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THE EFFECTS OF *HYPOXIS HEMEROCALLIDEA* ON BLOOD GLUCOSE LEVELS IN RATS WITH TYPE 2 DIABETES

By

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KEYWORDS

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Type 2 diabetes mellitus

Hypoxis hemerocallidea

High-fat diet

Streptozotocin

Intraperitoneal Glucose Tolerance

Free fatty acid

Pancreatic β-cells

Insulin secretion

Insulin resistance

ABSTRACT

About 180 million people have been estimated to suffer from type 2 diabetes (T2DM) in 2006 and the annual death rate due to this disease was 3 million by that time. More than 400 medicinal plants used for the treatment of diabetes mellitus have been recorded, but only a small number of these plants have received scientific and medical evaluation to assess their efficacy. The most common plant used to treat diabetes mellitus is *Hypoxis hemerocallidea (HH)*. The present study was undertaken to investigate the effects of *Hypoxis hemerocallidea* (*HH*) on T2DM in rats.

Male Wistar rats weighing 200-250 g were used in this experiment. *Hypoxis hemerocallidea* (*HH*) corm was used as plant material in the experiment. The study was based on three parts, an acute diabetes study, chronic diabetes study and insulin secretion study.

In the acute study, the rats were randomly divided into 2 groups (control and diabetes). The saline solution was added to different concentrations of *HH* corm to produce concentration of (50, 200, 400, 800 mg/ml). Diabetes was induced by intraperitoneal injections of STZ (65mg/kg). Two weeks after the injection (STZ 65 mg/kg), different concentrations of *HH*S was administered intraperitoneally after an overnight fast. The blood glucose levels were monitored in the diabetic and control rats at, 30, 60, 120, 180 and 240 minutes post injection.

In the chronic study, the rats were randomly divided into 6 different groups (control, HFD, DM, DM-*HH*, DM-PT*HH*, and *HH*). Diabetes mellitus was then induced in the groups of diabetic rats by intraperitoneal injections of STZ (40 mg/kg) and rats were fed a high fat diet (HFD). The body weight of the rats were measured weekly for 7 weeks. An intraperitoneal glucose tolerance test (IPGTT) was performed at the end of week 6. At the end of week 7, rats were killed and serum sample were collected for determination of fatty acid and insulin. Liver and

pancreatic tissue was collected for histological evaluation. In the insulin secretion study, *Hypoxis hemerocallidea* was tested for its effects on insulin secretion by pancreatic islet cells exposed to low (3mM) and high (20mM) glucose medium.

Results of the acute study indicated that HHS at a dose 800 mg/ml decreased blood glucose levels fastest in both normal and diabetic rats reaching significance after 30 minutes and 60 minutes respectively and remained below the baseline value until 240 minutes.

In the chronic study, it was illustrated that *HH* had no effect in normal rats on any of the parameters evaluated. Animals in the DM group gained weight the first two weeks, but thereafter began to lose weight. At the end of seven weeks the animals gained significantly less weight than the rest. Animals fed a HFD have more visceral fat compared to the control group. The visceral fat gain occurred in the absence of a significant increase in body weight. We found a markedly lower fasting glucose level in *HH* treated diabetic animals compared to untreated DM animals. At time zero the blood glucose level of the HFD group (5.8±0.5mmol/l) and the *HH* group (4.9±0.7mmol/l) were in the normal range, and were not significantly different ($P > 0.05$) from the control group (5.0±0.2mmol/l). After glucose load peak blood glucose levels was measured after 30 minutes in the control group (9.0±0.6mmol/l), the HFD group (9.8±0.4 mmol/l), the DM-*HH* group (21±5.7 mmol/l) and the DM-HHPT group (27.8±5.3 mmol/l). In the *HH* group the blood glucose level reached a peak at 60 minutes (7.6±0.6 mmol/l). In the DM group two peaks were recorded one after 10 minutes (27.2±7.1mmol/l) and another after 60 minutes (31±5.2 mmol/l). In the groups control, HFD, DM-HH, DM-HHPT and *HH* groups the blood glucose level after 120 minutes were not significantly different from the time zero value. The blood glucose level after 120 minutes in the DM group (28.2±7.1 mmol/L) was significantly higher ($P \leq 0.01$) than from the time zero value. Serum fatty acid levels were increased in all groups fed a high fat diet. The serum insulin levels in the HFD group (6.2 ± 0.76 μUI/ml protein; $P \leq 0.05$), the DM group (2.0 ± 0.9 μUI/ml protein; $P \leq 0.001$), the DM-*HH* group (3.4 ± 0.7 μUI/ml protein; $P \leq 0.001$) and the DM-*HHPT* group (3.0 ±

1.1 μUI/ml protein; $P \leq 0.001$) were significantly lower than the control group. The β-cell function in the HFD group (62 ± 8 %; $P \leq 0.001$), the DM group (3 ± 1 %; $P \leq 0.001$), the DM-*HH* (11 ± 9 %; $P \leq 0.001$) group and the DM-*HH*PT group (4 ± 2 %; $P \leq 0.001$) were significantly lower than the control group. The histological observation of the liver and the pancreas in rats after 7 weeks on different dietary regimes showed some morphological changes within the liver and pancreas parenchyma of some rats.

In the insulin secretion study, glucose stimulated insulin secretion in low (3mM) and high (2mM) glucose concentration. Furthermore, insulin secretion was significantly higher when the glucose concentration was increased from 3mM to 20 mM (1.10 ± 0.13 μUI/ml protein and 1.5 ± 0.17 mIU/mg protein respectively $P \leq 0.01$). In the presence of low *HH* (100 μg/ml), there was a marked increase in insulin secretion when exposure to high glucose compared to low glucose concentration, while in the presence of high *HH* (500 μg/ml), there was no significant different in insulin secretion in the presence of low or high glucose.

In conclusion, the results of this experimental study indicate that a concentration 800 mg/kg of *HH*S produces maximal hypoglycaemic effect in fasted normal and diabetic rats. *HH* has an antidiabetic activity as it lowers serum glucose levels in T2DM rats and significantly increases glucose tolerance. It also increases body weight of diabetic rats. *HH* treatment was found to improve insulin secretion in pancreatic islet cells.

DECLARATION

I declare that *the effect of Hypoxis hemerocallidea on blood glucose levels in rats with type 2 diabetes* is my own work, that it has not been submitted for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Mohamed Abdallah Elshawesh

Signed: الشاوش

DEDICATED TO

My Mother and Father

My Wife and Children

My Brother and Sister

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TABLE CONTENT

LIST OF FIGURES

LIST OF TAB LES

LIST OF ABBREVIATIONS

ADA	American Diabetes Association
ANOVA	Analyses of variance
AP	African potato
ATP	Adenosine triphosphate
BV	Blood vessel
BW	Body weight
CHD	Coronary heart diseases
Cv	Central vein
DM	Diabetes mellitus
FBS	Fetal bovine serum
FFA	Free fatty acid
g	Gram
G	Glucose
GB	Glibenclamide
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter two
GTT	Glucose Tolerance Test
HbA1c	Glycated haemoglobin
HBSS	Hank's balanced salt solution

HFD	High-fat diet
HH	*Hypoxis hemerocallidea*
***HH*+STZ**	*Hypoxis hemerocallidea* plus streptozotocin
***HH*PT**	*Hypoxis hemerocallidea* pre-treatment
***HH*S**	*Hypoxis hemerocallidea* solution
***HH*T**	*Hypoxis hemerocallidea* tea
HOMA	Homeostatic model assessment
IAPP	Islet amyloid polypeptide
IDDM	Insulin-dependent diabetes mellitus
IPGTT	Intraperitoneal glucose tolerance test
IL	Islets of Langerhans
IQR	Interquatile range
IR	Insulin resistance
K^+	Potassium channels
LA	Lipid accumulation
NaCl	Sodium chloride
NaH2PO4	Sodium dihydrogen phosphate
NaHPO4	Sodium phosphate
NC	Necrotic cells
NIDDM	Non-insulin-dependent diabetes mellitus
PBS	Phosphate buffered salin
RPMI 1640	Roswell Park Memorial Institute-1640 medium

STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
WHO	World Health Organisation
WR	Working reagent
β-cell	Beta cell

CHAPTER I

1 Introduction

Diabetes mellitus (DM) is a condition with an estimated worldwide prevalence, in 2011, of 366 million, and a United Kingdom (UK) prevalence of 2.9 million people (American diabetes association, 2012). DM is defined as a chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Hyperglycemia, or raised blood sugar, is a common consequence of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels (American diabetes association, 2012).

There are two main classifications of DM type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is an auto-immune disease resulting in the destruction of pancreatic β-cells. The primary risk factor for T1DM is a genetic predisposition. Injury or diseases of the pancreas can inhibit its ability to produce insulin and lead to T1DM. A range of relatively rare infections and illnesses can damage the pancreas and cause T1DM (Daneman, 2006). T2DM is strongly associated with obesity and occurs as a result of failing pancreatic β-cell function, responsible for the production of insulin, often alongside insulin resistance (Chen *et al.*, 2011).

Hypoxis hemerocallidea (*HH*) is commonly known as African potato is one of Southern Africa's most common and popular medicinal plants. It is used for a wide a range of medical treatments including urinary tract infections, heart disease, infertility and anxiety (Brown *et al.*, 2008). It has recently been the subject of several scientific studies (Nair *et al.*, 2007). It's most popular contemporary use is for prostate disorders for which there is increasingly good evidence about its efficacy. Phytosterols are thought to be the main bioactive compounds for this indication and also for anti-lipidemic, anti-diabetic and anti-inflammatory properties exhibited by hypoxis extracts (Boukes *et al.*, 2008)

Despite the popularity of *HH* as a herbal and botanical medicine, the research into its pharmacological application has not been of sufficient depth and width to prove it's utility in modern medicine (Mahomed & Ojewole, 2003; Boukes *et al.*, 2008). *HH* has been shown to have hypoglycemic effects in both normal and diabetic rats (Mahomed & Ojewole, 2003). The mechanism by which the hypoglycemic effect is achieved is however not clear.

The plant aqueous extract for *HH* caused significant reductions in the blood glucose levels of the fasted normal and STZ-induced diabetic rats (Mahomed & Ojewole, 2003). While it is likely that the hypoglycemic effects of the plant extract are largely due to its phytosterols and/or sterolin content, the exact mechanism of its hypoglycemic action is still obscure and will have to await further studies (Mahomed & Ojewole, 2003). In a study with orally administered aqueous extracts of African potato, Ojewole (2006) found significant anti-inflammatory effects in rats, starting at a dose of 50 mg/kg and significant antidiabetic effects starting at 100 mg/kg in both normoglycaemic and hypoglycaemic rats.

1.1 The purpose of the study

The aim of this study was to investigate the effects of *Hypoxis hemerocallidea* (*HH*) on type2 diabetes mellitus in rats. In particular, the study seeks:

- To determine whether *HH* can control blood glucose levels in diabetes animal models.
- To determine whether *HH* can increase pancreatic insulin secretion

CHAPTER II

2 Literature review

Diabetes mellitus (DM) is one of the most widely known diseases. The word diabetes originates from the Greek for `siphon', which means `flow through'. This describes the two main symptoms: a great thirst and a need to urinate persistently. The Latin word mellitus was later added. It means `honeyed', describing the sugary urine a diabetic excretes when the condition is uncontrolled, due to high levels of glucose circulating in the bloodstream (Barnett, 2010).

Diabetes has probably been known to medical science longer than any other ailment yet, in many respects, it is still poorly understood. It is characterized by hyperglycaemia (high blood glucose) which occurs because the liver and skeletal muscle cannot store glycogen and the tissues are unable to utilize glucose (Tuchman, 2009). Diabetes mellitus is caused by the pancreas producing insufficient insulin (Srinivasan *et al.*, 2008; Roden, 2004).

Diabetic prevalence for all age-groups across the world has been found to be 2.8% in 2000 and it is speculated that it will increase to 4.4% in 2030 (Wild *et al.*, 2004). It has further been speculated that the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al.*, 2004). When considering gender, it has been found that men have a higher diabetic prevalence then women (Wild *et al.*, 2004).

2.1 Classification of DM

In 1980 the World Health Organisation (WHO) classified diabetes into type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) and revised its classification in 1985 into two types, insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (Ohkubo *et al.*, 1995). The WHO also

categorized DM into other types like malnutrition-related diabetes, which is now omitted from the new classification due to its unknown aetiology, and diabetes which is diagnosed during pregnancy, as gestational diabetes (Alberti & Zimmet, 1998).

2.1.1 Type 1 diabetes mellitus (T1DM)

T1DM is also known as insulin dependent or early onset diabetes and is caused by an autoimmune destruction of the islets of Langerhans, resulting in decreased insulin production (Adler *et al.*, 2003; D'Souza *et al.*, 2009). T1DM is becoming a worldwide epidemic and evidence suggests that changing environmental factors may be responsible. Viral infections, nutritional exposures, perinatal factors, childhood growth, and other environmental factors have been explored as potential risk factors for T1DM (Maahs *et al*., 2010; Snell-Bergeon *et al*., 2010).

2.1.2 Type 2 diabetes mellitus (T2DM)

Formerly called adult-onset diabetes, is the most common type of diabetes affecting about 90 % of people with diabetes (Drouin *et al*., 2009). People can develop T2DM at any age, even during childhood, but this type of diabetes is most often associated with age (Adler *et al*., 2003; Chen *et al*., 2011). T2DM is also associated with obesity, physical inactivity, a family history of diabetes, previous history of gestational diabetes, and ethnicity (Committee and Classification, 2010; Pickup, 2004). The pancreas in T2DM loses its ability to produce enough insulin in response to meals, and blood glucose levels rise (Marshall *et al.*, 1991; Nyman *et al.*, 2011).

2.2 Symptoms of DM

Both T1DM and T2DM have similar symptoms. However, as for T1DM the symptoms develop more rapidly in days or weeks. These symptoms include polyuria, polydipsia, unexplained weight loss, muscle wasting, fatigue, cramps, constipation, blurred vision and skin infections. Long term complications caused by DM include microvascular and

nephropathic disease, and are the major causes of morbidity and mortality in DM patients (Wolfs *et al.*, 2009; Song & Hardisty, 2008; Ahmed, 2005).

Hypoglycaemia is the limiting factor in the glycaemic management of diabetes. Hypoglycaemia is a result of the interplay of insulin excess and compromised glucose counter regulation in T1DM (Graveling & Frier, 2009) caused by over medication, excessive exogenous insulin and strenuous exercise. It may lead to seizures, coma and irreversible brain death as blood glucose regulates the functioning of the body organs, including the brain. Symptoms due to hypoglycaemia include irritability, sweating, tingling lips, weakness, hunger and nausea (Cryer, 2002; McAulay *et al.*, 2001).

Hyperglycaemia is a result of increased blood glucose levels. This can be caused by factors such as not taking medication, exogenous insulin, an imbalanced diet or a lack of exercise (Dungan *et al.*, 2009). It may also occur due to autoimmune destruction of β-cells in the pancreas. Lack of insulin causes ketoacidosis when ketones are released into the blood causing a rise in the plasma acidity levels. Both hypo and hyperglycaemia are reversible and can be treated (Luitse *et al.*, 2012; Ahmed, 2005).

2.3 Complications associated with DM

Persistent high blood glucose levels could result in the glycosylation of proteins resulting in the irreversible damage of tiny blood vessels and nerve endings in the areas furthest from the heart, retina, renal glomerulus such as the nerve sheath and the cellular membranes (Deshpande *et al.*, 2008).

Macrovascular complications (which affect the larger blood vessels in the body), are common among T2DM patients. These patients are more likely to suffer from hypertension and coronary heart diseases (CHD) including, atherosclerosis and thrombosis. About 75% of macrovascular diabetic mortality is due to coronary heart disease. Figure 2.1 shows the most important organs affected by diabetes.

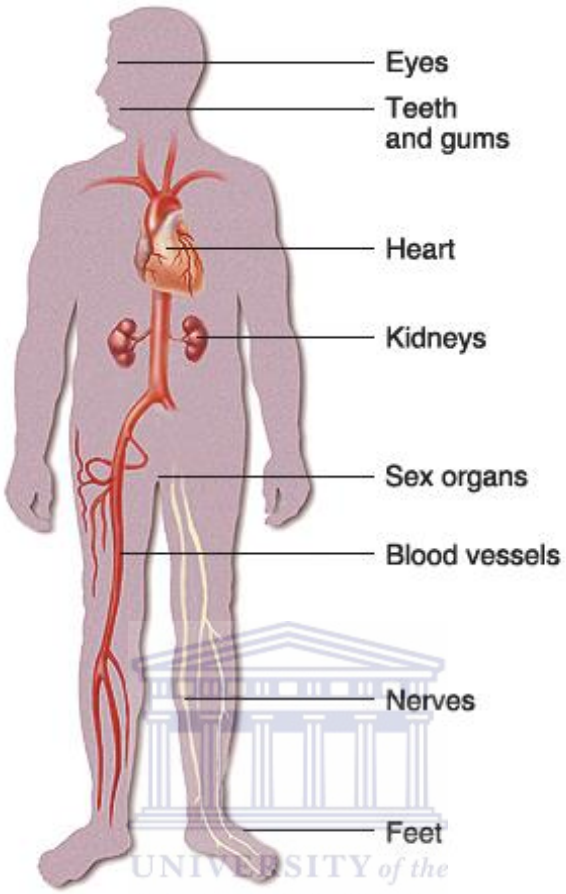

Figure 2.1. The organs affected by diabetes complication (Adapted from Deshpande *et al*., 2008).

2.4 Epidemiology of DM

According to the WHO, 180 million people have been estimated to suffer from T2DM in 2006 and the annual death rate due to this disease was 3 million at that time. Both numbers are expected to double over the next 25 years (Wolfs *et al*., 2009). An interesting fact about T2DM is that the prevalence of this disease varies widely among various racial and ethnic groups as shown in figure 2.2 below (Selvin *et al.*, 2011), which suggests that a genetic component plays a pivotal role in the disease development.

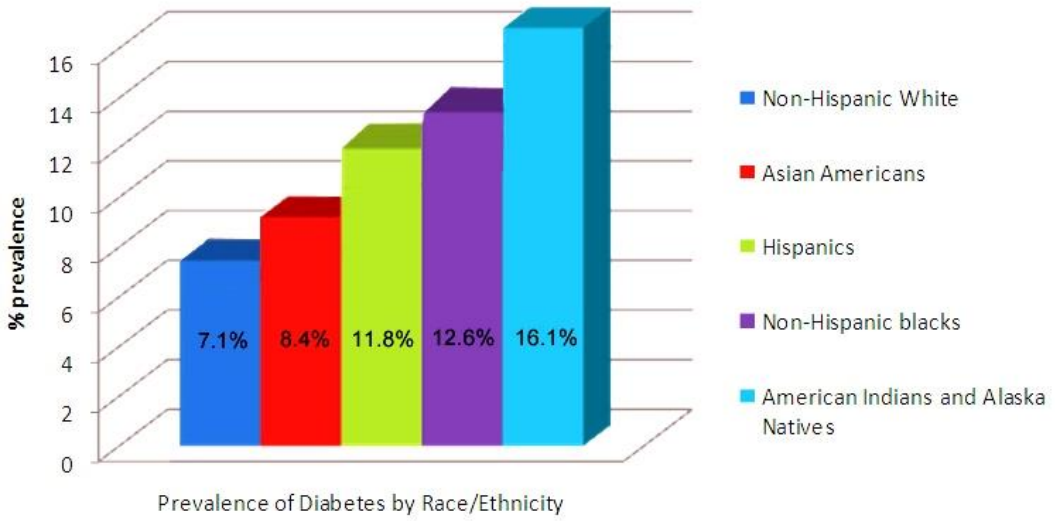

Figure 2.2. Prevalence of Diabetes by Race/Ethnicity in the United States (Adapted from Kappala, 2012).

The prevalence of T1DM is approximately 1 in 300 in the US by 18 years of age (Wild *et al.*, 2004). Research on risk factors for T1DM is an active area of research to identify genetic and environmental triggers that could potentially be targeted for intervention. While significant advances have been made in the clinical care of T1DM with resultant improvements in quality of life and clinical outcomes, much more needs to be done to improve care of diabetes, and to ultimately find a cure for T1DM. Epidemiologic studies have an important on-going role to investigate the complex causes, clinical care, prevention, and cure of T1DM (Lamb *et al.*, 2008; Snell-Bergeon *et al.*, 2010).

2.5 Diagnosis of DM

There was major perplexity regarding the criteria for diagnosis of DM before the 1970s. The WHO has put forward its first statement on the diagnosis of DM in 1965, which was later tailored and made simple. The American Diabetes Association (ADA) proposed its first diagnostic criteria, which emphasised the concentration of fasting glucose. Figure 2.3 shows a flow diagram for the diagnosis and screening of DM including Glucose Tolerance Test (GTT) (Kappala, 2012). A report by the WHO

recommends glycated haemoglobin (HbA1c) as an additional test to diagnose diabetes (Association, 2006) .

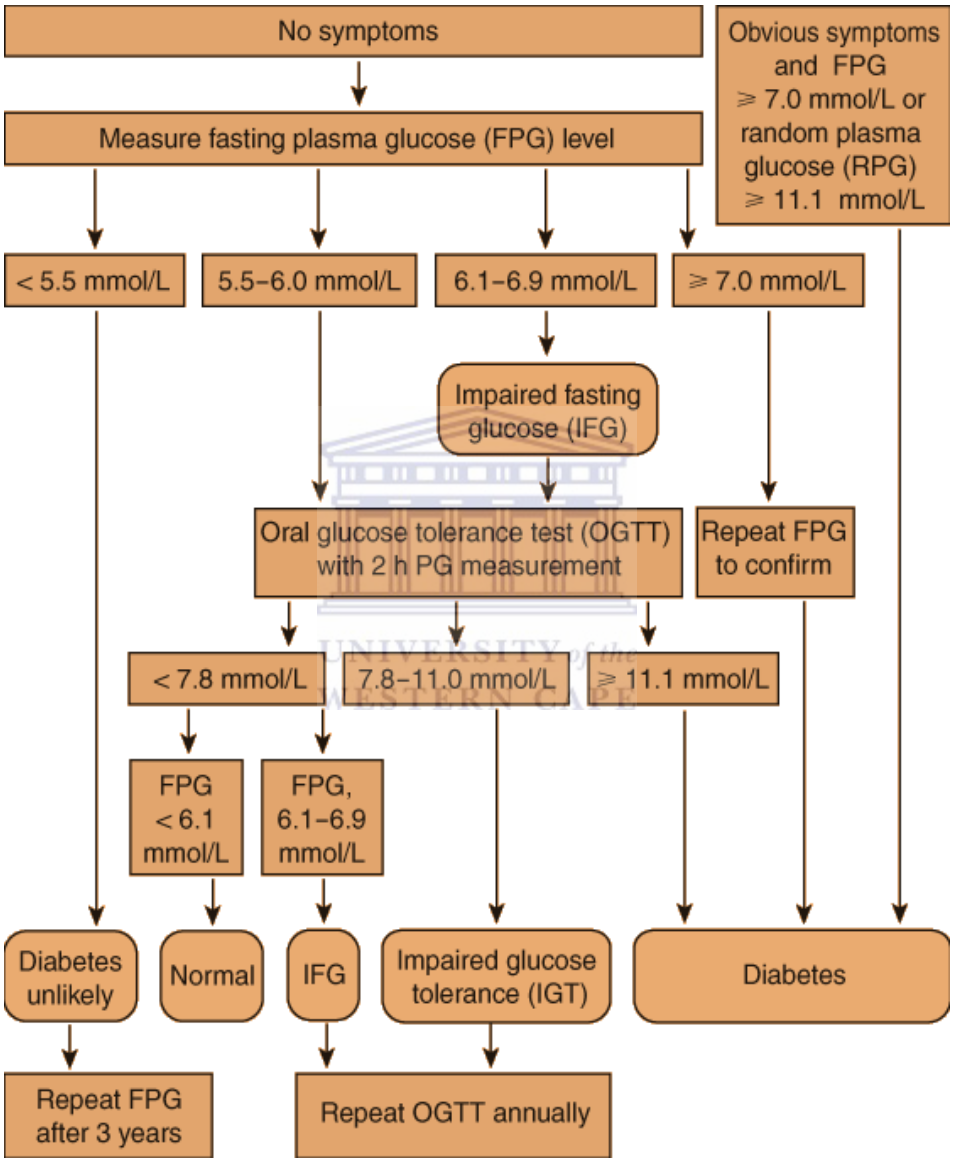

Figure 2.3. Diagnosis and screening for diabetes and impaired glucose metabolism (Adapted from Kappala, 2012).

2.6 Pathogenesis of DM

T1DM produces profound β-cell failure with secondary insulin resistance, whereas T2DM is associated with less severe insulin deficiency but greater impairment of insulin action. Given their similarities overall, it is not surprising that the two major forms of diabetes share many pathophysiologic features. Despite the apparent phenotypic similarity, the underlying pathogenic mechanisms leading to T1DM and T2DM are strikingly different. T1DM most likely results from an interplay of genetic, environmental, and autoimmune factors. Figure 2.4 shows a modern model of the pathogenesis and natural history of T1DM. The modern model expands and updates the traditional model by inclusion of information gained through an improved understanding of the roles for genetics, immunology, and environment that selectively destroy insulin-producing β-cells (Atkinson, 2012).

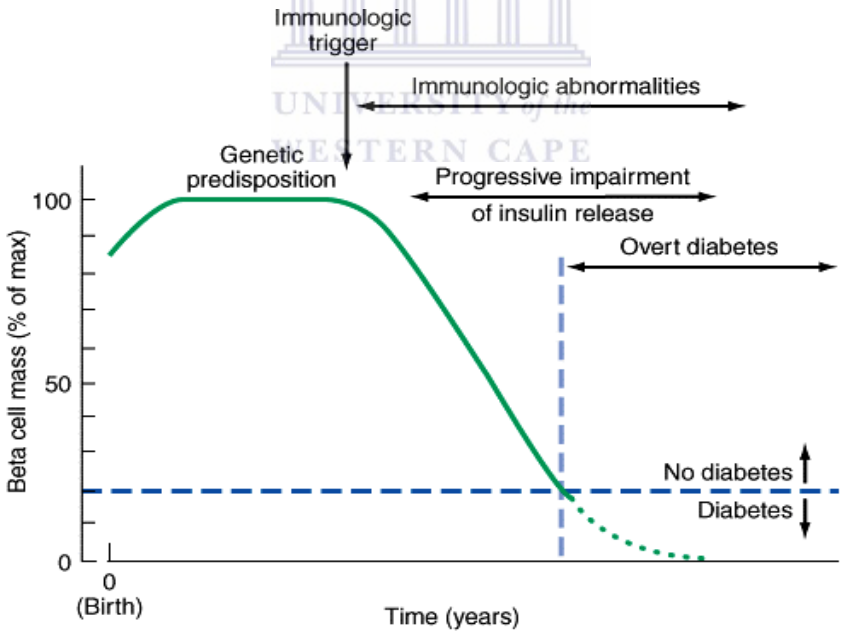

Figure 2.4. Summary of the sequence of events that lead to pancreatic β-cell loss and ultimately to the clinical evolution of type 1 diabetes (Adapted from Atkinson, 2012)**.**

The two main reasons underlying the pathophysiology of T2DM are impaired insulin secretion due to the dysfunction of pancreatic beta cells and impaired insulin action because of insulin resistance (DeFronzo, 2004). A breakdown of these two mechanisms consequently results in abnormally high levels of plasma glucose concentrations that are usually maintained in a definite range irrespective of the various changes for the requirement and supply which is regulated by a dynamic process between tissue sensitivity and insulin (Esmaillzadeh *et al.*, 2007). Figure 2.5 shows T2DM results from an array of dysfunctions characterized by hyperglycemia and results from the combination of resistance to insulin action, inadequate insulin secretion, and excessive or inappropriate glucagon secretion (Meshkani & Adeli, 2009) .

Inflammation and inflammatory cytokines have been suggested to be the reason for the development of T2DM. Insulin resistance develops from physical inactivity (Kahn, 1998). Insulin resistance is commonly accompanied by other cardiovascular risk factors like dyslipidaemia, hypertension and pro-thrombotic factors These factors together in an individual are called the metabolic syndrome, which precedes T2DM (Inzucchi *et al.*, 2012; DeFronzo, 2004).

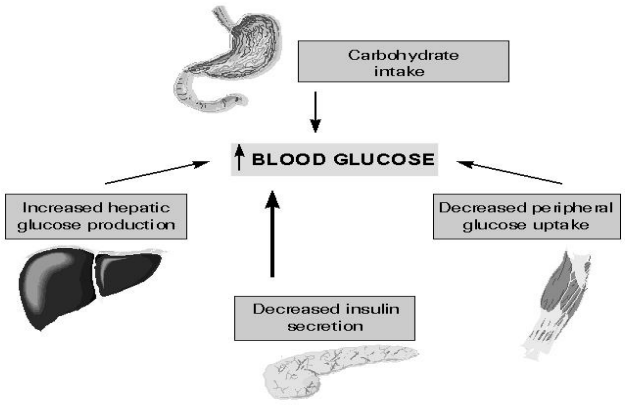

Figure 2.5. Simplified scheme for the pathophysiology of type 2 diabetes mellitus (Adapted from Kappala, 2012)**.**

2.7 Treatment of DM

Individuals suffering from T1DM are insulin dependent. Insulin are normally injected *8different forms, like fast affecting insulin, which is vastly absorbed. The structural difference between normal human insulin and the synthetic form of insulin is the conversion or replacement of few amino acids to slow down its absorption or to extend its action. Rapid insulin has to be given at eating period. While, short effecting insulin is exerting its effect within one hour after subcutaneous injection. While long acting insulin may act for more than twelve hours.

T2DM treatment depends mostly on different hypoglycaemic drugs (Ganesh et al., 2010). There are about five categorizes of available drugs. Sulfonylureas show their hypoglycaemic effect through enhancing insulin release from pancreatic β-cell. The mechanism of action of sulfonylureas is via its binding with ATP-sensitive potassium channels and thus preventing the release of potassium from the cell leading to influx of calcium via the voltage-dependent calcium channels. The elevated cytoplasmic calcium levels lead to the secretion of insulin. Meglitinide is a non-sulfonylureas drug that is linked to different β-cell receptors promoting a similar action to sulfonylureas in enhancing insulin secretion (Ganesh et al., 2010).

Metformin is one of the biguanides anti-diabetic drug categories and it exerts its effect by inhibiting glucose release from the liver via suppressing gluconeogenesis and glycolysis. Metformin does not lead to a condition of hypoglycaemia since it has no effect on insulin release (Ganesh et al., 2010). Thiazolidine increases glucose entrance into different tissues including skeletal muscle (Oiknine, and Mooradian, 2003). A last class of anti-diabetic drugs is the α-glycosidase inhibitors like miglitol and acarbose. Their effect is mainly through postponing carbohydrate absorption in the epithelial cells in the lining wall of the small intestine (Oiknine, and Mooradian, 2003).

2.8 Animal models for DM

Typically animals have been used intensively in DM research. Early studies used pancreatectomized dogs to confirm the pivotal role of the pancreas in glucose

homeostasis, crowned in the discovery and purification of insulin. Today animal experimentation is contentious and subject to restriction and ethical restrictions that vary all over the world. Most experiments are carried out on rodents, however some studies are still performed on larger animals. Several toxins, including alloxan and streptozotocin are used to stimulate hyperglycaemia in rats and mice (Coskun *et al.*, 2005). Selective breeding has produced many strains of animals that are considered reasonable models of Type 1 diabetes, Type 2 diabetes and related phenotypes such as obesity and insulin resistance. Out of their use in researches the pathogenesis of the disease and its complications, all new treatments for diabetes, including islet cell transplantation and precaution strategies, were originally achieved in animals. In recent years, molecular biological techniques have produced a large number of new animal models for the study of diabetes, including knock-in, generalized knock-out and tissue-specific knockout mice (Rees & Alcolado, 2005).

2.8.1 Models for T1DM

The main feature of T1DM is an autoimmune destruction of the β-cell, causing to lack of insulin production. In animal models for T1DM, this defect in insulin production is achieved using a variety of different mechanisms, ranging from chemical ablation of the beta cells to breeding rodents that spontaneously evolve autoimmune diabetes (King, 2012; Rees & Alcolado, 2005). Some of the most commonly used models of T1DM are outlined in Table2.1 (King, 2012).

Table 2.1. Summary of rodent models of type 1 diabetes (Adapted from King, 2012).

Induction mechanism	Model	Main features	Possible uses
Chemical Induction	High dose streptozotocin	Simple model of hyperglycaemia.	New formulations of insulin
	Alloxan		Transplantation models.
	Multiple low dose streptozotocin	Model of induced insulitis.	Treatments that may prevent beta cell death
Spontaneous autoimmune	NOD mice	Beta cell destruction due to an autoimmune process	Understanding genetics of type 1 diabetes
	BB rats		Understanding mechanism of type 1 diabetes
	LEW.1AR1/-iddm rats		Treatments that may prevent beta cell death
			Treatments that may manipulate autoimmune process
Genetically induced	AKITA mice	Beta cell destruction due to ER stress. Insulin dependent.	New formulations of insulin
			Transplantation models.
			Treatments to prevent ER stress
			(could also be used in type 2 diabetes research)
Virally-induced	Coxsackie B virus	Beta cell destruction induced by viral infection of beta cells	Establish potential role of viruses in the development of type 1 diabetes
	Encephalomyocarditis virus		
	Kilham rat virus		
	LCMV under insulin promoter		

2.8.2 Models for T2DM

T2DM is caused by insulin resistance coupled by a failure of the beta cell to compensate insulin secretion, therefore, typical animal of T2DM tend to include models of insulin resistance or models of β-cell dysfunction. Many T2DM animal models are reflecting the human condition and are obese, since obesity is closely related to T2DM development (King, 2012; Rees & Alcolado, 2005). Some of the most commonly used models for T2DM are outlined in Table 2.2 (King, 2012).

Animal models have dramatically contributed to the researches of T2DM. Most researcher is carried out on rodents, despite that the other species with human-like biological characteristics are also used. Animal models develop T2DM, either automatically or by using surgical, chemical, genetic or other techniques, and describe many clinical features or related phenotypes of the disease (Chatzigeorgiou *et al.*, 2009; Srinivasan & Ramarao, 2007). The combination of HFD fed and low-dose STZ treated rat serves as an alternative animal model for T2DM simulating the human syndrome that is also suitable for testing anti-diabetic agents for the treatment of T2DM (Srinivasan *et al.*, 2005).

Table 2.2. Summary of rodent models of type 2 diabetes (Adapted from King, 2012).

Induction mechanism	Model	Main features	Possible uses
Obese models (monogenic)	$Lep^{ob/ob}$ mice	Obesity-induced hyperglycaemia	Treatments to improve insulin resistance
	$Lepr^{db/db}$ mice		Treatments to improve beta cell function
	ZDF Rats		
Obese models (polygenic)	KK mice	Obesity-induced hyperglycaemia	Treatments to improve insulin resistance
	OLETF rat		Treatments to improve beta cell function
	NZO mice		Some models show diabetic complications
	TallyHo/Jng mice		
	NoncNZO10/LtJ mice		
Induced obesity	High fat feeding (mice or rats)	Obesity-induced hyperglycaemia	Treatments to improve insulin resistance
	Desert gerbil		Treatments to improve beta cell function
	Nile grass rat		Treatments to prevent diet-induced obesity
Non-obese models	GK rat	Hyperglycaemia induced by insufficient beta cell function/mass	Treatments to improve beta cell function
			Treatments to improve beta cell survival
Genetically induced models of beta cell dysfunction	hIAPP mice	Amyloid deposition in islets	Treatments to prevent amyloid deposition
			Treatments to improve beta cell survival
	AKITA mice	Beta cell destruction due to ER stress.	Treatments to prevent ER stress
			Treatments to improve beta cell survival

2.9 The Pancreas

The pancreas is a retroperitoneal secretory organ and the pancreas secretes several hormones including insulin and glucagon (Nobukawa, 2007). It is critical to the production of insulin and glucagon which regulate glucose levels in the blood. (Gromada *et al.*, 2007).

2.9.1 Anatomy of the Pancreas

The humans pancreas is found posterior to the stomach, in the curve of the duodenum which is the first part of the small intestine. It is a 12-15–cm long, soft, lobulated and weighs between 70-110 grams (Coales, 2000).

The structure of a pancreas includes a head, uncinate process, neck, body and tail. The head lies within, and closely attached to the 'C' of the duodenum. The main pancreatic duct (Wirsung) runs the length of the gland. It usually joins the common bile duct at the ampulla of Vater to enter the second part of the duodenal papilla (Horiguchi & Kamisawa, 2010) (Figure 2.6).

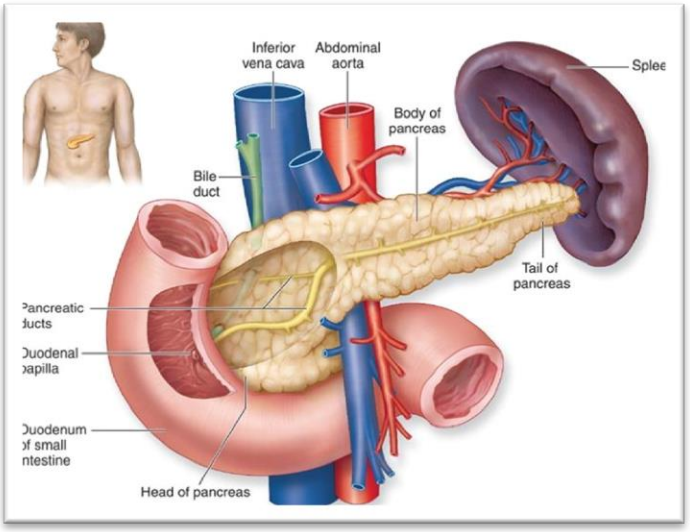

Figure 2.6. Diagramme of the Pancreas in situ (Adapted from Heijerman *et al.*, 1991).

The accessory duct (Santorini) opens into the duodenum proximal into the main duct. The gland is lobulated and produces the exocrine secretion of digestive hormones from serous-secreting cells, arranged in alveoli. Between these lie the islets of Langerhans, containing α-cells (glucagon secreting cells), β-cells (insulin secreting cells) and δ-cells (gastrin and somatostatin secreting cell) (Nobukawa, 2007). The arterial supply occurs through the splenic artery. The corresponding veins channel into the portal system. The splenic artery is characteristically tortuous and runs along the upper border of the pancreas; the vein lies behind the gland. Embryologically, the pancreas derives from a large dorsal diverticulum from the duodenum and a small ventral bud from the side of the bile duct. The ventral bud swings posteriorly to fuse with the dorsal, trapping the superior mesenteric vessels between hence forming the uncinate process. The ducts of the two buds communicate, which of the ventral bud takes over the main duct, leaving the original duct of the larger dorsal bud as the accessory duct (Ellis, 2007).

2.9.2 Histology of the Pancreas

The pancreas contains tissue with an endocrine and exocrine role, and this division is also visible when the pancreas is viewed under a microscope. The tissues with an endocrine role can be seen as lightly-stained clusters of cells, called islets of Langerhans (Young *et al.*, 2006).

Darker-staining cells form clusters called acini, which are arranged in lobes separated by a barrier. The secretory cells of each sinus surround a small intercalated duct. As a result of their secretory function, these cells have many small granules of zymogens that are visible. The intercalated ducts drain into larger ducts within the lobule, and eventually interlobular ducts. The ducts are lined by a single layer of columnar cells. With an increase in cells diameter, several layers of columnar cells may be seen (Young *et al.*, 2006).

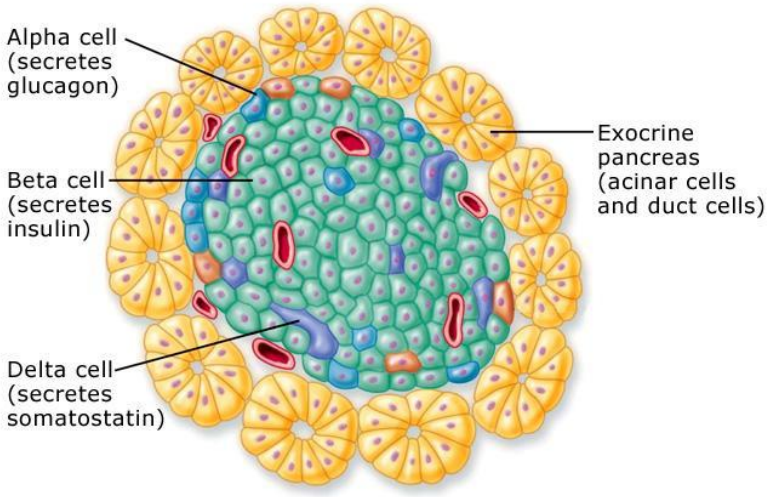

Figure 2.7. Schematic representation of islets of Langerhans embedded in the exocrine parenchyma (Adapted from Cendrowski, 2013).

The islets of Langerhans (Figure 2.7) consist of insulin- releasing β-cells (65-90%) forming the core of the islet, glucagon-releasing α-cells (15-20%), somatostatin-producing δ-cells (3-10%), and pancreatic polypeptide-producing PP-cells (1%) are usually located on the surface (Elayat *et al.*, 1995). Of these, β-cells, which couple nutrient metabolism with electrical activity to modulate the synthesis and release of insulin, have been most frequently studied. The islets of Langerhans play an imperative role in glucose metabolism and regulation of blood glucose concentration (Jo *et al.*, 2007).

2.9.3 Hormones Secreted by Pancreas

2.9.3.1 Insulin

Insulin is a small protein composed of two polypeptide chains containing 51 amino acids (Aronoff *et al.*, 2004). The primary action of insulin is to reduce blood glucose concentration. Beta cells can respond quickly to spikes in blood glucose concentrations by secreting some of their stored insulin while simultaneously producing more (Wilcox, 2005).

Insulin is synthesized in the ribosomes of the rough endoplasmic reticulum as preproinsulin which is the cleaved to proinsulin. Proinsulin is transported to the Golgi apparatus where it is packaged into secretory granules located close to the cell membrane. Proinsulin is cleaved into equimolar amounts of insulin and C-peptide in the secretory granules. The process of insulin secretion involves fusion of the secretory granules with the cell membrane and exocytosis of insulin and C-peptide Figure 2.8 (Skelin *et al.*, 2010).

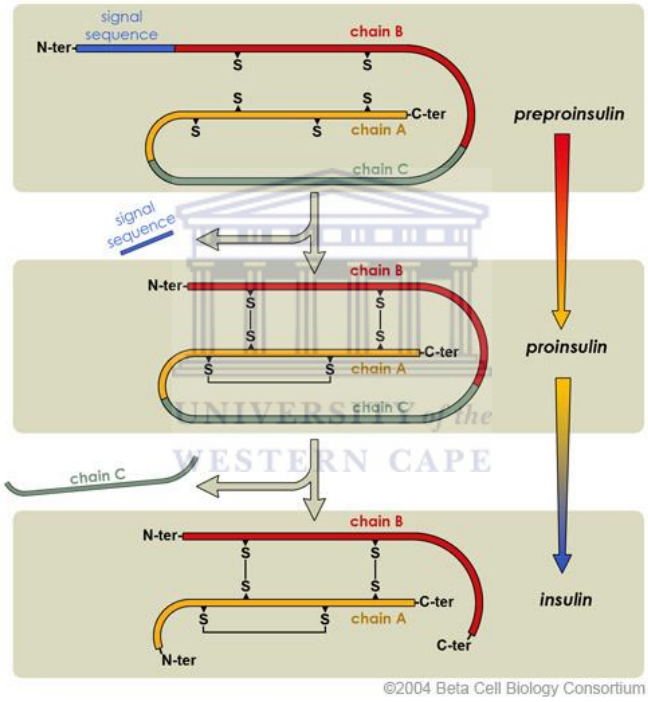

Figure 2.8. Insulin biosynthesis (Adapted from Skelin *et al.*, 2010).

2.9.3.1.1 **Insulin Secretion**

Glucose transporter two (GLUT2), the facilitative glucose transporter isoform expressed in pancreatic beta cells, is believed to play a role in glucose-stimulated insulin secretion. GLUT2 are a large group of membrane proteins that facilitate the transport of glucose

over a plasma membrane (Figure. 2.9) (Röder *et al.*, 2014; Guillam *et al.*, 2000). Glucose phosphorylation by glucokinase is the rate-limiting step that controls glucose-regulated insulin secretion. Further metabolism of glucose-6-phosphate via glycolysis generates Adenosine triphosphate (ATP) (Teague *et al.*, 2011).

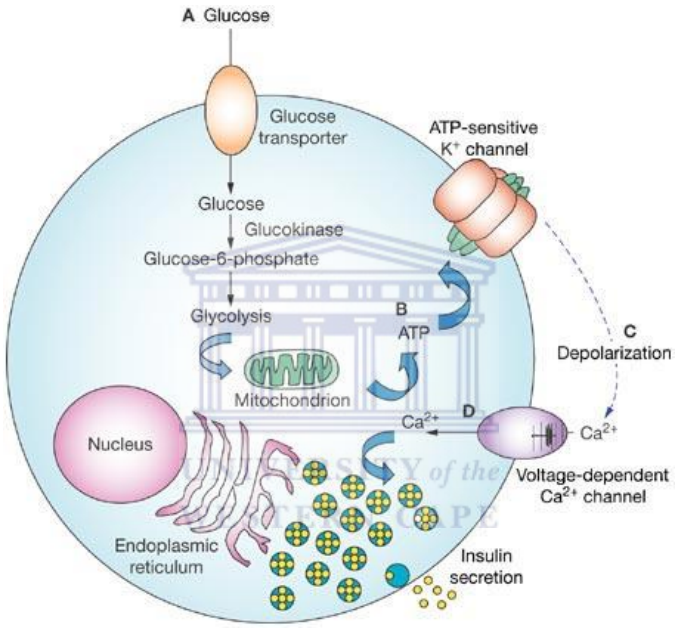

Figure 2.9. Glucose stimulated insulin secretion in pancreatic β-cells (Adapted from Teague *et al.*, 2011).

Potassium (K^+) channels consist of two separate proteins: one is the binding site for certain oral hypoglycemics (e.g., sulfonylureas, meglitinides); the other is an inwardly rectifying K^+ channel protein. Inhibition of this K^+ channel induces beta cell membrane depolarization, which opens voltage-dependent calcium channels (leading to an influx of calcium), and stimulates insulin secretion. Insulin secretory profiles reveal a pulsatile pattern of hormone release, with small secretory bursts occurring about every 10 minutes, superimposed upon greater amplitude oscillations of about 80–150 minutes. Incretins are released from neuroendocrine cells of the gastrointestinal tract following

food ingestion and amplify glucose-stimulated insulin secretion and suppress glucagon secretion (Lee, 2001).

Glucagon-like peptide 1 (GLP-1), the most potent incretin, is released from L cells in the small intestine and stimulates insulin secretion only when the blood glucose is above the fasting leve (Maechler & Wollheim, 2000).

2.9.3.1.2 **Insulin resistance**

In insulin resistance, muscle and fat do not respond properly to insulin and thus cannot easily absorb glucose from the bloodstream. As a result, the body needs higher levels of insulin to help glucose enter cells, the β-cells in the pancreas try to keep up with this increased demand for insulin by producing more (Giugliano *et al.*, 2008; Wilcox, 2005). The pathophysiology of insulin resistance involves a complex network of signalling pathways, activated by the insulin receptor, which regulates intermediary metabolism and its organization in cells (Giugliano *et al.*, 2008).

2.9.3.2 C-peptide

C-peptide is secreted into the blood in equimolar quantities to insulin and helps to prevent neuropathy and other vascular deterioration related symptoms of diabetes mellitus (Wahren *et al.*, 2000).

2.9.3.3 Amylin

Amylin is also known as islet amyloid polypeptide (IAPP). The function of amylin is to slow the rate of glucose entering the bloodstream. Amylin can be described as a synergistic partner to insulin, where insulin regulates long term food intake and amylin regulates short term food intake (Bennett *et al.*, 2003).

Amylin and insulin are co-localized within the same secretory granules of pancreatic β-cells. Amylin is also found in much lesser quantities in the gut and other tissues (Young, 2005).

2.10 Medicinal plants used for diabetes mellitus

Around 400 herbal medicines used to treat many diseases such as diabetes mellitus have been reported, but only a small number of these plants have received scientific and medical evaluation to assess their efficacy (Kavishankar, 2011). Traditional treatments are prescribed by practitioners of alternative medicine or sometimes taken by patients as supplements to conventional therapy (Arumugam *et al.*, 2013). Herbal medicine remedies are the mainstay of treatment in underdeveloped regions. (Arumugam *et al.*, *2013).* The hypoglycemic action of some treatments has been confirmed in animal models and non-insulin-dependent diabetic patient A botanical substitute for insulin seems unlikely, but herbal medicine may provide many clues for the development of new oral hypoglycemic agents and simple dietary adjuncts (Kavishankar, 2011; Arumugam *et al.*, 2013).

Table 2.3. Medicinal plants with antidiabetic and their reported effect on experimental models (Adapted from Kavishankar, 2011).

Botanical Name	Family	Antidiabetic and other beneficial effects
Achilieasantolina[L	Asteraceae	Hypoglycemic, antioxidant
Artemisia patterns	Asteraceae	Hypoglycemic, increases peripheral glucose utilization
Areca catechu L.	Arecaceae	Hypoglycemic
Beta vulgaris L.	Chenopodiaceae	Increases glucose tolerance in OGTT
BoerhaaviadiffusaL.	Nyctaginaceae	Decreases blood glucose level and increases plasma insulin levels, antioxidant
BombaxceibaL.	Malvaceae	Hypoglycemic
Butea manosperma(Lam)	Caesalpinaceae	Anti-hyperglycemic
CarumcarviL.	Apiaceae	Potent anti-hyperglycemic
CapparisspinosaL.	Capparidaceae	
CogniauxiapodoleanaBaillon Bail Ion	Cucurbitaceae	Hypoglycemic and anti-hyperglycemic
CommelinacommunisL	Conimelinaceae	Anti-hyperglycemic, management of non-insulin-dependent diabetes.

Croton cajucaraBenth	Euphorbiaceae	Anti-hyperglycemic
Curcuma longa L.	Zingiberaceae	Hypoglycemic, plays a role in PPAR-gamma activation
Cynodondactylon	Pers	Poaceae Anti-hyperglycemic
Enicostemmalittorale Blume	Gentianaceae	Decreases plasma glucose level, glycosylated haemoglobin and glucose-6-phosphatase activity in liver
Eriobotrya japonica	Lindl.	RosaceaeHypoglycemic
GentianaolivieriL.	Gentianaceae	Hypoglycemic, anti-hyperlipidemic
Ginkgo bilobaL.	Ginkgoaceae	Hypoglycemic, increases pancreatic beta-cell in NIDDM
GlobulariaalypumL.	Globulariaceae	Hypoglycemic, increases plasma insulin levels
GlycyrrhizauralensisFish.	Papilionaceae	PPAR-gamma ligand-binding activity, decreases the blood glucose levels
GymnemanwntanumHook	Asclepiadaceae	Anti-peroxidative, antioxidant, may prevent the cholinergic neural and retinal complications of hyperglycemia in diabetes
GymnemasylvestreR. Br.	Asclepiadaceae	Hypoglycemic. Hypolipidemic
Hintoniastandleyana	Rubiaceae	Anti-hyperglycemic
IbervilleasonoraeS.	Cucurbitaceae	Acute and chronic hypoglycemic
Ipomoea aquatic Forsk.	Convolvulaceae	Decreases serum glucose concentration by 29.4% in Type II diabetic patients. hypoglycemic
Kalopanaxpictus	Thumb. Araliaceae	Anti-diabetic activity, hypocholesterolmic and hypolipidemic
Lagerstroemia speciosaL.	Lythraceae	Insulin-like actions, glucose uptake, anti-adipogenesis
Medicago saliva L.	Fabaceae	Anti-hyperglycemic, insulin-releasing and insulin-like activity
Morus alba L.	Moraceae	Protects pancreatic beta cells from degeneration anddiminishes lipid peroxidation
Morusindica. L.	Moraceae	Hypoglycemic
MorusinignisL.	Moraceae	Hypoglycemic
MurrayakoenigiiL.	Rutaceae	Hypoglycemic, increases glycogenesis,decreases gluconeogenesis and glycogenolysis

NelumbonuciferaL.	Neluntbonaceae	Improves glucose tolerance and potentiates the action of exogenouslyinjected insulin
Nigella saliva Gaertn.	Ranunculaceae	Decreases oxidative stress and preserves pancreatic beta-cell integrity
Ocimumgratissinuim	L. Var. Lamiaceae	Hypoglycemic
Pandanus odorusRidl	Pandanaceae	Hypoglycemic, increases serum insulin levels and liver glycogen
ParmentieruedulisA.DC	Bignoniaceae	Hypoglycemic
Phyllanthussellowianus Mull.Arg.	Euphorbiaceae	Hypoglycemic
Psacaliumdecompositum(Gray) H.	Asteraceae	Hypoglycemic
Psacaliumpeltatum(Kunth)	Asteraceae	Anti-hyperglycemic
PunicagranatumL.	Punicaceae	Improves postprandial hyperglycemia in type 2 diabetes and obesity by inhibiting intestinal alpha-glucosidase activity
Solaria oblonga	Celastraceae	Hypoglycemic and possess anti-oxidant activity
SambucusnigraL.	Adoxaceae	Insulin-releasing and insulin-like activity
Sanguisdraxonis	Apocynaceae	Increase insulin sensitivity and improve the development of insulin resistance in rats
Sclerocaryabirea(A.Rich)	Anacardiaceae	Hypoglycemic
ScoparіadulcisL.	Scrophariaceae	Hypoglycemic, antihyperlipidemic, antidiabetic
Swertiachirayita(Roxb)	Gentianaceae	Stimulates insulin release from islets
Syzygiumalternifolium(Wt) Walp.	Myrtaceae	Hypoglycemic, antihyperglycemic and antihyperlipidemic
Terminalia bellirica(Gaertn)	Combretaceae	Stimulates insulin secretion. Enhances insulin action andinhibits both protein glycation and starch digestion.
Terminalia chebulaRetz.	Combretaceae	Dose-dependent glucose lowering effect, antidiabetic and renoprotective,decreases hepatic and skeletal muscle glycogen content, increases insulin release from the pancreatic islets

Teucriumpolium	Lamiaceae	Increases insulin release, antioxidant and hypoglycemic
TinosporacordifoliaMiers..	Menispermaceae	Hypoglycemic
Tinosporacrispa(L) Hook.	Menispermaceae	Anti-hyperglycemic, stimulates insulin release from islets
UrticadioicaL.	Urticaceae	Anti-hyperglycemic
UrticapiluliferaL.	Urticaceae	Hypoglycemic
VincaroseaL.	Apocynaceae	Anti-hyperglycemic
Withaniasoimifera(L) Dunal	Solanaceae	Hypoglycemic, antioxidant, diuretic and hypocholesterolemic
WithaniacoagulansDunal	Solanaceae	Anti-hyperglycemic, anti-hyperlipidemic and hypoglycemic
ZizyphussativaGaertn	Rhamnaceae	Hypoglycemic
Zizyphusspina-christiL	RhamnaceaeInsulinotropic,	hypoglycemicanddepressant effect on the central nervous system
ZygophyllumgaetulumEmb	Zygophyllaceae	Hypoglycemic, increases plasma insulin levels

2.11 *Hypoxis hemerocallidea* (*HH*)

Hypoxis hemerocallidea (*HH*) is more commonly known as hypoxis, African potato (AP), *Hypoxis rooperi*, Yellow star flower (English), Inkomfe (Zulu) and Lilabatseka, Zifozonke (Swazi) (Nicoletti *et al.*, 1992).

Hypoxis hemerocallidea (*HH*) is one of southern Africa's most important and popular medicinal plants (Nicoletti *et al.*, 1992). *HH* has a long history of traditional use for a diversity of ailments and more recently has been the subject of several scientific studies. In many parts of Africa the corms of this attractive yellow flowered herb have been used in the treatment of urinary tract diseases, prostate hypertrophy and cancer (Mahomed & Ojewole, 2003).

HH is geophytic and overcome winter conditions in the form of an underground rootstock called a corm. It also has adventitious roots attached to the corms that are thick, fleshy and which arise from the base of young corms. The flowering stems are unbranched, with 2-12 flowers per stalk. Flowers are symmetrical with 6 petals, which

are bright yellow, giving the plant its common name Yellow Stars, shown in Figure 2.10 (Nair, 2006; Nair & Kanfer, 2006).

Figure 2.10. Photograph of a Young Corm of *Hypoxis hemerocallidea* (Adapted from Nair, 2006).

The corms of these plants are graded on size. The small (200 g) corms are generally from one year old seedlings and the medium (450 g) corms are at least three years old whereas the large corms (800 g) are older. The fleshy corms of *HH* are yellow in colour in the inside, but soon turn to dark brown because of oxidation, when exposed to air (Figure: 2.11.) (Nair, 2006).

Figure 2.11. Photograph of the Tran-section of a Corm of *Hypoxis hemerocallidea* (Adapted from Nair, 2006).

2.11.1 Geographical distribution

HH is widely distributed all over the world, with reports of existence in various countries including South Africa, New Zealand, Malawi, Madagascar, Mauritius, Mozambique, America, Zimbabwe and other Central Africa countries (Street & Prinsloo, 2013; Mogatle, 2008).

In South Africa, all of the genus is distributed in the provinces KwaZulu-Natal, Limpopo, Eastern Cape, Mpumalanga, and Gauteng (Singh, 2007).

2.11.2 Traditional use

The corm of *HH* has been used in folk medicine to treat different diseases which include the common cold, flu, hypertension, adult-onset diabetes, psoriasis, urinary infections, testicular tumours, prostate hypertrophy, cancer and HIV/AIDS (Laporta *et al.*, 2007; Nair & Kanfer, 2006). Table 2.4 shows the most important traditional uses of *HH* (Mogatle, 2008).

Table 2.4. Traditional uses of *HH* (Adapted from **Mogatle, 2008)**

Reported Use	Part of plant	Preparation	Area
Diabetes	Corms	Fresh corms boiled and taken orally	Eastern Cape South Africa
Prostate Hypertrophy	NS*	NS*	Maputo Mozambique
Urinary or venereal Diseases	NS*	NS*	KwaZulu Natal South Africa
Cancer	Corms	Pulverised corms are boiled and taken orally	Eastern Cape Southern Africa
Wound management	Leaves corms	Dry powder sprinkled on wounds, Fresh or dried Material extracted and used as wash	Eastern Cape South Africa
HIV/AIDS management	NS*	NS*	Johannesburg, South Africa

*NS – not stated.

2.11.3 Phytochemistry and biological activity

The use of *HH* could be attributed to a few of the medicinal agents found in the plant of which hypoxoside, sitosterol, and its aglycone derivative rooperol are the most well-known compounds (Street & Prinsloo, 2013). Hypoxoside is the trivial name for (E)-1,5bis (4′-B-D-glucopyranossyloxy-3′-hydroxyphenyl) pent-1-en-4-yne which is a norlignan diglucoside isolated from the rootstock of the family Hypoxidaceae. In addition to hypoxoside and rooperol, the root stocks are reported to contain $\beta\beta$-sitosterols, sterol, monterpeneglycosides, stanols, and stigmastanols (Laporta *et al.*, 2007). The result of the chemical analysis of *HH* showed that the species have different classes of secondary metabolites, namely, glycosides, polyphenols, saponnins, steroids

and tannins. The rootstock of *HH* has yielded three cytokinins, identified as zeatin, zeatin riboside, and zeatin glucoside (Street & Prinsloo, 2013). The high concentration of phytosterols such as $\beta\beta$-sitosterols has proven to be effective against benign prostate hypertrophy (Steenkamp, 2003), and a strong decoction of the rootstocks is also used as purgatives (Mills *et al.*, 2005). Rooperol may be obtained by treating hypoxoside with a $\beta\beta$-glucosidase remove the attached glucose groups. $\beta\beta$-glucosidase is an enzyme found predominantly in the gastrointestinal tract (Mills *et al.*, 2005). While it is likely that the hypoglycemic effect of the plant extract is largely due to its phytosterols or sterolin content, the exact mechanism of its hypoglycemic action is still obscure and will have to await further studies (Nair, 2006; Street & Prinsloo, 2013).

2.11.4 Toxicity

A lethal dose (LD50) in mice was found to be 1948 ± 57 mg/kg of *HH* aqueous extract after oral administration (Ojewole, 2006). The LD50 of *HH* aqueous extracts after oral administration in mice 1948 ± 57 mg/kg as a lethal dose. Lower dose at (≤ 1600 mg/kg) of *HH* aqueous extracts is safe while relatively higher doses were toxic and/or lethal to the mice (Ojewole, 2006).

HH when infused in anaesthetized chacma baboons had no effect on the cardiovascular system. The cardiac output increased together with systemic and pulmonary arterial pressures and these changes were not accompanied by changes in heart rate, vascular resistances or in the filling pressures of the heart (Albrecht & Theron *et al.*, 1995).

.One should also bear in mind that the traditional use of the *HH* plant goes back a long time, this in itself constitutes a form of clinical trial, since evidence of toxicity would have led to its abandonment by traditional healers long ago. It is of interest to note that Vahrmeijer, 20 in his book Poisonous Plants of Southern Africa, makes no mention of Hypoxis species(van Wyk, 2008).

2.11.5 Registered Patents

Several patents of Hypoxis, hypoxoside, rooperi and rooperol have been registered in the Europe and the USA (Mogatle, 2008). A patent registered under the title “method of treating viral infections” was registered by Liebenberg . The patents include methods of extraction and preparation of derivatives (Mogatle, 2008).

CHAPTER III

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Drugs

All the chemicals used in the study were of standard grade. Ethanol, xylene , glucose, fructose and formaldehyde were obtained from Merck Chemicals Sodium citrate, sodium chloride (NaCl) and calcium chloride where purchased from Kimix Laboratories, South Africa. The sodium phosphate ($NaHPO_4$), sodium dihydrogen phosphate (NaH_2PO_4), streptozotocin (STZ), casein, cholesterol, histopaque and collagenase XI were obtained from Sigma-Aldrich. Hank's balanced salt solution (HBSS), Roswell Park Memorial Institute-1640 medium (RPMI 1640), L-glutamine, Penicillin–streptomycin and Fetal bovine serum (FBS) were obtained from GIBCO. Phosphate buffered salin (PBS) was obtained from Lonza and cooking fat (HUDSON & KNIGHT) was obtained at a local supermarket.

3.1.2 Instrument and Equipment

This is listed equipment used in these experiments:

- Glucose meter (Contour TS).
- Glucose strip (Contour TS).
- Tissue baths.
- Automated Leica TP 1020 tissue processor.
- Vacuum system.
- Microtome (Leica 2125 RT).
- GloMax Multi Detection System plate reader.
- water bath

- Centrifuge (Eppendorf, 5810R).
- Dissecting microscope.
- 50-ml sterile tube (SPL Life sciences supply).
- 96 sterile well plates (SPL Life sciences supply).
- Test tube 15cm-50cm (SPL Life sciences supply).
- Sterile pipittes 10ml (SPL Life sciences supply).
- 25-ml sterile pipettes (SPL Life sciences supply).
- 200 µl sterile pipette (SPL Life sciences supply).
- 100 mm Petri-dishes (SPL Life sciences supply).
- Filter paper (240nm thick).

3.2 Animals

Male Wistar rats were used in this experiment. *Rattus norvegicus* (Wistar rats) weighing 200–300g were obtained from the University of Stellenbosch, South Africa and housed in the Medical Bioscience Department, University of the Western Cape, in a temperature controlled room (23±1°C) with a 12-hour light dark cycle. Light hours were from 08h00 to 20h00 and dark hours from 20h00 to 08h00. The rats received food and water *ad libitum* during a 2-week acclimatization period.

3.3 Plant Material

The plant materials used in this experiment was *Hypoxis hemerocallidea* (*HH*) corm purchased, which was purchased from Global Fusion Trading Cape Town- South Africa.

3.4 Acute Studies

3.4.1 Experimental Protocol

3.4.1.1 Preparation of *Hypoxis hemerocallidea* solution (*HH*S)

The saline solution was added to different concentrations of HH corm (50, 200, 400, 800 mg/ml). Thereafter, it was filtered through a cheese cloth and what man filter paper (no 4 and 1 respectively) using a vacuum system and subsequently also using syringe filter (0.22 µm). The HHS was allowed to cool at room temperature and then intraperitoneal injected into the rats.

3.4.1.2 Treatment Protocol

The rats were randomly divided into 2 groups, with eight animals assigned to each group (n=8) and each group housed in separate cages.
Group (1), control group: the rats in this group were fed the normal laboratory pellets.
Group (2), Diabetes group (DM): the rats in this group were injected with streptozotocin (STZ) 65 mg/kg to induce diabetes mellitus and fed the normal laboratory pellets.

3.4.1.3 Parameters Assessed

Two weeks after STZ injection (65 mg/kg), the blood glucose levels were monitored in all the rats using a handheld Contour TS blood glucose meter. Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p) and blood glucose level was determined (at time zero) from a small drop of blood collected by tail prick. Rats then received a single bolus HHS intraperitoneal injection (50, 200, 400 or 800 mg/ml). The blood glucose levels were again monitored by tail prick after 30, 60, 120, 180 and 240 minute post injection.

3.5 Chronic Studies

3.5.1 Experimental Protocol

3.5.1.1 Preparation of *Hypoxis hemerocallidea* Tea (*HH*T)

Freshly boiled tap water was added to *HH* corm (3.81g/500 ml) for 5 minutes. Thereafter, it was filtered through a cheese cloth and Whatman filter paper (no 4 and 1 respectively) using a vacuum system. The *HHT* was allowed to cool at room temperature and given in drink water. Fresh tea was prepared every second day.

3.5.1.2 Preparation of the high-fat diet (HFD)

The high-fat diet (HFD) was prepared in-house and contained the ingredients stipulated in Table 3.1.

Table 3.1. The compositions of the diet prepared in-house

Content	Quantity (g)
Cooking fat	400
Fructose	100
Casein	100
Cholesterol	10
Rat pellets (Chow)	390

3.5.1.3 Treatment Protocol

The rats were randomly divided into 6 different groups, with six rats assigned to each group (n=6) and each group housed in separate cages. Each group received the following treatment ad libitum.

Group (1), control group: the rats in this group were feed normal laboratory pellets.

Group (2), the high-fat diet group (HFD): the rats in this group were given the HFD.

Group (3), the diabetes group (DM): the rats in this group were injected with STZ (40mg/kg) and fed the HFD for 7 weeks.

Group (4), the Diabetes with *Hypoxis hemerocallidea* treatment (DM-*HH group*): diabetes was induced by STZ injection (40mg/kg) in rats. The rats were then fed the HFD and treated with *HH* (800mg/kg) for 7 weeks.

Group (5), the *Hypoxis hemerocallidea* pre-treatment group (*HHPT*): rats were given *HH* and fed the HFD for 3 weeks. Diabetes was then induced by STZ (40mg/ml). Rats continued on the HFD and *HH* treatment another 4 weeks.

Group (6), the *Hypoxis Hemerocallidea* group (*HH*): started with treatment of *HHT* and fed normal laboratory pellets for 7 weeks. Figure 3.1 shows the diagram illustrating treatment protocols in chronic study.

The body weight of the rats were measured weekly for 7 weeks. Average intake water by the rats in each group was also measured. The data was used to calculate the *HH* concentration needed in the drinking water. An intraperitoneal glucose tolerance test (IPGTT) was performed at the end of week 6. After being on the high fat diet for 7 weeks, the animals fasted overnight. The next day the rats were anesthetized and dissected.

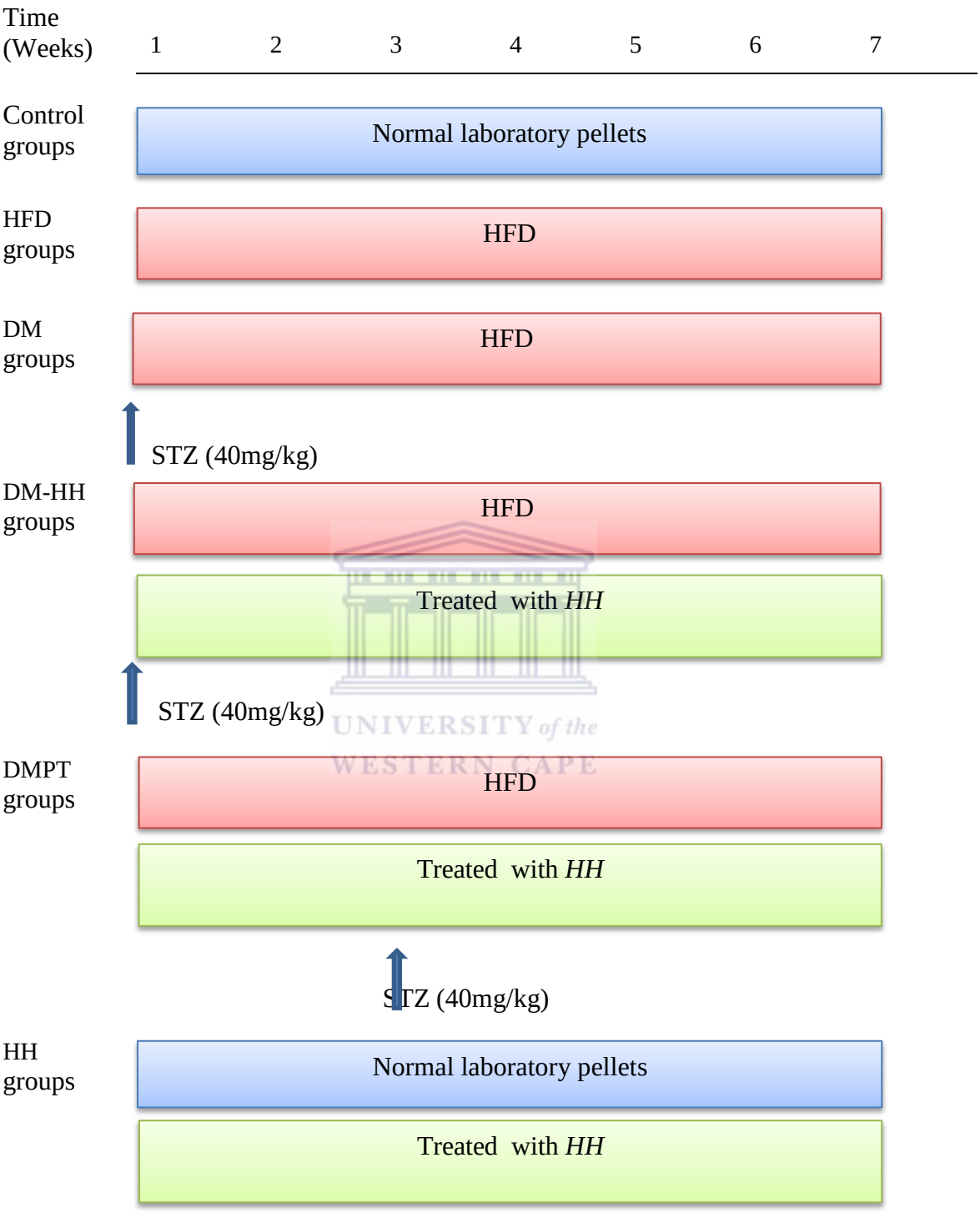

Figure 3.1. The diagram illustrating treatment protocols in chronic study.

3.5.1.4 Blood Collection

At the end of treatment, rats were anesthetized by sodium pentobarbital and killed by exsanguination. Blood samples were collected and allowed to clot for 30 minutes. A clear serum was separated by centrifuge (3000 x g, 15 minutes) and stored at -20 °C for further use and for the determination of insulin and fatty acids.

3.5.1.5 Parameters Assessed

3.5.1.5.1 Intraperitoneal Glucose Tolerance Test (IPGTT)

At the end of 6 weeks of treatment, an IPGTT tests were performed on all the rats using a handheld Contour TS blood glucose meter. After an overnight fast, the rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p) and time zero fasting blood glucose level determined from a drop of blood collected by snipping the tail. The Rats were then injected with 1.5 g/kg glucose and the blood glucose levels were monitored at 10, 30, 60, and 120 minutes post injection.

3.5.1.5.2 Tissue Histology

After the 7 week treatment, the rats were dissected. Liver, adipose tissue and pancreas was removed for histological evaluation. These organs were fixed in 10% neutral buffered formalin solution for about 7 days.

The 10% neutral buffered formalin solution was prepared in the following way; Formaldehyde 37% (100 mL), distilled water (900 mL), anhydrous monobasic sodium phosphate (NaH_2PO_4) (4.0 g) and disodium hydrogen phosphate (Na_2HPO_4) (6.5 g).

After fixation, the tissues were cut into small sections and placed in properly labelled cassettes. The tissues sample were then dehydrated through a series of graded ethanol solutions and cleared in xylene. Table 3.2 below shows time cycle of processing tissues.

Table 3.2. Time cycle of processing tissues.

Steps	Solutions	Time (hour)
1	*70*% ethanol	2
2	80% ethanol	2
3	90% ethanol	2
4	100% ethanol	2
5	100% ethanol	2
6	Xylene 1	2
7	Xylene 2	2
8	Wax bath 1	2
9	Wax bath 2	2

At the end of the 18 hour cycle, the tissues were embedded in paraffin wax and cut into sections of 6 µm. Each section was then placed on a staining rack and put into a hot air oven at 60°C for approximately 30 minutes to further fix the tissues.

After that, the wax was melted and the section were stained with haematoxylin (1g haematoxylin, 50g potassium alum, 0.2 g sodium iodate, 1 L dH20, 50g choral hydrate and 1g citric acid) and eosin (1% eosin, 1% phloxine and distilled water (2:1:3)), as shown in Table 3.3 below.

Table 3.3. Procedure for Haematoxylin and Eosin Stain.

Steps	Solutions	Time (minutes)
1	Xylene (x2)	5 each
2	100% ethanol (x2)	5 each
3	90% ethanol	5
4	80% ethanol	5
5	Haematoxylin	15
6	Rinsed in tap water	1
7	Scott's tap water	2
8	1% acid alcohol	2
9	Eosin	1
10	Rinsed in tap water	1
11	80% ethanol=	2
12	90% ethanol	2
13	100% ethanol	2
14	Xylene (x2)	2each

3.5.1.5.3 **Determination of insulin**

Insulin was measured in the plasma using a commercially available (Human Insulin ELISA -Enzyme-Linked Immunosorbent Assay, Abcam USA) kit. This assay employs an antibody specific for Human Insulin coated on a 96-well plate. A volume of 100 µl of Standard (0 µUI/ml – 300 µUI/ml) and 10 µl serum samples were pipetted into the wells and insulin present in a sample is bound to the wells by the immobilized antibody. The wells were washed 4 times with wash solution and 100 µl biotinylated anti-human insulin antibody (HRP) was added. After washing away unbound biotinylated antibody,

HRP-conjugated streptavidin was pipetted to the wells. The wells were washed again. 100 µl a TMB substrate solution was added to the wells and colour develops in proportion to the amount of insulin bound. A volume of 50 µl of stop solution was added to change the colour from blue to yellow, and the intensity of the colour is measured at 450 nm. Insulin content was determined by an ELISA.

3.5.1.5.4 **Determination of free fatty acid (FFA)**

Fatty acid was measured in the serum using a commercially available (Free Fatty Acid Quantification Kit Biovision USA). This assay employs fatty acid coated on a 96-well plate. 50 µl Standards and 50 µl serum samples were pipetted into the wells. The wells were added 2 µl ACR regent was added to each well and plant were incubated at 37°C for 30 minutes. 50 µl reaction mix was added plates incubated at 37°C for 30 minutes and the intensity of the colour was measured at 570 nm. FFA content was determined by an ELISA.

3.5.1.5.5 **Determination of insulin resistance (IR) and the β-cell function (%β)**

The insulin resistance and the β-cell function were determined using the Homeostatic model assessment (HOMA). The insulin resistance and β-cell function can be estimated from fasting glucose and insulin levels. The approximating equation for insulin resistance in the early model used a fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant (Wallace *et al.*, 2004; Yin *et al.*, 2009).

$$\text{HOMA} - \text{IR} = \frac{\text{Insulin levels} \times \text{Glucose}}{14.1}$$

$$\text{HOMA} - \beta\% = \frac{20 \times \text{Insulin levels}}{\text{Glucose} - 3.5}$$

3.6 Insulin secretion Studies

3.6.1 Preparation of *Hypoxis hemerocallidea* solution (*HHS*)

The phenol red free RPMI- 1640 medium was added to HH corm (2.38g/100 ml) to get low *HH* concentrations (100 µg/ml) and (5.95g/100 ml) to get high *HH* concentrations (500µg/ml). Thereafter, it was filtered through a 0.22µm syringe filter.

3.6.2 Isolation of rat pancreatic islets

The isolation of islets was conducted by using the collagenase digestion method modified in the laboratory at the University of Stellenbosch. The pancreas was removed and placed in a siliconized vial containing 2 ml of collagenase solution. Digestion was performed at 37°C for 20 minutes. Subsequently, the digestion was stopped by the addition of cold HBSS and the suspension was washed twice with HBSS. Islet separation was done by centrifugation on the histopaque discontinuous gradient (HBSS/1.083 g/ml, 16 minutes, 800 x g, and 4 °C). To ensure 100% purity of the preparation, islets were handpicked and counted under an inverted microscope. The islets were cultured in petri dishes containing phenol free RPMI- 1640 medium supplemented with 0.5mM- glucose, 340 µl L-glutamine and 5% fetal calf serum. at 37 °C in a humidified 5 % CO2 incubator..

3.6.3 Experimental protocol

Islets were collected and washed twice with PBS. Ten pancreatic islets was had suspended three times for 60 min in 2 ml RPMI-1640 solution supplemented with 10 % fetal calf serum and 3mM-glucose, after that the entire medium was discarded.

In preliminary studies, the Islets were divided into 2 groups which were incubated on low glucose RPMI-1640 medium supplemented with low glucose (3mM) or high glucose (20mM). After 60 minute incubation period 1ml of the medium was removed

for insulin determination. After a further 60 minutes the incubation medium was again removed for determination of insulin. The purpose of the preliminary study was to determine whether insulin secretion was the same in the first and second 60 minutes incubation period.

In the main study, the Islets were divided into 4 different groups. Group (1), islets were incubated in 2ml RPMI-1640 medium supplemented with 3mM glucose, control group. Group (2), islets were incubated in 2ml RPMI-1640 medium supplemented with 3mM glucose + 100mg/ml, low HH. Group (3), islets were incubated in 2ml RPMI-1640 medium supplemented with 3mM glucose + 500mg/ml, high HH. Group (4), islets were incubated in 2ml RPMI-1640 medium supplemented with 3mM glucose + 10mg/ml glibenclamide.

Islets were incubated for 60 minutes at 37°C. One ml of the incubation medium was removed from each group and stored for insulin determination. One ml medium supplemented with glucose, to adjust the glucose concentration in each well to 20 ml was added to each well. After further 60 minute incubation, the medium was removed from the wells for insulin determination. Protein content of islets was determined.

3.6.4 Parameters Assessed

3.6.4.1 Determination of insulin level

Insulin levels in the supernatant was measured in the culture supernatant using a commercially available (ab100578 Human Insulin ELISA -Enzyme-Linked Immunosorbent Assay, Abcam USA) kit as described under 3.5.1.5.3.

3.6.4.2 Determination protein concentration

A volume of 25 µl of each protein standard (25 µg/ml – 2000 µg/ml) added to the wells of a 96 well plate after that the samples were prepared and added to the wells of a 96

well plate. The working reagent (WR) was prepared by mixing 5 ml of Reagent A (500 ml containing /7, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and 100 µl of reagent B (25 ml containing 4 % cupric sulphate), then 200 µl of WR per well was added per well. The plate was covered and placed in an incubator for 30 minutes at 37 ^{o}C. Subsequently the absorbance was read at 560 nm in a 96 well plate reader. Each sample was analysed in triplicate.

3.7 Data analysis

All data are reported as means and SEM or median and interquatile range (IQR). Statistical differences among groups were analysed by analyses of variance (ANOVA). $P< 0.05$ was considered significantly different. Data analysis was done using GraphPad Prism version 5.1 for Windows (GraphPad Software, San Diego, CA, USA).

3.8 Ethical considerations

All experimental procedures were conducted after ethical clearance was obtained from the University of the Western Cape Senate Research Committee.

CHAPTER IV

4 Results

4.1 Acute Studies

We determined the effects of a single bolus administration of *HH* on blood glucose level in normal and diabetic rats, after 12 hours fast. Diabetes was induced in the group of diabetic rats by intraperitoneal injection of STZ (65 mg/kg). *HH* was tested at four different concentrations, i.e. 50, 200, 400 and 800 mg/kg.

4.1.1 Normal rats

The results are shown in Figure 4.1 and in Table 4.1. 50 mg/kg *HH* did not decrease the blood glucose level significantly over the 240 minute period. *HH* at a concentration of 200 mg/kg or 400 mg/kg decreased blood glucose level at 60 minutes and 120 minutes respectively ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$), and the blood glucose level remained below the time zero value (Table 4.1) for the rest of the 240 minutes duration.

HH at a dose of 800 mg/kg decreased blood glucose the fastest. It was significantly lower after 30 minutes, and remained significantly below the time zero value ($P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$), (Table 4.1) for the rest of the 240 minutes duration.

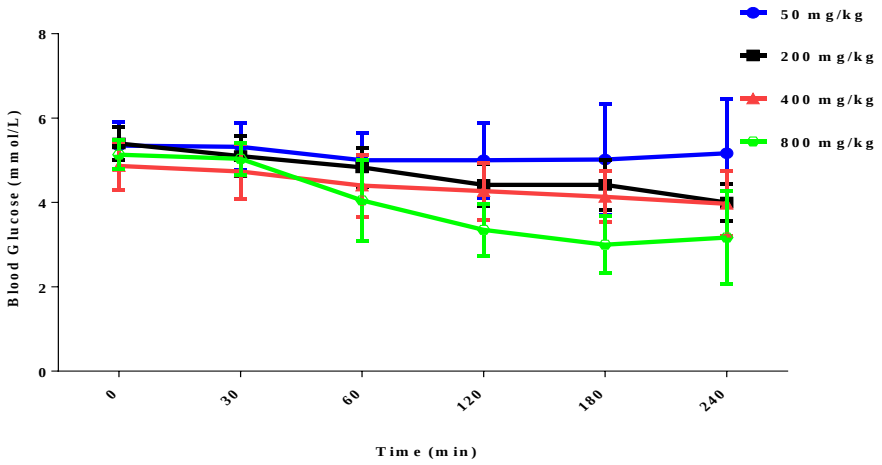

Figure 4.1. Effect of *HH* on blood glucose levels in normal rats.
Data is shown as mean ± SD of 7 animals per group. The statistics is given in Table 4.1

Table 4.1. Effect of *HH* on blood glucose levels in normal rats.

HH concentration injected / Time		0min	30min	60min	120min	180min	240min
50mg/kg	Blood Glucose (mmol/L)	5.4±0.5	5.3±0.6	5±0.6	5±0.9	5±1.3	5.2±1.3
	P(Comparison vs 0 min)		> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
200mg/kg	Blood Glucose (mmol/L)	5.4±0.4	5.1±0.5	4.8±0.5**	4.4±0.5***	4.4±0.6***	4±0.4***
	P(Comparison vs 0 min)		> 0.05	< 0.01	< 0.001	< 0.001	< 0.001
400mg/kg	Blood Glucose (mmol/L)	4.9±0.6	4.7±0.6	4.4±0.7	4.3±0.8*	4.1±0.6**	4±0.8***
	P(Comparison vs 0 min)		> 0.05	> 0.05	< 0.05	< 0.01	< 0.001
800mg/kg	Blood Glucose (mmol/L)	5±0.6	3.9±1.1*	3.6±1.3**	3.6±1.0***	3.8±1.2**	3.4±1.1***
	P(Comparison vs 0 min)		< 0.05	< 0.01	< 0.001	< 0.01	< 0.001

Values are expressed as the mean ± SD. N = 7 rats.
At each dose of *HH* blood glucose level were compared to zero min using ANOVA (*P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

4.1.2 Diabetic rats:

The results for diabetic rats are shown in Figure 4.2 and in Table 4.2. 50 mg/kg *HH* decreased blood glucose level between 120 minutes and 180 minutes ($P \leq 0.01$, $P \leq 0.001$). *HH* at a concentration of 200 mg/kg or 400 mg/kg decreased blood glucose levels between 60 minutes and 180 minutes. The values of blood glucose level at 240 minutes were not significant from time zero ($P > 0.05$).

HH at a dose of 800 mg/kg decreased blood glucose the fastest and was significantly lower after 60 minutes ($P \leq 0.001$). The blood glucose level remained significantly below the time zero value (Table 4.2) until 240 minutes.

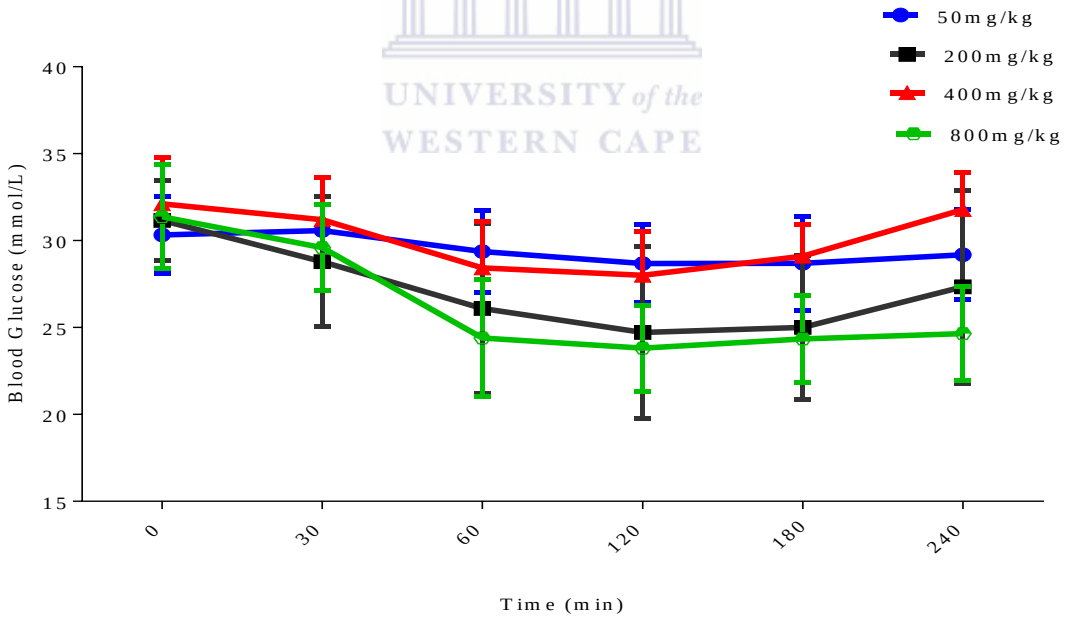

Figure 4.2. Effect of *HH* on blood glucose levels in diabetic rats.
Data is shown as mean ± SD of 7 animals per group. The statistics is given in Table 4.2.

Table 4.2. Effect of *HH* on blood glucose levels in DM rats

Time / *HH* concentration injected		0min	30min	60min	120min	180min	240min
50mg/kg	Blood Glucose (mmol/L)	30.3±2.2	30.6±2.0	29.4±2.4	28.7±2.2**	28.7±2.7**	29.2±2.6
	P(Comparison vs 0 min)		> 0.05	> 0.05	< 0.01	< 0.01	> 0.05
200mg/kg	Blood Glucose (mmol/L)	31±2.3	28.8±3.7	26.1±4.8**	24.7±4.9***	25±4.1***	27.3±5.5
	P (Comparison vs 0 min)		> 0.05	< 0.01	< 0.001	< 0.001	> 0.05
400mg/kg	Blood Glucose (mmol/L)	32.1±2.7	31.2±2.4	28.4±2.7***	28±2.5***	29.1±1.9**	31.7±2.1
	P(Comparison vs 0 min)		> 0.05	< 0.001	< 0.001	< 0.01	> 0.05
800mg/kg	Blood Glucose (mmol/L)	31.3±2.9	29.6±2.4	24.4±3.3***	23.8±2.4***	24.3±2.5***	24.6±2.7***
	P(Comparison vs 0 min)		> 0.05	< 0.001	< 0.001	< 0.001	< 0.001

Values are expressed as the mean ± SD. N = 7 rats.
At each dose of *HH* blood glucose level were compared to zero min using ANOVA (*P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

4.2 Chronic Studies

4.2.1 Body weight gain

The initial mean body weights of the six groups of animals were not significantly different before the dietary intervention as shown in Table 4.3.

Animals in the control group, HFD group and *HH* group statistically increased in weight over the seven week period. The weekly weight gain in the HFD group and the *HH* group were not significantly different from the control group. The total weight gain in the DM group ($P \leq 0.001$), the DM-*HH* group $P \leq 0.01$) and the DM-*HHPT* group $P \leq 0.01$) were significantly lower than the control group as shown in Table 4.3.The total weight gain in the DM-*HH* group and the DM-*HHPT* group were significantly higher than the DM group ($P \leq 0.001$).

Animals in the DM group gained weight the first two weeks, but thereafter began to lose weight. At the end of seven weeks the animals gained significantly less weight than the rest (Table 4.3).

Animals fed a HFD have more visceral fat compared to the control group, as shown in Figure 4.4.

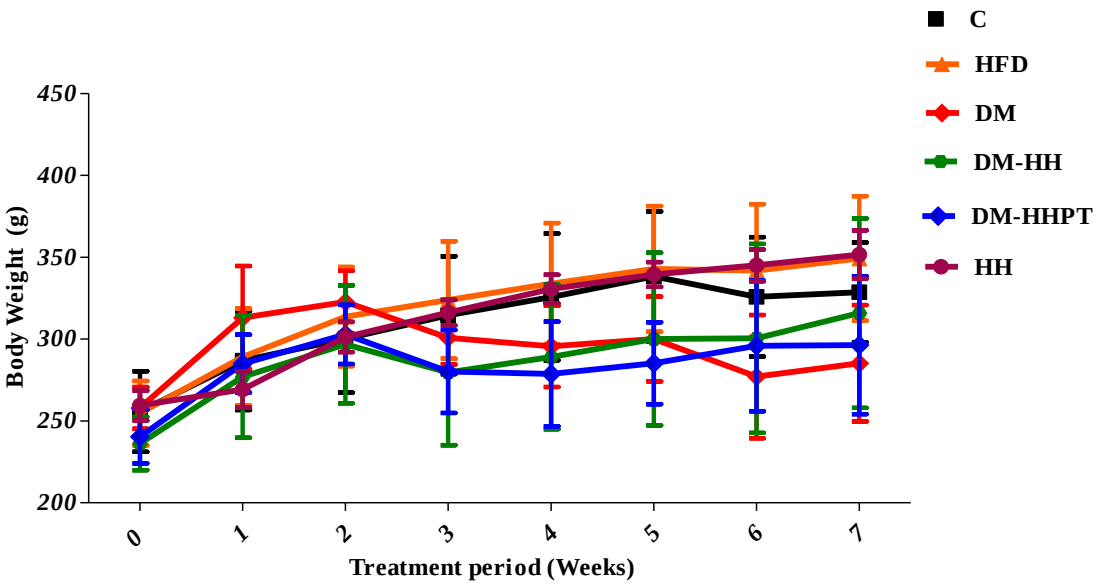

Figure 4.3. Weekly weight gain for the period of treatment in rats.
Values are the mean ± SD of 6 animals per group. Abbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HH*PT, Diabetes Mellitus-*Hypoxis hemerocallidea* pre-treatment; *HH*, *Hypoxis hemerocallidea*. Statistics are indicated in Table 4.3.

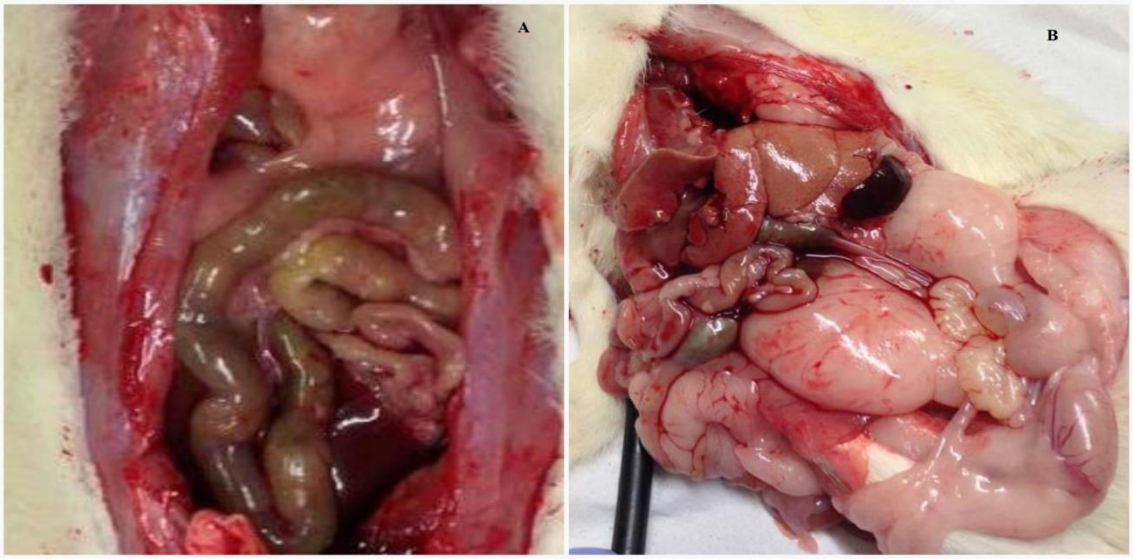

Figure 4.4. Visceral fat in rats from the control or diabetic groups.
(A) Normal rats (B) HFD rats.

Table 4.3. Weekly weight gain for the period of treatment in rats.

Weeks Groups	**Control**	**HFD**	**DM**	**DM-*HH***	**DM-*HHPT***	***HH***
0 weeks	256±25	255±20	258±13	249±15	240±16	259±9
P (Comparison to Control)		> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
7 weeks	329±30	349±38	285±36	302±58	296±42	352±15
P (Comparison to Control)		> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Body weight gain	73± 11	95± 20	27± 32***	53± 35**,#	56± 30**,#	92± 10
P (Comparison to Control)		> 0.05	< 0.001	< 0.001	< 0.001	> 0.05

Values are expressed as the mean ± SD. N = 6 rats. Weight (g). Significant differences between the treated and the control groups (ANOVA) are indicated *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 compared to control group, # P ≤ 0.05 compared to DM group.

4.2.2 Intraperitoneal Glucose Tolerance Test (IPGTT)

The results of the IPGTT performed at week 6 are shown in Figure 4.5 and Table 4.4. At time zero the blood glucose level of the HFD group (5.8±0.5mmol/l) and the *HH* group (4.9±0.7mmol/l) were in the normal range, and were not significantly different ($P > 0.05$) from the control group (5.0±0.2mmol/l). The blood glucose level of all the diabetic groups were significantly higher *(*DM group 19.3±3.3mmol/l, DM-*HH* group 11.0±3.6 mmol/l and DM-*HHPT* group 17.9±1.9 mmol/l) compared to the control group ($P \leq 0.001$). The blood glucose levels in the DM-*HHPT* group were not significantly different from the DM group and the blood glucose level in DM-*HH* group were significantly lower than the DM group ($P \leq 0.001$).

After glucose load peak blood glucose levels were measured after 30 minutes in the control group (9.0±0.6mmol/l), the HFD group (9.8±0.4 mmol/l), the DM-*HH* group (21±5.7 mmol/l) and the DM-*HHPT* group (27.8±5.3 mmol/l). In the *HH* group the blood glucose level reached a peak at 60 minutes (7.6±0.6 mmol/l). In the DM group two peaks were recorded one after 10 minutes (27.2±7.1mmol/l) and another after 60 minutes (31±5.2 mmol/l).

In the control, HFD, DM-*HH*, DM-*HHPT* and *HH* groups the blood glucose level after 120 minutes were not significantly different from the time zero value. The blood glucose level after 120 minutes in the DM group ($P \leq 0.01$) was significantly higher than from the time zero value

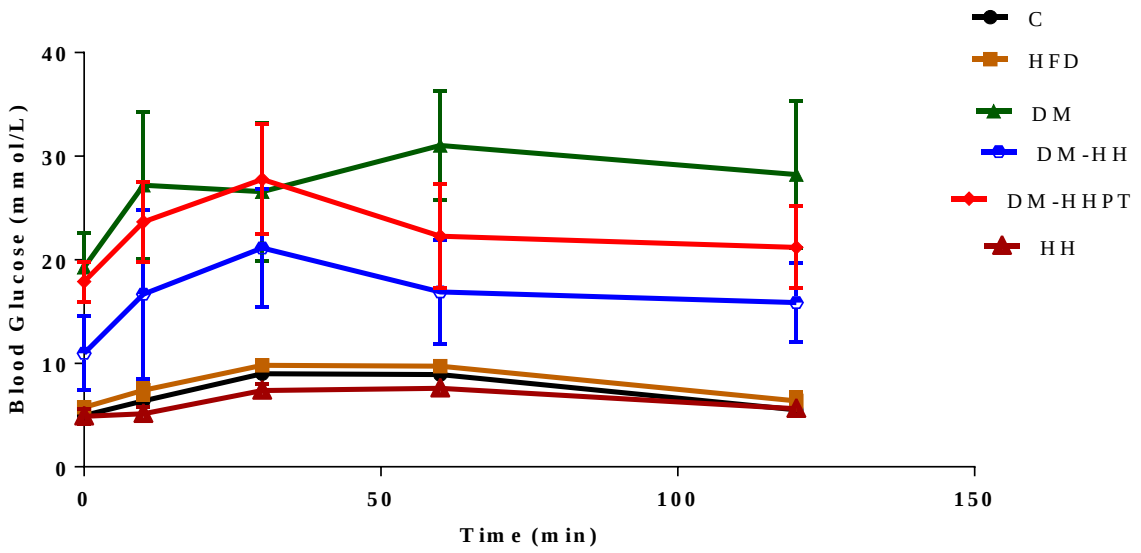

Figure 4.5. Intraperitoneal glucose tolerance tests (IPGTT) performed at week 6.
Values are the mean ± SD of 6 animals per groupAbbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HHPT*, Diabetes Mellitus-*Hypoxis hemerocallidea* pre-treatment; *HH*, *Hypoxis hemerocallidea*. The statistics are given in Table 4.4.

Table 4.4. Intraperitoneal glucose tolerance tests (IPGTT) performed at week 6

Time Groups	Control	HFD	DM	DM-*HH*	DM-*HHPT*	*HH*
0 min	5.0±0.2	5.8±0.5	19.3±3.3	11.0±3.6	17.9±1.9	4.9±0.7
10 min	6.4±0.5	7.4±0.8	27.2±7.1	16.7±8.2	23.7±3.9	5.1±0.7
30min	9.0±0.6	9.8±0.4	26.0±6.7	21.1±5.7	27.8±5.3	7.4±0.6
60 min	8.9±0.5	9.7±0.4	31.0±5.2	16.9±5.0	22.3±5.0	7.6±0.6
120 min	5.5±0.4	6.4±0.7	28.2±7.1[#]	15.9±3.8	21.2±4.0	5.6±0.5
Area under the curve of the IPGTT						
	29.24±1.9	32.47±1.8	108.5±4.4***	73.06±3.3***	96.11±4.1***	26.01±1.3
P (Comparison to Control)		> 0.05	< 0.001	< 0.001	< 0.001	> 0.05

Values are expressed as the mean ± SD. N = 6 rats per group. Blood Glucose (mmol/L).

Significant differences between the treated and the control groups (ANOVA) are indicated ***P ≤ 0.00. [#]P ≤ 0.01 compared between 0 min and 120 min

4.2.3 Serum free fatty acids (FFA) in the rats on the different dietary regimes

Figure 4.6 shows the serum free fatty acid levels after seven weeks on the respective diets. Statistically the FFA in the *HH* group (0.23±0.03 mmol/l; $P > 0.05$) was not significantly different from the control group (0.23±0.04 mmol/l). The FFA in the HFD group (0.37±0.05 mmol/l; $P \leq 0.001$), the DM group (0.47±0.05 mmol/l; $P \leq 0.001$), the DM-*HH* (0.39±0.06 mmol/l; $P \leq 0.001$) group and the DM-*HHPT* group (0.32±0.07 mmol/l; $P \leq 0.01$) were significantly higher than the control group. The FFA in the DM-*HH* group ($P \leq 0.01$) and the DM-*HHPT* group ($P \leq 0.001$) were significantly lower than the DM group.

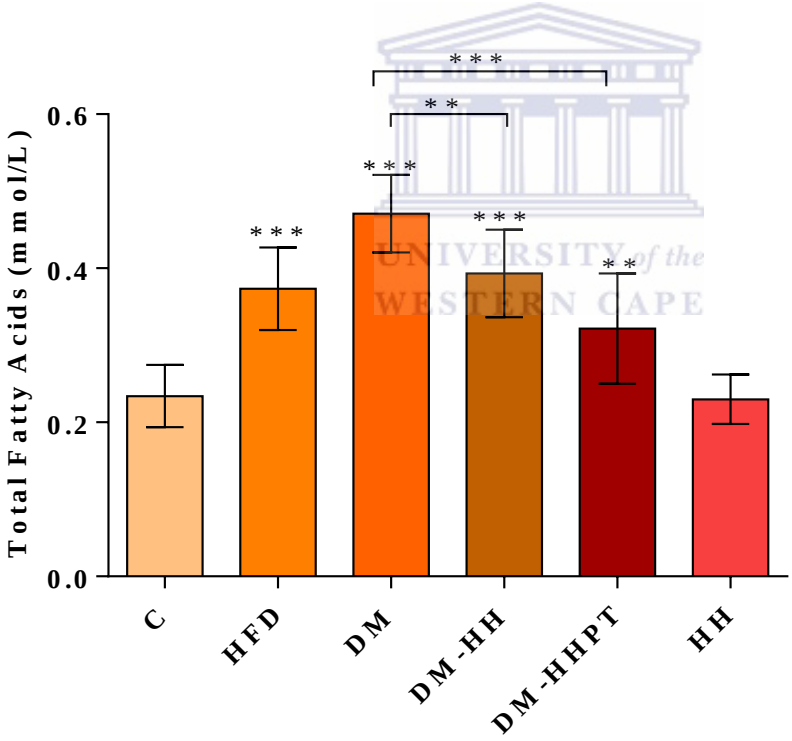

Figure 4.6. Serum free fatty acids in the rats on the different dietary regimes.
Values are the mean ± SD of 6 animals per group.Abbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HH*PT, Diabetes Mellitus- *Hypoxis hemerocallidea* pre-treatment; *HH*, *Hypoxis hemerocallidea*. Significant differences between the treated and the control groups (ANOVA) are indicated ** $P \leq 0.01$, *** $P \leq 0.001$.

4.2.4 Serum insulin levels in the rats on the different dietary regimes

Figure 4.7 shows the serum insulin levels after seven weeks on the respective diets. Statistically the serum insulin level in the *HH* group (8.2 ± 0.48 µUI/ml protein; P > 0.05) was not significantly different from the control group (7.7 ± 0.77 µUI/ml protein). The serum insulin levels in the HFD group (6.2 ± 0.76 µUI/ml protein; P ≤ 0.05), the DM group (2.0 ± 0.9 µUI/ml protein; P ≤ 0.001), the DM-*HH* group (3.4 ± 0.7 µUI/ml protein; P ≤ 0.001) and the DM-*HHPT* group (3.0 ± 1.1 µUI/ml protein; P ≤ 0.001) were significantly lower than the control group. The DM-*HH* group (P ≤ 0.05) were significantly higher than the DM group and the DM-*HHPT* group was not significantly different from the DM group.

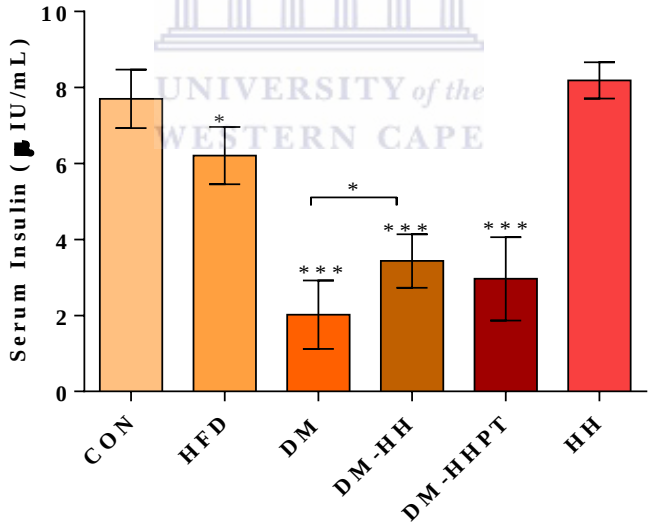

Figure 4.7. Serum insulin levels in the rats on the different dietary regimes.
Values are the mean ± SD of 6 animals per group.Abbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HH*PT, Diabetes Mellitus- *Hypoxis* hemerocallidea pre-treatment; *HH*, *Hypoxis hemerocallidea*. Significant differences between the treated and the control groups (ANOVA) are indicated * P ≤ 0.05, *** P ≤ 0.001.

4.2.5 β-cell function (β) in the rats on the different dietary regimes

Figure 4.8 shows the β-cell function after seven weeks on the respective diets. Statistically the β-cell function in the *HH* group (98 ± 9 %; P > 0.05) was not significantly different from the control group (117 ± 27 %). The β-cell function in the HFD group (62 ± 8 %; P ≤ 0.001), the DM group (3 ± 1 %; P ≤ 0.001), the DM-*HH* (11 ± 9 %; P ≤ 0.001) group and the DM-*HH*PT group (4 ± 2 %; P ≤ 0.001) were significantly lower than the control group.The β-cell function in the DM-*HH* group and the DM-*HH*PT group were not significantly different from the DM group.

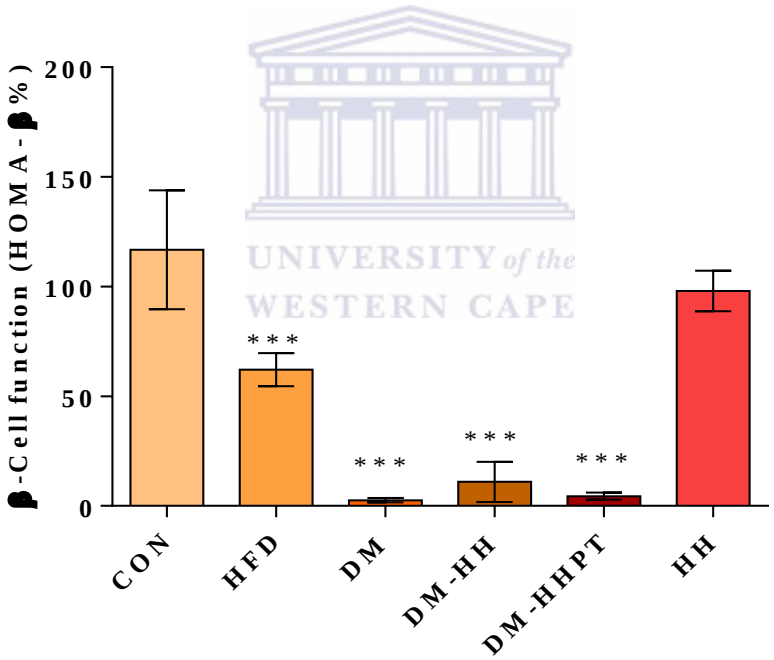

Figure 4.8. β-cell function in the rats on the different dietary regimes.
Values are the mean ± SD of 6 animals per group.Abbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HH*PT, Diabetes Mellitus-*Hypoxis hemerocallidea* pre-treatment; *HH*, *Hypoxis hemerocallidea*. Significant differences between the treated and the control groups (ANOVA) are indicated *** P ≤ 0.001.

4.2.6 Insulin resistance (IR) in the rats on the different dietary regimes

Figure 4.9 shows the Insulin resistance (IR) levels after seven weeks on the respective diets. Statistically the IR level varied between (2.42 ± 0.29 IR) in the HFD group and (3.59 ± 1.5 IR) in the DM-*HH*PT group. No significantly different was found between any of the groups (P > 0.05).

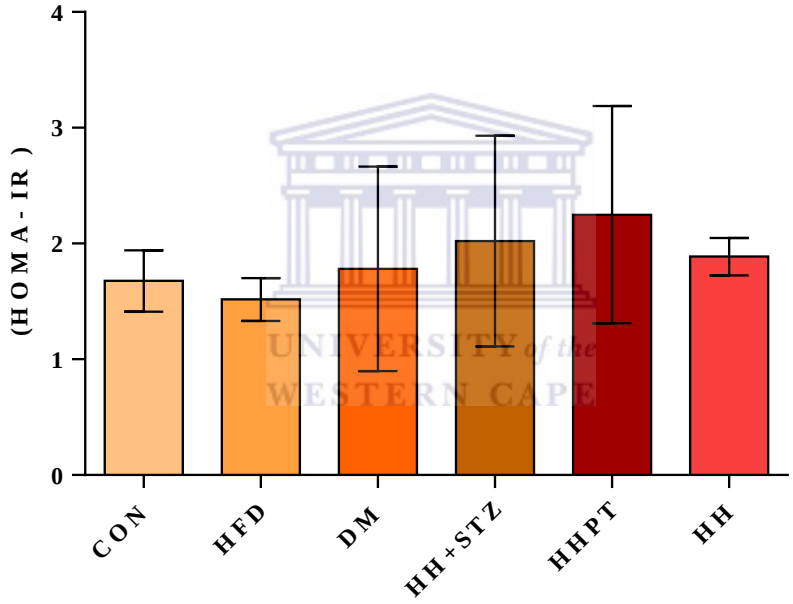

Figure 4.9. Insulin resistance (IR) in the rats on the different dietary regimes
Values are the mean ± SD of 6 animals per group.Abbreviations: C, control; HFD, High-Fat Diet; DM, Diabetes Mellitus; DM-*HH* Diabetes Mellitus-*Hypoxis hemerocallidea*; DM-*HH*PT, Diabetes Mellitus- *Hypoxis hemerocallidea* pre-treatment; *HH*, *Hypoxis hemerocallidea*. Significant differences between the treated and the control groups (ANOVA).

4.2.7 Morphology of liver and pancreas after 7 weeks on different dietary regimes

4.2.7.1 Liver

Figure 4.10 shows the gross appearance of the liver in the control rats and in the rats exposed to HFD. There was a marked enlargement of the liver in the HFD rats (Figure 4.10 B) compared to the liver in the control rats which exhibited a normal liver size and colour (Figure 4.10 A). Meanwhile, the beige colour observed in the liver of HFD rats is a gross characteristic feature of a fatty liver. The histological observation of the liver in rats after 7 weeks on different dietary regimes showed some morphological changes within the liver parenchyma of some rats. In the control rats the hepatocytes, the portal triads and the microvasculature appeared within normal limit (Figure 4.11 A). Similarly, the liver of the *HH* treated rats did not reveal any pathological alterations (Figures 4.11 F). However, there were obvious distortions in the architecture of the liver parenchyma of the rats that received an STZ injection (DM, DM-*HH*, DM-*HHPT* groups); the livers in these groups were presented with a dilatation of the sinusoids in the liver parenchyma, a dislocation of the wall of the central vein, a growing numbers of necrotic cells and lipid accumulation within the hepatocytes Figures 4.11 C, D, E). These observations were similar to what was observed in the liver of the rats exposed to HFD (Figures 4. 11 B).

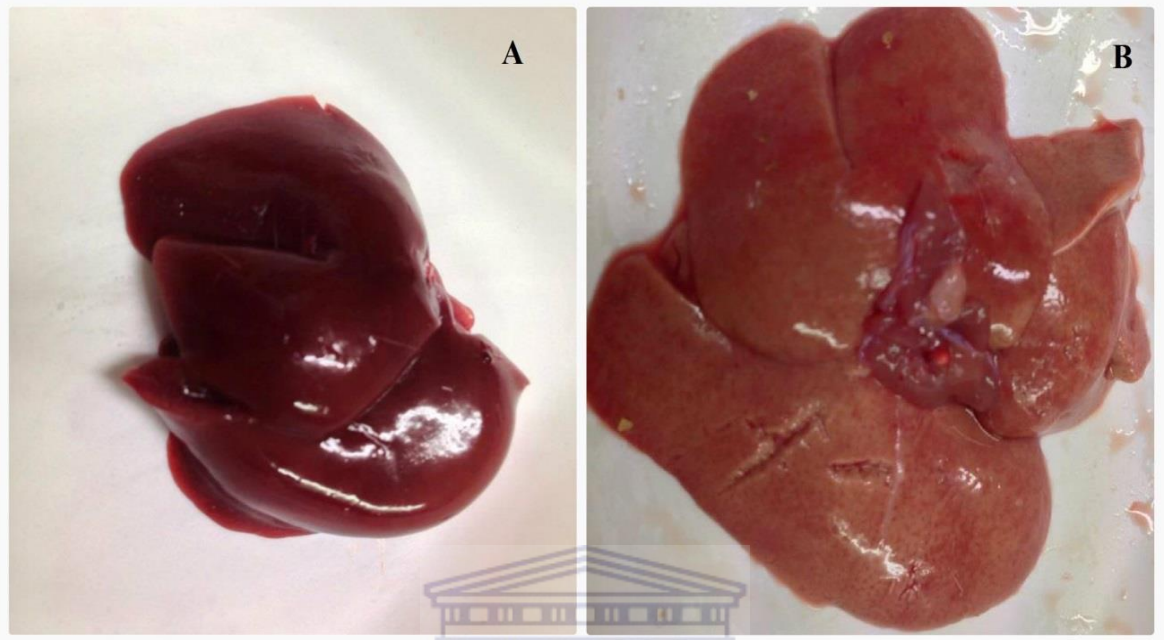

Figure 4.10. Gross appearance of the liver in Normal (A) and 7–week HFD (B) rats.

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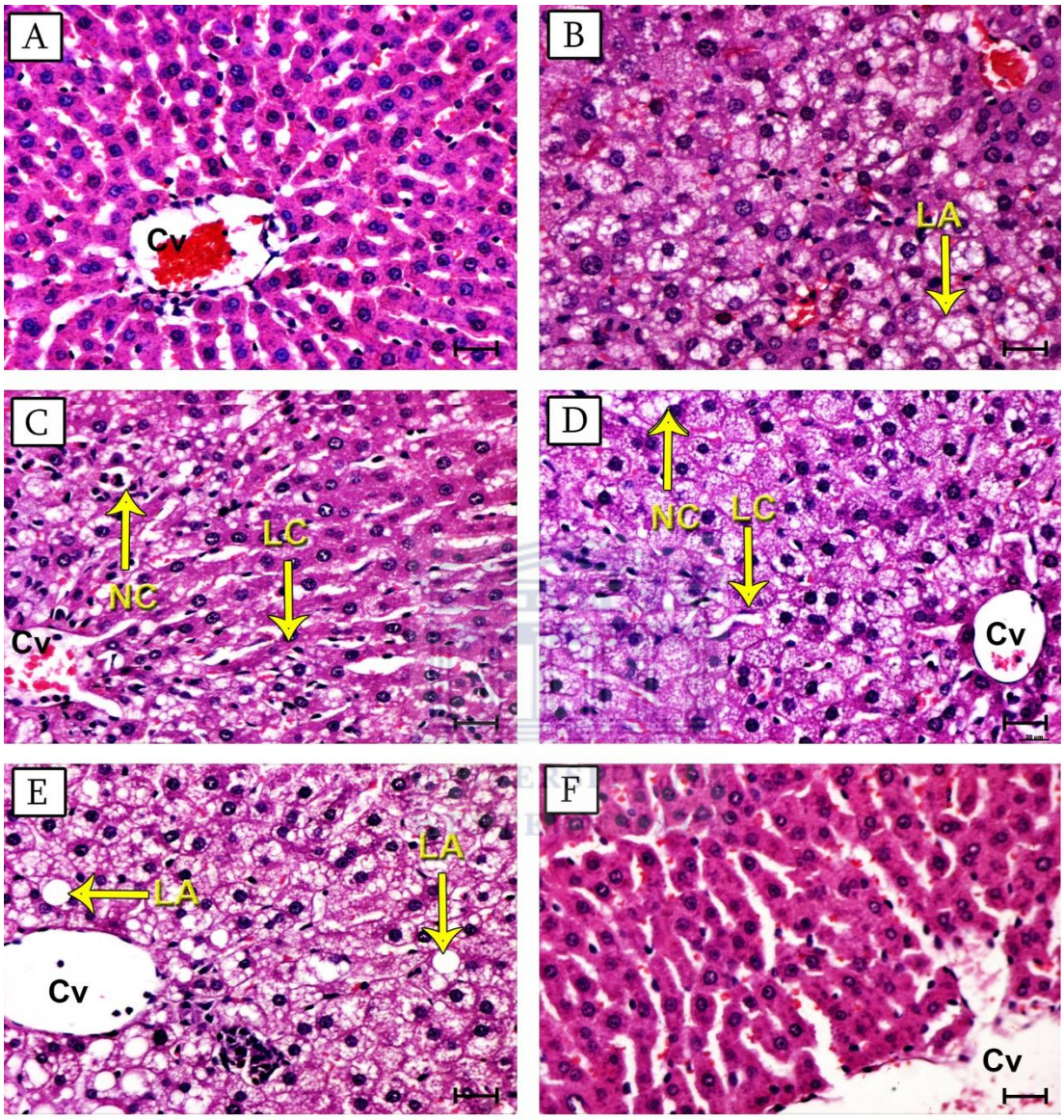

Figure 4.11. H&E staining showing morphology of the rat liver exposed to *Hypoxis hemerocallidea* (*HH*) for 7 weeks.

(A) control, (B) HFD, (C) DM, (D) DM-*HH*, (E) DM-*HHPT* and (F) *HH*; Cv central vein; LA lipid accumulation; NC necrotic cells; LC Lymphocytic cell; Bar = 20 µm.400X .

4.2.7.2 Pancreas

The histological observation of the pancreas in rats after 7 weeks on different dietary regimes is presented in Figure 4.12. The pancreatic tissues in the normal and *HH*-treated rats appeared normal with prominent and healthy islets of Langerhans (Figures 4.12 A, F); while there were high numbers of lymphocytic infiltrations observed in the other groups (Figures 4.12 B, C, D and E). The sizes of the islets of Langerhans were relatively reduced in the pancreas of rats treated with STZ with a massive distortion on their cellular architectures. This observation characterized the histological appearance of the islet of langerhans seen in the HFD rats. The Islets of langerhans in both the control and the *HH* groups were not affected

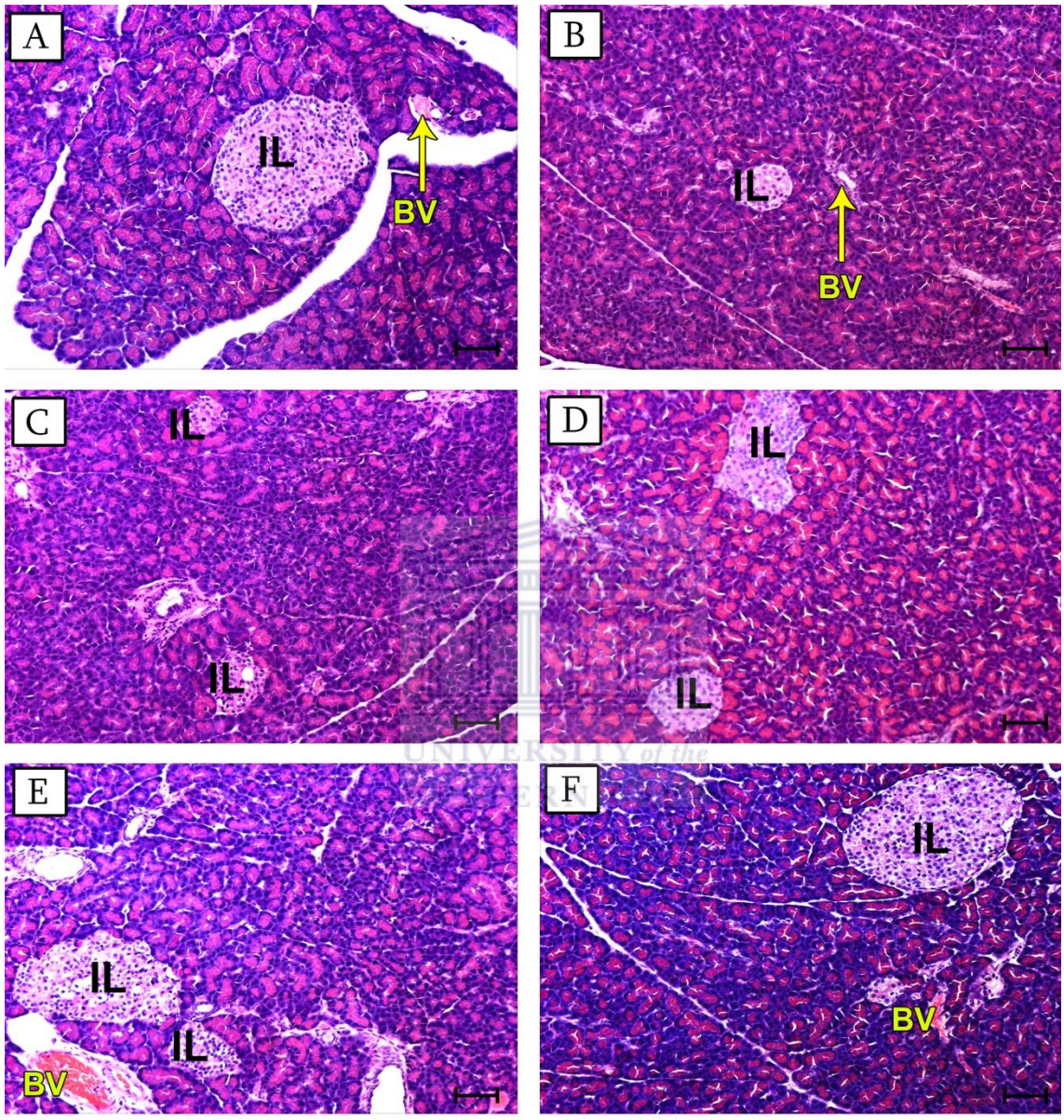

Figure 4.12. H&E staining showing morphology of the rat Pancreas exposed to *Hypoxis hemerocallidea* (*HH*) for 7 weeks.
(A) control, (B) HFD, (C) DM, (D) DM-*HH*, (E) DM-*HHPT* and (F) *HH*; BV blood vessel; Ex exocrine portion of pancreas; IL islets of Langerhans; Bar = 10 µm.400X.

4.3 Insulin secretion studies

Preliminary studies indicated that insulin secretion in low (3 mM) or high (20mM) glucose remained constant over the 120 minutes experimental period (Figure 4.13).

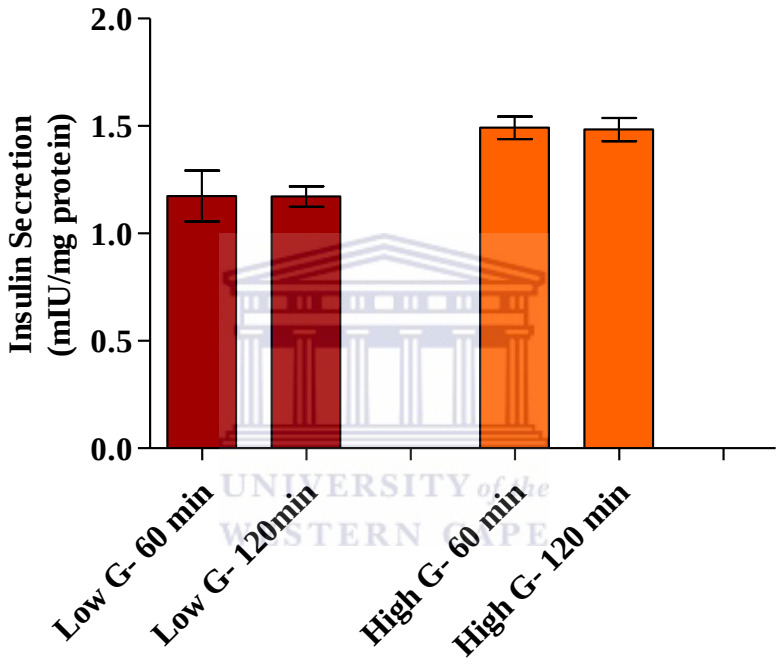

Figure 4.13. Preliminary studies for insulin Secretion in Isolated rat Islets.
Values are the mean ± SD of 10 islets per group. Abbreviations: low G= low glucose, high G= high glucose

Figure 4.14 shows insulin secretion by pancreatic islet cells. Statistically the insulin secretion in high glucose group (1.5 ± 0.17 mIU/mg protein; $P \leq 0.01$) was significantly higher than the low glucose group (1.10 ± 0.13 mIU/mg protein). Also, the insulin secretion in high glucose + low *HH* group (1.94 ± 0.11 mIU/mg protein; $P \leq 0.01$) was significantly higher than the low glucose + low *HH* group (1.5 ± 0.11 mIU/mg protein) and the insulin secretion in high glucose + glibenclamide 10 mg/ml group (2.3 ± 0.15 mIU/mg protein; P ≤

0.001) was significantly higher than the low glucose + glibenclamide 10 mg/ml group (1.75 ± 0.16 mIU/mg protein). The insulin secretion in high glucose + high *HH* (2.35 ± 0.14 mIU/mg protein; P > 0.05) was not significantly different from the low glucose + high *HH* group (2.34 ± 0.24 mIU/mg protein).

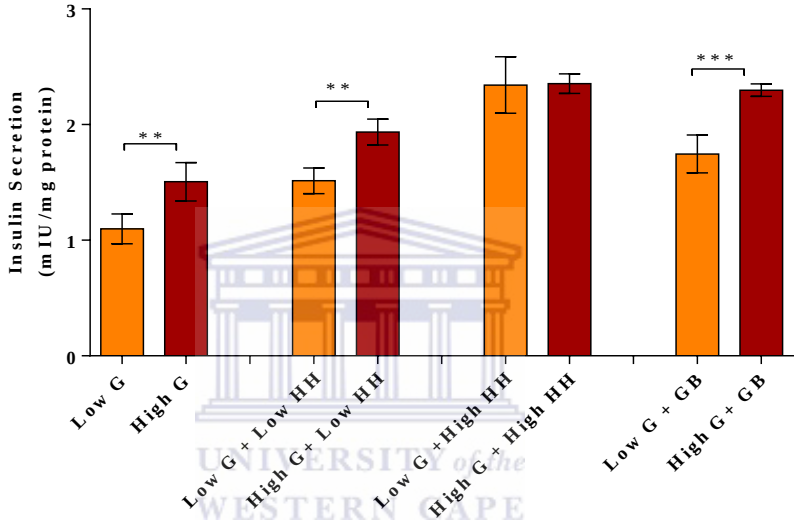

Figure 4.14. Insulin Secretion in Isolated rat Islets.
Values are the mean ± SD of 10 islets per group. Abbreviations: low G low glucose, high G high glucose, low *HH* low *Hypoxis hemerocallidea*; high *HH* high *Hypoxis hemerocallidea*; GB glibenclamide. Significant differences are indicated. ** P ≤ 0.01, *** P ≤ 0.001.

Figure 4.15 shows the effect of *HH* on insulin secretion at a low glucose concentration. Statistically the insulin secretion in low glucose + low *HH* group (1.5 ± 0.11 mIU/mg protein; P ≤ 0.01), low glucose + high *HH* (2.34 ± 0.24 mIU/mg protein; P ≤ 0.001) and low glucose + glibenclamide 10 mg/ml (1.75 ± 0.16 mIU/mg protein; P ≤ 0.001) were significantly higher than the low glucose group (1.10 ± 0.13 mIU/mg protein). The insulin secretion in low glucose+high *HH* (P ≤ 0.001) was significantly higher than low glucose+ low *HH* group.

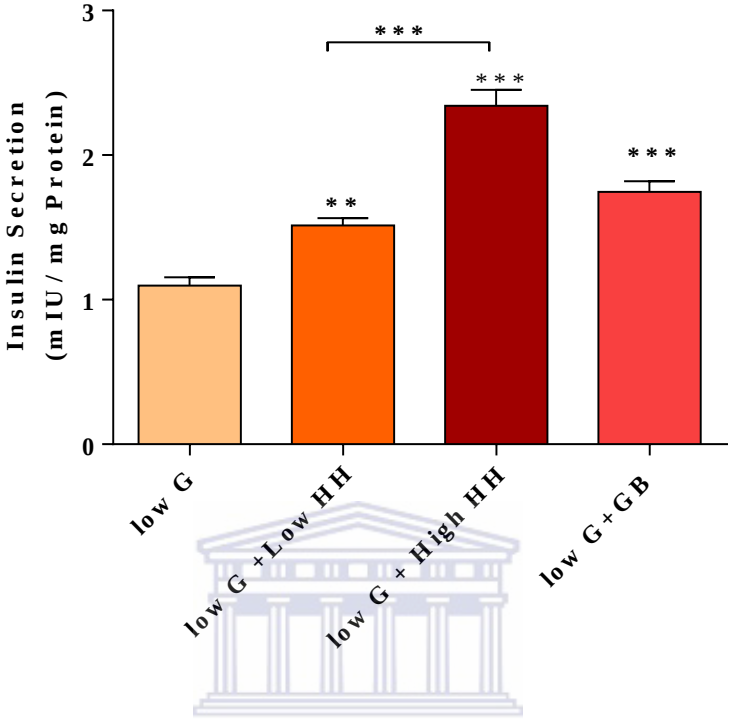

Figure 4.15. The Insulin Secretion at a low glucose concentration.
Values represented are the mean ± SD of 10 islets per group. Abbreviations: low G low glucose, low *HH* low *Hypoxis hemerocallidea*; high *HH* high *Hypoxis hemerocallidea*; GB glibenclamide. Significant differences (ANOVA) are indicated ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4.16 shows the effect of *HH* on insulin secretion at a high glucose concentration. Statistically the insulin secretion in high glucose + low *HH* group (1.94 ± 0.11 mIU/mg protein; $P \leq 0.001$), high glucose + high *HH* (2.35 ± 0.17 mIU/mg protein; $P \leq 0.001$) and high glucose + glibenclamide 10 mg/ml (2.3 ± 0.15 mIU/mg protein; $P \leq 0.001$) were significantly higher than the high glucose group (1.5 ± 0.17 mIU/mg protein). The insulin secretion in high glucose + high *HH* ($P \leq 0.001$) was significantly higher than high glucose + low *HH* group.

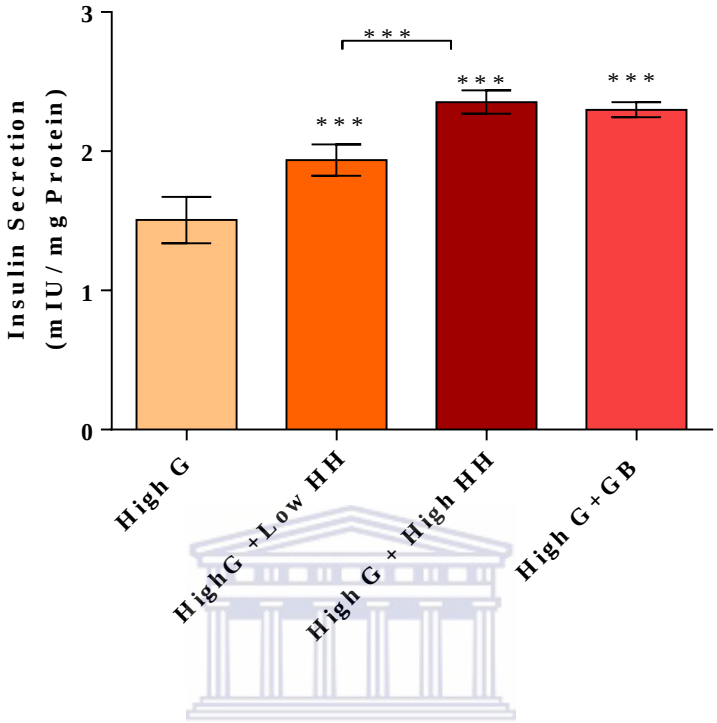

Figure 4.16. The insulin secretion at a high glucose concentration.
Values are the mean ± SD of 10 islets per group. Abbreviations: G; glucose, *HH*; *Hypoxis hemerocallidea*; GB glibenclamide. Significant differences are indicated *** $P \leq 0.001$.

CHAPTER V

5 Discussion

Diabetes mellitus is a metabolic disease that can result in chronic-hyperglycaemia due to an imbalance in the metabolism of carbohydrate, fat and protein which may result in an impaired secretion of insulin (Kitabchi *et al.*, 2001; Drouin *et al.*, 2009; Alsahli & Gerich, 2010). T1DM is caused by an autoimmune assault to the β-cells while the pathogenesis of T2DM is variable, whereby the degree of damage to the cells is relative to the varying degree of insulin resistance (Guidelines & Force, 2005; Donath & Shoelson, 2011; Atkinson *et al.*,2014). The main aim of this research was to study the possible effect of *HH* on blood glucose levels in rats with type 2 diabetes. The study was based on three parts acute, chronic and insulin secretion.

5.1 Acute Studies

This study has shown that *HH* had the ability to decrease blood glucose level in *STZ* induced diabetic rats. In normal rats (Figure 4.1), *HH* at concentrations of 200 mg/kg or 400 mg/ kg decrease the blood glucose level at 60 minutes and 120 minutes, while, *HH* at a concentration of 800 mg/ml *HH,* decrease the blood glucose level at a faster rate and significance was achieved after 30 minutes. In diabetic rats (Figure 4.2), the decrease in blood glucose level at concentrations of 200 mg/ kg or 400 mg/kg was observed between 60 minutes and 180 minutes, while at the high dose of 800 mg/kg the blood glucose level decreased at a faster rate and was significant after 60 minutes. *HH* at a dose of 800 mg/ml appears to be the most effective dose to reduce blood glucose level in diabetic rats when using the *HH* as solution (*HHS*), this is similar to what was reported by Mahomed & Ojewole (2003) to be the most effective dose in the study. However, the low dose of 100

mg/ kg of *HH* has been shown to have significant antidiabetic effects in both normoglycaemic and hyperglycaemic rats (Erlwanger & Cooper, 2008).

The antidiabetic properties of *HH* is probably due to its antioxidant properties (Katerere & Eloff, 2008). It appears that the combined effects of hypoxidae, together with other constituents like stigmasterols help combat the oxidative stress generated by chloroquine (Panda *et al.*, 2009). Both aqueous and ethanolic extracts of *HH* are able to scavenge hydroxyl radicals through their anti-oxidant property by the presence of hypoxidae that is converted to rooperol in vivo (Nair, 2006; Street & Prinsloo, 2013).

5.2 Chronic Studies

A low dose of STZ (40 mg/kg) and HFD was used to induce T2DM in the rats. The HFD and low dose of STZ simulates natural disease progression and metabolic characteristics typical of individuals at increased risk of developing T2DM (Srinivasan *et al.*, 2005). In DM rats treated with *HH*, there was no significant difference in all parameters evaluated between DM-*HH* group and DM-*HH*PT group (except the glucose tolerance). Furthermore, one should however keep in mind that the duration of diabetes was shorter in the DM-*HH*PT group than in the DM-*HH* group. *HH* did not show significant effects in the non-diabetic rats on the different parameter evaluated. The body weight, glucose tolerance, histological observation of liver and pancreas and FFA levels were the same as those of the control group.

Our study demonstrated a small but not significant gain in body weight of normal rats and normal rats treated by *HH*. HFD rats also had increased body wright, which was most likely due to normal body growth and ageing during the 7 weeks of the experiment (Figure 4.3). On the other hand, the results of the present study showed a significant decrease in body weight in diabetic rats compared to control rats. In diabetic rats, a loss in body weight occurs because glucose is not available, resulting in excessive breakdown of tissue protein

(muscle wasting) (Pareek *et al.*, 2009). Treatment of diabetic rats with *HH* as reported by Musabayane *et al.* (2005) caused the body weight in diabetic rats to increase and this can be attributed to the improvement in insulin secretion and glycemic control (Musabayane *et al.*, 2005). After seven weeks of HFD feeding the rats, the visceral fat mass was increased. Interestingly, blood glucose was within the normal range and insulin resistance was absent. These are probably due to the short duration of the experiment and confirm previous results obtained in our laboratory. In order to limit the duration of our experiments, we opted to induce diabetes by injecting a low dose of STZ. The visceral fat gain occurred in the absence of a significant increase in body weight. The lack of significant increased body weight under conditions of high-fat feeding for 7 weeks could be due to enhanced activity of the sympathetic nervous system (Moan *et al.*, 1995; Reaven *et al.*, 1996).

Prior to *HH* treatment, the STZ treated rats were randomly divided into two groups (DM and DM-*HH*). The result of the IPGTT performed at week 6 of the experiment is presented in (Figure 4.5) and (Table 4.4). We found a markedly lower fasting glucose in *HH* treated diabetic animals compared to untreated DM animals. The DM group presented a higher blood glucose level followed by the DM-*HH*PT group. The DM-*HH* group showed the lowest blood glucose level in diabetic rats. Hence, these results demonstrated the hypoglycaemic activity of *HH*. Regarding the glucose tolerance test, all diabetic *HH* treated animals had improved glucose tolerance compared to untreated DM rats, and the 120 min post glucose load level was closer to baseline levels. Rats in control, HFD and *HH* groups had a good tolerance for blood glucose level, probably due to improved insulin secretion. Contrary to our findings, results from a 13-week study (Bates *et al*., 2007) have shown that glucose tolerance in HFD rats did not differ from DM rats. However, our study was conducted over a period of 6 week start period, which may explain the observed differences regarding glucose tolerance between the two studies.

This study appears to support the concept that FFA levels are elevated in DM rats. The results presented in Figure 4.6 showed significant increase in serum FFA levels of DM rats.

FFA inhibit insulin stimulated peripheral glucose uptake in a dose-dependent manner in controls and in patients with T2DM (Boden, 1997). We found that serum FFA increased in DM rats treated with *HH* compared to normal rats, while FFA decreased in DM rats treated with *HH* compared to untreated DM rats. The result of this study is in agreement with that of Storlien *et al.,* (1986) central adipose level elevates plasma FFA, which exacerbates insulin resistance. *FFA* also depresses the activity of pancreatic β-cells, which could result in a rapid progression of diabetes (Storlien *et al.,* 1986). FFA might mediate the insulin resistance and impaired glucose tolerance associated with central obesity (Bergman & Ader, 2000). Insulin resistance is associated with an abnormal lipid profile. In the Insulin resistance state the body uses stored fat as metabolic substrate in preference to glucose, resulting in dyslipidemia. Enhanced lipid breakdown causes an increase in the plasma free fatty acid levels with subsequent changes in lipoprotein composition (Van Gaal *et al.,* 2006).

T1DM and T2DM are characterized by β-cell depletion, with a 70–80% loss in β-cell function in T1DM and 25–50% in T2DM; although common to both they occur through distinctly different mechanisms (Green *et al.,* 2003; Young *et al.,* 2000). Diabetic pancreatic fragments produce about 50% less insulin compared to the normal healthy pancreas (Lotfy, 2012). The present study has shown a pronounced reduction in levels of serum insulin in untreated diabetic rats compared to normal rats (Figure 4.7). In diabetic rats, the serum insulin levels were decreased because the β-cell is not able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia (Reaven, 1988). The changes in insulin secretion would be entirely explained by disorder of β-cell function (Kahn *et al.,* 1989). Apoptosis is probably the main form of β-cell death in both forms of the disease. It has been suggested that the mechanisms leading to nutrient and cytokine induced β-cell death in T2DM and T1DM, respectively (Green *et al.,* 2003). Treatment of diabetic rats with *HH* showed a significant elevation in serum insulin levels compared to diabetic rats. These results suggest the *HH* may improve insulin levels in serum via enhancement of insulin secretion in rats only when the blood glucose level was above the

normal range. The increase serum insulin level is accompanied increase in β-cell function (Park *et al.*, 2007). Figure 4.8 showed the β-cell function in HFD rats and DM rats was significantly lower than the normal rats. Also, β-cell function was similar between the two *HH* treatment groups and DM group. The HOMA-IR model has become a widely used clinical and epidemiological tool and, when used appropriately, it can yield valuable data (Wallace *et al.*, 2004). However, as with all models, the primary input data need to be robust, and the data need to be interpreted carefully (Lan *et al.*, 2011), The results obtained and shown in Figure 4.9 does not support the development of insulin resistance in the *DM* rats. The large standard errors, due to the variation in fatty acid and serum insulin levels measured, make it very difficult to make much inference from the data.

The study also investigated the effect of *HH* on liver and pancreatic function in diabetic and HFD rats by histological observation. There was a marked enlargement in the liver with in HFD and DM rats (Figure. 4.11. B, C), dilation and congestion of central vein, lipid accumulation and hepatic sinusoids were observed in the hepatic sections. The incidence and intensity of changes in *HH* rats was much lower compared with HFD and DM rats (Figure. 4.11. D, E). However, the DM rats treated by *HH* presented with some lipid accumulation. On the other hand, the liver of normal rats treated by *HH* did not show any pathological alteration (Figure. 4.11. F). Similar observations were made by Sahin et.al (2007) in DM and HFD rats, this study the histopathology of the liver had vacuolated eosinophilic cytoplasm with ground-glass appearance and fatty inclusions (Sahin *et al.*, 2007), the histopathological examination of the livers of control mice revealed severe hepatic fat accumulation along with increased liver weight; however, this was accompanied with only mild elevation of liver specific enzymes (Mookkan *et al.*, 2014).

The HFD rats and the DM rats presented with high number of Lymphocytic infiltration and massive distortion of the pancreatic cellular architecture (Figure. 4.12. B, C), while the normal rats treated by *HH* had a healthy pancreatic morphology similar to that of the normal control rats (Figure. 4.12. F). Treatment with *HH* showed a slight hypertrophy of

Langerhans islets in pancreas compared to DM rats (Figure. 4.12. D, E). The relevant reduction in the size of the islet cell in DM rats was similar to that observed in the HFD rats. The result of this study is in agreement with that of Yan *et al.,* (2006) who also did study the histological changes in pancreatic DM rats. The histological observation in pancreatic diabetic rats treated by *M. emarginata* showed the β-cells regeneration (Gandhi & Sasikumar, 2012).

Following acute treatment, relatively moderate to high doses of *HHT* (800 mg/kg) produced significant reductions in the blood glucose concentrations normal and *DM* rats. While it is likely that the hypoglycemic effect of the *HH* is largely due to its phytosterols or sterolin content, the exact mechanism of its hypoglycemic action is still obscure and will have to await further studies (Mahomed & Ojewole, 2003). The present study reports that the *HH* can have a protective effect against a *HFD* induced obesity in rats through an enhanced expression of uncoupling proteins and elevated phosphorylation in the visceral adipose tissue (Pareek *et al.*, 2009).

5.3 Insulin secretion studies

This study also showed the effect of *HH* on pancreatic islet cell insulin secretion. When the demand for insulin is chronically increased by physiological or pathological changes, beta cells can adapt by enhancing insulin secretion via increased beta cell function and/or increased beta cell mass (Unger, 1991; Aronoff *et al.*, 2004). Inadequate adaptation leads to the development of hyperglycemia and eventually diabetes mellitus (Chiasson *et al.*, 2003). An antidiabetic agent could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and/or by improving/mimicking insulin action (Gray & Flatt, 2007). This is the first time that it shown that *HH*, increase insulin secretion from pancreatic β-cells isolated from rats.

Glucose stimulated insulin secretion in low (3mM) and high (20mM) concentrations (Figure 4.14). Furthermore, insulin secretion was significantly higher when the glucose

concentration was increased from 3mM to 20 mM (1.10 ± 0.13 µUI/ml protein and 1.5 ± 0.17 mIU/mg protein respectively $P \leq 0.01$). The result of this study is in agreement with that of Purrello *et al.* (1989) who also observed increased insulin secretion in the presence of low (5.5mM) and high (16.7mM) glucose concentrations. Henquin (2004) demonstrate of that the blockade of K_{ATP} channels in the plasma membrane of β-cells is a key step in the stimulation of insulin release by glucose and by many pharmacological components. This results in stimulation of the Ca^{+2} influx, and increase the cytosolic concentration of free Ca^{+2}, which constitutes the triggering signal to induce exocytosis of insulin granules. The mechanism of glucose-induced desensitization in β-cells is not known. Evidence indicates that this phenomenon is not a consequence of impaired insulin synthesis (Purrello *et al.*, 1989). Farmer *et al.* (2012) which demonstrate that the mechanism of glucose stimulated the insulin secretion in a dose-dependent manner. The present study has also shown the beneficial use of *HH* to induce insulin secretion from pancreatic islet cells. In the presence of low *HH* (100 µg/ml), there was a marked increase in insulin secretion when exposed to high glucose compared to low glucose concentration, while, in the presence of high HH (500 µg/ml), there was no significant different in insulin secretion in the presence of low or high glucose. Interestingly, in the presence of glibenclamide (10 mg/ml), the insulin secretion increased after exposure to high glucose compared to low glucose.

Figures 4.15 and 4.16 illustrates that at the higher *HH* concentration (500 µg/ml), insulin secretion is higher than at the low *HH* concentration (100 µg/ml) at both low (3mM) and high (20mM) glucose levels. On closer inspection it appears that at 500 µg/ml *HH*, insulin secretion is the same at both 3mM glucose (2.34 ± 0.24 mIU/mg protein) and 20mM glucose (2.35 ± 0.17 mIU/µg protein) (Figure 4.15). Furthermore, these values also correspond with the maximum insulin secretion achieved with 10 mg/ml glibenclamide in the study (Figure 4.15), It thus appears as if this is the maximum amount of insulin that can be secreted over the hour measured. We do not know whether the rate of insulin secretion remained constant over the 60 minutes. It is possible that the β-cells became either desensitized or exhausted. There are two clear concepts for glucose-induced

desensitization, the first is a consequence of functional changes in the β-cell that impair glucose-recognition. The second, results from increased secretory activity in the β-cell that leads to a depletion of releasable insulin (Rustenbeck, 2002). Increase insulin secretion from β-cells occurs by two mechanisms. The first, under high-glucose conditions, proceeded by a classical secretory pathway involving K_{ATP} channel closure, membrane depolarization, calcium channel gating, and calcium mobilization. The second mechanism, under low-glucose conditions, may occurs independently of glucose metabolism (Ammälä *et al.*, 1993; Haidar *et al.*, 2004).

5.4 Conclusions

Our objectives were:

- To determine whether *HH* can control blood glucose levels in diabetes animal models.
- To determine whether *HH* can increase pancreatic insulin secretion

The results of this experimental study indicate that a dose 800 mg/kg of *HHS* produces maximal hypoglycaemic effects in fasted normal and diabetic rats in our acute experiment. Also, *HH* in chronic doses has an antidiabetic activity as it lowers serum glucose levels in diabetic rats and significantly increases glucose tolerance. It also increases body weight of diabetic rats. We also found that *HH* treatment improved insulin secretion in pancreatic islet cells. Further studies are necessary to investigate the exact mechanism by which HH stimulates insulin secretion of pancreatic islet cells.

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