control at same glucose concentration (no extract) (3.51 ng/mL/10 islets vs 4.67 ng/mL/10 islets) (Figure 3.8 c), which was more marked at 25 mM glucose (4.81 ng/mL/10 islets vs 6.99 ng/mL/10 islets, $p < 0.001$) (Figure 3.8 d). Interestingly, at concentrations below and above 1 µg/mL of *T. iboga*, there is no significant potentiation of insulin secretion levels, at 0.001 µg/mL (0.11 ng/mL/10 islets), 0.01 µg/mL (0.75 ng/mL/10 islets), 0.1 µg/mL (1.60 ng/mL/10 islets) and at 10 µg/mL (1.28 ng/mL/10 islets) and 100 µg/mL (0.84 ng/mL/10 islets) compared to control (no extract) (0.90 ng/mL/10 islets) at 2.8 mM glucose. Similarly, at 11.1 mM of glucose (Figure 3.8 b), there is a no significant potentiation of insulin release at 0.1 µg/mL (2.83 ng/mL/10 islets) and 10 µg/mL (2.29 ng/mL/10 islets) compared to control (no extract) (3 ng/mL/10 islets). However, there is a significant inhibition of insulin release at 0.001 µg/mL (0.21 ng/mL/10 islets), 0.01 µg/mL (1.28 ng/mL/10 islets) and 100 µg/mL of *T. iboga* (1.23 ng/mL/10 islets) compared to control at same glucose concentration (no extract) (3 ng/mL/10 islets, $p < 0.01$). At 16.7 mM of glucose, low (0.001 to 1 µg/mL) and high (10 and 100 µg/mL) *T. iboga* extract concentrations inhibited significantly insulin release compared to control at same glucose concentration (no extract) which was more marked at 25 mM of glucose. Indeed, at

16.7 mM of glucose (Figure 3.8 c), *T. iboga* extract at 0.001 µg/mL (0.31 ng/mL/10 islets), 0.01 µg/mL (1.16 ng/mL/10 islets), 0.1 µg/mL (2.50 ng/mL/10 islets), 1 µg/mL (3.52 ng/mL/10 islets), 10 µg/mL (2.11 ng/mL/10 islets) and 100 µg/mL (0.94 ng/mL/10 islets) inhibited significantly insulin release compared to control at the same concentration (no extract) (4.67 ng/mL/10 islets, $p < 0.05$). Similarly, at 25 mM of glucose *T. iboga* extract concentrations (0.001 to 100 µg/mL) inhibited significantly insulin release compared to control at the same glucose concentration (no extract) (6.99 ng/mL/10 islets, $p < 0.001$). At low (2.8 mM) and high (11.1, 16.7 and 25 mM) glucose concentrations, *T. iboga* extract at 1 µg/mL was the concentration optimal compared to control (no extract, no glucose).

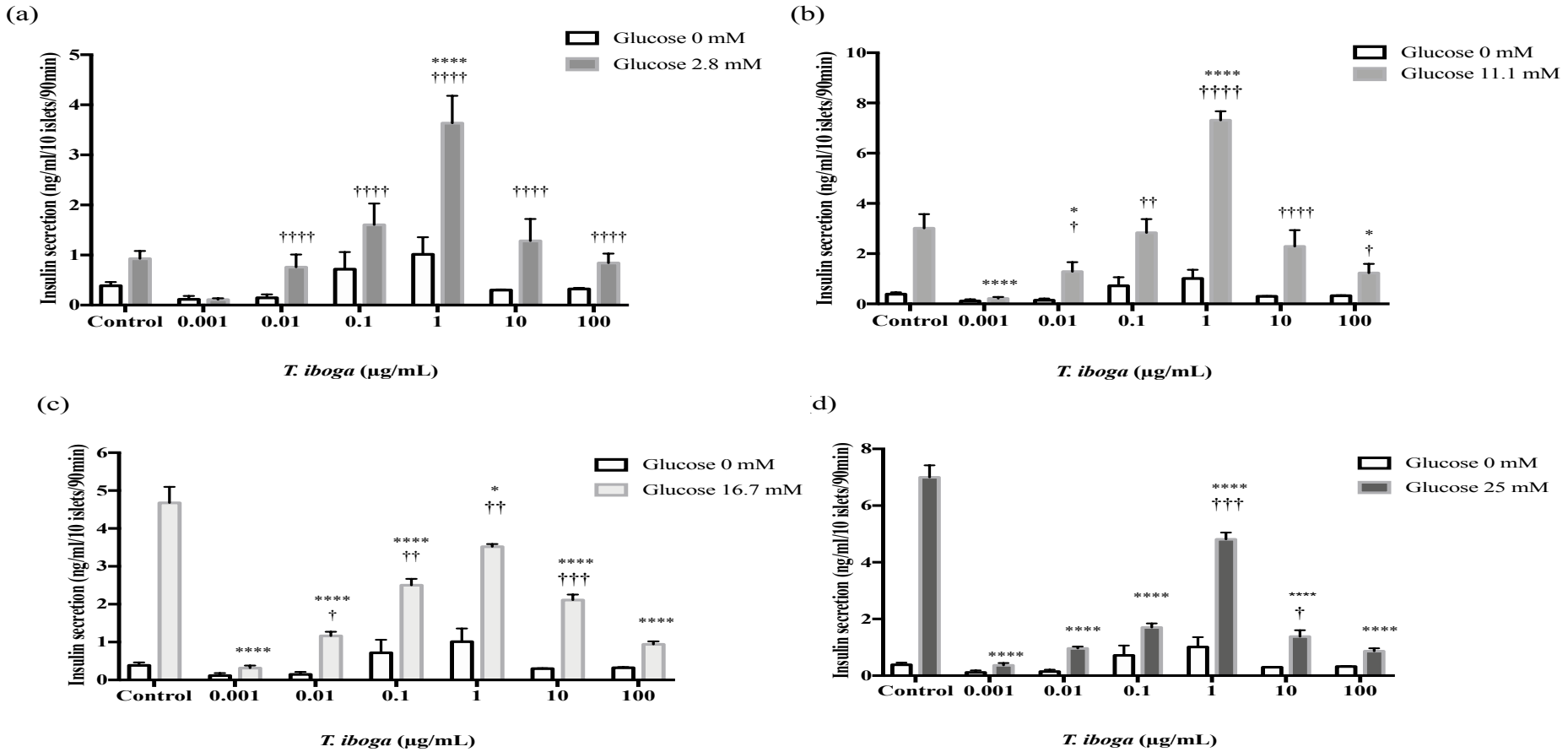


Figure 3.8. Effects of the aqueous extract of *T. iboga* (0.001 to 100 µg/mL) on insulin release from rat pancreatic islets. Dose-response effect of *T. iboga* on insulin release in the presence of both non-stimulatory (0, 2.8 mM) and stimulatory concentrations (>11.1 mM) of glucose. Values are means of 21 observations. Data were analysed by two-way ANOVA followed by Dunnett's test, effect of *T. iboga* and effect of panel a) 2.8 mM b) 11.1 mM c) 16.7 mM d) 25 mM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ relative to respective glucose (no extract). † $p < 0.05$, †† $p < 0.01$ and †††† $p < 0.001$, compared with incubations at 0 mM glucose (control) and at same concentrations of *T. iboga* extract

3.3.5. Effects of *T. iboga* optimal concentration (1 µg/mL) on insulin release from isolated rat pancreatic islets in the presence of increasing glucose concentrations

Figure 3.9 below shows the dose-response effect of increasing glucose concentrations (0, 2.8, 11.1, 16.7, 25 mM) upon insulin release in the presence or absence of *T. iboga* at 1 µg/mL. At 2.8 and 11.1mM of glucose, *T. iboga* induced a significant increase of insulin secretion by 4-fold and 2-fold compared to the respective controls (3.63 vs 0.92 ng/mL/10 islets and 7.31 vs 3 ng/mL/10 islets, at 2.8 and 11.1 mM glucose respectively, $p < 0.0001$). However, at 25 mM of glucose, *T. iboga* potentiation of insulin release decreased from 6.99 to 4.81 ng/mL/10 islets, this inhibition of the extract potentiation was significant $p < 0.05$. At 16.7 mM glucose, there is a decrease from 4.67 to 3.52 ng/mL/10 islets. Although, this variation was not significant. The optimal effect of *T. iboga* at 1 µg/mL on glucose induced insulin release compared to the control was seen at glucose concentration of 11.1 mM.

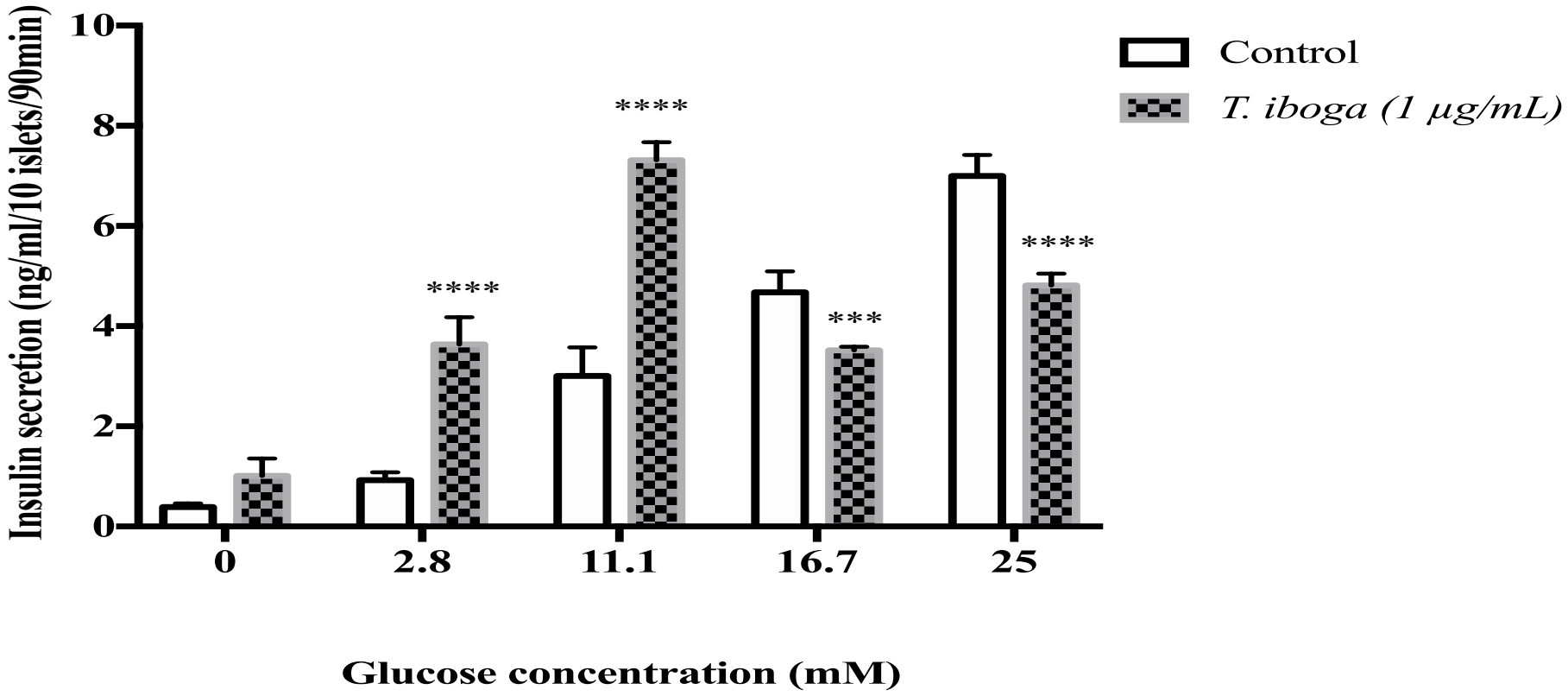


Figure 3.9. Dose-response effect of glucose upon insulin release in the presence and absence of *T. iboga* extract (1 µg/mL). Values are means ± SEM for groups of 15 observations and were analysed by two-way ANOVA followed by Tukey's test. * $p < 0.05$, **** $p < 0.0001$, compared with incubations at the same glucose concentration without *T. iboga* extract (control).

3.3.6. Effects of *T. iboga* (1 µg/mL) on insulin release from isolated rat pancreatic islets in the presence of Tolbutamide

The Figure 3.10 below shows the effect of optimal *T. iboga* (1µg/ml) concentration in the presence of tolbutamide (200 µM), an insulin secretagogue, on glucose-induced insulin secretion. Both tolbutamide and *T. iboga* aqueous extract stimulated insulin secretion at glucose stimulatory and non-stimulatory concentrations, however, the magnitude of their effects was totally different. Tolbutamide enhanced significantly glucose-induced insulin secretion at concentrations greater than 2 mM glucose in a glucose concentration-dependent manner. Whereas *T. iboga* aqueous extract potentiated glucose-induced insulin secretion on a bell shape manner. Indeed, at 2.8 and 11.1 mM glucose *T. iboga* aqueous extract induced more potentiation of glucose-induced insulin release compared to tolbutamide at the same glucose concentrations (3.63 vs 2.73 and 7.31 vs 6.07 ng/ml/10 islets, respectively), although this potentiation is not significant. However, at 16.7 and 25 mM glucose there is a significant inhibition of *T. iboga* potentiation of glucose-induced insulin secretion while, the effects of tolbutamide were steadily increased (3.51 vs 7.02 ng/ml/10 islets, $p < 0.0001$ and 4.81 vs 8.79 ng/ml/10 islets, $p < 0.0001$ respectively). Also, glucose-induced insulin secretion was significantly increased in presence of both *T. iboga* and the secretagogue, tolbutamide at stimulatory glucose concentrations 2.8 and 11.1 mM compared to their solely effects (5.56 vs 2.73 ng/ml/10 islets for tolbutamide alone and vs 3.63 ng/ml/10 islets for *T. iboga* alone at 2.8 mM glucose, and 9.03 vs 6.07 for tolbutamide alone and vs 7.31 ng/ml/10 islets for *T. iboga* alone at 11.1 mM glucose). This suggests a combination effect of the plant extract and the anti-diabetic drug and more potentiation of glucose insulin release compared to the secretagogue alone. However, there is a decrease on glucose-induced insulin release levels at 16.7 mM when islets are incubated with both *T. iboga* aqueous extract and tolbutamide comparing to the effect of tolbutamide alone (5.25 vs 7.02 ng/ml/10 islets, $p < 0.0001$). The effect of tolbutamide remained unchanged at 25 mM glucose.

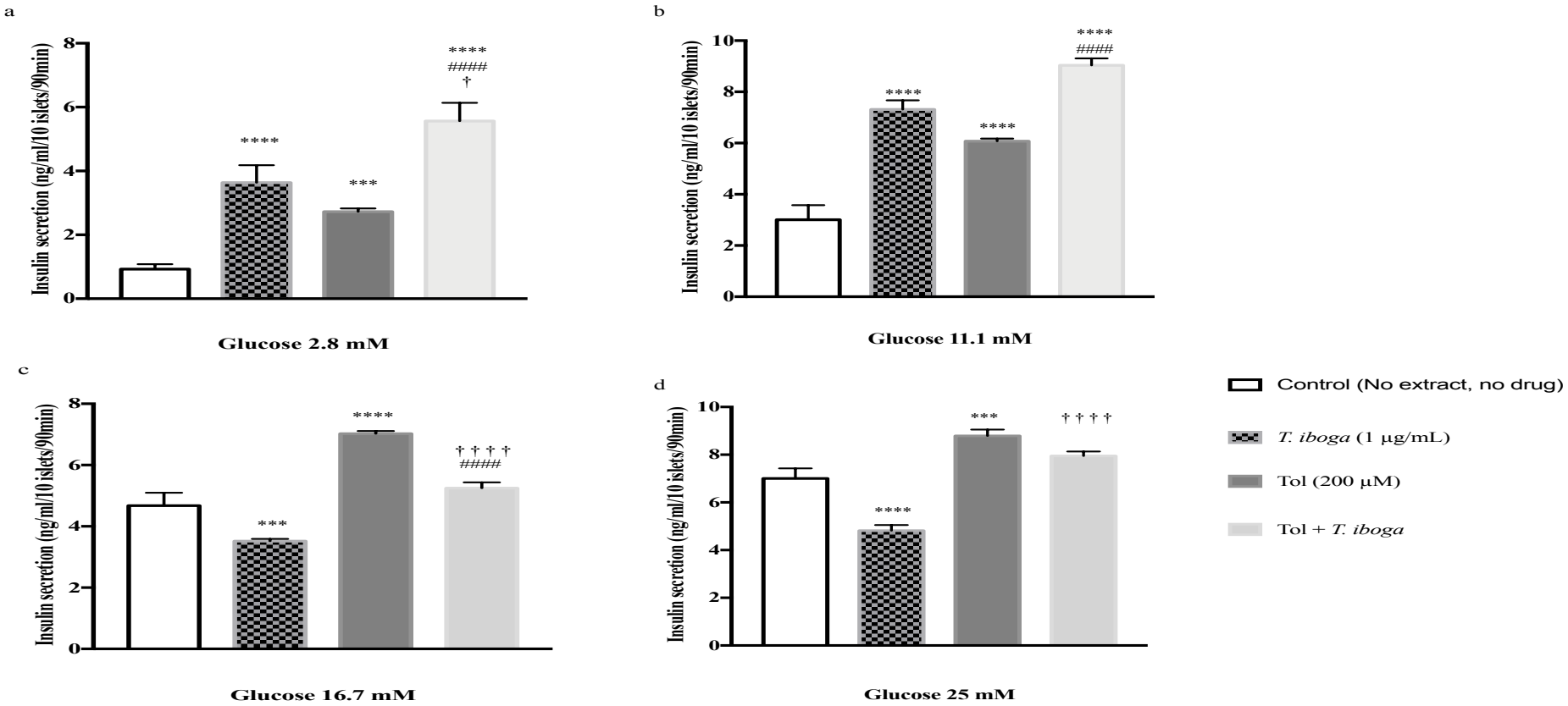


Figure 3.10. Effect of *T. iboga* (1 µg/mL) on insulin release from rat pancreatic islets in the presence of Tolbutamide (200 µM) in the presence of 2.8 mM, 11.1 mM, 16.7 mM and 25 mM glucose. Data is presented as ± SEM of 10-22 independent experiments and were analysed by one-way ANOVA followed by Tukey's test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control no extract, no drug, † $p < 0.05$, † † † † $p < 0.0001$ compared with *T. iboga* (1 µg/ml), #### $p < 0.05$ compared with Tolbutamide (200 µM)

3.3.7. Effects of *T. iboga* (1 µg/mL) on insulin release from isolated rat pancreatic islets in the presence of σ receptor antagonist, SM-21

To investigate mechanism of action of *T. iboga* aqueous extract on insulin secretion, we examined its effect in presence of selective σ2 receptor antagonist SM-21. Firstly, SM-21 effects were assessed on glucose-induced insulin release. The data below (Figure 3.11) shows that SM-21 significantly inhibited insulin secretion at 11.1, 16.7 and 25 mM glucose ($p < 0.001$). At low concentration of SM-21 (1 µM), insulin release decreased from 3 vs 1.31, 4.67 vs 1.52 ng/ml/10 islets and 6.99 vs 0.61 ng/ml/10 islets at 11.1, 16.7 and 25 mM glucose, respectively $p < 0.001$ (Figure 3.11 c). However, there is little or no effect of SM (1 µM) at 0 mM and 2.8 mM glucose (Figure 3.11 c). Similarly, at high concentration, SM-21 (10 µM) significantly inhibited glucose-induced insulin release at 11.1, 16.7 and 25 mM glucose ($p < 0.0001$). SM-21 at 10 µM divided by 6-fold the insulin levels compared to control at 11.1 mM and 25 mM glucose, and by 4 at 16.7 mM ($p < 0.0001$) glucose compared to control (Figure 3.11 d).

The effect of optimal *T. iboga* concentration (1 ug/ml) was then assessed in presence of low and high concentrations of SM-21 (Figure 3.11 e, f). Data in Figure 3.11 e, f shows that at low and high glucose concentrations 2.8, 11.1 and 25 mM, there is a significant decrease in *T. iboga* potentiation of insulin release at both low and high SM-21 concentrations. Indeed, insulin levels in presence of *T. iboga* (1 µg/ml) at 2.8 mM glucose decreased from 3.63 to 1.65 ng/ml/10 islets in presence of SM-21 at 1 µM (Figure 3.11 e) and to 0.97 ng/ml/10 islets in presence of SM-21 at 10 µM (Figure 3.11 f). At 11.1 mM, *T. iboga* insulin secretion potentiation decreased by 65% in presence of low and high SM-21 concentrations (1 and 10 µM) and by more than 20% at 25 mM glucose in presence of both SM-21 concentrations. However, *T. iboga* aqueous extract effect was not altered in presence of the antagonist SM-21 at 16.7 mM glucose.

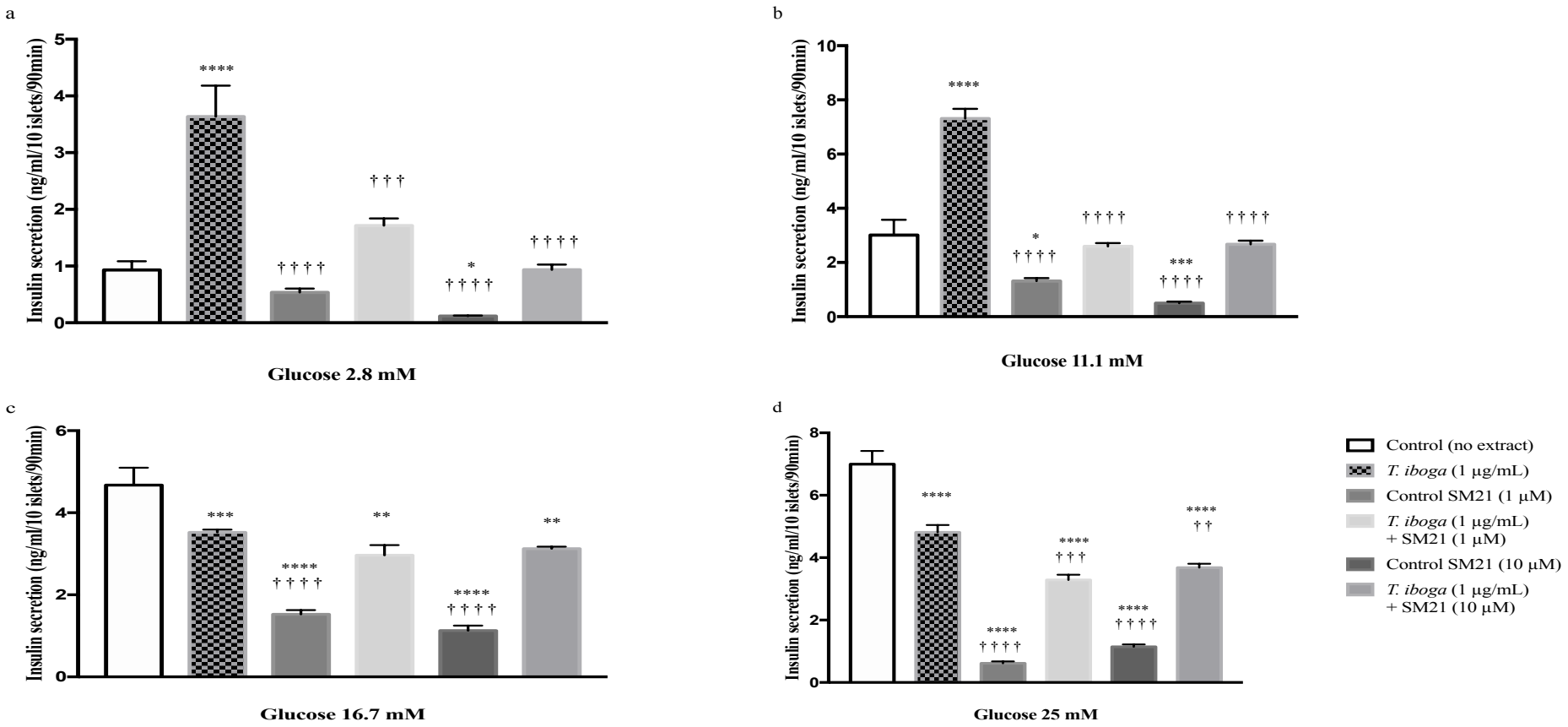


Figure 3.11. Effect of *T. iboga* (1 µg/mL) on insulin release from rat pancreatic islets in the presence of SM-21 (1 and 10 µM) at increasing 2.8 mM, 11.1 mM, 16.7 mM and 25 mM glucose. Data is presented as ± SEM of 10-21 independent experiments and were analysed by two-way ANOVA followed by Tukey's test * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ compared with control (no extract, no drug). †† $p < 0.01$, †††† $p < 0.0001$ compared with *T. iboga* (1µg/ml).

3.3.8. Effects of *T. iboga* (1 µg/mL) optimal concentration and increasing glucose concentrations on nitrite production from rat isolated pancreatic islets

Griess assay was performed on cells that were treated with *T. iboga* optimal concentration (1 µg/mL) at increasing glucose concentrations (0, 2.8, 11.1, 16.7 and 25 mM). Levels of total nitrite are shown on Figure 3.12 below, which reflects the amount of nitric oxide (NO), one major marker of oxidative stress. Total nitrite productions are low in cells treated with 0, 2.8 and 11.1 mM glucose (0.01, 0.01 and 0.02 µM respectively, $p > 0.05$ versus 0 mM) and increased significantly at 16.7 and 25 mM glucose (0.14 and 0.27 µM, $p < 0.05$ and $p < 0.0001$, respectively, compared with 0 mM and 11.1 mM). Similarly, total nitrite productions in presence of *T. iboga* (1 µg/ml) are low at 0, 2.8 and 11.1 mM glucose (0.03, 0.04 and 0.05 µM respectively, $p > 0.05$ versus 0 mM) and significantly increased at 16.7 and 25 mM glucose (0.163 and 0.29 µM, $p < 0.05$ and $p < 0.0001$, respectively compared with 0 mM and 11.1 mM). Nitrite levels in the presence of *T. iboga* were not significantly altered compared to respective controls (no extract).

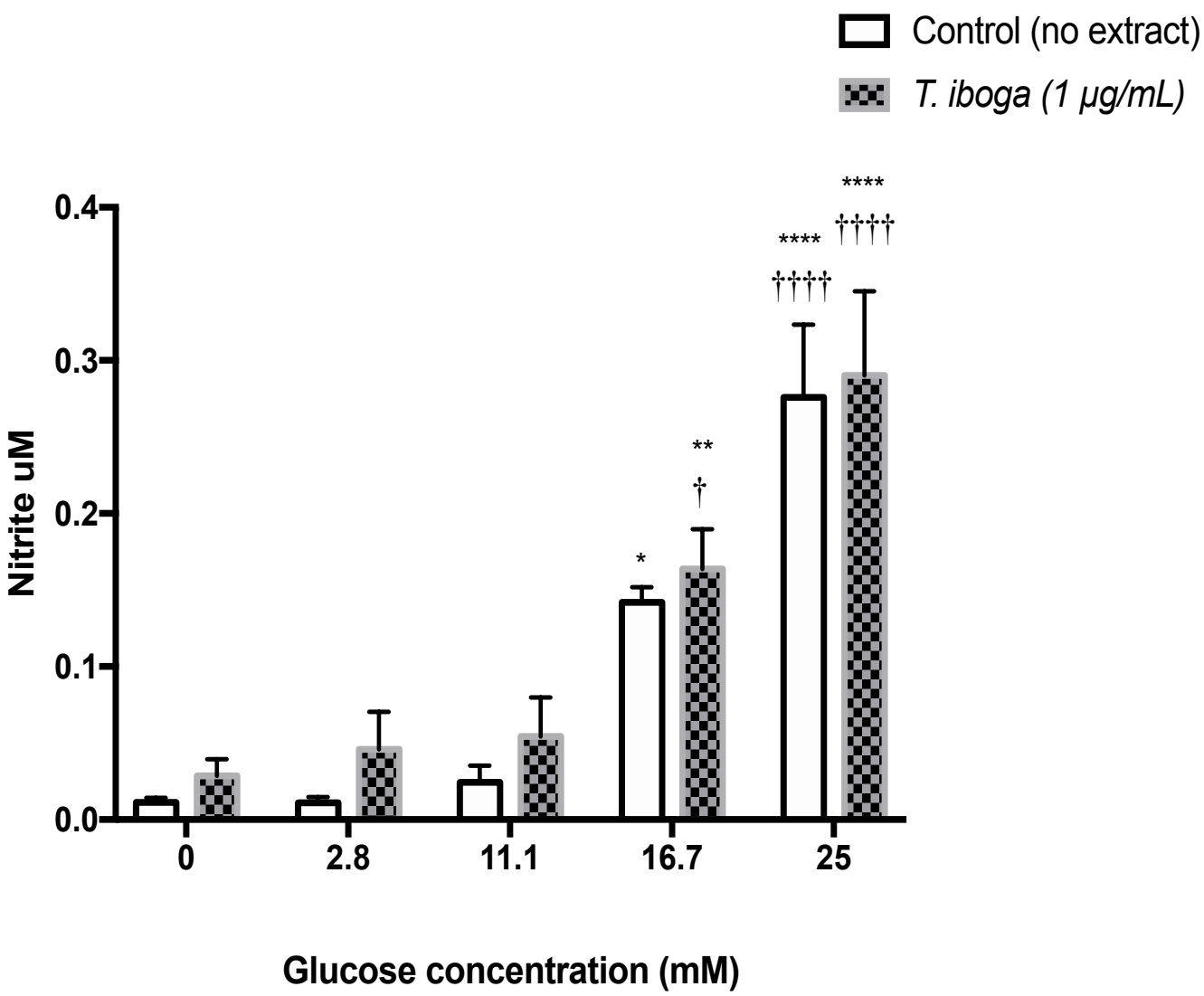


Figure 3.12. Effect of the aqueous extract of *T. iboga* (1 µg/mL) optimal concentration on nitrate production from rat pancreatic islets in the presence of increasing glucose concentrations (0, 2.8, 11.1, 16.7, 25 mM). The levels of total nitrite were recorded which reflects the amount of nitric oxide (NO) in samples. Data is presented as ± SEM of 7 independent experiments and were analysed by two-way ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ relative to 0 mM glucose alone. † $p < 0.05$, †††† $p < 0.0001$ relative to stimulatory glucose (11.1 mM), $p > 0.05$ relative to respective control (no extract).

3.3.9. Concentration dependent effects of *G. tessmannii* (1 µg/mL to 10 mg/mL) aqueous extract on low and high glucose induced insulin secretion from isolated rat pancreatic islets

Figure 3.13 below shows the effect of the aqueous plant extract from 1 µg/mL to 10 mg/mL on insulin secretion in presence of increasing glucose concentrations. Islets were incubated over 90 min at 37^{o}C with increasing glucose concentrations (0, 2.8, 11.1, 16.7 and 25 mM) and in presence of *G. tessmannii*, as described previously. The data shows that in the absence of glucose (Figure 3.13 a) *G. tessmannii* aqueous extract do not influence insulin release. However, at non-stimulatory and stimulatory glucose concentrations (from 2.8 to 25 mM), a bell shape of the effects of *G. tessmannii* is shown. At 2.8 mM of glucose, the aqueous extract triggered significant increase of glucose-induced insulin release compared to control at 50 µg/mL and 70 µg/mL (2.38 vs 0.90 ng/mL/10 islets $p < 0.05$ and 4.23 vs 0.90 ng/ml/10 islets $p < 0.0001$, respectively) (Figure 3.13 a). Moreover, the effect of *G. tessmannii* aqueous extract was increased in a glucose concentration-dependant manner. At 11.1 mM glucose, *G. tessmannii* showed a marked increase in glucose-induced insulin release at 70 µg/mL (6.66 vs 2.94 ng/mL/10 islets, $p < 0.0001$) (Figure 3.13 b). At 16.7 mM of glucose, *G. tessmannii* potentiation of glucose-induced insulin secretion was also seen at 70 µg/mL with 7.12 vs 4.67 ng/mL/10 islets, $p < 0.0001$ (Figure 3.13 c). However, at 25 mM glucose, the effect of *G. tessmannii* at 70 µg/mL was not significant comparing to control. At non-stimulatory glucose concentration (2.8 mM), *G. tessmannii* concentrations lower than 50 µg/mL and higher than 70 µg/mL failed to potentiate glucose-induced insulin release comparing to controls (Figure 3.13 a). Furthermore, a significant inhibition was observed at 16.7 and 25 mM of glucose at *G. tessmannii* low (1 to 50 µg/mL) and high (1 and 10 mg/mL) concentrations (Figure 3.13 c, d). At 11.1 mM of glucose, *G. tessmannii* at 30 and 50 µg/mL did not potentiate insulin secretion. At low (2.8 mM) and high (11.1, 16.7 and 25 mM) glucose concentrations, *G. tessmannii* extract at 70 µg/mL was the concentration optimal compared to control (no extract).

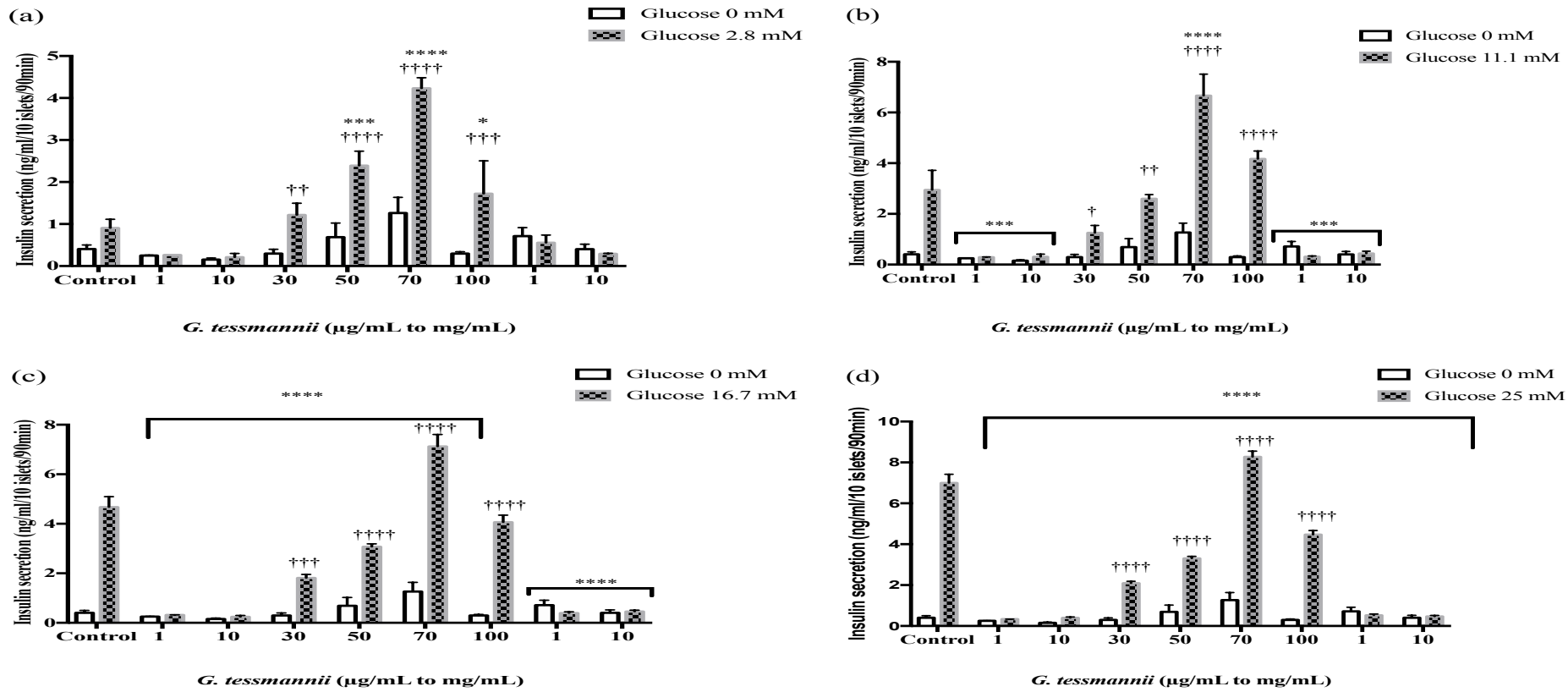


Figure 3.13. Effects of the aqueous extract of *G. tessmannii* (1 µg/mL to 10 mg/mL) on insulin release from rat pancreatic islets. Dose-response effect of *G. tessmannii* on insulin release in the presence of both non-stimulatory (0, 2.8 mM) and stimulatory concentrations (>11.1 mM) of glucose. Values are means of 15 observations. Data were analysed by two-way ANOVA followed by Dunnett's test, effect of *G. tessmannii* and effect of panel a) 2.8 mM b) 11.1 mM c) 16.7 mM d) 25 mM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ relative to respective glucose alone (no extract). † $p < 0.05$, ††† $p < 0.001$ and †††† $p < 0.001$, compared with incubations at 0 mM glucose and at same concentrations of *G. tessmannii* extract.

3.3.10. Effects of *G. tessmannii* (70 µg/mL) on insulin release from isolated rat pancreatic islets in the presence of increasing glucose concentrations

Following the assessment of the effects of *G. tessmannii* in the presence of increasing glucose concentrations, we compared the effects of optimal *G. tessmannii* concentration (70 µg/mL) to those of glucose in isolated pancreatic islets. The figure 3.14 below shows a concentration-dependent increased of insulin levels when cells are incubated with increasing glucose concentrations (2.8 to 25 mM). At non-stimulatory and stimulatory glucose concentrations (2.8 to 16.7 mM), glucose-induced insulin secretion was significantly higher in presence of *G. tessmannii* at 70 µg/mL compared to controls. Indeed, *G. tessmannii* (70 µg/mL) significantly potentiated insulin release from 0.93 to 4.23 ng/ml/10 islets at 2.8 mM ($p < 0.001$), from 3 to 6.66 ng/ml/10 islets at 11.1 mM ($p < 0.0001$) and from 4.67 to 7.12 ng/ml/10 islets at 16.7 mM ($p < 0.05$). However, at 25 mM glucose there was no potentiation by the plant extract compared to the control.

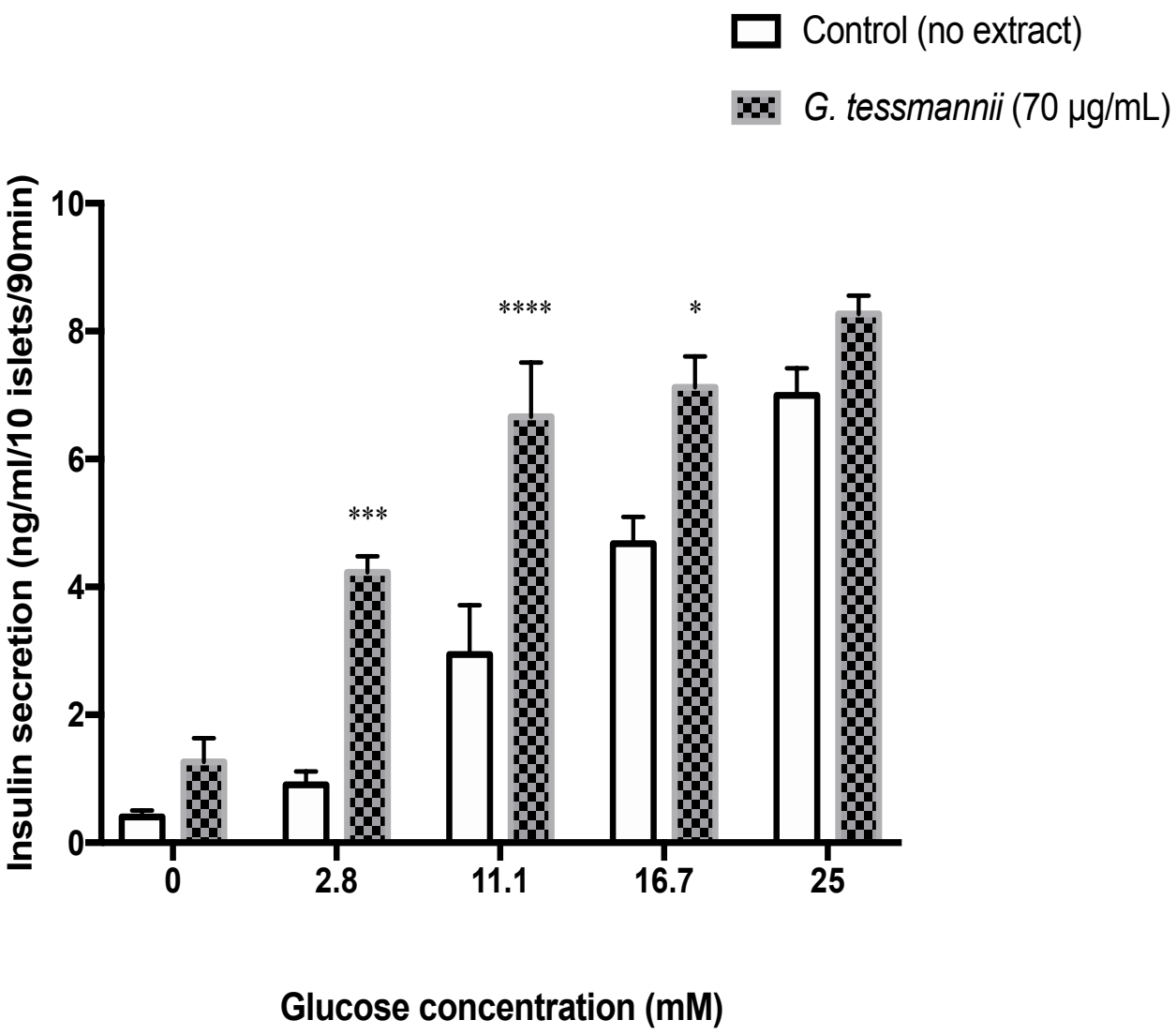


Figure 3.14. Dose-response effect of glucose upon insulin release in the presence and absence of *G. tessmannii* extract (70 µg/mL). Values are means ± SEM for groups of 15 observations and were analysed by two-way ANOVA followed by Tukey's test. * $p < 0.05$, **** $p < 0.0001$, compared with incubations at the same glucose concentration without *G. tessmannii* extract.

3.3.11. Effects of *G. tessmannii* (70 µg/mL) on insulin release from isolated rat pancreatic islets in the presence of Tolbutamide

The effect of optimal *G. tessmannii* (70 µg/mL) concentration in presence of tolbutamide (200 µM) is presented in Figure 3.15 below. The data shows that both tolbutamide and *G. tessmannii* aqueous extract stimulated insulin secretion at glucose non-stimulatory and stimulatory concentrations (2.8, 11.1, 16.7 and 25 mM). Indeed, tolbutamide increased significantly glucose-induced insulin secretion at concentrations greater than 2 mM glucose in a dose-dependent manner. Similarly, *G. tessmannii* at 70 µg/mL potentiated glucose-induced insulin secretion in a glucose concentration dependent manner. Indeed, at glucose concentrations from 2.8 to 16.7 mM glucose *G. tessmannii* aqueous extract induced a higher but not significant potentiation of glucose-induced insulin release compared to tolbutamide at the same glucose concentrations (4.23 vs 2.73, 6.66 vs 6.07 ng/ml/10 islets and 7.12 vs 7.02 ng/ml/10 islets, respectively at 2.8, 11.1 and 16.7 mM glucose), except at 25 mM glucose (8.26 vs 8.79 ng/ml/10 islets). The effects of both tolbutamide and *G. tessmannii* were significantly enhanced compared to the effects of tolbutamide alone at 2.8, 11.1 and 16.7 mM glucose (5.31 vs 2.73, 7.93 ng/ml/10 islets vs 6.07, and 8.59 vs 7.02 ng/ml/10 islets, respectively, $p < 0.001$). A combination effect of the plant extract to the anti-diabetic drug at these concentrations was suggested. However, the effect of tolbutamide was unchanged at 25 mM glucose in presence of *G. tessmannii*.

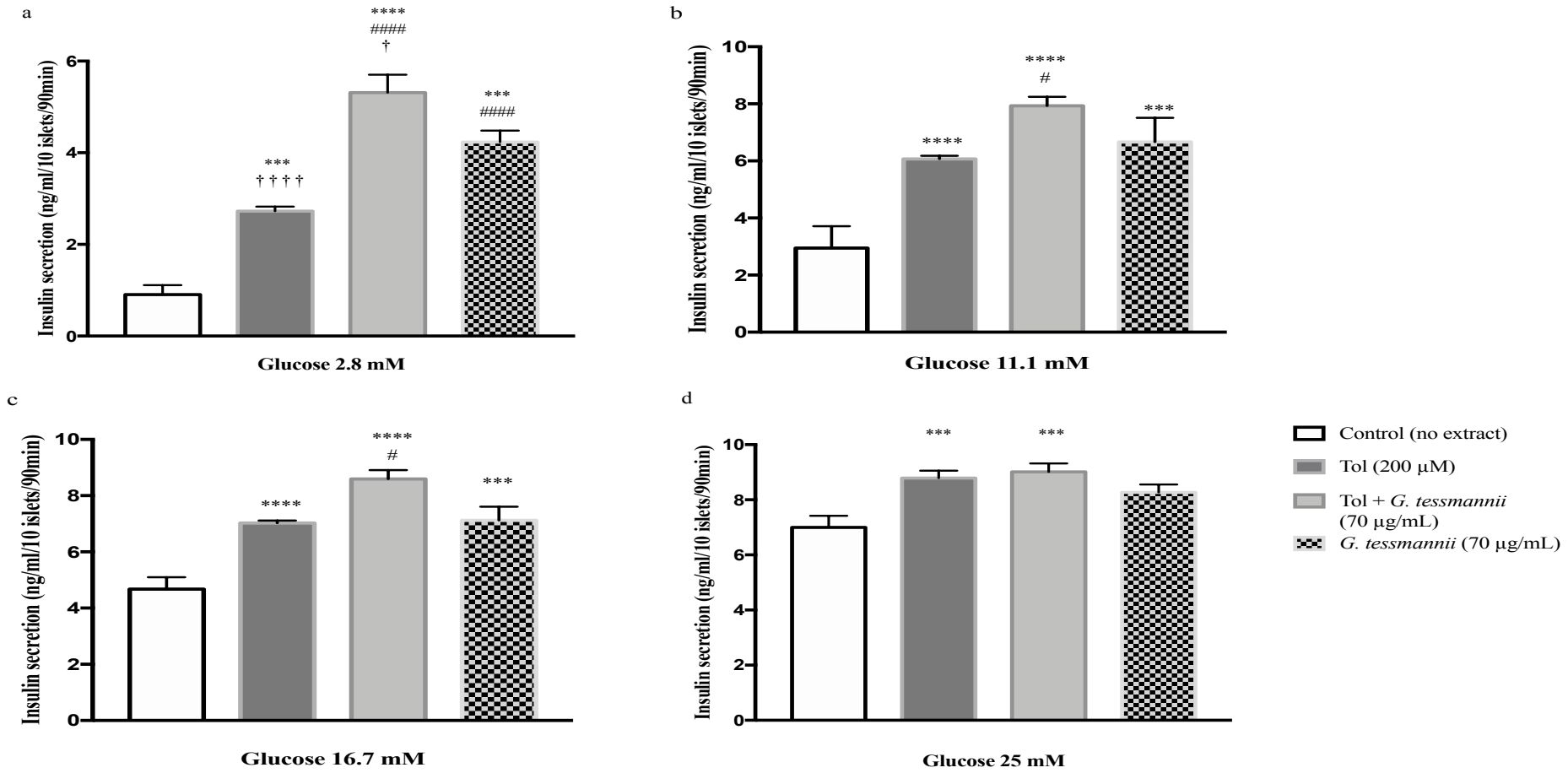


Figure 3.15. Effect of *G. tessmannii* (70 µg/mL) on insulin release from rat pancreatic islets in the presence of Tolbutamide (200 µM) in the presence of 2.8 mM, 11.1 mM, 16.7 mM and 25 mM glucose. Data is presented as ± SEM of 10-22 independent experiments and were analysed by two-way ANOVA followed by Tukey's test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control without extract or drug, #### $p < 0.0001$ compared with Tolbutamide

3.3.12. Effects of *G. tessmannii* (70 µg/mL) optimal concentration and increasing glucose concentrations on nitrite production from rat isolated pancreatic islets

Griess assay was also performed on cells that were incubated with *G. tessmannii* optimal concentration (70 µg/mL) and at increasing glucose concentrations (0, 2.8, 11.1, 16.7 and 25 mM). Levels of total nitrite are shown on Figure 3.16 below, and reflects the amount of nitric oxide (NO), an oxidative stress major marker. Data shows that total nitrite productions are low at 0, 2.8 and 11.1 mM glucose (0.01, 0.01 and 0.02 µM respectively, $p > 0.05$ versus 0 mM) and increased significantly at 16.7 and 25 mM glucose (0.14 and 0.25 µM respectively, $p < 0.05$). Similarly, total nitrite productions in presence of *G. tessmannii* (70 µg/mL) are low at 0, 2.8 and 11.1 mM glucose (0.03, 0.06 and 0.07 µM respectively, $p > 0.05$ versus 0 mM) and significantly increased at 16.7 and 25 mM glucose up to 0.21 µM, $p < 0.001$ for both concentrations. However, nitrite levels in the presence of *G. tessmannii* were not altered when compared to controls (no extract).

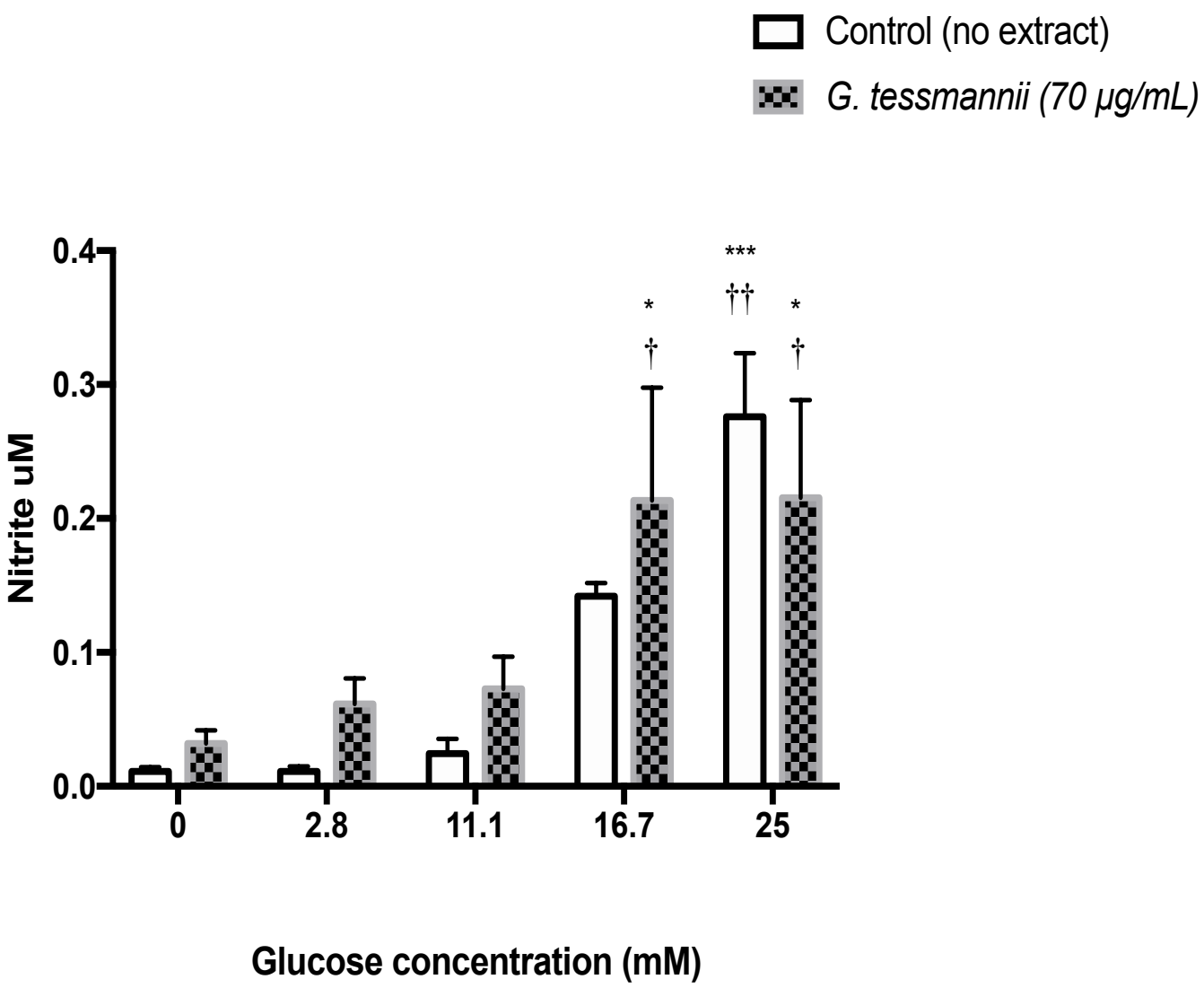


Figure 3.16. Effect of the aqueous extract of *G. tessmannii* (70 µg/mL) optimal concentration on nitrate production from rat pancreatic islets in the presence of increasing glucose concentrations (0, 2.8, 11.1, 16.7, 25 mM). The levels of total nitrite were recorded which reflects the amount of nitric oxide (NO) in samples. Data is presented as ± SEM of 7 independent experiments and were analysed by two-way ANOVA followed by Tukey's test. * $p < 0.05$, *** $p < 0.001$ relative to 0 mM glucose alone. † $p < 0.05$, †† $p < 0.01$ relative to stimulatory glucose (11.1 mM), $p > 0.05$ relative to respective control (no extract).

3.4. Discussion

In this chapter, we explored the mechanism of actions of *T. iboga* and *G. tessmannii* for their anti-diabetic potential activity *in vitro*. We used the same method of extraction for *T. iboga* and *G. tessmannii* as the one used by traditional practitioner's (Bading-Taika *et al.*, 2018). The yield obtained for 75 g of *T. iboga* root barks powder was 2.11 g of aqueous extract, which was consistent to previous data, 2.2 g dry material was obtained for 75 g of *T. iboga* (Souza *et al.,* 2011). In parallel, the yield obtained for 100 g of *G. tessmannii* stem barks powder was 12.49 g of aqueous extract, which was twice more than the yield obtained in previous studies. The discrepancy might be due to technical skills during filtration process (Koumba Madingou *et al.,* 2016). Moreover, this method was chosen in order to deal in our experiments, with the similar compounds given to Gabonese population by traditional therapists for the management of diabetes (Bading-Taika *et al.,* 2018).

Following the aqueous extraction of the medicinal plants, we investigated for *in vitro* activity of the aqueous extracts of *T. iboga* and *G. tessmannii*. Firstly, as the main organ involved with diabetes is pancreas and the pancreatic islets of Langerhans, the isolation of the glandular organ was fundamental. Several techniques have been developed over the years to enable isolation of viable and functional pancreatic islets (Szot *et al.,* 2007; Qader *et al.,* 2007; Carter *et al.,* 2009). The procedure of islets of Langerhans isolation is a well-established series of successive mechanical and enzymatic separations of connected cells, followed by a gradient dissociation for islets purification (Neuman *et al.,* 2014; Miceli *et al.,* 2018). Among the method used in this chapter, histopaque provided better yield of rat islets comparing to Ficoll 400, as it enhanced islets purity (Szot et al., 2007; Carter *et al.,* 2009; Miceli *et al.,* 2018). Also, mechanical damage to the islets was reduced as inflated pancreas was excised and digested without been cut or mechanically digested (Carter *et al.*, 2009). The isolated islets yielded 1887 ± 428, which is close to previous results suggesting a number between 2000 to 5000 isolated islets per pancreas for young rats, this number increases with age and is influenced by the expertise of the technician (Carter *et al.,* 2009). Although, an ultimate expected islets yield is hard to quantify as numerous parameters must be taken into account such as the strain and age of rodent (Qader *et al.,* 2007; Carter *et al.,* 2009). The isolated islets obtained were selected after visual inspection under a light microscope and were similar to previous isolated islets pictures (Carter *et al.,* 2009). The use of primary isolated rat islets in this project allowed a more precise comprehension of how islets respond

to certain stimuli comparing to insulinoma-derived cell lines such as INS-1 and MIN6 ß-cell lines (Neuman *et al.,* 2014, Carter *et al.,* 2009). Indeed, *in vitro* tissue culture models are useful tools for studying specific ß-cells functions. However, the interactions between different cell types within islets including the paracrine and somatostatin which may regulate ß-cells functions, and gap junction between ß-cells that potentiates insulin release may be altered in cell line purified cultures. Thus, the response of these immortalised ß-cell lines to stimuli can consistently differ from fresh cells (Carter *et al.,* 2009; Neuman *et al.,* 2014). Furthermore, using pancreas in whole animal models could be an alternative option than the use of primary isolated islets, however, this method presents its own set of issues including the accurate measure of insulin as liver plays also an important role in the metabolism of insulin (Neuman *et al.,* 2014).

The function of isolated rat pancreatic islets was then tested in presence of increasing glucose concentrations and insulin release measured using ELISA kits. As pancreatic rat islets contain insulin-secreting ß-cells, the ultimate method to confirm the type of cells isolated in this chapter, was by testing their capability to secrete insulin in presence of stimulatory glucose concentrations. Figure 3.7 shows a glucose-induced insulin release in a concentration-dependent manner at 2.8, 11.1, 16.7 and 25 mM glucose. These results were consistent with other results obtained in the same conditions (Souza *et al.,* 2011; Vilches-Flores *et al.,* 2010) and confirmed the isolation of pancreatic islets containing ß-cells by the method selected in this chapter. At this point, glucose-induced insulin release was then assessed in the presence of increasing concentrations of *T. iboga* and *G. tessmannii* aqueous extracts, insulin secretagogue tolbutamide and σ2 receptor antagonist SM-21.

In the same glucose environment than previously, *T. iboga* increasing concentrations (0.001 to 100 µg/mL) exhibited a bell-shape effect on glucose-induced insulin secretion at all glucose concentrations (Figure 3.8). Indeed, at high concentrations of *T. iboga* (10 and 100 µg/mL) no potentiation of insulin secretion was seen at low glucose concentrations (0 and 2.8 mM). However, at high glucose concentrations, *T. iboga* aqueous extract high concentrations (10 and 100 µg/mL) inhibited significantly insulin secretion at 11.1, 16.7 mM glucose and 25 mM compared to control at same glucose concentrations with no extract (1.23 vs 3 ng/mL/10 islets for 100 µg/mL at 11.1 mM glucose, 2.11 vs 4.67 and 0.94 vs 4.67 ng/mL/10 islets for 10 and 100 µg/mL at 16.7 mM glucose and 1.58 vs 6.99 and 0.86 vs 6.99 ng/mL/10 islets for 10 and 100 µg/mL at 25 mM glucose $p < 0.0001$). This negative effect of the σ2 ligand (*T.*

iboga) was already seen in previous data with σ2 receptor agonist PB28 in Cassano *et al*. 2006 experiment. Indeed, in this experiment, high concentrations of *T. iboga* inhibited insulin release and this is thought to be an abolition of the increase of cytosolic Ca^{2+} triggered by the stimulation of high glucose concentrations. Thus, after 60 min incubation of *T. iboga* (10 and 100 µg/mL), high extract concentrations may saturate σ2 receptors of endoplasmic reticulum (ER), mitochondria and other organelles where occur σ2 receptors, resulting in high concentrations of Ca^{2+} intracellular that may lead to cell depletion and decrease of insulin release (Cassano *et al.*, 2006). This could easily be checked using fura-2 test and Griess assay at high *T. iboga* concentrations in further experiments. *T. iboga* (>1 µg/mL) is specifically inhibiting glucose stimulated insulin release at 16.7 and 25mM (no extract) which is in contrast with previous data at 16.7 mM glucose concentration (Souza *et al.,* 2011) but support other observations by Cassano *et al.* (2006).

Interestingly, at the lowest *T. iboga* extract concentrations tested (0.001 to 0.1 µg/mL) and at stimulatory glucose concentrations (11.1, 16.7 and 25 mM), a marked inhibition of insulin secretion was observed compared to controls at the same glucose concentrations. These observations suggested that *T. iboga* aqueous extract may contain antagonistic molecules which have an opposite effect to those that potentiate insulin secretion and inhibit glucose-induced insulin secretion. These molecules at stimulatory glucose concentrations seem more efficient at low concentrations of *T. iboga*, efficacy which is gradually reduced with increasing concentrations of the plant extract (0.01, 0.1 and 1 µg/mL). However, at higher concentrations of *T. iboga* (from 10 µg/mL) there is an antagonism effect of insulin potentiation and secretion at stimulatory glucose concentrations, which was already seen at high concentrations of plant extracts in contrast with low concentrations effects (Djomeni Dzeufiet *et al.,* 2006).

At non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM), *T. iboga* aqueous extract potentiated glucose-induced insulin release with an optimal effect at 1 µg/mL which was significantly increased at 11.1 mM glucose ($p < 0.0001$) compared to the effect at 2.8 mM glucose (Figure 3.8, panel a-b). This effect was in agreement with previous study (Souza *et al.*, 2011). Interestingly, at higher glucose concentrations (16.7 and 25 mM), the effect of *T. iboga* (1 µg/mL) on glucose-induced insulin secretion decreased significantly compared to controls (no extract) ($p < 0.01$ and $p < 0.01$, respectively) and compared to the effect at 11.1 mM ($p < 0.0001$). This drop on the plant potentiation of glucose-induced

insulin release was not seen in Souza *et al.* (2011) at 16.7 mM. However, this discrepancy cannot be discussed as in Souza paper the highest glucose concentration used was 16.7 mM, thus we cannot predict the effect at glucose concentration above than 16.7 mM from their results and compare with our results which could have helped to construct a hypothesis on the contradictory results observed.

The effect of the optimal concentration of *T. iboga* aqueous extract (1 µg/mL) was then compared to tolbutamide, a sulfonylurea commonly used for type 2 diabetes treatment. Sulfonylureas lower blood glucose through a direct insulin secretion stimulation from pancreatic ß-cells. Our data show that *T. iboga* (1 µg/mL) at non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM) potentiated glucose-induced insulin secretion in a glucose-dependent manner. These effects are similar to well-known insulin secretagogue including sulfonylurea, such as tolbutamide (Ball *et al.,* 2004). Moreover, these results are in accordance with previous findings (Souza *et al.,* 2011). In our data, *T. iboga* aqueous extract has shown to be more efficient at glucose stimulatory concentrations (11.1 mM) than tolbutamide and failed to potentiate glucose-induced insulin release at high glucose concentrations (16.7 and 25 mM). Sulfonylureas are known to stimulate insulin secretion from islets of Langerhans by direct binding to the sulfonylurea receptor SUR1, one subunit of K_{ATP} channel in ß-cell plasma membrane. This binding leads to the inactivation of K_{ATP} channel, promoting the membrane potential depolarisation and electrical activity with rise in intracellular Ca^{2+} and insulin secretion (Ball *et al.,* 2004; Souza *et al.,* 2011). However, *T. iboga* aqueous extract has been reported to exhibit an insulinotropic action in a different way. Indeed, previous data have demonstrated that K_{ATP} channels were involved in the effects of *T. iboga* aqueous extract, as those were suppressed in the presence of diazoxide (K_{ATP} channel blocker) which also inhibited, glucose and sulfonylurea-induced insulin release by preventing K_{ATP} channels closure (Souza *et al.,* 2011). Our data shows that *T. iboga* at 1 µg/mL significantly potentiated the effects of Tolbutamide (200 µM) at non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM), this result is in agreement with previous data (Souza *et al.,* 2011). Thus, as mentioned previously, *T. iboga* aqueous extract insulin potentiation is not purely mediated by the same binding sites as sulfonylurea (Souza *et al.,* 2011). In parallel, it has been also shown that *T. iboga* aqueous extract insulinotropic effects were Ca^{2+}-dependent. Indeed, the Ca^{2+}dependency was evidenced by the inhibition of insulin release potentiation by the plant in a Ca^{2+}-free medium and in presence of Ca^{2+}channels blocker (Souza *et al.,* 2011). Moreover, additional evidence of Ca^{2+}

involvement in *T. iboga* glucose-induced insulin potentiation was demonstrated when the plant extract exhibited enhanced Ca^{2+}uptake in islets in the presence of increasing glucose concentrations in Fura-2 test (Souza *et al.,* 2011). It has been previously suggested that *T. iboga* active principles may interact with σ2 receptors (Bowen *et al.,* 1995; Souza *et al,* 2011), which are present in endocrine tissues and have a putative role in modulating the synthesis of enzymes/hormones such as insulin, cytotoxicity and cell death (Bowen, 2001; Ahn *et al.,* 2015).

As *T. iboga* has been suggested to be σ2 receptors agonist (Bowen, 2001; Souza *et al.,* 2011), we assessed *T. iboga* effects at a concentration of 1 µg/mL, that significantly potentiated glucose-induced insulin release in presence of SM-21 at low and high concentrations, along with the involvement of σ2 receptors in insulin secretion. SM-21 or the 3 alpha-tropanyl-2-(4-chorophenoxy)- butyrate have been reported to possess high and preferentially affinity to σ2 receptors and act as functional antagonist at σ2 receptors (Matsumoto and Mack, 2002). Our data show that, there is significant reversal of glucose stimulating insulin release at 2.8 mM, 11.1 mM, 16.7 mM and 25 mM glucose at high concentration of SM-21 (Figure 3.11). Indeed, at 11.1 and 16.7 mM glucose, glucose-induced insulin secretion was inhibited by more than 2-fold, while the insulin secretion was almost totally inhibited at 25 mM glucose at both SM-21concentrations (1 and 10 µM) compared to controls. At low concentration of SM-21 (1 µM) glucose-induced insulin secretion was significantly partially inhibited at stimulatory and high glucose concentrations (11.1, 16.7 and 25 mM) by more than 2-fold at 11.1 mM and 16.7 mM glucose and by 11-fold at 25 mM glucose concentrations, $p < 0.0001$, Figure 3.11. However, at low glucose concentration (2.8 mM), SM-21 (1 µM) inhibition of glucose-induced insulin secretion was not significant but inhibited significantly *T. iboga* effects at the same glucose concentration, indicating a functional antagonism. In the presence of 1 µg/mL *T. iboga*, insulin secretion potentiation was partially inhibited by 3-fold in the presence of SM-21 at 10 µM, Figure 3.11. At 11.1 mM glucose, the effect of both SM-21 concentrations on insulin secretion was inhibited up to 3 ng/ml/10 islets compared to control at same glucose concentration, with no extract (~8 ng/ml/10 islets), while the insulin levels were not altered at 16.7 mM and slightly inhibited at 25 mM glucose. The σ2 receptors involvement in the effect of *T. iboga* aqueous extract was evidenced by the action of SM-21. There are few data in the literature on σ2 receptors mediated insulin secretion experimentations and no known endogenous ligand has been firmly identified yet (Souza *et al.,* 2011; Nicholson *et al.,* 2016; Zeng *et al.,* 2014). However, the inhibition of glucose-

induced insulin secretion by the antagonist SM-21 might be partly explained by the involvement of σ2 receptors in the modulation of intracellular Ca^{2+} flux, which is major in insulin secretion, as mentioned previously (Vilner and Bowen, 2000; Ashcroft and Rorsman, 2012). Furthermore, previous experiments have revealed that σ ligands produce a dual modulation of intracellular Ca^{2+} via the release of Ca^{2+} from intracellular stores and the prevention of depolarisation-dependent Ca^{2+} influx (Vilner and Bowen, 2000). Indeed, in the absence of *T. iboga*, σ2 antagonist SM-21 may alter Ca^{2+} influx in ß-cells and decrease insulin secretion induced by glucose stimulatory concentrations (Ahn *et al.,* 2015). However, in the presence of *T. iboga* aqueous extract, there is a partial inhibition by SM-21 compared to controls with no extract. σ2 receptors were already suggested to block all Ca^{2+} channel subtypes in the body cells (N-, L-, P/G- R-type) and modulate cell-to-cell signalling in sympathetic and parasympathetic neurons, since in neonatal rat intracardiac and superior cervical ganglia, σ2 receptors rapidly depress peak of Ca^{2+} channel currents (by more than 95%) in a reversible manner (Cassano *et al.,* 2006). Hence, taken together, the effects shown at high concentration of SM-21 (10 μM) and with *T. iboga* aqueous extract in our study support the hypothesis that σ2 receptors may negatively modulate an increase in cytosolic Ca^{2+}, which could counterbalance the positive effect on intracellular Ca^{2+} observed after σ2 receptors activation (Cassano *et al.,* 2006).

The indication of oxidative stress on isolated islets from increased glucose and *T. iboga* extract was assessed via Griess assay, a colorimetric assay which detects total nitrite reflecting the amount of nitric oxide (NO) produced, to ensure that the insulin secretion measured was due to treatment-induced insulin secretion and not cell death because of oxidative stress. Indeed, it has been mentioned that pancreatic islets isolation may induce an oxidative stress (Carter *et al.,* 2009; Miceli *et al.,* 2018). Oxidative stress is also a common characteristic of both type 1 and type 2 diabetes mellitus as biomarkers of oxidative stress are strongly elevated in pancreas of diabetics (Miceli *et al.,* 2018). Numerous *in vitro* and *in vivo* studies have shown that mechanical and enzymatic separations of pancreatic islets during isolation, as well as elevated glucose exhibition, may lead to oxidative stress and affecting ß-cells survival and function (Miceli *et al.,* 2018). This issue is due to the imbalance between the production of reactive species (ROS) and the weak antioxidant capacity in ß-cells. As mentioned previously, pancreatic ß-cells are extremely sensitive to oxidative stress due to their frail antioxidant system, caused by a low expression and activity of the hydrogen peroxide (H_2O_2)- inactivating enzymes in insulin-producing cells comparing to other tissues (Miceli *et al.,* 2018). Figure 3.12, showed that at high glucose concentrations (16.7 and 25

mM), levels of total nitrite, thus NO production were significantly ($p < 0.0001$) elevated compared to no glucose or stimulatory glucose concentration of 11.1 mM. These results were already observed in other studies when isolated islets were exposed to high glucose concentrations. Indeed, glucose concentrations of 16.7 and 28 mM are physiological stimulatory concentrations of ROS production, thus elevated NO production compared to controls (2.8 and 11.1 mM), which is consistent to our results (Armann *et al.,* 2007; Zhao *et al.,* 2018). Further the evidence for oxidative stress (elevated nitrates) of islets at concentrations >16 mM glucose may explain the reduction in insulin secretion in the presence of *T. iboga* concentrations > 10 µg/mL insulin secretagogue. High concentrations of *T. iboga* which enhance Ca^{2+} levels via σ2 receptor activation may induce cytotoxicity in cells already sensitive to oxidative stress.

The effects of *G. tessmannii* aqueous extract were assessed under the same conditions as *T. iboga.* At increasing glucose concentrations (0 to 25 mM glucose), data show that the optimal concentration of the aqueous extract of *G. tessmannii* is 70 µg/mL with insulin levels reaching 6.66 ng/ml/10 islets at 11.1 mM glucose and 7.12 ng/ml/10 islets at 16.7 mM glucose compared to controls. The bell-shape effect was also observed in the data and suggest that at stimulatory glucose concentrations (11.1, 16.7 and 25 mM), *G. tessmannii* aqueous extract at low concentrations (1 µg/mL and 10 µg/mL for 11.1 mM glucose, 1 µg/mL to 50 µg/mL for 16.7 and 25 mM glucose) may contain antagonistic molecules which inhibited glucose-induced insulin secretion, as seen with *T. iboga*, exhibiting a dual effect of the plant extract. In addition, at high concentrations of *G. tessmannii* (1 mg/mL and 10 mg/mL at 11.1 and 16.7 mM glucose and from 100 µg/mL to 10 mg/mL at 25 mM glucose) the insulin potentiation effect was also inhibited indicating an opposite effect of the plant extract at these concentrations as seen with *T. iboga* high concentrations (10 and 100 µg/mL) and other plant extracts (Djomeni Dzeufiet *et al.,* 2006).

Interestingly, *G. tessmannii* optimal concentration (70 µg/mL) effects on insulin secretion were increasing with glucose concentrations, compared to *T. iboga* optimal concentration (1 µg/mL). These effects were similar to those produced by insulin secretagogue tolbutamide at same glucose concentrations and those produced by *T. iboga* aqueous extract at 1 µg/mL at stimulatory glucose concentration (7.31 ng/ml/10 islets at 11.1 mM). *G. tessmannii* aqueous extract at 70 µg/mL potentiated glucose-induced insulin secretion in presence of insulin secretagogue tolbutamide at 200 µM, however, the effects were less amplified than those with *T. iboga* (1 µg/mL) under the same conditions, except at glucose concentrations 16.7

and 25 mM. Indeed, at 11.1 mM and 16.7 mM *G. tessmannii* aqueous extract (70 µg/mL) in presence of tolbutamide (200 µM) induced insulin levels to reach 7.93 and 8.59 ng/ml/10 islets respectively compared to 8.04 and 5.24 ng/ml/10 islets for the effects with *T. iboga* at 1 µg/mL, a relatively low concentration compared to *G. tessmannii* optimal concentration of 70 µg/mL. *G. tessmannii* observed effects in presence of tolbutamide suggest that the plant extract may bind at the same receptor of the insulin secretagogue, but not at the same site.

This is the first evidence that *G. tessmannii* has insulin secretagogue activity. *G. tessmannii* phytochemical analyses has revealed bioactive compounds such as flavonoids, alkaloids and triterpenes saponins (Madingou *et al.,* 2012). These molecules have been investigated for several activities including antimicrobial, antioxidant, cytotoxicity, anti-inflammatory, immune-stimulatory and hypoglycaemia (Keller *et al.,* 2011; Cui *et al.,* 2015; Nyangono Beyegue *et al.,* 2013; Hameed *et al.,* 2018). Moreover, saponin-rich fraction from *Momordica charantia*, compound also present in *G. tessmannii*, (Madingou *et al.,* 2012) has shown *in vitro* insulin secretion stimulation in pancreatic ß-cells (MIN6) in a concentration dependent-manner which may support the insulinotropic effect observed with *G. tessmannii* in primary isolated ß-cells (Keller *et al.,* 2011). Furthermore, a previous study has shown that triterpenoid saponins from *Aralia taibaiensis* a Chinese traditional medicine, have potently stimulated the release of insulin from BTC3 cells at stimulatory glucose concentrations (Cui *et al.,* 2015), supporting the effect of *G. tessmannii* aqueous extract (rich in triterpenoid) (Madingou *et al.,* 2012) in pancreatic ß-cells. However, the mechanisms of action of those bioactive compounds are only beginning to be explored, and some of their effects are suggested to be mediated via GPR40 mediated Ca^{2+} and PKC pathways, while PKA pathway has also been suggested to mediate insulin secretion enhancement by flavonoids (Hameed *et al.,* 2018).

As with *T. iboga*, indications of *G. tessmannii* inducing oxidative stress was assessed. (Figure 3.16) shows little or no effect on primary isolated rat islets after treatment with various concentrations of the plant extract.

In this chapter, I demonstrated that *T. iboga* aqueous extract at 1 µg/mL potentiated insulin release at non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM), this effect was more potent than those of insulin secretagogue, tolbutamide (200 µM). Unlike sulfonylureas, *T. iboga* aqueous extract mediated the increase of insulin release through σ2 receptors via rise of intracellular Ca^{2+}. This mechanism was confirmed by the inhibition of *T.*

iboga effects by SM-21, a potent and selective σ2 receptor antagonist, at low and high glucose concentrations. Also, it was demonstrated for the first time, that *G. tessmannii* aqueous extract at 70 µg/mL potentiated insulin release at non-stimulatory and stimulatory glucose concentrations (from 2.8 to 16.7 mM) and these effects were similar to those of tolbutamide. Both plant extracts have additional effect on insulin release when incubated with tolbutamide at 11.1 mM glucose. Moreover, optimal concentrations of *T. iboga* (1 µg/mL) and *G. tessmannii* (70 µg/mL) did not induce oxidative stress at non-stimulatory and stimulatory glucose concentrations (2.8 and 11.1 mM).

Chapter 4: *In vivo* investigation of aqueous extracts of *T. iboga* in Type 2 fructose-fed hyperglycaemic diabetic rats

4.1. Introduction

DM as described in chapter 1, is a disease mainly characterised by elevated levels of blood glucose as a result of defects in insulin secretion and/ or insulin action (Motala and Ramaiya, 2010). T2DM is the most prevalent form of diabetes which and results largely from the body's ineffective use of insulin, insulin resistance, excess of body weight and physical inactivity (Motala and Ramaiya, 2010) leading to glucose intolerance, hyperglycaemia and overt diabetes causing blindness, end-stage renal disease as a consequence of microvascular pathology, numerous debilitating neuropathies increasing morbidity and mortality in diabetics (Tripathy *et al.,* 2006; Domingueti *et al.,* 2016).

The root bark of *T. iboga* is widely used in Gabonese traditional medicine for the management of T2DM, as mentioned previously (Bading Taika *et al.,* 2018). Previous *in vitro* studies have demonstrated that the aqueous extract of *T. iboga* has sulfonylureas-like action on insulin release (Souza *et al.,* 2011). *T. iboga* aqueous extracts (1 µg/ml) potentiated the stimulation of insulin secretion at glucose stimulatory concentrations (11.1 and 16.7 mM) *in vitro* via the closure of K^+-$_{ATP}$ channels and the increase of Ca^{2+} influx in isolated pancreatic islets triggering glucose-induced insulin release, in the same way as the medicine tolbutamide (200 µM) (Souza *et al.,* 2011). Furthermore, as shown in chapter 3, we confirm that *T. iboga* aqueous extract (1 µg/ml) potentiates the insulin secretion at glucose non-stimulatory and stimulatory concentrations (2.8 and 11.1 mM) from isolated rat pancreatic cells via a novel interaction with σ2 receptors.

T. iboga aqueous extract is reported to be consumed for the management of T2DM among Gabonese population (Tjeck *et al.,* 2016; Bading Taïka *et al.,* 2018). Although, to date, there is a lack of scientific information regarding the appropriate range of concentrations for *T. iboga* extract to be used safely for diabetes management in animals and in patients (Bading Taïka *et al.,* 2018).

Animal models of diabetes are widely used to investigate *in vivo* efficacy, mode of action and side effects of anti-diabetic plants (Eddooks *et al.,* 2012). Wilson and Islam, (2012) have characterised a non-genetic, cost effective, model for T2D in rats with a fast induction time

and a stable pathogenesis of T2D of insulin resistance and partial pancreatic ß-cell dysfunction over an 11 weeks' experimental period. One goal of this chapter was to determine the *in vivo* anti-diabetic effects of a range of concentrations of *T. iboga* (50, 100 and 200 mg/kg b.w.) in this rat model of T2D. That alternative non-genetic model for T2D has shown similar clinical pathogeneses than those seen in human especially insulin resistance (IR) and partial pancreatic ß-cells dysfunction and, can be developed in an easy way and within a shorter period of time (Wilson and Islam, 2012). Given the insulin potentiation effects of *T. iboga in vitro* (Chapter 3), here we have determined the effect of *T. iboga* on oral glucose tolerance and intraperitoneal fasting insulin tolerance *in vivo* using the STZ/Fructose T2D models, as both techniques are useful tools routinely used to evaluate the influence of plant extracts or drugs on insulin sensitivity in laboratory animals *in vivo* (Guarino *et al.,* 2013). However, the neurotoxic effects of ibogaine, one major active principle of *T. iboga*, was reported to be due, in part to agonist at the same σ2 receptor (Bowen, 2001; Glick *et al.,* 2001).

Evidence has shown the main constituent in *T. iboga*, ibogaine, exhibits toxicity with LD50 ranging from 145 to 175 mg/kg via i.p. and 263 to 327 mg/kg p.o. in rats (Kubiliene *et al.,* 2008). Despite the neurotoxic and cardiotoxic properties of ibogaine, there may be other compounds in *T. iboga* aqueous extract with hypoglycaemic properties that prevent or reduce the development of T2D (Gonzales-Castejon *et al.,* 2011; Bading-Taïka *et al.,* 2018), supported by in vitro studies (Chapter 3, Souza *et al.,* 2011).

The main criterion/ feature, characteristic/property in the selection of a medicinal plants for use in health purposes is safety, as plant extracts should not only be considered efficacious but also safe for consumption (Bulus *et al.,* 2011). To determine a safe non-toxic range of concentrations of *T. iboga* aqueous extract, we first evaluated the toxicity in healthy rats (objective 1). These results provided information for selecting a safe pharmacological concentration range of 50 to 200 mg/kg for evaluating *in vivo* hypoglycaemic efficacy in healthy rats and T2D rats (objective 2 and 3) (Elufioye and Onoja, 2015).

In this Chapter, we aimed to evaluate *T. iboga* toxicity (from 50 to 200 mg/kg) and optimal dose regimen *in vivo* in rats, to evaluate the *in vivo* hypoglycaemic effect of *T. iboga* (50-200 mg/kg) in naïve control rats, to investigate the *in vivo* anti-diabetic effect of *T. iboga* (50-200 mg/kg) in Fructose-induced STZ type diabetic animal model, to evaluate the potential adverse effects in Fructose-induced STZ diabetic model of long time (28 days) administration

of *T. iboga* (50-200 mg/kg). The animal doses were calculated based on the reported quantity of *T. iboga* administered daily by traditional healers to their patients. This dose was considered as the equivalent human dose (HED) (100 ml of the aqueous extract of *T. iboga* per day) which was used to calculate back the range of doses for toxicological and *in vivo* studies (Hosseini *et al*., 2018).

4.2. Materials and methods

4.2.1. Animals and environment

Wistar rats (180-250 g) from the Animal Unit of the Institute of Pharmacopoeia and Traditional Medicine (IPHAMETRA), were used for toxicological and pharmacodynamics studies. Wistar rats (9 weeks age) were housed in Group of same genders (3 each) and similar weight in standard cages (Techniplast 2000P) with sawdust and shredded paper wool bedding with free access to water and food (Labdiet 5001). All study protocols were approved by the National Committee of Ethics in Scientific Research of Gabon (0013/2019/MESRSTT/USTM/VR).

4.2.2. Preparation of *T. iboga* aqueous extract

The root barks of *T. iboga* were collected in Gabonese forest during harvest time by botanists from IPHAMETRA (the institute of pharmacopoeia and traditional medicine) and were authenticated (Gabon National Herbarium). Voucher specimens were deposited in the National Herbarium of Gabon as sample no. (20358). The air-dried root barks were grinded using a traditional mortar and pestle, and the fine powder was macerated as described in Chapter 3 (materials and methods- Plant material and preparation of *T. iboga* and *G. tessmannii* aqueous extracts).

4.2.3. Toxicological studies – Acute and sub-acute toxicities (dosing schedule) of *T. iboga* aqueous extract in Wistar rats

Acute toxicity in Wistar rats over 24 hours

Toxicity studies were conducted in accordance to the method recommended by UNICEF/UNDP/World Bank/ WHO special program for research guidance on non-clinical testing strategies, 2002 for the assessment of toxicity of medicinal plants, with slight modifications for *T. iboga*. Acute oral toxicity and toxicity by intraperitoneal route were carried out according to modified Lorke's method (Lorke, 1983). This method was simple, reproducible and used minimum number of animals.

- *Lorke's modified method for oral toxicity consists of two phases 1 and 2 respectively*

The phase 1 requires nine animals, divided into three Groups of three animals each. Each Group of animals is administered a single different dose of the plant extract tested (10, 100, 1000 mg/kg body weight) (Table 4.1). The animals are placed under observation for their gross behavioural, neurologic (such as tremors, seizures), autonomic and toxic effects up to 24 hr, as well as mortally.

Table 4.1. Doses ranges in animal models and equivalence to human dose (Muhammad Ahmed, 2015)

No	Dose	Ratio
1	10 mg/kg	Less than human dose
2	100 mg/kg	App similar to human dose
3	200 mg/kg	2 times greater than human dose
4	500 mg/kg	4 times greater than human dose
5	1000 mg/kg	7 times greater than human dose
6	5000 mg/kg	35 times greater than human dose
7	10000 mg/kg	70 times greater than human dose
8	Saline (10 ml/kg)	control Group

In the phase 2, further specific doses of the extract were administered once to determine the correct LD_{50} value. The phase 2 consisted of three animals divided into three Groups of one

animal each. The animals received higher doses 1600, 2900, and 5000 mg/kg b.w. of tested plant extract and then observed for 24 hr. Animals were observed frequently on the day of treatment and surviving animals were monitored for signs of acute toxicity - the gross behavioural, neurologic, autonomic and toxic effects, as well as mortality. Food consumption, faeces and urine were also examined at 2 hr and then at 6 hr intervals for 24 hr. For the surviving animals, food consumption and body weight were monitored for up to three weeks to established recovery from any toxicity.

Then the medium Lethal Dose (LD_{50}: the dose that kills 50% of test population) was calculated using the following formula:

$LD_{50} = \sqrt{(D0 \; x \; D100)}$

D_0 = Highest dose that gave no mortality

D_{100} = Lowest dose that lead to mortality

Hodge and Sterner scale (Table 4.2, below) was used for the evaluation of toxicity associated with the LD_{50}.

Table 4.2. Toxicity scale (Hodge and Sterner, 2005)

No	Term	LD50 (rat, oral)
1	Extremely toxic	Less than 1mg/kg
2	Highly toxic	1-50 mg/kg
3	Moderately Toxic	50-500 mg/kg
4	Slightly Toxic	500-5000 mg/kg
5	Practically Non-toxic	5000-15,000 mg/kg

Subacute oral toxicity in Wistar rats over 28 days

The subacute oral toxicity was conducted on rats of both gender, divided into eight Groups of three animals each (three females and three males). The doses were selected regarding the acute toxicity results in rats' dose (Table 4.4). The highest subacute dose was $1/5^{th}$ of the

highest dose of the acute toxicity study. All animals were treated daily p.o. (Dose volume = Xml/kg) for twenty-eight days and animals were observed twice daily for any adverse effect or toxic signs and behavioral alterations, mortality and morbidity until the completion of the experiment (Adewale *et al.,* 2016). Extract or distilled water were administered by oral gavage. Body weights (weekly), food and water consumption (daily) were recorded. The Groups were distributed as followed:

Group 1 and 2: Female and Male Control Group (vehicle p.o. equivalent dose volume)

Group 3 and 4: Female and male 50 mg/kg b.w. of *T. iboga p.o.*

Group 5 and 6: Female and male 100 mg/kg b.w of *T. iboga p.o.*

Group 7 and 8: Female and male 200 mg/kg b.w. of *T. iboga p.o.*

On the 29^{th} day, the animals were sacrificed by cervical dislocation after CO_2 exposure following overnight fasting. Animal weight was recorded, organs such as heart, liver, kidneys, spleen, lungs, testis and ovaries were carefully dissected out and weighed. Serum biochemical parameters were measured (AST, ALP, ALT, TC, Urea, TG, Blood Glucose, Creatinine).

4.2.4. Experimental design for the assessment of hypoglycaemic activity of *T. iboga* aqueous extract

20 healthy Wistar rats were divided and treated in the following pattern according to Jaiswal *et al*. (2009): 5 per Group

- Group 1: Normal control, vehicle, distilled water DV ml/kg, p.o. NC (N=5)

- Groups 2: *T. iboga* 50 mg/kg p.o.

- Group 3: *T. iboga* 100 mg/kg p.o.

- Group 4: *T. iboga* 200 mg/kg p.o.

For all experiments, Fasting Blood Glucose (FBG) was checked initially (overnight fast 12 hr), then Blood Glucose Level (BGL) was taken 90 min before treatment, which was considered as time "0 hr" value. An oral glucose tolerance test (OGTT) was performed to assess the hypoglycaemic activity of *T. iboga* on healthy rats.

- OGTT

After a 12 hr overnight fast, rats were weighed before taking a blood glucose measurement at time 0 hr (90 min before administration of the glucose solution with treatment). We returned rats from each Group in their respective Group cage (no food or water).

One drop of tail vein blood was obtained by a needle prick of conscious rats and measured using a glucometer (Accu-check active, @Roche, Germany). Glucose meter was calibrated according to the manufacturer's instructions before each experiment.

We used gavage tube with rounded tip 20 gauge for glucose oral administration.

Following FBG, a glucose solution of 20% D-Glucose in 0.9% NaCl (2 g/kg rat was given at a DV of 20 ml/kg rat), or 20 µl of 20% glucose/g rat (Table 4.3).

Blood glucose at 1 hr, 2 hr and 3 hr after glucose injection at 30 sec intervals were measured. We returned rats at completion of 180-minute time point to their respective cages with food and water. We dissolved 10 g of D-glucose (Sigma) in 50 ml of 0.9% NaCl before use.

0.9% NaCl: Dissolve 0.9 g NaCl (Sigma) in 100 ml of deionized water.

Doses of *T. iboga* were calculated according to the subacute toxicity test results. We used values below the 10% of oral DL_{50} (1442.77 mg/kg b.w.) in rats, we obtained a value of 144.28 mg/kg b.w. value that gives no adverse effect and good recovery after a long-term treatment with *T. iboga* aqueous extract.

Table 4.3**.** Experimental treatment design for the effect of *T. iboga* on OGTT (FBG: Fasting blood glucose; dH2O: distilled water; BG: Blood glucose; BGL: Blood glucose level; T0: Time 0 hr; T1: Time 1 hr; T2: Time 2 hr; T3: Time 3 hr; b.w.: body weight)

Treatment Group	**Group 1 (Normal Control, NC)**	**Group 2 *T. iboga* (50 mg/kg)**	**Group 3 *T. iboga* (100 mg/kg)**	**Group 4 *T. iboga* (200 mg/kg)**
Route	Oral	Oral	Oral	Oral
Dose	10 ml of dH2O + D-glucose (20%)	50 mg/kg b.w. *T. iboga* + D-glucose (20%)	100 mg/kg b.w. *T. iboga* + D-glucose (20 %)	200 mg/kg b.w. *T. iboga* + D-glucose (20 %)
FBG	Initial BG value after fasting	Initial BG value after fasting	Initial BG value after fasting	Initial BG value after fasting
BGL (T0)	BGL 90 min before treatment administration	BGL 90 min before treatment administration	BGL 90 min before treatment administration	BGL 90 min before treatment administration
BGL (T1)	BGL 1 hr after T0	BGL 1 hr after T0	BGL 1 hr after T0	BGL 1hr after T0
BGL (T2)	BGL 2 hr after T0	BGL 2 hr after T0	BGL 2 hr after T0	BGL 2 hr after T0
BGL (T3)	BGL 3 hr after T0	BGL 3 hr after T0	BGL 3 hr after T0	BGL 3 hr after T0

4.2.5. Experimental design of the assessment of anti-diabetic activity of *T. iboga* aqueous extracts on type 2 fructose diabetes according to Wilson and Islam (2012)

A non-genetic rat model T2D was generated in accordance with Wilson and Islam (2012). 38 male Wistar rats weighing 180-250 g were used. Water supplemented with 10% fructose (FR10) for an initial 2 weeks' period was provided followed by normal water regimen and Labdiet 5001 *ad libitum* throughout the experimental period. The normal control Group (NC, Group 1) was provided with normal drinking water throughout the study.

On the day of STZ injection, STZ was dissolved in a citrate buffer (pH 4.4) at a concentration of 4 mg/ml and allowed to stand in the fridge for 30 min prior to injection, as this time period allows for the more toxic anomer of STZ to equilibrate with the less toxic anomer, thereby standardising the toxic quality of the preparation (De la Garza-Rodea *et al.* 2010). All animals were fasted overnight and injected intraperitoneally (i.p., DV 10 ml/kg) with a low dose of STZ 40 mg kg b.w. except for Group 1 (normal control, NC) animals that were injected with vehicle citrate buffer, pH 4.4. Blood glucose was measured by using a glucometer on a droplet of blood collected from tail vein. Rats with Non-Fasting Blood Glucose (NFBG) level ≥ 300 mg/dl were considered as diabetic and selected for experimental study.

Weekly FBG and NFBG were measured throughout the experimental period. For 48 hr following the STZ or vehicle injection 2% sucrose solution was provided to avoid initial hypoglycaemia observed following STZ treatment. All treated animals were giving a choice of 2% sucrose or water (providing 1 bottle of water and 1 bottle of 2% sucrose per cage) during the 48 hr period after STZ or buffer injection and then returned to normal drinking water *ad libitum* throughout the study.

In the experiment, a total of 42 rats, including 10% of possible non-diabetic rats (30 diabetic surviving rats and 5 normal rats) were used. Animals were randomly divided based upon mean body weight into 8 groups of 5 rats each after the confirmation of diabetes (blood glucose ≥ 300 mg/dl) and drugs/ extracts were administered as in Figure 4.1. Doses were selected according to the sub-acute toxicity test results and hypoglycaemic efficacy (Figure 4.2).

Group 1: Normal Control: no Fructose, no STZ

Group 2: STZ control: STZ (40 mg/kg i.p.), no Fructose

Group 3: STZ/ Fructose 10% Control STZ/FR10 (40 mg/kg i.p.)

Group 4: *T. iboga* (50 mg/kg p.o.) + Fructose (10%, + STZ (40 mg/kg i.p.)

Group 5: *T. iboga* (100 mg/kg p.o.) + Fructose (10% + STZ (40 mg/kg i.p.)

Group 6: *T. iboga* (200 mg/kg p.o.) + Fructose (10% + STZ (40 mg/kg i.p.)

Group 7: Glibenclamide 5 mg/kg b.w. + Fructose (10%), + STZ (40 mg/kg i.p.)

Group 8: Fructose control: Fructose (10%), no STZ

Treatments (*T. iboga*, Glibenclamide, Vehicle) were orally administered once daily (8 am every day) for four weeks (28 days).

Blood glucose was measured at 0, 30, 60 min then 120 min after first treatment administration and then daily (NFBG) during the treatment period. A droplet of blood was withdrawn from the tail vain each time and blood glucose measured with a glucometer (Accu-Check active, @Roche, Germany)

Oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (i.p.ITT) were performed at the 2^{nd} and 4^{th} weeks of treatment.

- OGTT procedure was detailed in section 4.2.4.
- ipITT

Intraperitoneal insulin tolerance test or i.p.ITT is designed to determine the sensitivity of insulin-responsive tissues in the rodent. This is determined by the measurement of glucose remaining in the circulation over time after a bolus i.p. insulin injection.

Stock of Humulin R solution (100 U/ml) was diluted with saline to 0.5 U/ml (1/200 dilution) by adding 10 µl stock (100 U/ml) to 1990 µl 0.9% (w/v) sterile saline.

Working insulin solution (0.075 U/ml) of 12 ml was prepared by adding 1.8 ml of 0.5 U/ml insulin solution to 10.2 ml of sterile physiological saline (0.9% NaCl) in a sterile 15-ml centrifuge tube.

Rats fasted overnight before the day of experiment. We measured blood glucose prior insulin treatment. Then, administered the rat an intraperitoneal injection of 10 µl of 0.075 U/ml insulin solution per gram body weight (dose of 0.75 U/kg, 10 mL/kg) with a 28 G x ½" needle.

Blood samples were taken from the tail vein by a needle prick of conscious rats and measured using a glucometer before the insulin injection and at 30, 60, 90 and 120 min. Between each of these time points, we returned the rat to its cage with no food and water.

Food and water intake, and body weights were recorded on a daily basis (morning) during the 28 days' experiment. At the end of 4 weeks' experiment, rats were anaesthetised by CO_2 asphyxiation and opened at the abdomen. Blood was withdrawn from the abdominal aorta and centrifuged at 3000 rpm for 10 min to obtain serum.

Organs (heart, lungs, liver, kidneys, testis) were weighed and serum biochemical parameters measured (HDL, LDL, Hba1c, AST, ALP, ALT, TC, Urea, TG, Blood Glucose, Creatinin) and also insulin resistance (HOMA-IR, HOMA-β scores).

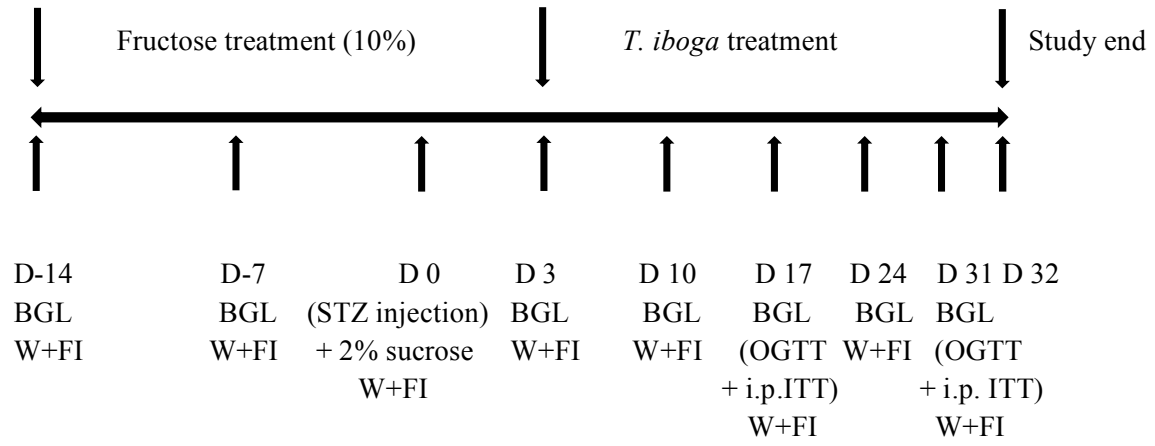


Figure 4.1. Experimental schedule for the induction and development of STZ/FR10 T2D model and *T. iboga* treatment. D: day, BGL: blood glucose levels, OGTT: oral glucose tolerance test, ITT: insulin tolerance test, W: weight, FI: fluid intake

4.2.6. Biochemical analyses

Biochemical analyses in Gabon were performed following blood samples collection from the heart of the animal using the heart puncture technique. Blood was collected in EDTA tubes which contain the anticoagulant heparin. The blood samples were rapidly centrifuged at 3000 rpm for 10 min using a bench-top centrifuge (CYAN CL008, Cypress Diagnostics, Belgium) to obtain plasma. The plasma obtained was used for biochemical analyses using the biochemistry analyzer, CYANsmart (Cypress Diagnostics, Belgium), which is an easy-to-operate semi-automatic biochemistry analyzer.

Analyses carried out included the measurement of the activity of the serum alanine aminotransferase (ALT), the aspartate aminotransferase (AST) and the alkaline phosphatase (ALP), along with the serum Total Protein (TP), urea, and creatinine concentrations which are indicators of kidney function. Besides, the serum Triglyceride (TG) (fat carried in the blood), High-Density Lipoprotein (HDL) also called "good" cholesterol, Low-Density Lipoprotein (LDL) also called "bad" cholesterol, and Total Cholesterol (TC) concentrations were assessed to obtain an indication of the extract/drug effects on lipid profile. Also, serum glucose and glycated hemoglobin (for long period blood sugar level involved in diabetes conditions) concentrations gave indications and monitoring of diabetes. All these parameters were assessed using commercially available test kits (Biomed-Gabon, Cypress Diagnostics, Belgium). Glucose was measured using a glucometer (Accu-Check active, @Roche, Germany).

4.2.7. Data analysis

All experimental data are presented as mean ± standard error mean SEM. Data analysis was performed with GraphPad Prism 7.0 software to determine the level of statistical significance. When the level of probability (p) was less than 0.05 (*), 0.01 (**), 0.001 (***), or 0.0001 (****) the effect of the difference was considered statistically significant, very significant or extremely significant compared to an independent variable. One-way ANOVA was conducted to examine the effect of a single independent variable (e.g. blood glucose) on more than two Groups (e.g. extract concentrations, vehicle) followed by Dunnett's test for comparison between treatment Groups.

Two-way ANOVA was conducted to evaluate the effect of two independent variables in specific experiments followed by Tukey's tests, in terms of the effect of treatment (e.g., STZ) in addition to the effect of the tested drug concentration (*T. iboga* or Glibenclamide). Body weight, food intake, OGTT, and i.p.ITT data were analysed using a repeated-measures-in-time design ANOVA. Repeated measures within animals were modelled using a first-order autoregressive variance-covariance structure (body weight and food intake over time) or an unstructured variance-covariance structure (OGTT and i.p.ITT data over time). Baseline values were used as linear covariate for body weight and food intake data.

4.3. Results

4.3.1. Toxicological studies - Acute and sub-acute toxicities

4.3.1.1. Acute toxicity of *T. iboga* in rats

Table 4.4. Acute toxicity of *T. iboga* aqueous extract administered by oral route to Wistar rats (b.w.: body weight)

Experiment	Dose (mg/kg b.w.)	Mortality after 24 hr	Symptoms after 2 hr	Mortality after 14 days
Phase 1	10	0/3	Nil	0/3
	100	0/3	Light abdominal cramps	0/3
	1000	0/3	Light abdominal cramps and lost balance	0/3
Phase 2	1600	0/1	Light abdominal cramps and lost balance	0/1
	2900	1/1	Abdominal cramps, turning around, lost balance	1/1
	5000	1/1	Strong abdominal cramps, respiratory distress, trembling	1/1

We obtained by oral route a maximum dose of the extract that kills 0% of the Group (LD_0) of 784.71 mg/kg b.w. and the minimum dose of the extract that kills 100 % of the population (LD_{100}) of 2000 mg/kg b.w.

LD_{50} by oral route was 1442.77 mg/kg b.w.

T. iboga aqueous extract was found to be slightly toxic by oral route, according to Hodge and Sterner scale (Table 4.2).

A maximum dose of the extract that kills 0% of the population by intraperitoneal (i.p.) route was (LD_0) of 350 mg/kg b.w. and the minimum dose of the extract that kills almost 100 % of the Group (LD_{100}) of 375 mg/kg b.w.

LD_{50} by i.p. route was 362.28 mg/kg b.w.

T. iboga aqueous extract was found to be moderately toxic by i.p. route, according to Hodge and Sterner scale (Table 4.2).

4.3.1.2. Sub-acute toxicity study in rats

4.3.1.2.1. Mortality and general behaviour

Table 4.5. Sub-chronic toxicity of *T. iboga* administered by oral route to rats for 28 days (dw: distilled water; +: level of irritability)

Doses (mg/kg)	**Toxic symptoms**
0 (1 ml/ 100 g of dw)	None
50	None
100	None
200	Irritability (+)

Oral ingestion of the aqueous extract of *T. iboga* at 50, 100 and 200 mg/kg b.w. once daily for 28 days did not induce any mortality in rats of both genders. No significant difference in food and water consumption was recorded in treated and control Groups.

Body weight was recorded once a week during the 28 days' treatment, as shown on Table 4.6 and organs weight were measured on the 29th day (Table 4.7).

Table 4.6. Effect of *T. iboga* root barks during 28 day's treatment on healthy rats' body gain weights % (Values are mean ± SEM (n=5). Two-way repeated measures ANOVA followed by Dunnett's multiple comparisons test. Statistical difference between groups are indicated with different letters (* $p < 0.05$ compared to respective control group)

Day/ *T. iboga* doses (mg/kg)		**0**	**50**	**100**	**200**
7	**Male**	+ 7.16	+ 17.24*	+ 12.55*	+ 18.6*
	Female	- 12.6	+ 1.48*	+ 12.74*	- 4.16
14	**Male**	+ 0	+ 8.23*	+ 2.55*	+ 6.96*
	Female	- 2.42	+ 5.65*	+ 2.54*	+ 1.91
21	**Male**	+ 0.07	+ 10.58*	+ 20.92*	+ 4.62
	Female	+ 0	+ 2.75*	+ 5.70*	- 0.52
28	**Male**	+ 6.15	+ 4.99*	+ 13.20*	+ 0.8
	Female	- 0.08	- 2.11*	- 9.38*	- 0.17

Male rats in control Group gained weight throughout the study. However, female rats in control Group lost weight during the first two weeks and then did not gain or lost weight (Table 4.6). In Group 1, treated with 50 mg/kg b.w. of *T. iboga*, male rats gained significantly more weight from the first week until the end of the study compared to control ($p < 0.05$) Group (Table 4.6). Similarly, female rats of the same Group gained weight throughout the study (Table 4.6). In Groups treated with 100 and 200 mg/kg b.w., male and female rats gained weight throughout the experimental period (table 4.6). The data in Table 4.6 shows weight gain in all experimental Groups throughout the study.

Table 4.7. Organ weights of healthy rats (male and female) after 28 days of oral treatment with *T. iboga* (Values are mean ± SEM (n=5). Two-way ANOVA followed by Dunnett's multiple comparisons test. Statistical difference between Groups are indicated as follow: ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared with control Group)

Organs		**Control (no extract)**	***T. iboga* 50 mg/kg b.w.**	***T. iboga* 100 mg/kg b.w.**	***T. iboga* 200 mg/kg b.w.**
Heart	**Male**	1000.6 ± 69.1	1285.6 ±21.6	978.4 ± 14.2	1045.4 ± 22.8
	Female	866.2 ± 33.2	745.6 ± 2.1	681 ± 8.3	999.2 ± 12.5
Lungs	**Male**	1817.2 ± 153.2	2407.6 ± 235.7	1685.6 ± 82.7***	2996.2 ± 499.7****
	Female	1128 ± 150.6	1305.2 ± 2.5	1682 ± 18.5**	1484.4 ± 311.4
Liver	**Male**	7501.4 ± 118.2	7285.8 ± 46.4	6844 ± 92.6***	7993.2 ± 86.8**
	Female	5637.2 ± 255.1	5694.2 ± 37.5	5492.4 ± 39.7	7331 ± 175.1****
Kidney	**Male**	896 ± 44.3	1043.8 ± 33.1	811 ± 17.1	899 ± 6.1
(L)		712.2 ± 46.3	584.6 ± 4.8	648.6 ± 4.4	900.8 ± 12.1
Female					
Kidney	**Male**	946.2 ± 68.0	1054.8 ± 43.2	799 ± 22.7	917.8 ± 26.0

(R) Female	722.2 ± 39.2	639.6 ± 14.0	653.6 ± 9.4	928.8 ± 13.2
Spleen Male	627.2 ± 19.2	764.4 ± 6.0	479.8 ± 11.1	819.2 ± 78.3
Female	428.2 ± 22.0	383.4 ± 5.7	533 ± 9.0	504.8 ± 9.4
Testis	3152.8 ± 201.0	3288.6 ± 11.1	3171.4 ± 100.6	3094.8 ± 61.5
Ovaries and Uterus	1239.8 ± 27.3	1382.2 ± 7.3	1733.4 ± 32.0**	1444.4 ± 50.0

The table 4.7 shows that male lungs weights in Groups treated with 100 mg/kg and 200 mg/kg of *T. iboga* aqueous extract were significantly higher than control after 28 days ($p <$ 0.001 and $p <0.0001$, respectively). Male liver weights in Group treated with 200 mg/kg were significantly higher than control, however, in Group treated with 100 mg/kg of *T. iboga*, liver weights significantly decreased compared to control ($p <0.01$ and $p <0.001$, respectively). Similarly, female lungs weights in Group treated with 100 mg/kg of *T. iboga* were significantly higher than control ($p <0.01$) and female liver weights in Group treated with 200 mg/kg of *T. iboga* were significantly increased compared to control ($p <0.0001$). Ovaries and uterus weights were also significantly increased in female rats Group treated with 100 mg/kg of *T. iboga* aqueous extract compared to control ($p <0.01$). No change in tissue weights were seen in Groups treated at 50 mg/kg ($p > 0.05$) in both males and females.

Table 4.8. Biochemical parameters of healthy rats treated with *T. iboga* for 28 days (Values are mean ± SEM (n=6, male or female). One-way ANOVA followed by Dunnett's multiple comparisons test. Statistical difference between groups are indicated **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$ vs control)

Parameters	**Control (no extract)**	***T. iboga* 50 mg/kg b.w.**	***T. iboga* 100 mg/kg b.w.**	***T. iboga* 200 mg/kg b.w.**
AST (IU/L)				
Male	205.6 ± 3.7	201.0 ± 5.1	169.200 ± 7.2 ****	203.6 ± 3.6
Female	199.0 ± 0.7	195.6 ± 2.3	118.8 ± 0.6***	194.2 ± 1.6
ALT (IU/L)				
Male	53.2 ± 0.8	49.0 ± 2.0	53.0 ± 0.7	53.2 ± 0.6
Female	61.5 ± 0.5	63.000 ± 1.356	39.080 ± 0.037****	58.400 ± 0.510
ALP (IU/L)				
Male	484.6 ± 2.6	483.4 ± 2.0	473.6 ± 3.5 ****	486.8 ± 2.1
Female	258.4 ± 1.2	253.8 ± 1.4	239.6 ± 1.5****	253.6 ± 0.7
TC (mmol/L)				
Male	2.1 ± 0.0	2.1 ± 0.0	2.2 ± 0.0	2.4 ± 0.0
Female	2.0 ± 0.0	2.0 ± 0.0	2.1 ± 0.0	2.3 ± 0.1
Urea (mmol/L)				
Male	9.5 ± 0.1	9.9 ± 0.1	9.1 ± 0.6	8.7 ± 0.0
Female	9.3 ± 0.0	15.1 ± 0.0*	9.7 ± 0.1	9.0 ± 0.0

Glycemia (mmol/L)	2.6 ± 0.4	1.6 ± 0.2	1.0 ± 0.0	1.1 ± 0.1
Male				
Female	2.2 ± 0.1	1.3 ± 0.0	0.9 ± 0.1	0.7 ± 0.1
Blood glucose (mmol/L)	4.5 ± 0.5	2.3 ± 0.1	7.0 ± 0.3	5.0 ± 0.1
Male				
Female	3.4 ± 0.2	3.1 ± 0.0	3.1 ± 0.0	8.3 ± 0.1
Creatinine (µmol/L)	56.6 ± 3.0	51.2 ± 1.2*	58.2 ± 2.6	50.4 ± 2.3*
Male				
Female	83.0 ± 0.7	70.6 ± 0.4****	61.8 ± 0.4****	63.4 ± 0.5****
Hb (g/dL)	15.1 ± 0.3	15.4 ± 0.1	13.2 ± 0.2	13.5 ± 0.1
Male				
Female	12.2 ± 0.2	12.1 ± 0.0	15.1 ± 0.0	15.4 ± 0.1

TC: Total cholesterol, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, Hb: Haemoglobin

The table 4.9 indicates a significant drop of AST and ALP levels in male and female Groups treated with *T. iboga* at 100 mg/kg (p <0.0001) and ALT levels only in females Group. Creatinine levels in male and female Groups treated with *T. iboga* at 50 and 200 mg/kg were significantly lowered compared to control Groups, and at the 100 mg/kg dose in females only (p< 0.0001).

4.3.2. Hypoglycaemic effect of *T. iboga* doses (50, 100 and 200 mg/kg b.w.) during a 3 hr glucose tolerance test in healthy rat

Figure 4.2 illustrates the effect of daily oral administration for 28 days with variable doses of aqueous extract of *T. iboga* (50, 100 and 200 mg/kg) on OGTT of normal rats. There is a significant increase in blood glucose at 50 mg/kg of *T. iboga* up to 2 hr after glucose load, followed by a significant fall in blood glucose by 3 hr. At higher doses of *T. iboga* (100 and 200 mg/kg), blood glucose is elevated at 2 hr after glucose load and drop at 3 hr. All doses show same temporal hypoglycaemic effect after 3 hr post dose, dose of 50 mg/kg showed a maximum fall of 48.2% at 3hr after glucose load, whereas fall of 44.4% and 38.5% was observed with dose of 100 and 200 mg/kg respectively at the corresponding time.

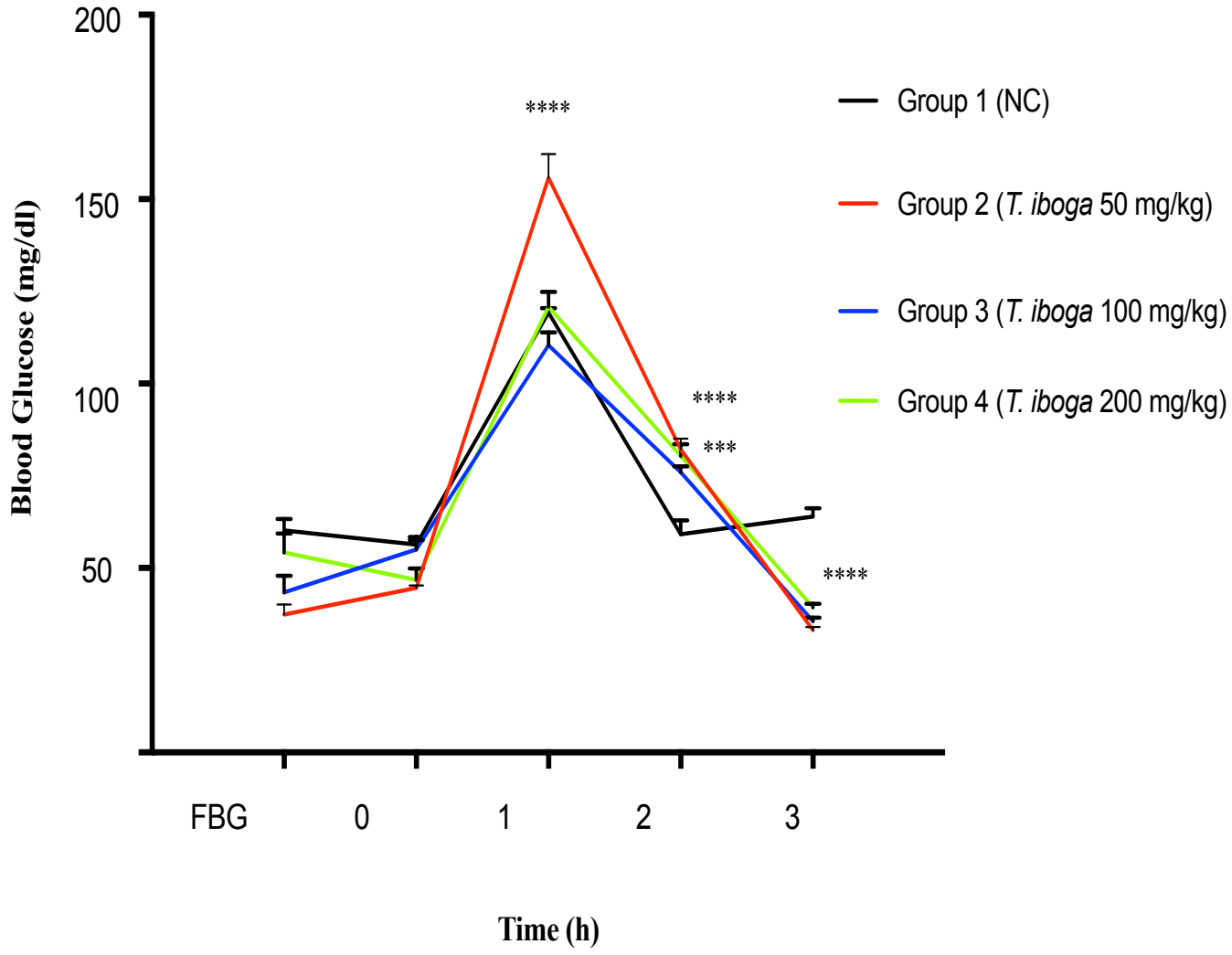


Figure 4.2. Hypoglycaemic effect of graded doses of aqueous extract of *T. iboga* on blood glucose of normal rat during OGTT. Data is presented as mean ± SEM (n=5) and were analysed by two-way ANOVA repeated measures followed by Dunnett's multiple comparison test. Statistical significance is represented as *** $p < 0.001$ and **** $p < 0.0001$ compared with NC (no control, no extract).

4.3.3. Effect of *T. iboga* doses (50, 100 and 200 mg/kg) on STZ/FR10 type 2 diabetic rats - body weight, food and fluid intake, and blood glucose

Figure 4.3 a, b below shows that all experimental Groups gained weight during the 2 first weeks of the experiment. However, from 2 weeks onward, weight gain significantly decreased in Group 3 (STZ/FR10) compared to normal control ($p < 0.05$) and to STZ control Group ($p < 0.05$). Group 2 treated with STZ control significantly gained weight compared to Group 3 (STZ/FR10) and Group 8 (FR10, no STZ) ($p < 0.05$) from week 3. Group 8 (FR10, no STZ) gained weight significantly from week 3 onward compared to Group 2 (STZ control), Group 3 (STZ/FR10) and normal control ($p < 0.05$). Groups treated with STZ/FR10 and *T. iboga* at 50, 100 and 200 mg/kg gained significantly less weight than normal control, while Groups treated glibenclamide gained significantly more weight than Group 3 (STZ/FR10 control) Figure 4.3 b.

Food intake did not fluctuate throughout the experimental period Figure 4.4, however, the fluid intake increased significantly in Group 3 (STZ/FR10 control) compared to normal control and Group 2 (STZ control) ($p < 0.05$). Also, fluid intake increased significantly in Group 2 (STZ control) and Group 8 (FR10, no STZ) compared to normal control ($p < 0.05$).

FBG in all Groups did not fluctuate during the 2 first weeks of the experimental period (Fructose treatment period). However, from week 4, after STZ injection, NFBG increased significantly in all groups compared to normal control. In Group 3 (STZ/FR10) NFBG levels increased steadily during the remaining experimental period compared to normal control Group and Group 2 (STZ control) Group ($p < 0.05$) (Figure 4.5 a). However, in Group 2 (STZ control) NFBG decreased significantly compared to Group 3 (STZ/FR10) and remained low until the end of experimental period ($p < 0.05$) Group 8 (FR10, no STZ) NFBG remained low throughout the experimental period (Figure 4.5 a). Also, NFBG significantly increased in Groups treated with *T. iboga* (50 to 200 mg/kg) and Group 7 (Glibenclamide) compared to NC (normal control). From week 3, in Group 4 (*T. iboga* 50 mg/kg) NFBG levels decreased significantly during the remaining experimental period compared to Group 3 (STZ/FR10 control) and Group 2 (STZ control) ($p < 0.05$) to reach levels comparable to those of NC Group (Figure 4.5 b). Also, in Group 6 (*T. iboga* 200 mg/kg), NFBG levels followed the same pattern as Group 4 (*T. iboga* 50 mg/kg), although NFBG levels remained significantly higher than those of NC ($p < 0.05$) in a similar way of Group 3 (STZ/FR10) (Figure 4.5 b). In Group 5 (*T. iboga* 100 mg/kg), NFBG levels remained significantly higher than NC,

(STZ/FR10 control) and (FR10, no STZ) Groups from week 4 up to week 6 after STZ injection at week 2 ($p < 0.05$) (Figure 4.5 b). In Group 7 (Gli), NFBG levels increased significantly after STZ injection (at week 2) compared to NC ($p < 0.05$) and decreased gradually from week 3 up to the end of experimental period, however, NFBG levels remained significantly higher than those of NC ($p < 0.05$) (Figure 4.5 b).

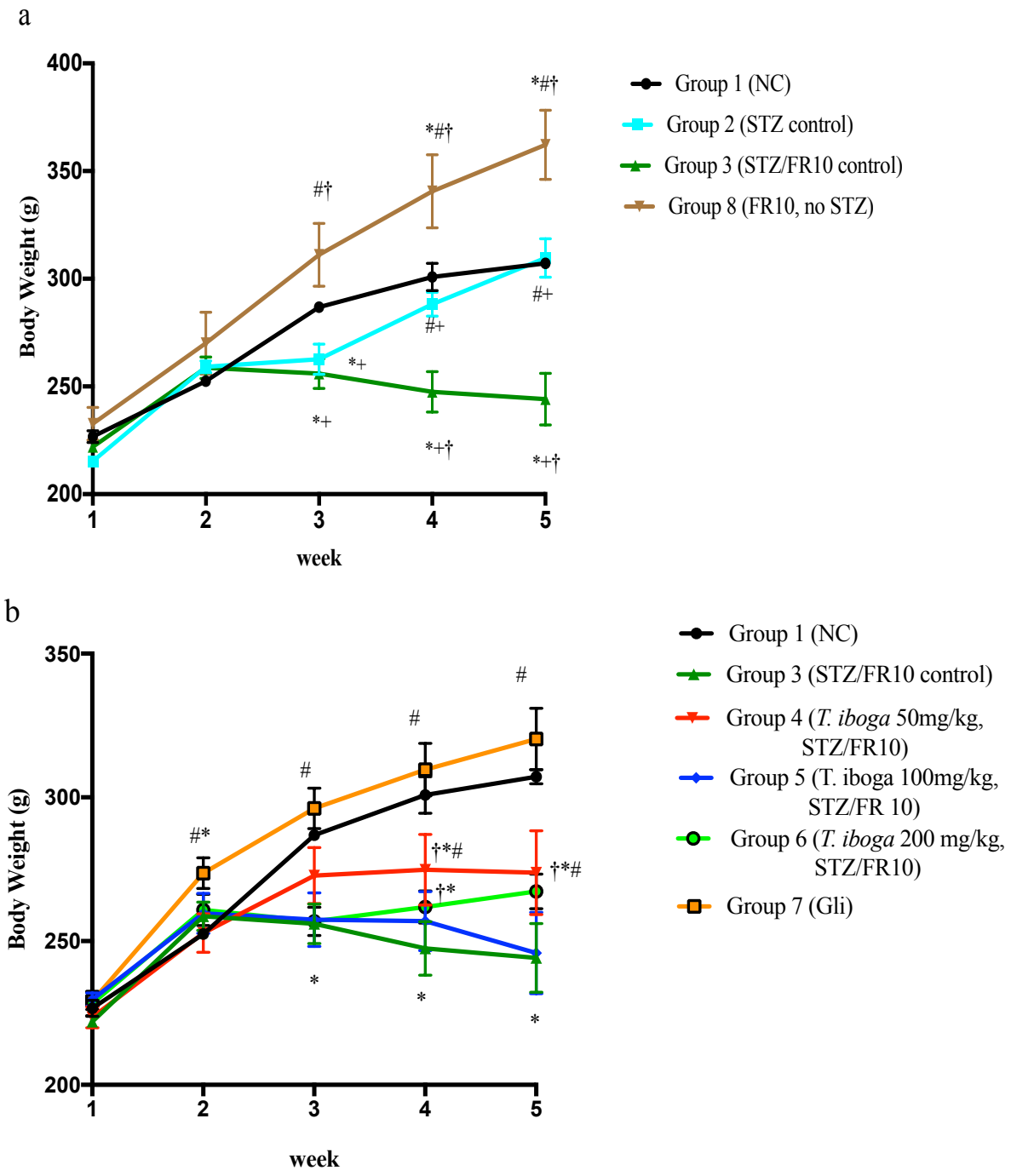


Figure 4.3. Mean body gain weight over 6 weeks of experimental period. (a) Control Groups N=4 and (b) Treated Groups N=6- Fructose 10% treatment from week 0 to week 2, STZ injection at week 2, *T. iboga* treatment from week 2 to week 6. Data are presented as mean ± SEM (n=5 per group), and were analysed by two-way ANOVA with post-hoc Tukey's multiple comparison test. Statistical significance is represented as * $p < 0.05$ vs NC, # $p < 0.05$ vs STZ/FR10 control, † $p < 0.05$ vs STZ control, + $p < 0.05$ vs FR10, no STZ

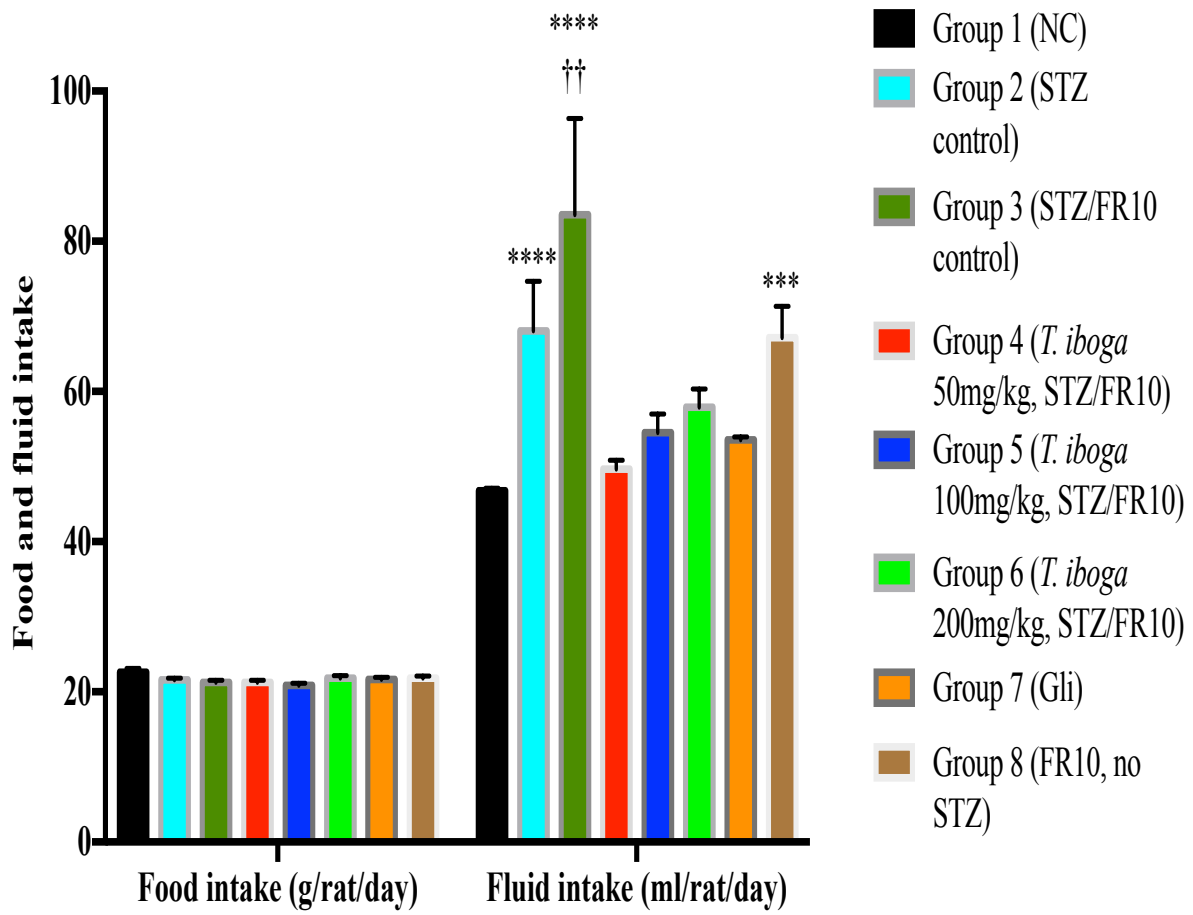


Figure 4.4. Mean food intake and fluid intake over 6 weeks of experimental period. Data are presented as mean ± SEM (n=5 per group), and were analysed by two-way ANOVA with post-hoc Tukey's multiple comparison test. Statistical significance is represented as* $p < 0.05$ vs NC, # $p < 0.05$ vs STZ/FR10 control, † $p < 0.05$ vs STZ control

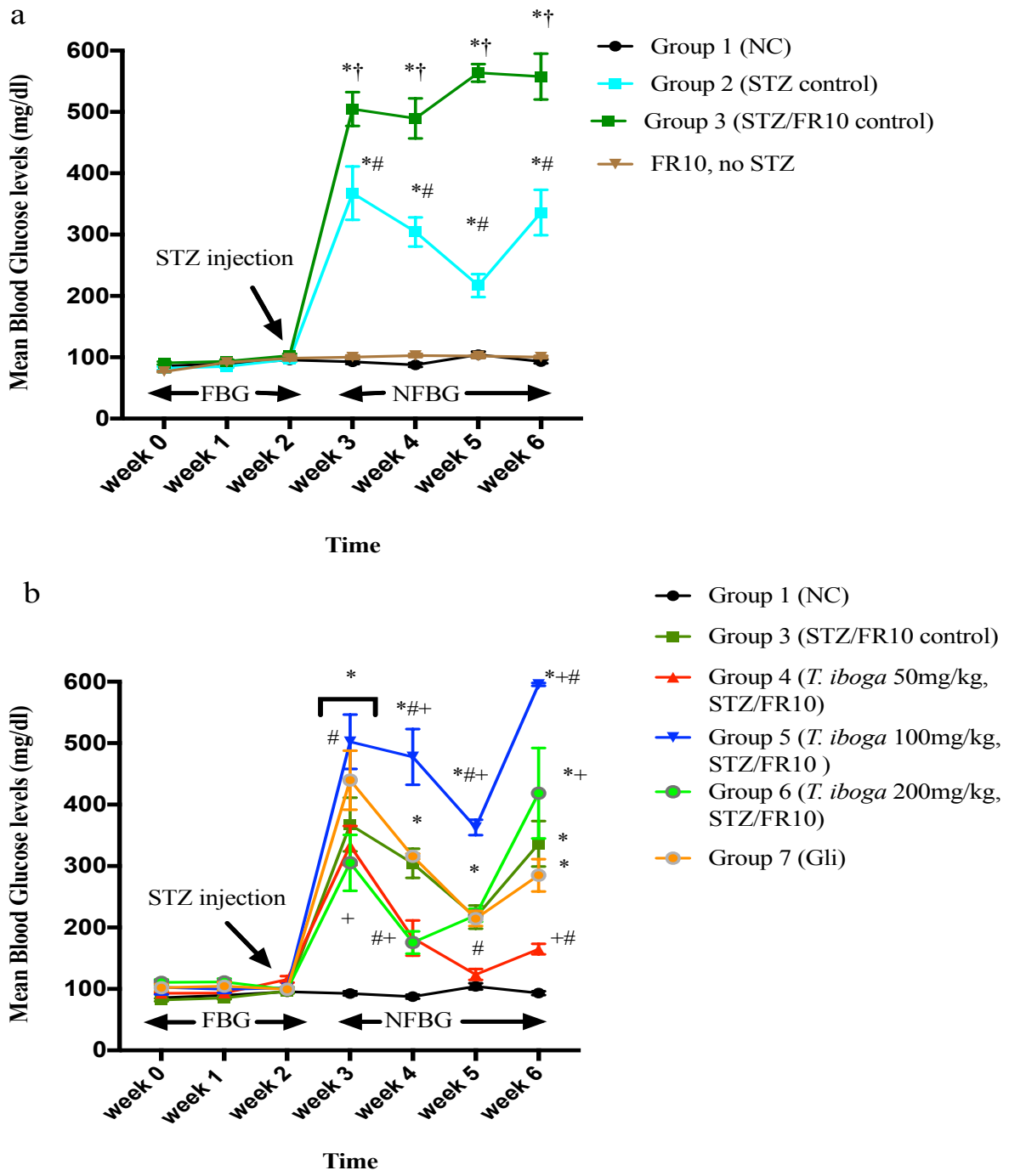


Figure 4.5. Mean blood glucose over 6 weeks of experimental period. (a) Control Groups N=4 and (b) Treated Groups N=6 - (FR10) Fructose 10% treatment was indicated from week 0 to week 2 with FBG (Fasting Blood Glucose), STZ injection was indicated at week 2 with NFBG (Non-Fasting Blood Glucose) levels, *T. iboga* treatment from week 2 to week 6. Data are presented as mean ± SEM (n=5 per group), and were analysed by two-way ANOVA with post-hoc Tukey's multiple comparison test. Statistical significance is represented as * $p <$ 0.05 vs NC, # $p <$0.05 vs STZ/FR10 control, † $p < 0.05$ vs STZ control, + $p < 0.05$ vs Gli (5 mg/kg)

4.3.4. Effect of *T. iboga* (50 to 200 mg/kg p.o.) and Glibenclamide on glucose tolerance and insulin sensitivity on STZ/FR10 type 2 diabetic rats

One of the major aims of this study was to determine whether ingestion of *T. iboga* extract has any beneficial effect on T2D induced by STZ/FR10. The results of the glucose tolerance tests conducted at 2 weeks of treatment with *T. iboga* showed that Groups treated with the plant aqueous extract cleared glucose slower after glucose bolus dose compared to the negative control Group (Figure 4.6 a, b). At week 2, Group 3 (STZ/FR10 control) showed significantly higher levels of blood glucose compared to NC and Group 2 (STZ control) ($p < 0.05$) (Figure 4.6. a, b). Similarly, in Group 7 (Gli), blood glucose levels were significantly higher than NC and STZ control Group 2 up to 60 min after glucose load and decreased significantly after 120 min compared to Group 3 (STZ/FR10 control) ($p < 0.05$) (Figure 4.6 a, b). The area under the curve (AUC) of Groups 3 (STZ/FR10 control), 2 (STZ, control) and 8 (FR10, no STZ) showed marked glucose intolerance compared to NC ($p < 0.0001$). The area under the curves (AUC) show marked glucose intolerance in Group 3 (STZ/FR10 control), 2 (STZ, control) and 8 (FR10, no STZ) compared to NC ($p < 0.0001$) (Figure 4.6 b).

At 4 weeks of *T. iboga* treatment, the glucose tolerance tests showed that Group treated with 50 mg/kg of *T. iboga* cleared glucose in the same manner of NC Group. However, Groups treated with 100 and 200 mg/kg of *T. iboga* cleared slower glucose compared to the NC Group (Figure 4.6 c, d). Group 5 (*T. iboga* 100 mg/kg), exerted significantly higher blood glucose than NC and Group 2 (STZ control) at 30 min ($p < 0.05$) in the same pattern than Group treated with Glibenclamide (Figure 4.6 c, d). However, Group 4 exhibited significant lower blood glucose levels compared to Group 3 after 60 min ($p < 0.05$) but these levels remained significantly higher than those of NC ($p < 0.05$) (Figure 4.6 c, d). Also, Group 6 (*T. iboga* 200 mg/kg) exerted significantly lower levels of blood glucose compared to Group 3 (STZ/FR10 control) from 30 min to 120 min after glucose load ($p < 0.05$) (Figure 4.6 c, d). The AUC results show marked glucose intolerance in Groups 3 (STZ/FR10, control), 5 (*T. iboga* 100 mg/kg, STZ/FR10) and 7 (Gli) ($p < 0.0001$) compared to NC (Figure 4.6 d).

The results of the insulin sensitivity tests conducted at 2 weeks of treatment with *T. iboga* showed that the plant extract did not improve insulin sensitivity compared to negative control Group (Figure 4.6 a, b) at 2 weeks and 4 weeks of plant extract treatment. Also, Group treated with glibenclamide did not either improve insulin sensitivity during this experiment (Figure 4.7 a, b, c, d). At 2 weeks, Group 5 (*T. iboga* 100 mg/kg) at 30 min after insulin

injection exerted significantly higher levels of blood glucose compared to NC, Group 2 (STZ control) and Group 3 (STZ/FR10 control) ($p < 0.05$) (Figure 4.7 a, b). Similarly, at week 4, blood glucose levels in Group 5 (*T. iboga* 100 mg/kg) significantly increased at 30 min after insulin injection compared to NC and Group 2 (STZ control) ($p < 0.05$) and decreased rapidly to reach levels close to those of NC at 120 min (Figure 4.7 c, d). In Group 7, at 60 min after insulin injection, blood glucose levels remained significantly higher than those of Group 2 (STZ control) and NC ($p < 0.05$), to increase again after 60 min to levels significantly higher than those of Group 3 (STZ/FR10 control), Group 2 (STZ control) and NC ($p < 0.05$) (Figure 4.7 c, d). Groups 4 and 6 exhibited blood glucose significantly lower than Group 3 throughout the insulin tolerance time ($p < 0.05$) (Figure 4.7 c, d).

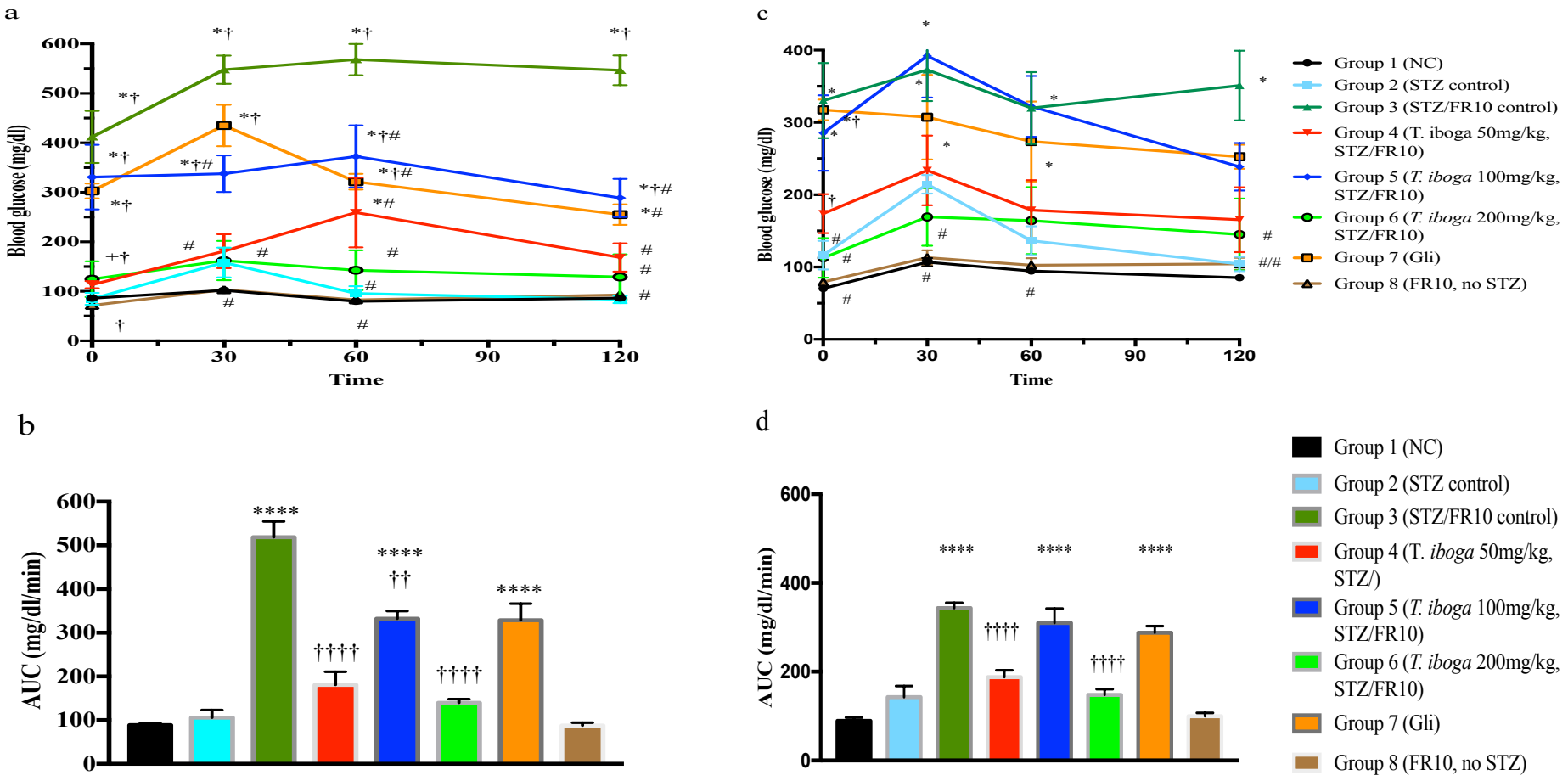


Figure 4.6. Effect of *T. iboga* aqueous extract on glucose tolerance in T2D rats at 2 weeks (a, b) and 4 weeks (c, d). Data are presented as mean ± SEM (n=5 per group), and were analysed by two-way ANOVA with post-hoc Tukey's multiple comparison test. Statistical significance is represented as **** $p < 0.0001$, * $p < 0.05$ vs NC, # $p < 0.05$ vs STZ control, †††† $p < 0.0001$, † $p < 0.05$ vs STZ/FR10 control and + $p < 0.001$ vs Gli (5 mg/kg)

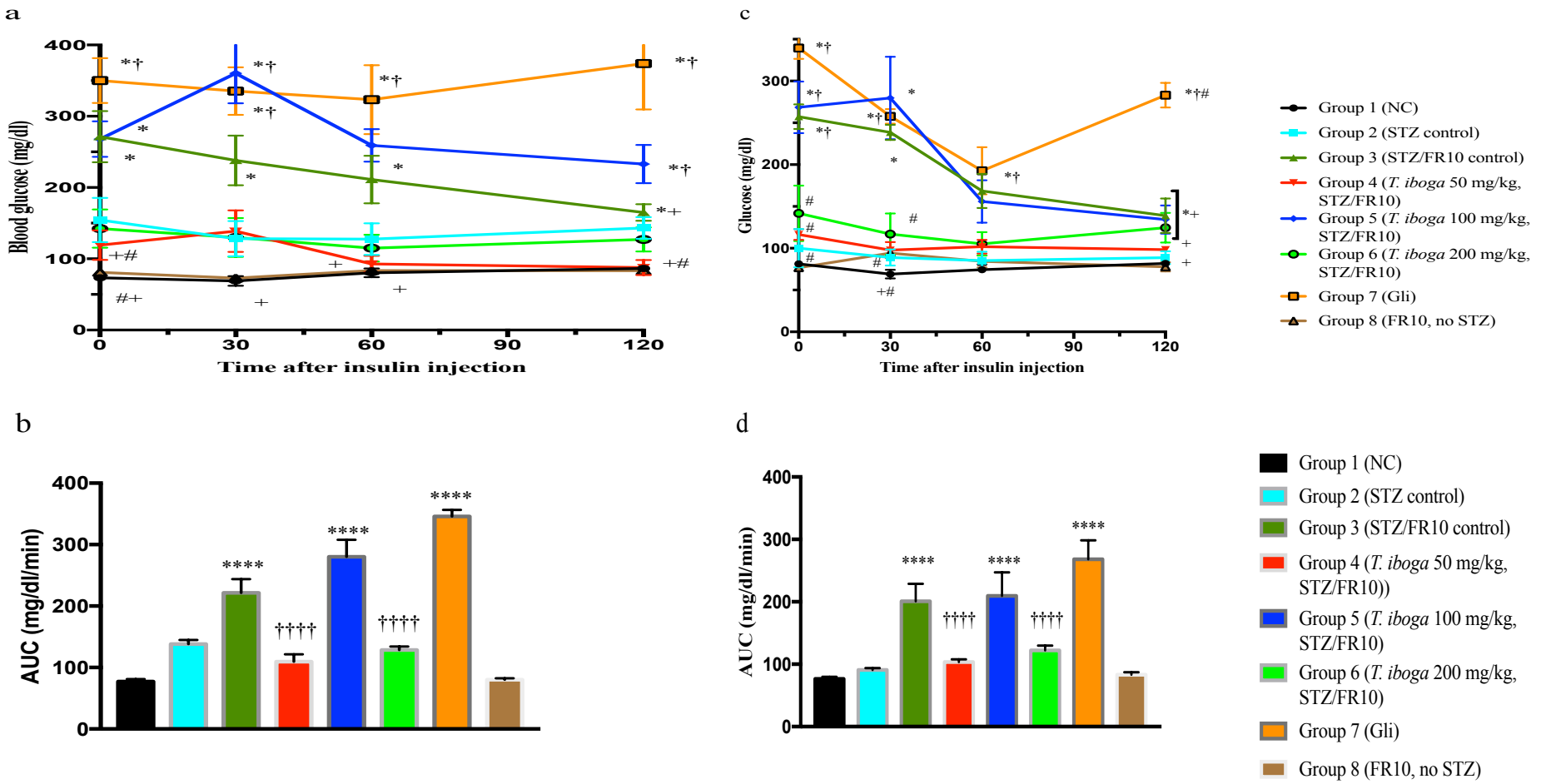


Figure 4.7. Effect of *T. iboga* aqueous extract on insulin tolerance at 2 weeks (a, b) and 4 weeks (c, d). Data are presented as mean ± SEM (n=5 per group), and were analysed by two-way ANOVA with post-hoc Tukey's multiple comparison test. Statistical significance is represented **** $p < 0.0001$, * $p < 0.05$ vs NC, # $p < 0.05$ vs STZ control, †††† $p < 0.0001$, † $p < 0.05$ vs STZ/FR10 control and + $p < 0.001$ vs Gli (5 mg/kg)

4.3.5. Effect of *T. iboga* doses (50, 100 and 200 mg/kg b.w.) on organs weights and biochemical parameters of STZ/FR10 T2D rats

As shown on Table 4.10, *T. iboga* (50 to 200 mg/kg) did not influence tissue weight during the 4 weeks' experimental period except for Group 6 at 200 mg/kg in which liver weight was significantly increased compared to Group 3 (STZ/FR10 control) ($p < 0.05$). However, in Group 3 (STZ/FR10 control), liver weight was significantly decreased compared to NC ($p < 0.05$) and increased in Group 8 (FR10, no STZ) compared to NC, Group 2 (STZ control) and Group 3 (STZ/FR10 control) ($p < 0.05$).

The Table 4.11 shows the Group 3 (FR10/STZ control) and Group 5 (*T. iboga* 100 mg/kg) have significantly higher plasma levels of glucose compared to NC ($p < 0.05$). Also, Group 3 (STZ/FR10) and Group 2 (STZ control) have significant higher levels of creatinine compared to NC ($p < 0.05$), HbA1c compared to NC and Group 7 (Gli) ($p < 0.05$) and ALT compared to NC and Group 2 (STZ control) ($p < 0.05$). However, AST levels was significantly lower compared to NC ($p < 0.05$) in the same Group (STZ/FR10 control). Glucose levels were significantly lower in Groups 4 (*T. iboga* 50 mg/kg), 6 (*T. iboga* 200 mg/kg) and 8 (FR10, no STZ) ($p < 0.05$). Creatinine levels decreased significantly in Groups treated with *T. iboga* (50 to 200 mg/kg), Group 7 (Gli) and Group 8 (FR10, no STZ) compared to Group 3 (STZ/FR10 control) and Group 2 (STZ control) ($p < 0.05$). HbA1c levels were significantly increased in Group 3 (STZ/FR10 control) compared to NC and Group 7 (Gli) ($p < 0.05$) and in Group 8 (FR10, no STZ) compared to NC and Group 2 (STZ control) ($p < 0.05$). Whereas, AST levels were significantly lower in Groups treated with *T. iboga* (50 to 200 mg/kg) and Group 7 (Gli) compared to NC, Group 2 (STZ control) and Group 3 (STZ/FR10 control) ($p < 0.05$).

As shown on Table 4.12, *T. iboga* treatment in T2D rats for a period of 4 weeks did not influence lipid profile, except for the Group 6 (*T. iboga* 200 mg/kg) which exhibited significant increase in HDL-c levels compared to Group 3 (STZ/FR10 control) ($p < 0.05$). However, triglycerides levels were significantly increased in Group 3 (STZ/FR10 control) compared to NC ($p < 0.05$).

Table 4.9. Tissue weights in different animal groups at the end of experimental period (All values are mean ± SEM of 5 animals. NC: normal control, Group 2: STZ treatment, Group 3: STZ/FR10 treatment, Group 4: *T. iboga* 50 mg/kg treatment, Group 5: *T. iboga* 100 mg/kg treatment, Group 6: *T. iboga* 200 mg/kg treatment, Group 7: Gli 5 mg/kg treatment, Group 8: FR10, no STZ treatment. * $p < 0.05$ vs NC. † $p < 0.05$ vs STZ control. # $p < 0.05$ vs STZ/FR10 control)

Parameter (g)	**NC**	**Group 2 (STZ)**	**Group 3 (STZ/FR10)**	**Group 4 (*T. iboga* 50 mg/kg**	**Group 5 (*T. iboga* 100 mg/kg)**	**Group 6 (*T. iboga* 200 mg/kg)**	**Group 7 (Gli)**	**Group 8 (FR10, no STZ)**
Heart	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	1.2 ± 0.0
Liver	10.3 ± 0.2	10.0 ± 0.5	9.2 ± 0.7*	9.7 ± 0.4	9.7 ± 0.2	10.7 ± 0.6#	10.0 ± 0.0	12.3 ± 0.5*†#
Kidneys	2.1 ± 0.0	2.2 ± 0.1	2.3 ± 0.1	2.1 ± 0.0	2.2 ± 0.0	2.3 ± 0.2	2.8 ± 0.1	3.0 ± 0.1
Testis	2.7 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.3 ± 0.1	2.6 ± 0.1	3.0 ± 0.2	3.0 ± 0.0	3.7 ± 0.1*†#
Lungs	.3 ± 0.1	1.2 ± 0.0	1.3 ± 0.0	1.6 ± 0.1	1.3 ± 0.0	1.4 ± 0.1	1.7 ± 0.1	2.0 ± 0.1

Table 4.10. Plasma glucose, urea, creatinine, HbA1c, AST, ALT and ALP in different animal groups at the end of experimental period

Parameter (mM)	**NC**	**Group 2 (STZ control)**	**Group 3 (STZ/FR10 control)**	**Group 4 (*T. iboga* 50 mg/kg)**	**Group 5 (*T. iboga* 100 mg/kg)**	**Group 6 (*T. iboga* 200 mg/kg)**	**Group 7 (Gli)**	**Group 8 (FR10, no STZ)**
Glucose	5.0 ± 0.3	6.1 ± 1.6	16.5 ± 1.2*	4.6 ± 0.6$^{\#}$	15.6 ± 2.8*	6.9 ± 2.4$^{\#}$	11.0 ± 2.7*	3.3 ± 0.5$^{\#}$
Urea	3.3 ± 0.1	3.5 ± 0.2	4.0 ± 0.4	3.6 ± 0.1	3.5 ± 0.1	3.3 ± 0.2	3.4 ± 0.3	3.1 ± 0.1
Creatinine	58.3 ± 2.4	79.1 ± 2.3*	82.9 ± 4.3**	62.0 ± 2.5$^{\dagger\#}$	63.4 ± 4.0$^{\dagger\#}$	57.9 ± 3.8$^{\dagger\#}$	62.3 ± 4.7†	54.8 ± 1.9$^{\dagger\#}$
HbA1c	2.0 ± 0.0	2.2 ± 0.2	4.2 ± 0.7*†	2.9 ± 0.5	4.3 ± 0.7	3.0 ± 0.4	3.3 ± 0.4	2.0 ± 0.0
AST (U/L)	96.2 ± 4.4	72.0 ± 7.2*	114.0 ± 1.9*†	17.9 ± 2.6$^{*\dagger\#}$	21.2 ± 4.4$^{*\dagger\#}$	15.4 ± 1.4$^{*\dagger\#}$	19.5 ± 0.4$^{*\dagger\#}$	116.6 ± 2.0*
ALT (U/L)	31.0 ± 3.3	37.6 ± 4.6	44.2 ± 0.9*†	17.0 ± 1.8$^{*\dagger\#}$	20.1 ± 4.4	14.2 ± 1.4$^{*\dagger\#}$	16.5 ± 0.8$^{*\dagger\#}$	42.9 ± 3.1*†
ALP (U/L)	123.9 ± 4.3	112.7 ± 6.7	121.5 ± 5.7	102.8 ± 6.4$^{*\#}$	118.2 ± 10.1	107.1 ± 5.9$^{*\#}$	102.0 ± 11.1$^{*\#}$	71.8 ± 5.2$^{*\dagger/\#}$

Table 4.11. Plasma lipid profile in different animal groups at the end of the experimental period

Parameter (mM)	**NC**	**Group 2 (STZ control)**	**Group 3 (STZ/FR10 control)**	**Group 4 (*T. iboga* 50 mg/kg)**	**Group 5 (*T. iboga* 100 mg/kg)**	**Group 6 (*T. iboga* 200 mg/kg)**	**Group 7 (Gli)**	**Group 8 (FR10, no STZ)**
Cholesterol	1.4 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.0	1.6 ± 0.0
HDL-C	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0#	0.4 ± 0.0	0.5 ± 0.0
LDL-C	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.2 ± 0.1
Triglycerides	4.3 ± 0.2	3.7 ± 0.1	7.4 ± 0.6*	0.7 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.0

4.4. Discussion

In vivo investigations were performed in this Chapter only with *T. iboga* aqueous extract in healthy and T2D animal models, since fewer mechanistic data were available for *G. tessmannii*. Although, toxicity results of *G. Tessmannii* aqueous extract gave some indications of the dose range that could be used to conduct *in vivo i*nvestigations in T2D animal models (Koumba Madingou *et al.,* 2016).

In this chapter, we first investigated the range of safe concentrations of *T. iboga* aqueous extract to be used for *in vivo* studies using toxicological approaches. We then investigated the hypoglycaemic effects of *T. iboga* in healthy rats using these selected concentrations from sub-acute toxicity study results. Lastly, the anti-diabetic potential of these selected concentrations of aqueous extract of *T. iboga* were investigated in T2D models.

The acute toxicity study showed that oral administration of *T. iboga* root barks aqueous extracts induced mild toxicity by oral route in Wistar rats according to Hodge and Sterner scale (Table 4.4) with the LD_{50} dose of 1442.77 mg/kg b.w. Thus, after a 28 days' subacute toxicity study in rats, doses lower than the acute dose of 10% of the oral LD_{50}, which is an equivalent to the no-observable adverse effect level (NOAEL), were used (Bulgheroni *et al.,* 2009).

In the subacute toxicity study, we observed no-significant differences in food and water consumption at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg of *T. iboga* and the control Group in both male and female rats and in control Group during 28-day oral administration of *T. iboga* aqueous extract administration may indicate that the animals were having healthy growth based on their food and intake consumption. Also, rats in all experimental Group gained body weight over the course of this study, except for female rats in group treated with 200 mg/kg of *T. iboga* (Table 4.7). Thus, it can be suggested that growth inhibition did not occur during this course of repeated administration of *T. iboga* aqueous extract root barks. Likewise, alteration in organ weights is an indication of toxicity in animals which are easily determined by toxicity tests (Adewale *et al.,* 2016). There is a very high possibility that ingested plant extract into the body may be toxic to major organs, such as the kidney, liver, spleen, heart and brain since it has diverse roles in the body depending on its bioavailability. Here in this study, there is no major change in the organ weights of all experimental Groups (Table 4.8).

Biochemical analyses performed on blood samples give an important index for general physiological and pathological status in animals and humans (Adewale *et al.,* 2016). Since blood is the major means of transport for a large number of nutrients and foreign elements within the body, blood constituents such as red blood cells, white blood counts, platelets and haemoglobin are first exposed to concentrations of toxic compounds (Adewale *et al.,* 2016). *T. iboga* aqueous extract of root barks did not induce any damage to the blood cells of healthy Wistar rats since haemoglobin levels in all Groups were not altered after the plant administration (Table 4.8). Furthermore, aspartate and alanine transaminases (AST and ALT) which are good indicators of liver damage, alkaline phosphatase (ALP), that indicates bile ducts obstruction or liver and gallbladder inflammation, creatinine and urea which are known to be good indicators of renal function (Adewale *et al.,* 2016) were measured (Table 4.8 and Table 4.11). In healthy male and female Groups treated with *T. iboga* at 100 mg/kg transaminases levels were significantly decreased ($p < 0.0001$), as well as ALP in female Groups (Table 4.8) indicating a non-toxicity of the plant extract to the liver. Moreover, creatinine levels also significantly decreased ($p <0.0001$) in male Groups treated with *T. iboga* at 100 and 200 mg/kg and female Groups treated with *T. iboga* at 50, 100 and 200 mg/kg. This may probably be an indication that the extract did not interfere with the capacity of the liver and the kidney to excrete these plant metabolites and may potentiate/stimulate these organs functions. Also, *T. iboga* may improve insulin resistance as creatinine levels were significantly decreased at selected doses (50, 100 and 200 mg/kg) (Wilson and Islam, 2012). Thus, these results suggest that *T. iboga* aqueous extract does not have negative effect on liver and kidney, but rather seems to have a protective effect on these organs.

Our study revealed an *in vivo* hypoglycaemic effect of *T. iboga* aqueous extract (50 to 200 mg/kg p.o.) on healthy rats after 3 hours. Indeed, a glucose dose of 2 g/kg administered to healthy rats considerably increased blood glucose levels of all experimental Groups, which were significantly decreased by a single oral dose of *T. iboga* aqueous extract compared to control Group ($p < 0.0001$), with a maximum effect in Groups treated with *T. iboga* at 50 mg/kg for 180 min following glucose load (Figure 4.2). *T. iboga* aqueous extract did not show a dose-dependent hypoglycaemic effect; however, the hypoglycaemic effect was delayed and was shown after 3 hr, which indicates an intracellular action of *T. iboga* aqueous extract evidenced in Chapter 3, in isolated pancreatic ß-cells. Indeed, *T. iboga* aqueous extract (1 µg/ml) potentiated insulin secretion from isolated pancreatic islets Chapter 3, Figure 3.9).

Furthermore, we investigated the anti-diabetic effect of *T. iboga* aqueous extract (50 to 200 mg/kg p.o.) in a non-genetic fructose/STZ rat model of T2D (Wilson and Islam, 2012). The combination of 10% fructose (FR10) treatment with a low dose of STZ (40 mg/kg) in our Wistar rats, resulted in a rapid, stable hyperglycaemia, insulin resistance and sensitivity to type 2 anti-diabetic drugs such as glibenclamide. Common symptoms of T2D include polydipsia and weight loss, which is sometimes accompanied by polyphagia (Nagata *et al*., 2006). Significant polydipsia was present in diabetic control Groups in our experiment. The significantly higher fluid intake and significantly lower b.w. gain in the T2D compared to the control Groups might be due to the severity of diabetic conditions as well as higher energy expenditure via urinary glucose excretion in our experiment (Figure 4.4). In our experiment, the T2D (Group 3) did not exhibit actual weight loss as seen in T1D patients and animal models (King, 2012; Eddooks *et al.,* 2012) but rather decreased weight gain as compared to the control Groups. Furthermore, it has been reported in previous work that animals treated with STZ/FR10 exhibited less body weight gain compared to NC and STZ Groups, which is in agreement with our results (Wilson and Islam, 2012).

The ADA report that the FBG > 100 mg/dl but < 126 mg/dl, blood glucose at 2h post-glucose load > 140 mg/dl but < 200 mg/dl and fasting plasma glucose (FPG) > 140 mg/dl and/or 2 hr post-glucose load > 200 mg/dl are, respectively, considered as impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and diabetes (Wilson and Islam, 2012). The NFBG of T1 and T2 Groups were significantly higher than NC Group 1 (Figure 4.5 a). However, the NFBG range of T2D Group 3 was significantly higher than the T1D Group 2 (504.8 vs 367.6 mg/dl, Figure 4.5 a) similar to the Wilson and Islam (2012) study. Similar to other studies, (Mawa *et al.,* 2019), the NFBG > 300 mg/dl has been used as cut-off point for T2D, which has been maintained by fructose/STZ (Group 3) but not by STZ T1 Group 2 in our experiment, which was also seen in Wilson and Islam (2012) study. Additionally, our T2D rats demonstrated sensitivity to anti-diabetic drug glibenclamide, by significantly reducing blood glucose levels over a 3 hr period (Figure 4.6 a, b).

In the present study, animals treated with fructose alone and T1D rats treated with glibenclamide gained significantly more weight than negative control (NC) animals ($p < 0.0001$), while animals in Groups treated with T2D model (STZ/FR10) and *T. iboga* at 50, 100 and 200 mg/kg gained less weight than NC Group (Figure 4.3). These results are in agreement with the effects already reported of this T2 anti-diabetic drug and the negative impact of excessive fructose consumption within the body, including weight gain, fat

accumulation and circulating levels of triglycerides (Köseler *et al.,* 2018). Furthermore, in previous work using animals treated with STZ/FR10, less body weight gain was exhibited compared to NC and STZ groups, which is in agreement with our results (Wilson and Islam, 2012). A significant higher fluid intake ($p < 0.0001$) was observed in Groups treated with *T. iboga* at 200 mg/kg, STZ and STZ/FR10 compared to NC Group (Figure 4.4) and not lower doses of *T. iboga*. Thus, *T. iboga* aqueous extract at 50 and 100 mg/kg may exert some activity that prevent diabetic features such as polydipsia and avoid weight gain, which is a common side effect of T2 anti-diabetic drugs such as glibenclamide (Skliros *et al*., 2016). Hence, *T. iboga* may prevent excess of energy loss via urinary glucose excretion, commonly seen in diabetic condition (Wilson and Islam 2012).

The OGTT performed at 2 weeks showed significantly elevated blood glucose levels for T2D (STZ/FR10) Group ($p < 0.0001$), almost during the 2 hr period of the test (> 500 mg/dl) compared to NC, T1D STZ Group and Groups treated with *T. iboga* significant differences between glucose tolerance in T1 STZ diabetes and T2 fructose/STZ diabetes is also seen in previous work of Wilson and Islam, (2012). In Groups treated with *T. iboga* at 50 and 100 mg/kg, the peak of glucose level was reached only at 60 min after glucose load and decreased slowly after 1 hr period and was brought to levels nearly those of NC Group (Figure 4.7 a-b). These results suggest a rapid metabolism of glucose that may be potentiated by *T. iboga* at these doses (50 and 100 mg/kg) compared to T2D control rats. Moreover, the OGTT performed after 4 weeks' period treatment of *T. iboga* showed a more rapid peak of glucose level (after 30 min) and a sharp fall of glucose levels from 30 min to decrease slowly after 60 min and reached levels close to those of NC Group. The significant hypoglycaemic response of *T. iboga* aqueous extract was clearly shown at 50 mg/kg while, at higher doses (100 and 200 mg/kg), less hypoglycaemic responses were evidenced. The area under the curves (AUC) at the OGTT test at 2 weeks plant extract treatment showed significant glucose intolerance in T2D, in Group treated with *T. iboga* at 100 mg/kg and also Group treated with Glibenclamide, which suggest a contradictory effect of the anti-diabetic drug on Fructose/STZ-induced T2D rats after 2 weeks of *T. iboga* treatment. Indeed, in Groups treated with *T. iboga* at 100 mg/kg, blood glucose levels were significantly elevated after 120 min test compared to those of NC Group and Groups treated with *T. iboga* at 50 and 200 mg/kg (Figure 4.7 c, d). This reduced hypoglycaemic response at higher doses of plant extract has already been observed with numerous indigenous plants (Singh *et al*., 2007; Jaiswal *et al.,* 2009).

Similarly, ITT performed at 2 weeks showed a marked insulin resistance during the first 30 min of the test, in Groups treated with *T. iboga* at 50 and 100 mg/kg, while, in other experimental Groups glucose levels patterns showed moderate sign of insulin resistance (Figure 4.7 c-d). However, when ITT was performed at 4 weeks' period of *T. iboga* treatment, insulin resistance was increased in Groups treated with STZ/FR10, which was already shown in previous work and is a characteristic of T2D insulin resistance (Wilson and Islam, 2012), and in *T. iboga* at 100 mg/kg Group, while insulin resistance improvement was observed in Groups treated with *T. iboga* at 50 mg/kg and 200 mg/kg better than the anti-diabetic drug glibenclamide (Figure 4.8 c, d). These results support the hypoglycaemic effect of *T. iboga* at 50 and 200 mg/kg, however, there are in contradiction with those seen in Wilson and Islam (2012) work. The contradictory effect of anti-diabetic drug glibenclamide seen in our study, may be related to the length difference between our works, here 4 weeks while > 7 weeks in Wilson and Islam (2012). Moreover, in our study, plasma insulin could not be measured and HOMA-ß and HOMA-IR could not be calculated which would emphasis pancreatic ß-cells function and the state of insulin resistance in treated Groups.

Biochemical analyses results in Table 4.10 showed significant increase of liver weights in STZ/FR10 and FR10 Groups compared to control ($p < 0.05$) while testis weights of FR10 Group lowered significantly compared to control ($p < 0.05$). These organs weight alterations reflect the negative effect of FR10 treatment on liver and hepatic functions, already reported (Wilson and Islam, 2012; Köseler *et al.,* 2018) and suggest possible deleterious influence on sexual organs of high fructose diets. Plasma glucose was also significantly lower in FR10 Group compared to STZ/FR10 Group ($p < 0.05$), which support the well-known STZ consequences on glucose levels supplemented by fructose effects (Wilson and Islam, 2012). Furthermore, creatinine levels in STZ and STZ/FR10 Groups were significantly increased compared to NC Group ($p < 0.01$), while significant decreased in groups treated with *T. iboga* and glibenclamide was observed compared to STZ Group ($p < 0.01$) and in FR10 Group compared to STZ and STZ/FR10 Groups ($p < 0.01$). However, plasma urea levels in all Groups were not altered compared to control Group. Our results demonstrated kidney damage in STZ and STZ/FR10 Groups evidenced by elevated plasma creatinine levels, which is in agreement with previous data (Maya *et al*., 2019; Al Hroob *et al.,* 2018; Wilson and Islam, 2012). Additionally, plasma AST levels were significantly increased in Groups treated with STZ and FR10 compared to NC Groups ($p < 0.0001$), and STZ/FR10 compared to NC and STZ Groups. Whilst, Groups treated with *T. iboga* at 50, 100 and 200 mg/kg and

glibenclamide showed significantly lowered plasma AST levels compared to NC, STZ and STZ/FR10 Groups ($p < 0.05$ and $p < 0.0001$). In a similar way, plasma ALT levels were increased in Groups treated with STZ/FR10 compared to NC and STZ Groups ($p < 0.05$), while in Groups treated *T. iboga* at 50 and 200 mg/kg and glibenclamide a significant decrease of ALT levels was observed compared to NC, STZ and STZ/FR10 Groups ($p < 0.05$ and 0.0001). Plasma ALP levels were also decreased in Groups treated with *T. iboga* at 50 and 200 mg/kg, glibenclamide and FR10 compared to NC and STZ/FR10 Groups ($p < 0.05$). Hence, *T. iboga* aqueous extract at 50, 100 and mg/kg may ameliorate diabetes induced liver damage in a similar way of glibenclamide (Chukwunonso Obi *et al.,* 2015) and may exert a protective effect of liver and kidney organs by preventing deleterious change in transaminases and creatinine, as well as urea levels. These results are in agreement with previous data which demonstrated increased levels of AST, ALT and ALP parameters following STZ, STZ/FR10 or FR10 treatments (Köseler *et al.,* 2018; Saeed *et al.,* 2008).

Overall, T2D models was developed in this chapter over 7 weeks using fructose treatment and low dose STZ, which allowed T2D features to be assessed by *T. iboga* aqueous extract (50 to 200 mg/kg). Hypoglycaemic effects on healthy rats and anti-hyperglycaemic effects on fructose-fed T2D model were demonstrated. *T. iboga* aqueous extract at 50 mg/kg and mitigated effect at 100 and 200 mg/kg. The plant extract at these selected doses (50, 100 and 200 mg/kg), is moderately toxic and do not influence body and major organs weight or parameters reflecting any side effects after 28 days administration. Insulin resistance markers were not calculated and would have helped to understand the effect of *T. iboga* (50 to 200 mg/kg|) on insulin resistance after 4 weeks' treatment. This is the first *in vivo* study to demonstrated *T. iboga* aqueous extract hypoglycaemic and anti-hyperglycaemic effects which support the effect already shown in pancreatic ß-cells in Chapter 3. Taking into account the hypoglycaemic and anti-hyperglycaemic effects of *T. iboga* aqueous extract in T2D rat model, we wanted to evaluate the plant extract effect in another model of metabolic disorder, diet induced obesity (DIO) which exhibit features of multiple factor including obesity, dysglycaemia and dyslipidemia which characterise the metabolic syndrome (MetS).

Chapter 5: *In vivo* investigation of the aqueous extract of *T. iboga* on dysfunctional metabolism (glucose and lipid metabolism) in high-fat-fed C57BL/6J mice

5.1. Introduction

Metabolic syndrome is an emerging world-wide public health issue characterised by the occurrence of three or more of these conditions: abdominal obesity, insulin resistance, impaired glucose tolerance, hypertension, and inflammatory and thrombotic state. Some of these features are also seen in T2DM, as mentioned in previous Chapters. An estimated 11% of Gabonese population meet the criteria for people with metabolic syndrome which puts them at high risk for developing cardiovascular disease and T2DM (Siawaya *et al*., 2015; Grundy *et al.,* 2004; Kaur, 2014).

Since *T. iboga* aqueous extract (50, 100 and 200 mg/kg) has shown *in vivo* hypoglycaemic potential and reduction in metabolic markers sur as insulin resistance (Chapter 4) and is used in Gabonese traditional medicine for the management of diabetes (Tjeck *et al.,* 2016), the hypothesis that this medicinal plant can mitigate metabolic syndrome in male C57BL/6J mice, a mice model that develops obesity, hyperglycaemia and glucose intolerance/ insulin resistance when fed a high-fat-diet was considered.

Unfortunately, there are limited data available on the toxicity of *T. iboga,* hence, information on ibogaine toxicity, the most abundant indole alkaloid present in the root bark, have been used for doses selection in this study. Indeed, the (NOAEL) is defined as the highest dose of a chemical which does not cause an observable adverse effect on a test animal was 25 mg/kg body weight for ibogaine by i.p. (Kubiliene *et al.,* 2008), NOAEL for ibogaine has not been demonstrated for the oral route. Thus, the lowest dose that can cause lethality in animals remains 263 mg/kg body weight, which lead to a theoretical accepted dose of 0.87 mg/kg in humans after application of factors such as intra-species variability (division by 10), inter-species variability (division by 10) and people susceptibility (division by 3). Nevertheless, higher doses ranged from 6 to 30 mg/kg b.w. (within doses that have shown evidence of toxicity in animal models) have also been tested in humans and exhibited good results (Schep *et al.,* 2016).

A diet-induced obesity mouse model, which more accurately displays metabolic syndrome in humans was selected (Miranda *et al.,* 2016). The C57BL/6J mouse develops a metabolic

syndrome-like phenotype when fed a high fat diet. They develop hyperglycaemia, obesity, hypertension and hyperinsulinemia on a high-fat diet but remain lean when fed a low-fat diet (Miranda *et al.,* 2016).

The goal of this study was to determine the effects of *T. iboga* on various endpoints of metabolic syndrome in male C57BL/6J mice fed a high fat diet. This study advances the knowledge on the *in vivo* anti-diabetic effects of *T. iboga* (Chapter 4) in a non-genetic, diet-induced rodent model of metabolic syndrome. This study has been recently published in the Journal of Food Biosciences (Bading-Taika *et al.,* 2018)

5.2. Materials and Methods

5.2.1. *T. iboga* extraction

Root bark of *T. iboga* was provided by the Institute of Pharmacopoeia and Traditional Medicine (IPHAMETRA) in Libreville, Gabon. *T. iboga* extract for the mouse feeding study was prepared following the method by of Sadoon *et al.* (2014). The method was detailed in Chapter 2, section 2.2.1.

5.2.2. Animals and environment

Male C57/6J mice, 8 weeks of age, weighing 20-25 g were purchased from The Jackson Laboratory, Bar Harbor, ME, USA. Animals were maintained on a 12h dark/light cycle and fed a regular mouse chow diet and individually housed in labeled standard cages with bottled tap water in plastic containers and fed their respective diets ad libitum (Techniplast 2000P) with sawdust (Datesand grade 7 substrate) and shredded paper wool bedding with free access to bottled tap water and lab food (Labdiet 5001). All animal studies were conducted with the approval from the Institutional Animal Care and Use Committee of Oregon State University, Corvallis, USA. At the end of 10 week's experiment, all mice, after an overnight fast, were euthanized via CO_2 inhalation and blood was collected for endpoint markers of metabolic syndrome, including diabetes

5.2.3. Experimental design of the assessment of the effects of bioactive compounds from *T. iboga* for the improvement of dysfunctional metabolism in DIO mouse

After one week of acclimation, the mice were randomly assigned into 4 Groups of 12 animals, namely:

Each mouse was housed individually in labeled plastic cages with bottled tap water in plastic containers and fed their respective diets *ad libitum*.

- Group 1: normal control (LFD)

- Group 2: High-fat diet control (HFD)

- Group 3: High-fat diet + *T. iboga* aqueous extract at low dose (HFD + *T. iboga* 0.83 mg/kg)

- Group 4: High-fat diet + *T. iboga* aqueous extract at high dose (HFD + *T. iboga* 2.07 mg/kg)

Group 1 control mice were fed a regular chow diet. Group 2 mice were given HFD as control. The diet fed to mice was as powder) 3 mice were given HFD containing 0.83 mg of ibogaine as *T. iboga* extract/kg body weight/day (low-dose *T. iboga* aqueous extract). Group 4 mice were given HFD containing 2.08 mg of ibogaine as *T. iboga* extract/kg body weight/day (high-dose *T. iboga* aqueous extract).

The HFD contained 60 %, 20% and 20 % total calories from fat, carbohydrate and protein, respectively. This HFD diet has been used to induce obesity and metabolic syndrome in male C57BL/6J mice (Miranda *et al.,* 2016). The LFD contained 10%, 70% and 20 % total calories from fat, carbohydrate and protein, respectively. Both diets were obtained from Dyets Inc, Bethlehem, PA, USA. The HFD was fortified with *T. iboga* extract on a weekly basis by dissolving a calculated amount of extract in a very small volume of ethanol for incorporation into the diet, so that it would provide an ibogaine dose of 0.83 (low dose) or 2.08 mg/kg b.w. per day. To obtain a daily dose of 49.5 µg of ibogaine. The amount of *T. iboga* extract containing 49.5 ibogaine was dissolved in ethanol and mixed with 2.5 g of Diet that the mouse would consume per day. The ethanol in the pellets of diet was allowed to evaporate in the fume hood. The fortified amount of *T. iboga* extract was adjusted each week based on the previous week's body weight and food intake.

The dose of 2.08 mg/kg body weight was 126 times lower than the LD_{50} (263 mg/kg body weight) of ibogaine in mice (Kubiliene *et al.,* 2008). As a treatment for opioid dependence in humans, ibogaine is used at doses between 15 and 20mg/kg body weight. Mouse doses of 0.83 mg/kg/day (Group 3) and 2.08 mg/kg/day (Group 4) were equivalent to human doses of 10 mg/kg/day and 25 mg/kg/day, calculations obtained from the FDA formula for interspecies scaling dose (Bading-Taika et al., 2018).

Table 5.1 Allometric scaling dose of ibogaine and *T. iboga* extract (total alkaloids) (for a person of 70 kg)

Ibogaine	***T. iboga* extract (total alkaloids)**	**Human equivalent dose (for a person of 70 kg)**
	mg/kg/day	
0.83	19.6	0.28
2.07	49	0.70
0.21	50	0.70
0.42	100	1.4
0.84	200	2.8

Body weights were recorded weekly and food intake was monitored every two days during 10-week feeding study in order to reach the target dose regimes. All animals were fed their respective diets for 10 weeks, before the study was terminated.

All animal studies were conducted with the approval from the Institutional Animal Care and Use Committee of Oregon State University, Corvallis, Oregon, USA.

5.2.4. Intraperitoneal glucose tolerance test (i.p. GTT)

During the 4th and 9th week of study, a glucose tolerance test (GTT) was performed. Five C57BL/6J mice in each treatment Group were fasted for 4 hr and given an intraperitoneal (i.p.*)* injection of D-glucose (2 g/kg body weight). A drop of blood was collected from a tail prick at 30, 60, 90 and 120 min after the glucose bolus injection for measuring glucose using a Johnson and Johnson One Touch® Ultra Blood Glucose Monitoring System.

After the 6-hr fast, mice were weighed before taking glucose measurement at time 0 (before injection of the glucose solution). We prepared mice by labelling tails (1-5 lines across tail) for easy order identification using marker, in the same manner as in chapter 4. We placed five mice from each group in one cage (no food or water) properly labelled with the group ID after weighing. We prepared syringe for each mouse before doing the glucose measurement at time 0. We calculated appropriate glucose dosage per mouse according to the following formula: dose in µl = weight in grams x 10. We measured blood glucose level at time 0. Wiped the tail first with 70% ethanol and then using a 23-gauge syringe needle, pricked the skin near the end of the tail. Stroke base of tail to encourage blood flow. We measured glucose with a glucose test strip on a drop of blood coming out from the pricked tail. Then, we recorded readings. After finishing the glucose time 0 measurements, we injected a glucose solution to the selected mice from each group at 30-sec intervals. Record starting time.

We injected mouse with 10 µl of 10% glucose per gram body weight (equivalent to 1 g glucose/kg body weight). Sampled blood glucose at 15, 30, 60 and 120 minutes after glucose injection at 30-sec intervals. Returned mice at completion of 120 min time point to their respective cages with food and water.

5.2.5. Intraperitoneal insulin tolerance test (i.p. ITT)

Intraperitoneal insulin tolerance test or i.p.ITT is designed to determine the sensitivity of insulin-responsive tissues in the rodent. This is determined by measurement of glucose remaining in the circulation over time after a bolus (i.p.) insulin injection.

An insulin tolerance test (ITT) was also conducted at 9th weeks of the feeding study. Another set of five mice in each treatment Group were fasted for 4 hr before giving them an i.p. dose

of insulin (0.75 U/kg b.w.). Blood glucose was determined using the same protocol as the glucose tolerance test.

Food and water intake, and body weights were recorded on a daily basis during the 28 days' experiment.

At the end of the 10-week feeding mice were anaesthetised by CO2 asphyxiation and opened at the abdomen. Blood was withdrawn from the abdominal aorta and centrifuged at 3000 rpm for 10 min to obtain serum.

Organs (heart, lungs, liver, kidneys, testis) were weighed and serum biochemical parameters measured (HDL, LDL, Hba1c, AST, ALP, ALT, TC, Urea, TG, Blood Glucose, Creatinin) also including markers of insulin resistance (HOMA-IR, HOMA-β scores).

5.2.6. Biochemical measurement of metabolic markers

Biochemical analyses were performed after blood samples collection in EDTA/heparinized-treated tubes from the heart of the animal using also the heart puncture technique. Cells were removed from plasma by centrifugation for 10 minutes at 2000 rpm and the supernatant were directly use for analysis. Blood plasma was analysed for insulin using a mouse insulin ELISA kit (Alpco Diagnostics, Salem, NH). Plasma Monocyte Chemoattractant Protein-1 (MCP-1) and Interleukin (IL)-6 were analysed by ELISA kits from Life Technologies Corporation (Carlsbad, CA). Plasma leptin levels and PCSK9 (Proprotein convertase 9) were estimated by ELISA kits obtained from Life Technologies Corporation (Carlsbad, CA) and R&D Systems, Inc. MN, USA, respectively; and inflammatory markers MMP-9 and ICAM-1 were determined using ELISA kits (ALPCO, Salem, USA; Thermo Fisher Scientific, USA, respectively). Total plasma cholesterol (TC) and triglycerides (TG) were determined following the same method of Savoldi *et al.,* 1976 using modified Trinder method, Infinity™ Cholesterol and Infinity™ Triglycerides reagents kits, respectively (Thermo Scientific, Middletown, USA). Plasma glucose was analysed by enzymatic method (Mutarotase-GOD) using Autokit Glucose (Wako Chemicals USA, Inc, Richmond, VA). Plasma low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) levels were measured using microplate-based (Bioo Scientific Corporation, Austin, TX) method. ALT and AST were analysed using manual enzymatic assay (Bioo Scientific Corporation, Austin, TX).

5.2.7. Data analysis

Data were analysed using SAS 9.2 software (SAS Institute Inc., Cary, NC) and GraphPad Prism 7.0 (San Diego, CA). Body weight, food intake, GTT, and ITT data were analysed using a repeated-measures-in-time design ANOVA. Repeated measures within animals were modelled using a first- order autoregressive variance-covariance structure (body weight and food intake) or an unstructured variance-covariance structure (GTT and ITT) data. Baseline values were used as linear covariate for body weight and feed intake data. The AUC data presented in Figure 5.1 were analysed using a one-way ANOVA and a post-hoc Tukey's multiple comparison test. A $p < 0.05$ was considered statistically significant.

5.3. Results

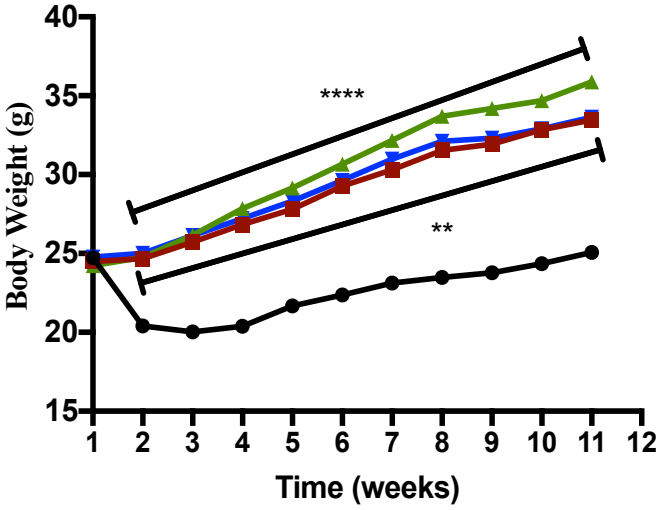
5.3.1. Rationale for the selection of *T. iboga* doses

We used the calculated ibogaine content of 1.93% in the bark to fortify the diets with iboga extract (Chapter 2, Table 2.1). The fortified diets delivered a daily ibogaine dose of 0.83 and 2.07 mg/kg body weight.

5.3.2. Effect of diet and *T. iboga* extract on body weight, food intake and liver weight

The LFD-fed mice gained significantly less weight than the mice fed the HFD with or without *T. iboga* extract (Figure 5.1 a) after 1 week up to 10 weeks. The LFD mice lost weight during the first week and then slowly gained weight until the end of the study. The LFD mice actually were eating significantly more food than the mice fed the HFD within the first week (Figure 5.1 b) but their feed efficiency was lower compared to the mice on the HFD (Table 5.2). The HFD mice treated with the low dose of *T. iboga* extract gained significantly more weight than the HFD mice and the HFD mice treated with the high dose *T. iboga*. Liver weights were much higher in the HFD-fed mice than the LFD-fed mice (Table 5.2).

a



b

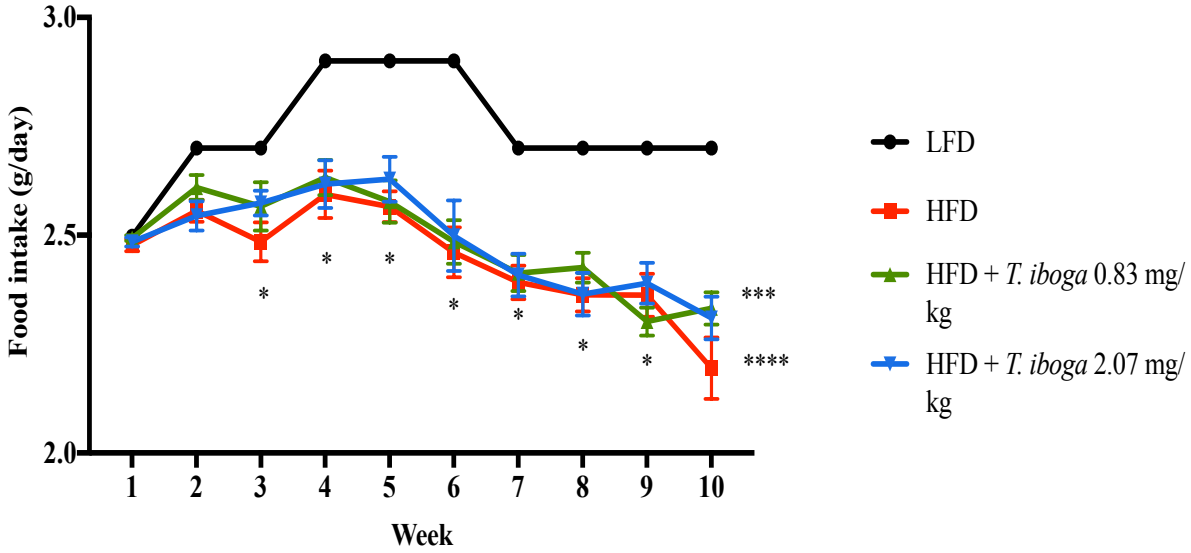


Figure 5.1. Effect of the HFD and iboga extract feeding on body weight (a) and food intake (b) of mice. LFD, low-fat diet; HFD, high-fat diet; Ib, Ibogaine. Data were analysed using a repeated-measures-in-time design ANOVA in PROC MIXED for repeated measures within animals (n = 12 per group). Statistical differences between Groups are indicated with different letters (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$**** $p < 0.0001$ vs LFD).

Table 5.2. Body weight gain, food intake, liver weight of mice fed experimental diets after 10 weeks (All values are means ± SEM of 11-12 animals. * $p < 0.05$ vs LFD. ** $p < 0.05$ vs HFD).

Parameter	**LFD**	**HFD**	**HFD + *T. iboga* 0.83 mg/kg**	**HFD + *T. iboga* 2.07 mg/kg**
Body weight gain(g)	0.33 ± 0.00	8.96 ± 0.72*	11.6 ± 0.71*	8.86 ± 0.67*
Food intake (g/day)	2.74 ± 0.04	2.45 ± 0.04*	2.48 ± 0.04*	2.48 ± 0.04*
Feed efficiency (g weight gain/food intake)	0.002 ± 0.000	0.052 ± 0.003*	0.067 ± 0.003*	0.051 ± 0.003*
Liver weight (g)	0.834 ± 0.0042	1.31 ± 0.196*	1.23 ± 0.044*	1.09 ± 0.042*

5.3.3. Effect of diet and *T. iboga* extract on glucose tolerance and insulin sensitivity

One of the major aims of this study was to determine whether ingestion of *T. iboga* extract has any beneficial effect on T2D induced by feeding a HFD. The results of the glucose tolerance tests conducted at 4 and 9 weeks of feeding showed that mice fed the HFD cleared glucose slower after the glucose bolus dose compared to the LFD group (Figure 5.2). Oral *T. iboga* administration did not improve the glucose tolerance of the HFD mice. In fact, the HFD mice treated with the low or high *T. iboga* dose were slightly less glucose tolerant than the HFD controls at 4 weeks of treatment (Figure 5.2 a). Similar results were obtained in the insulin tolerance test (Figure 5.2 e, f) in that the HFD-fed mice had higher blood glucose levels after insulin injection while *T. iboga* exposure did not improve the insulin sensitivity of the mice fed a HFD.

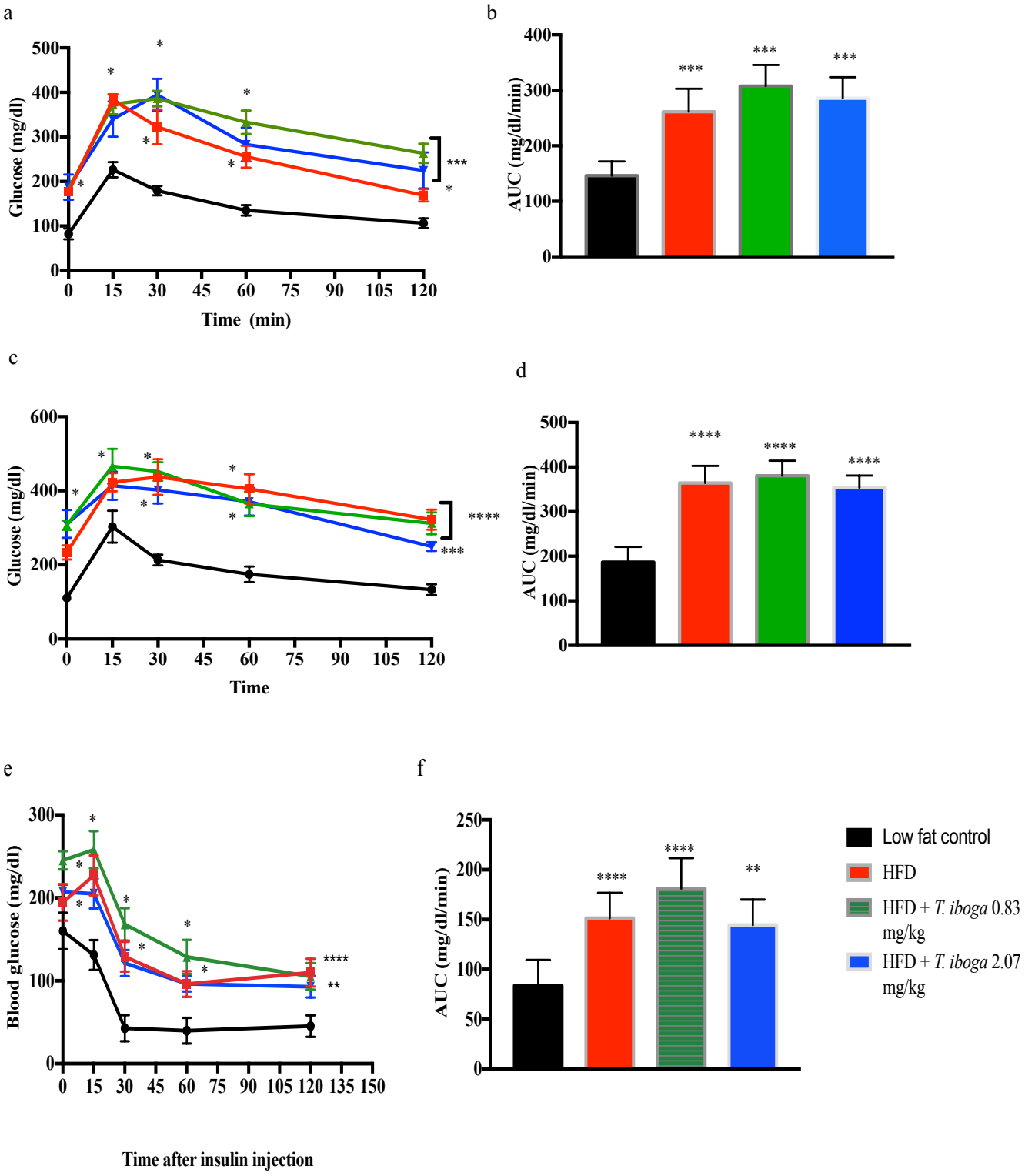


Figure 5.2. Influence of diet and *T. iboga* extract on glucose tolerance in mice at 4 weeks (a, b) and 9 weeks (c, d) and on insulin tolerance at 9 weeks (e, f) of the feeding study. The mice were fasted for 6 h before i.p. injection of glucose, 2 g/kg body weight, or of insulin, 0.75 U/kg body weight, before blood glucose was analysed at designated times. LFD, low-fat diet; HFD, high-fat diet; Ib, ibogaine. Data presented in panels a, c, and e were analysed using a repeated-measures-in-time design ANOVA in PROC MIXED for repeated measures within animals (n = 5 per group). Data presented in panels B, D, and F were analysed using a one-way ANOVA and a post-hoc Tukey's multiple comparison test. Statistical differences between groups are indicated with different letters (*$p < 0.05$ and *** vs LFD).

5.3.4. Effect of diet and *T. iboga* extract on other metabolic parameters

As shown in Table 5.2, no significant difference was observed in ALT and AST between LFD-fed mice and HFD-fed mice. Also, the results showed that mice fed a HFD had significantly higher plasma levels of glucose, triglycerides, LDL- cholesterol, HDL-cholesterol, leptin IL-6, ICAM-1, and MCP-1 compared to the group fed LFD (Table 5.2 and 5.3). The addition of *T. iboga* extract to the HFD did not have a significant effect on any of these metabolic parameters, except for the reduction of plasma levels of MCP-1 by low-dose *T. iboga* treatment (Table 5.3).

Table 5.3. Plasma lipids profile in mice fed experimental diets (All values are means ± SEM of 11-12 animals. * $p < 0.05$ vs LFD. # $p < 0.05$ vs HFD)

Mice groups/ Plasma lipids	**LFD**	**HFD**	**HFD + *T. iboga* 0.83 mg/kg**	**HFD + *T. iboga* 2.07 mg/kg**
	(mg/dl)			
Total cholesterol	170 ± 8.96	256 ± 8.18*	258 ± 8.96*	231 ± 14.0*
HDL cholesterol	54.7 ± 7.29	72.4 ± 3.36*	72.9 ± 1.99*	74.1 ± 3.37*
LDL cholesterol	43.9 ± 6.95	88.7 ± 13.6*	80.8 ± 7.82*	80.6 ± 7.17*
Triglycerides	48.8 ± 1.89	60.6 ± 4.56*	64.9 ± 4.57*	66.6 ± 5.17*

Table 5.4. Metabolic parameters of mice fed experimental diets (All values are means ± SEM of 11-12 animals. * $p < 0.05$ vs LFD. # $p < 0.05$ vs HFD)

Mice groups/ plasma parameters	**LFD**	**HFD**	**HFD + *T. iboga* 0.83 mg/kg**	**HFD + *T. iboga* 2.07 mg/kg**
Glucose (mg/dL)	243 ± 24.9	310 ± 24.4*	343 ± 12.4*	284 ± 24.1*
Insulin (ng/mL)	ND	1.35 ± 0.23	1.21 ± 0.16	1.33 ± 0.33
Leptin (ng/mL)	6.18 ± 1.67	26.1 ± 2.26*	30.8 ± 1.31*	26.8 ± 1.81*
IL-6 (pg/mL)	6.98 ± 0.46	9.96 ± 1.17*	8.55 ± 0.65*	8.75 ± 0.05*
ICAM-1 (ng/mL)	17.7 ± 3.48	34.3 ± 2.34*	33.8 ± 1.87*	34.1 ± 1.97*
MCP-1 (pg/mL)	134 ± 8.09	810 ± 284.2*	81.5 ± 23.6##	337 ± 119#
MMP-9 (ng/mL)	30.9 ± 8.09	45.6 ± 13.8	57.8 ± 14.3	30.6 ± 5.72
ALT (U/L)	50.2 ± 3.92	48.2 ± 1.92	54.7 ± 7.0	45.2 ± 3.79
AST (U/I)	156 ± 12.9	147 ± 8.81	118 ± 13.8	131 ± 21.2

5.4. Discussion

In this study, the mouse doses of 0.83 and 2.07 mg/kg body weight are equivalent to human doses of 4.7 mg/day and 12 mg/day for a 70 kg person by allometric scaling of dose (Sharma and McNeill, 2009). Thus, assuming that the total alkaloid content of the root bark is 7.8% (Chapter 2, Table 2.2), the human equivalent dose of 12 mg ibogaine per day would represent a total alkaloid dose of 49 mg per day (0.70 mg/kg/day for a 70 kg person) when given in the form of *T. iboga* bark extract. Moreover, the selected high dose in this study approaches the recently recommended human dose of 0.87 mg ibogaine/kg that is considered safe and appropriate for treatment of opioid addiction in humans (Schep *et al.,* 2016). Forsyth *et al*., 2016 team investigated the effects of a single oral dose of 20 mg ibogaine in humans on a number of psychological variables. A pharmaceutical preparation of *T. manii* (Lambarène), containing 8 mg ibogaine per tablet, was marketed in France as a neuromuscular stimulant, promotor of cell combustion, and anti-fatigue agent. The recommended dose was 16–32 mg ibogaine per day (2–4 tablets daily) (Goutarel *et al*., 1993). Assuming that other iboga alkaloids exert pharmacological activity, these doses are comparable to our low dose in mice (human equivalent dose for a 70 kg person: 20 mg total *T. iboga* alkaloids). However, these doses are more than twice lower than the lowest dose tested in healthy and T2D animals in Chapter 4 (50 mg/kg for *T. iboga*). As this is the first study designed to investigate the effects of *T. iboga* aqueous extract in an *in vivo* mouse model of metabolic syndrome induced glucose tolerance, we were unable to predict efficacious dose levels for ameliorating glucose intolerance. The data in this chapter, do not support the conclusions reached by Souza *et al.* (2011) that aqueous extracts of *T. iboga* root bark showed anti-hyperglycaemic activity. Using isolated islets of Langerhans from the pancreas of non-fasting female Wistar rats, these authors found that bioactives in the aqueous extract of *T. iboga* exerted an insulinotropic effect *ex vivo*, similar but not identical in mechanism compared to tolbutamide, a representative of the sulfonylurea class of T2 diabetic drugs. Similarly, we report the same concentration of aqueous extract of *T. iboga* exerted an acute insulinotropic effect *ex vivo* (Chapter 3). However, a daily consumption for 10 weeks of the plant extract incorporated in mice chow might induced an increased concentrations of *T. iboga* active constituents which might activate other pathways than the one leading to lowering blood glucose levels as seen in Chapter 4. With chronic administration of sulfonylureas, insulin levels do not change with treatment, consistent with our observations, but fasting plasma glucose levels decrease due to lowering of insulin resistance (Davis, 2006), which we did not observe with *T. iboga* treatment in this study. One explanation for the discrepancy is that the doses used in this

study were not optimal. In addition, we cannot exclude the possibility that *T. iboga* bioactives are converted into inactive metabolites by gut microbial or hepatic metabolism. For instance, ibogaine undergoes *O*- demethylation by the hepatic cytochrome P450 enzyme CYP2D9 (Obach *et al.,* 1998). It is possible that the metabolite, 12-hydroxyibogamine (syn. noribogaine), lacks insulinotropic activity or might be in concentrations (after chronic administration) too high which would induced an antagonism to the hypoglycaemic effect of the metabolite, as suggested in Chapter 3.

One major concern of the use of herbal or dietary supplements such as kava-kava (Stickel and Shouval, 2015) is liver damage. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are generally used as indicators of liver injury (Ozer *et al.,* 2008). As shown on Table 5.2, supplementation of the HFD with *T. iboga* extract did not cause a significant increase in ALT or AST suggesting that the extract is not hepatotoxic. Furthermore, other important parameters in MetS are pro-inflammatory cytokines including interleukin 6 (IL-6) and adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) which are involved in the progression and development of MetS complications such as cardiovascular diseases (Colina-Coca *et al.,* 2017). These parameters were increased in HFD, HFD + Low and High *T. iboga* Groups compared to LFD Group, thus *T. iboga* extract did not improve inflammatory disease processes (Colina-Cola *et al.,* 2017). Also, plasma leptin levels, which are well associated with body fat mass, fat accumulation and insulin resistance, were elevated in HFD, HFD + Low and High *T. iboga* (Table 5.2). Leptin is mainly expressed by adipocytes and promotes vascular inflammation and calcification, increased levels has been reported in subjects with high levels of TC and TG, as shown in our results, and are associated with endothelial derived inflammation (Parka *et al.,* 2017). In contrast, results in Table 5.1 showed in HFD + Low *T. iboga* Group, significantly lowered plasma levels of MCP-1 or CCL2 which is a chemokine that may contribute to low-grade inflammation in obesity by recruiting monocytes to adipose tissues (O'Connor *et al.,* 2015). Therefore, *T. iboga* aqueous extract may be beneficial in ameliorating low-grade inflammation in obesity when dosed prophylactically in DIO models. However, the extract did not improve lipid plasma profile as TC, LDL and HDL remained elevated in treated Groups compared to HFD Group.

Overall, feeding a HFD to male mice for 10 weeks resulted in obesity and high plasma levels of circulating glucose and insulin, which were not attenuated by treatment with *T. iboga*

extract at dose levels of 0.83 and 2.07 mg/kg/ day. However, plasma levels of MCP-1, a chemokine involved in inflammation in obesity, was significantly attenuated by low-dose *T. iboga* extract (0.83 mg/kg/day) suggesting an anti-inflammatory effect of the extract and suggesting a prophylactic activity of *T. iboga* extract on DIO models.

Chapter 6: General discussion

Since preliminary *in vitro* investigations of the root barks of *T. iboga* aqueous extract have shown potentiation of insulin secretion (Souza *et al.,* 2011) and this plant has been reported to be used in Gabonese traditional medicine for the management of DM and its complications, along with the stem barks of *G. tessmannii* aqueous extract (Bading Taika *et al*., 2018), the aim of this thesis was to (i) characterise the phytochemical composition of *T. iboga* root barks and identify potential insulinotropic agents in the plant, (ii) to investigate for the first time the effects of the aqueous extracts of *G. tessmannii* on glucose-induced insulin release from rat isolated pancreatic cells, (iii) to investigate the role of σ2 receptors in mediating *T. iboga* aqueous extract effects on glucose-induced insulin release from rat pancreatic cells, and (iv) to investigate for the first time the effects of *T. iboga* aqueous extract in naïve rats, in fructose-fed/STZ T2D animal model and in DIO animal model of dysfunctional metabolism in C57BL/6J mice.

6.1. Identification of five phenolic phytochemicals, and thirty alkaloid phytochemicals from *T. iboga* root bark

Phytochemical characterisation of the aqueous extract of *T. iboga* root barks was performed using LC-MS/MS technique in positive ionisation mode due to the presence of alkaloids which contain nitrogen atoms with high proton affinity. Several unknown and known compounds of the aqueous extract of *T. iboga* were revealed using a mass defect filtering approach specifically designed for screening of indole alkaloids. 30 alkaloids were detected, of which only 7 were previously reported in *T. iboga* including ibogaine, iboxygaine, ibogaline, alloibogamine, catharanthine, ibogamine, noribogaine (Chapter 2, Table 2.5) (Taylor, 1968; Alper, 2001; Gaignault and Delourme-Houdé, 1977; Bowen, 2001). This useful post acquisition data processing based on mass defect (difference between the nominal mass and the monoisotopic mass of a molecule) detected group of molecules with similar elemental compositions, including indole alkaloids. Previous studies have already revealed presence of indole alkaloids in *T. iboga* root barks (Gaignault and Delourme-Houdé, 1977), with ibogaine as its major constituent (Bartlett *et al.,* 1958). The principal alkaloid of *T. iboga*, ibogaine, has attracted attention in many countries around the world for providing relief of opioid craving in drug addicts (Glick *et al.,* 1996). The anti-addictive property of

ibogaine has been cited in Gabonese religious community 'Bwiti', who use the plant root barks as in traditional ritual to enter in trance and reach an in between state, to be able to contact ancestors (Mazoyer *et al.,* 2013) Among the unknown compounds revealed for the first time in this thesis, several have previously been identified in other plants also belonging to the Apocynaceae family including Yohimbine from *Rauwolfia* root bark (Funk *et al.,* 2019; Stavrinides *et al.,* 2015), Quinidine (Halperin *et al.,* 2018; Yang *et al.,* 2009; Stavrinides *et al.,* 2015), Coronaridine (Arias *et al*., 2011; Arias *et al.,* 2017), Affinine (Rosales *et al.,* 2019; Yang *et al.,* 2010), Jerantinine B isolated from *Tabernaemontana corymbosa* (Qazzaz *et al.,* 2016). Among the 23 known alkaloids listed in Chapter 2 (Table 2.5), only yohimbine, an alpha2-adrenoceptor antagonist, has been reported to exhibit *in vivo* anti-diabetic property (Abdel-Zaher *et al.,* 2001). This study revealed that yohimbine at 20 mg/kg b.w. potentiated glucose-induced insulin release in non-diabetic control rats and improved oral glucose tolerance, potentiated glucose-induced insulin release in T2D, but not in T1D rats. Moreover, pre-treatment of yohimbine enhanced the hypoglycaemic and insulinotropic effects of glibenclamide in an additive manner and antagonised hyperglycaemia and hyperinsulinemia effects induced by diazoxide in control and T2D rats (Abdel-Zaher *et al.,* 2001). The presence of the alkaloid, yomhibine in *T. iboga* aqueous extract may contribute to the antidiabetic activity we report in chapters 3 and 4. The investigation of potential additive antidiabetic effects of *T. iboga* and glibenclamide *in vitro* and *in vivo* will be of interest in future studies. 48 unknown alkaloids could not be characterised as these constituents were not listed in any libraries (Chapter 2, Table 2.5).
For the first time, phenolic compounds were revealed in significant quantity in *T. iboga* and the most prevalent was 3-*O*-caffeyolquinic acid (Chapter 2, Table 2.5). The amount of 3-*O*-caffeoylquinic acid in *T. iboga* root bark, extracted with 70% aqueous methanol, was determined to be 0.97 mg/g of plant powder (Table 2.5, figure 2.8). 3-*O*-caffeoylquinic acid is a phenolic compound naturally found in food which has exhibited numerous pharmacological properties including antioxidant, free radical scavenging, anti-inflammatory, radio protective, anti-tumour, antioxidant, analgesic and anti ulcerogenic activities (Bagdas *et al.,* 2015; Jabeur *et al.,* 2016). The amount of chlorogenic acid found in *T. iboga* aqueous extract, in this study was estimated at 0.97 mg/g in 100 mg of plant root barks (Chapter 2, section 2.3.2). A previous study has shown that 3-*O*-caffeyolquinic acid at 100 mg/kg b.w. orally administered to DIO model HFD-fed C57BL/6J mice increased insulin resistance and hepatic lipid accumulation compared to control HFD mice (Mubarak *et al.,* 2013). However, another study demonstrated that 3CQA at a lower dose of 20 mg/kg b.w administered orally

to HFD-fed ICR mice decreased their insulin levels, thus exhibiting improvement of insulin resistance (Cho *et al.,* 2010). The amount of 3-CQA in *T. iboga* doses used the *in vivo* anti-diabetic study in healthy, fructose/STZ T2D rats (Chapter 4), and DIO models C57BL/6J mice (Chapter 5), would give 0.05 mg in 50 mg/kg, 0.1 mg in 100 mg/kg, 0.2 mg in 200 mg/kg, 0.10 mg/kg in 107 mg/kg (dose containing 2.07 mg of ibogaine) of *T. iboga* by extrapolation, if the extraction yield were quantitative. These very low *T. iboga* extract amounts of chlorogenic acid are unlikely to have contributed to the effects of *T. iboga* seen in the *in vivo* studies in chapters 4 and 5. Furthermore, to test the hypothesis that alkaloids in *T. iboga* root barks and not 3-*O*- caffeoylquinic acids are responsible for the reported biological effects, alkaloid extracts devoid of phenolic acids were prepared for testing *in vivo* chapters 4 and 5. Thus, other compounds in *T. iboga* aqueous extract may in part, play a role in the anti-diabetic action of the plant. As all active compounds in *T. iboga* root barks aqueous extract have not been fully characterised, further investigations are needed to correlate the active principles contributing to the anti-diabetic effects of *T. iboga*.

6.2. *T. iboga* aqueous extract (1 µg/ml) stimulated glucose-induced insulin release from isolated rat pancreatic islets

In order to assess the effects of *G. tessmannii* and *T. iboga* aqueous extracts on glucose-induced insulin release in isolated rat pancreatic islets, a reproducible method of isolation was developed in Chapter 3 adapted from Carter *et al.* (2009) and Szot *et al.* (2007). The isolated rat pancreatic islets were responsive to glucose and functional throughout the experimental period of 90 min, in a similar manner reported in previous studies (Szot *et al.,* 2007; Carter *et al.*, 2009 and Souza *et al.,* 2011).

The finding that aqueous extract of *T. iboga* (1µg/ml) stimulates glucose-induced (2.8 to 11.1 mM) insulin release is in line with previous published work, in isolated rat pancreatic islets at stimulatory glucose concentrations (2.8 to 16.7 mM) (Souza *et al.,* 2011). These aqueous extract effects were more effective than the insulin secretagogue, tolbutamide (Souza *et al.,* 2011; Chapter 3). However, the bell-shape effects seen at glucose stimulatory concentrations (11.1 to 25 mM) suggested that certain *T. iboga* active constituents might have an antagonism activity to insulin secretion and those are efficient at low concentrations (0.001 to 0.1 µg/ml). Indeed, as crude plant extracts are complex mixtures with multiple pharmacological activities, the different constituents may exert antagonistic, allosteric and/ or synergistic

effects (Kennedy and Wightman, 2011; Rasoanaivo *et al.,* 2011). Depending on the concentration, an active constituent could have blunted the activity of a second bioactive constituent revealing a dual effect (Djomeni Dzeufiet *et al.,* 2006). Moreover, in certain instance, a potentially active constituent at higher concentration, might be stabilised by a second constituent, which could lead to the effects seen at high concentrations of the plant extract (10 to 10 µg/ml) (Rasoanaivo *et al.,* 2011).

6.2.1. *T. iboga* aqueous extract (1 µg/ml) stimulated glucose-induced insulin release was blocked by the σ2 receptor antagonist, SM-21 (1 and 10 µM)

At high glucose concentrations (16.7 and 25 mM) *T. iboga* (0.01 to 100 µg/ml) inhibited glucose-induced insulin secretion. Furthermore, low (state concentrations 0.001 to 0.01 µg/ml) and high (10 to 100 µg/ml) concentrations of *T. iboga* aqueous extract did not alter glucose-induced insulin secretion, except for *T. iboga* at 0.1 µg/ml and low glucose concentrations (2.8 and 11.1 mM), which is not in agreement with previous findings (Souza *et al.,* 2011). The discrepancy seen with high concentrations of *T. iboga* (10 and 100 µg/ml) may be explained by its effect via σ2 receptors which are involved in apoptosis process (Bowen, 2001). Hence, the results support the insulinotropic effect of *T. iboga* aqueous extract (1 µg/ml), but not in the same dose- and glucose-dependent manners as stated previously (Souza *et al.,* 2011).

T. iboga insulinotropic effects have been suggested to be mediated by σ2 receptors (Souza et al., 2011), and its main active constituents (ibogaine and related iboga congeners) are selective σ2 receptors ligands (Bowen *et al.,* 1995). In the presence of the σ2 receptor antagonist, SM-21 at 1 and 10 µM, the insulinotropic effects of glucose and *T. iboga* (1 µg/ml) were inhibited. This confirmed for the first time the involvement of σ2 receptors in the action of *T. iboga* aqueous extract in isolated rat pancreatic islets. σ2 receptors antagonism may act directly or indirectly on K^+-$_{ATP}$ channels, as diazoxide, which prevents K^+-$_{ATP}$ closure inhibits glucose-induced insulin secretion and *T. iboga* potentiation of glucose-induced insulin release (Souza *et al.,* 2011). The involvement of σ2 receptors on intracellular Ca^{2+} flux in *T. iboga* aqueous extract insulin release is suggested by previous study, where intracellular Ca^{2+} flux was increased in the presence of *T. iboga* aqueous extract (1 µg/ml), and *T. iboga* aqueous extract insulin stimulatory effects were inhibited in the presence of cobalt, an inorganic Ca^{2+} channel blocker (Souza *et al.,* 2011). Latent and

sustainable rise of Ca^{2+} by σ2 ligands may lead to toxicity and cell death (Bowen, 2001). Given that σ2 receptors increase intracellular Ca^{2+} levels in apoptosis induced cell death (Bowen, 2001), this may explain, the contradictory effect of high concentrations (> 10 µg/ml) of *T. iboga* aqueous extract inhibiting glucose-stimulated insulin release from isolated rat pancreatic islets. *T. iboga* aqueous extract (1 µg/ml) did not increase the levels of nitrates indicating no cell toxicity at stimulatory glucoses concentrations (2.8 and 11.1 mM), however higher concentrations of *T. iboga* (> 10 µg/ml) on nitrate production from isolated rat pancreatic islets was not investigated. Neurotoxic effects of ibogaine, one major active principle of *T. iboga*, are reported to, in part, be due to agonist action at the same σ2 receptors (Bowen, 2001; Glick et al., 2001). Elevated glucose levels (> 16 mM) as seen in diabetic conditions, may chronically activate this pathway, leading to pancreatic islet cell desensitization and glucotoxicity (Hall *et al.,* 2018). Glucose concentrations greater than 16.7 mM significantly elevated nitrate production from isolated rat pancreatic islets, compared with no glucose, and although insulin secretion increased glucose dependently up to 25 mM, elevated nitrates may indicate these cells are under oxidative stress (akin to other reports Arman *et al.,* 2007; Zhao *et al.,* 2018). Furthermore, high concentrations of *T. iboga* (> 10 µg/ml) elevating Ca^{2+} levels via σ2 receptors activation may induce cytotoxicity in isolated pancreatic islets already sensitive to oxidative stress, leading to the reduction in insulin production seen in Chapter 3.

6.2.2. *G. tessmannii* aqueous extract potentiates glucose-induced insulin release from isolated rat pancreatic islets

Using isolated rat pancreatic islets, this thesis has demonstrated for the first time that the aqueous extract of *G. tessmannii* (70 µg/ml) has insulinotropic effects at stimulatory glucose concentrations (2.8 to 25 mM), supporting its traditional medicinal use in managing diabetes (Chapter 3). The insulin secretion potentiation of *G. tessmannii* (70 µg/ml) was seen at higher glucose concentrations (16.7 mM) compared with *T. iboga* (up to 11 mM). These effects were similar to those of tolbutamide (200 µM) and enhanced the effect of tolbutamide when incubated in the same medium. At the highest glucose concentration (25 mM) *G. tessmannii* (70 µg/ml) did increase insulin secretion, which was close to statistical significance. Low (1 to 60 µg/ml) and high (100 µg/ml to 10 mg/ml) concentrations of *G. tessmannii* inhibited insulin release at all glucose concentrations, producing a bell-shaped dose-dependency, similar to *T. iboga.* In future studies, it will be interesting to evaluate if *G. tessmannii*

aqueous extracts stimulatory and inhibitory effects on glucose-stimulated insulin release and additive effect with tolbutamide may be explained by an interaction with σ2 receptors and modulation of intracellular Ca^{2+}. Moreover, a phytochemical characterisation of *G. tessmannii* aqueous extract might allowed the identification of other active constituents that may exert inhibitory activity of insulin secretion at 1 to 50 µg/ml. *G. tessmannii* aqueous extract (70 µg/ml) did not increase the levels of nitrates over cells treated with no extract, indicting no cell toxicity at stimulatory glucose concentrations, however lower (<60 µg/ml) and higher (>100 µg/ml) concentrations of *G. tessmannii* on nitrate production from isolated rat pancreatic islets was not investigated.

G. tessmannii phytochemical analyses has revealed bioactive compounds such as flavonoids, alkaloids and triterpenes saponins (Madingou *et al.,* 2012). Moreover, a saponin-rich fraction (present in *G. tessmannii*) from *Momorcica charantia* stimulates insulin secretion in pancreatic ß-cells (MIN6) (Keller *et al.,* 2011). Furthermore, triterpenoid saponins from *Aralia taibaiensis* a Chinese traditional medicine, potently stimulate insulin release from BTC3 cells at stimulatory glucose concentrations (Cui *et al.,* 2015). The insulin potentiation mechanism of action of *G. tessmannii* aqueous extract in primary isolated rat pancreatic islets requires further investigation.

6.3. *T. iboga* aqueous extract (50 to 200 mg/kg b.w.) induces hypoglycaemia in healthy rats

T. iboga aqueous extract concentrations (50 to 200 mg/kg) were shown to be moderately toxic, however, after 28 days' daily treatment, the aqueous extract did not influence major organs weight and functions. *T. iboga* at 50 and 200 mg/kg induced hypoglycaemia activity over 3 hours fasted glucose tolerance.

6.3.1. *T. iboga* aqueous extract (50 to 200 mg/kg b.w.) an anti-hyperglycaemic in fructose-fed/STZ T2D models

The anti-diabetic effects of *T. iboga* concentrations (50, 100 and 200 mg/kg b.w.) were investigated in a fructose-fed/STZ T2D rat model according to Wilson and Islam (2012) protocol in Chapter 4. Feeding a 10% fructose solution over 2 weeks combined with a low intraperitoneal dose of STZ (40 mg/kg) to rats, led to the development of T2D, in agreement

with Wilson and Islam (2012) data. This model presented T2D features, including IR and glucose intolerance (significantly different from T1D group that received STZ alone) and allowed the assessment of the effects of *T. iboga* aqueous extract on T2DM. T2D rats exhibited significantly less body weight gain and higher blood glucose levels than T1D rats and normal control rats over 28 weeks, in agreement with Wilson and Islam, (2012). This is the first study to demonstrate *in vivo* anti-diabetic effects of *T. iboga* (50 mg/kg) comparable to glibenclamide (5 mg/kg), and with no side effect and normalisation of body weight. Also, this is the first study to demonstrate *in vivo* glucose tolerance improvement of *T. iboga* (50 mg/kg) with decreased creatinine levels compared to control and T1 and T2D (Wilson and Islam, 2012). Moreover, this is the first study to assess *T. iboga* (50 to 200 mg/kg) on hyperglycaemia, blood tissue and tissue markers of IR.

6.3.2. Antidiabetic effects of *T. iboga* on DIO model, C57BL/6J mice

As the time of the experiments in Chapter 5, efficacious and non-toxic concentrations of *T. iboga* for ameliorating glucose tolerance in DIO mouse models was not predicted and hence pharmacological low (0.83 mg/kg) and high (2.07 mg/kg) doses were selected based upon ibogaine toxicity in mice (LD_{50} 263 mg/kg) (Kubiliene *et al.,* 2008). *T. iboga* aqueous extract (0.83 and 2.07 mg/kg) indicated a trend of improvement of insulin resistance in HFD with a possible prophylactic effect on DIO models, suggesting that the doses selected may be too low compared to those which have exhibited hyperinsulinemia and anti-hyperglycaemia in rat diabetic models.

6.4. Final Conclusion

In this thesis, *T. iboga* phytochemical characterisation has revealed 23 unknown alkaloids, never reported for *T. iboga*, and 7 known alkaloids. Among the unknown alkaloids, only yohimbine has shown *in vivo* anti-hyperglycaemic and hypoglycaemic activities, which has been demonstrated for the first time with *T. iboga* at 50 mg/kg in fructose-fed/STZ T2D model and healthy rats. However, *T. iboga* at 107 mg/kg (or 2.07 mg of ibogaine) failed to improve metabolic syndrome in DIO C57BL/6J mice. The insulinotropic effect of *T. iboga* at 1 μg/ml in isolated pancreatic islets was shown for the first time to be mediated by σ2 receptors, which could also mediate toxic effects. Significantly, *T. iboga* aqueous extract is

regularly consumed by Gabonese population and controlled toxicology study has demonstrated that it is moderately toxic at 50, 100 and 200 mg/kg doses.

Overall, it has been demonstrated that *T. iboga* aqueous extract has hypoglycaemic and anti-hyperglycaemic properties when administered orally to rats and these activities may be mediated through an interaction with σ2 receptors. Collectively these *in vitro* and *in vivo* experiments support the continued use of *T. iboga* and *G. tessmannii* root extracts for the management of diabetes.

A key goal in managing diabetes is to improve blood glucose levels and to improve insulin levels in people who live with Type 2 diabetes. Further investigations are now needed to evaluate the 23 previously unknown alkaloids in *T. iboga* for insulinotropic activity and mechanism of action and subsequently evaluation of appropriate safe doses of active alkaloids in controlled long term clinical trials before validating the use of *T. iboga* aqueous extract for the management of diabetes and to have a real positive social impact by improving the health and wellbeing of people living with Type 2 diabetes in Gabon. Moreover, the few data collected so far on the mechanism of action of *G. tessmannii* aqueous extract are encouraging but not concluding for this plant extract to be used safely for the management of DM in Gabon.

6.5. Future works

6.5.1 Assessment of known and unknown alkaloids fractions of *T. iboga* at stimulatory concentrations (2.8 to 11.1 mM) on rat isolated pancreatic islets

6.5.2. Ca^{2+} imaging and assessment of high concentrations of *T. iboga* (10 and 100 µg/ml) in isolated pancreatic ß-cells in presence of low and high glucose concentrations (2.8 to 25 mM)

6.5.3. Ca^{2+} imaging in the presence of thapsigargin and *T. iboga* (1 µg/ml) from isolated pancreatic ß-cells at stimulatory glucose concentrations (2.8 and 11.1 mM)

6.5.4. Screening of *T. iboga* compounds in insulin producing cells at high and low glucose concentrations (2.8 and 25 mM)

6.5.5. Assessment of *T. iboga* optimal concentration (1 µg/ml) in σ2 receptors knockout cells

6.5.6. Phytochemical characterisation of *G. tessmannii* using LC/MS-MS and Progenesis software

6.5.7. Investigation of *G. tessmannii* stimulatory (70 µg/ml) and inhibiting concentrations (<60 µg/ml and >100 µg/ml) on σ2 receptors at glucose stimulatory concentrations and in presence of tolbutamide (200 µM) on isolated rat pancreatic islets

6.5.8. Investigation of *G. tessmannii* optimal concentration (70 µg/ml) on K^{+}_{-ATP} receptors in presence of diazoxide on isolated rat pancreatic islets

6.5.9. Modulation of intracellular Ca^{2+} in presence of *G. tessmannii* stimulatory concentration (70 µg/ml) on isolated rat pancreatic islets (Ca^{2+} imaging)

6.5.10. Griess assay with low (<60 µg/ml) and high (>100 µg/ml) concentrations of *G. tessmannii* on nitrate production at stimulatory glucose concentrations on isolated rat pancreatic islets

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