THE POSSIBLE EFFECT OF *HYPOXIS HEMEROCALLEDIA* (AFRICAN POTATO) ON BLOOD GLUCOSE LEVELS: AN *IN VITRO* STUDY

By

AMEL AHMED SWAYEB

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae, in the Department of Medical Bioscience, University of the Western Cape.

Supervisor: Prof. Daneel Dietrich.

Co supervisor: Prof. M de kock.

KEYWORDS

Blood glucose level

Diabetes

Glucose

Hypoxis hemerocalledia

Insulin secretion

Proliferation

RIN-5F (pancreatic tumour cell)

Viability

α- amylase

α- glucosidase

ABSTRACT

The plant *Hypoxis hemerocallidea*, also known as the African potato, is commonly used as a traditional medicine to treat diabetes in South Africa. The mechanism by which it lowers blood glucose levels is not known. The main aim of this research was to study the possible hypoglycemic effect of *HH* using RIN-5 F pancreatic tumor cells. To accomplish this, the study was divided into three parts: (1) to test whether exposure of RIN-5F cells to glucose and *HH* extract affect the cell proliferation and cell viability, (2) to test whether the *HH* extract have an effect on insulin secretion, and (3) to test whether the *HH* extract has an effect on alpha amylase and alpha glucosiadase enzyme activity.

The RIN-5F cells were exposed to different concentrations of glucose (5, 10, 20, 37.5, 50, 55, 74, and 92.3 mM) for different times (1, 3, 6 and 24 hours). The RIN-5F cells were also exposed to different concentrations of *HH* (50, 100, 150, 200 and 500 µ/ml) for different times (1, 3, 6 and 24 hours). Cell proliferation was evaluated using crystal violet staining and cell viability was evaluated using the XTT assay. To evaluate the effect of glucose and *HH* on RIN-5 F cell insulin secretion the cells were exposed to *HH* (100 µg/ml or 500 µg/ml) and / or glucose (2 mM or 50 mM) for 30 or 90 minutes. Insulin, α-amylase activity and α-glucosidase activity were evaluated by using commercially available colorimetric assays. Enzymatic activity in the presence of *HH* was compared with positive controls for α-amylase activity or α-glucosidase activity. Results are expressed as means ± SEM or median. Statistical differences among groups were analyzed by analyses of variance. $P < 0.05$ was considered as significant.

An increase in the cell viability and cell proliferation was found when RIN-5 F cells were exposed to high glucose concentrations and a high dose of *HH* extract for a short time period (1, 3 and 6 hours).When the cells were exposed to the *HH* extract over 24 hours, *HH* did not affect cell viability significantly.

Insulin secretion from RIN-5 F cells was increased when exposed to low glucose (2 mM) or high glucose (50 mM) for 30 minutes. Insulin secretion was increased from RIN5F cells after exposure to low *HH* (100 µg/ml) or high *HH* (500 µg/ml) for 30 minutes. Exposure of RIN5-F cells to *HH* for 90 minutes caused a further increase in insulin secretion from (4.3±0.17 mIU/mg protein; $P \leq 0.01$) in 100 µg/ml, to (7.87±0.17 mIU/mg protein; $P \leq 0.001$) in 500 µg/ml. At both 30 minutes and 90 minutes, insulin secretion was significantly higher when cells where exposed to 500 µg/ml *HH* compared to 100 µg/ml *HH*. Insulin secretion by cells exposed to 2 mM glucose + 100 µg/ml *HH* (4.69±0.16 mIU/mg protein; $P \leq 0.001$) was significantly higher than when exposed to 2 mM glucose only (2.27±0.17 mIU/mg protein), while the insulin secretion in 2 mM glucose + 500 µg/ml *HH* (2.56±0.17 mIU/mg protein; $P > 0.05$) was not significantly different from that in 2 mM glucose treated cells (2.27±0.17 mIU/mg protein). Similar results are obtained after 90 minutes. In the presence of high-glucose (50 mM), at both 30 minutes and 90 minutes, insulin secretion was significantly decreased when cells where exposed to low concentration of *HH* (100 µg/ml) and high concentration of *HH* (500 µg/ml).

The *HH* extract produced α-amylase enzyme inhibition. The maximum inhibition was at a concentration of 10µg/ml (922±117U/ml; $P \leq 0.01$). The 5 µg/ml concentrations failed to produce significant inhibition. The *HH* extract had significant α- glucosidase inhibitory activity at a concentration of 5µg/ml (0.12±0.3U/ml; $P \leq 0.001$) or 10µg/ml (0.13±0.3U/ml; $P \leq 0.001$).

In conclusion, based on its ability to inhibit α-amylase and α- glucosidase activity *HH* has the potential to be used in control of blood glucose levels. The *HH* aqueous extract increased insulin secretion under our basic experimental conditions and in the presence of low glucose levels, but not at high (50 mM) glucose concentrations. Insulin secretion in the presence of different glucose concentrations, in the presence of *HH*, needs further investigation. It is recommended that the ability of *HH* to stimulate insulin secretion be evaluated at 15-20 mM glucose.

DECLARATION

I declare that: The possible effect of *Hypoxis hemerocalledia* (African potato) on blood glucose levels an *in vitro* study is my own work, that has not been submitted before for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged as complete references.

Amel Ahmed Swayeb November 2015

Signed: امل أحمد سويب

DEDICATION

I dedicate this master's thesis to my brother (Mahmoud and Saleh).

I hope God (Allah) blesses them in the other life

ACKNOWLEDGEMENTS

In the name of God (Allah), the Most Beneficent, the Most Merciful

I make shukr to my Creator for presenting me the inner strength and ability to achieve my goals.

I would like to say jazakallah to my parents, Ahmed Swayed and Fatima Mohamed, and my brothers and sisters for their love affection, blessings, guidance, encouragement, support and sacrifices to make great life for me.

To my supervisor Prof. Daneel Dietrich and co-supervisor Prof. de.Kock, thank you for believing in me. I would also want to thank you for your continued patience and unconditional love. You guys are the best and trust me I wouldn't find any better supervisors in the world.

I would also want to say jazakallah to my husband (Mohamed), babies (Kadija, Abdullah, and Mahmoud) for all their support and understanding throughout my academic life. It is their love and continual encouragement that motivates me to do my best.

To Dr. Abdalla Elgenidi and his wife Dr. Asma Harrama, my friends, big THANKS for believing in and taking care of me. I am extremely grateful to Dr. Abdalla for providing me with the guidance and for being there for me in the lab.

To my colleague, Dr. Emhemed Aboukatala shukran for standing by me, you supported me in my post graduate experience.

To all my friends (Abeer, Monia, Fathiya, and Yasmin) thank you for always lending a helping hand in times when I needed it the most.

I would sincerely like to thank the Libyan Embassy\ South Africa for providing funds for this research. To the University of Western Cape and the staff, thank you.

I am eternally grateful to you all. Thank you for your support and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ADA	American diabetes association
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
APUD	Amine precursor uptake decarboxylation
Acetyl-CoA	Acetyl coenzyme A
BCA	Protein assay kit
Ca^{2+}	Calcium
cAMP	Cyclic adenosine 3',5cyclicmono phosphate
Cells/ml	Cells per millilitre
CHD	Coronary heart diseases
Cl^-	Chloride
CO_2	Carbon dioxide
DM	Diabetes mellitus
DMSO	Dimethyl sulphoxide
EC	Enzyme catalayse
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme-linked immune sorbant assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FBS	Foetal bovine serum
GIT	Gastrointestinal tract
GLUT1	Glucose transporter one

GLUT2	Glucose transporter two
HCl	Hydrochloride acid
HH	*Hypoxis hemerocalledia*
HHP	*Hypoxis hemerocallidea powder*
HIV	Human immune deficiency virus
IGF	Insulin growth factor
ILGF	Insulin-like growth factor
IRS-1	Insulin receptor substrate
IU	International unit
K^+	Potassium
Kg	Kilogram
L	Litre
mg	Milligram
mg/ml	Milligrams per millilitre
min	Minute
mM	Millimolar
M-PER	Mammalian protein extraction reagent
MRC	Medical research council
mU	Milliunit
NADH	Nicotinamide adenine dinucleotide hydrate
NPY	Neuropeptide Y
NS	Not significant
O_2	Oxygen
OD -	Optical density
PBS	Phosphate buffered saline
pg/ml	Picograms per millilitre
PP	Pancreatic polypeptide
RIN-5F	Pancreas islet tumor cell
RPMI	Roswell park-memorial institute
RT	Room temperature

SD	Standard deviation
STZ	Streptozotocin
TCA	Tricarboxylic acid
T1DM	Type 1diabetes
T2DM	Type 2 diabetes
TNFα	Tumour necrosis factor α
u/ml	Micromole per millilitre
WHO	World health organisation
WR	Working reagent
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide

Latin Abbreviations

α	Alpha
β	Beta
°C	Degrees Celsius
dl	Decilitre
g	Gram
µl	Microliter
µM	Micromole

Chapter I

1 Introduction

The blood glucose level is regulated as part of metabolic homeostasis (Young, 1977). The glucose in the plasma is regulated between 3.5-8.0 mM (Arciero *et al.,* 1999). Blood sugar levels outside the normal range may be an indicator of a medical condition. A persistently high level in blood glucose is referred to as hyperglycemia and low glucose levels are referred to as hypoglycemia (Colberg & Colberg, 2009; Lee *et al.,* 2005). Glucose transported from the intestines or liver to the body cells is made available for uptake by absorption cells and metabolism (Reddy, 2012).

The islets of Langerhans in the pancreas coordinates the secretion of glucagon and insulin (Lowell & Shulman, 2005; Quesada *et al.,* 2008). In the event that the blood glucose levels rise the β-cells import and oxidise glucose to support mitochondrial ATP synthesis. This process prompts a rise in the cytoplasmic ATP/ ADP ratio, which consequently induces the Ca^{2+} uptake from mitochondria followed by a release of insulin (Maechler *et al.,* 2010; MacDonald *et al.,* 2005). After its release the insulin ensures the uptake of glucose from sensitive tissues, metabolism and storage of glucose as glycogen or lipids. Insulin has the ability to counteract the effects of glucagon in the liver. This occurs by inhibition of gluconeogenesis and glycogenolysis. Low blood glucose levels prompt the secretion of glucagon which in turn induces glyconeogenesis and glucose mobilization in the hepatic tissue. This process then restores normoglycemia and at the same time inhibiting insulin action (Quesada *et al.,* 2008).

In the event that there is a disruption in the coordinated secretion of insulin and glucagon, it may lead to lasting pathological effects, including the development of type1 diabetes (T1DM) and type2 diabetes (T2DM). Both T1DM and T2DM are characterized by gradual failure and destruction of the β-cells. T2DM develops via a

complex interplay between genetics and environmental factors such as exposure to toxins, diet and alcohol consumption (Cnop *et al.,* 2005). T1DM is characterized by an auto-immune assault, which occurs with the invasion of the islets by mononuclear cells and induction of intra-islet inflammation and cell apoptosis (Knip *et al.,* 2005). Development of T1DM can also be facilitated by exposure to chemicals. Chemical substances such as alloxan and streptozotocin are often administered to selectively eradicate β cells and induce T1DM. The two chemical substances eradicate β cells by inducing oxidative stress and damage by disrupting pancreatic ion transport and calcium levels (Szkudelski, 2001).

The major source of blood glucose is dietary carbohydrates such as starch, which are hydrolyzed by α-glucosidases and pancreatic α-amylase, so as to be absorbed by the small intestine. Inhibitors of the enzymes could retard the uptake of dietary carbohydrates, which suppress postprandial hyperglycaemia. Alpha-glucosidase inhibitors such as acarbose, miglitol, and voglibose are known to minimize postprandial hyperglycemia primarily by interfering with the activity of carbohydrate-digesting enzymes and decrease glucose absorption (Maurus *et al*., 2005).

Hypoxis hemerocallidea (*HH*) is one of the most commonly used medicinal plants in South Africa (Nair *et al.,* 2007). It is used for the treatment of numerous ailments which include urinary tract infections, heart disease, infertility and anxiety (Brown *et al.,* 2008). Due to its usefulness in traditional medicine, H*ypoxis hemerocallidea* has attracted a lot of interest in scientific studies (Nair *et al.,* 2007). *Hypoxis* extracts contain phytosterols which are thought to be the main pharmacological ingredient responsible for its anti-lipidemic, anti-diabetic and anti-inflammatory properties (Boukes *et al.,* 2008). An aqueous extract of H*ypoxis hemerocallidea* was found to exhibit a significant reduction in the blood glucose levels of fasted normal and Streptozotocin (STZ) induced diabetic rats (Mahomed & Ojewole, 2003). It is thought that the blood glucose lowering effect observed is due to the presence of phytosterols and/or sterolin. The mechanism of the blood glucose lowering action is

still not well understood. Ojewole (2006) in a study in which an aqueous extracts of H*ypoxis hemerocallidea* were orally administered found anti-diabetic effects starting at 100 mg/kg in both normoglycaemic and hypoglycaemic rats.

1.1 The purpose of the study

The plant *Hypoxis hemerocallidea,* also known as the African potato, is commonly used as a traditional medicine to treat diabetes in South Africa. The mechanism by which it lowers blood glucose levels is not known. The purpose of this study is thus to explore some of the hypoglycemic effects of this plant.

1.1.1 Specific objectives

- To determine the toxicity of *Hypoxis hemerocallidea* and glucose on pancreatic RIN-5 F cell viability
- To determine whether *Hypoxis hemerocallidea* can increase insulin secretion from the pancreatic RIN-5 F cells.
- To determine whether *Hypoxis hemerocallidea* can inhibit the activity of α-amylase and α -glucosidase.

Chapter II

2 Literature review

The normal range of glucose in the plasma is regulated between 3.5-8.0 mM (Arciero *et al.,* 1999). The level of the sugar in the blood outside the normal range may be indicative of a medical condition. A persistently high level in blood glucose is referred to as hyperglycemia and low glucose levels are referred to as hypoglycemia (Colberg & Colberg, 2009; Lee *et al.*, 2005). Glucose levels are usually lowest in the morning before the first meal of the day and rise after meals for an hour or two by a few millimolar (Colberg & Colberg, 2009; Reddy, 2012). Glucose transported from the intestines or liver to the body cells is made available for uptake by absorption cells and metabolism (Reddy, 2012).

Two hormones secreted from the pancreas are mainly responsible for blood glucose regulation, insulin and glucagon. Ingestion of carbohydrate elicits a rapid rise in insulin concentration and a reduction in glucagon concentration. The increase in insulin concentrations happen before a rise in the blood glucose concentration and is thought to be mediated largely by hormonal signals arising in the digestive system (Aronoff et al., 2004; Kuznetsov, 1978). Glucagon is considered as a hyperglycemic hormone which work on the liver to elevate hepatic glucose production when blood glucose levels drop.

2.1 The Pancreas and blood glucose level control

The pancreas is a glandular organ in the upper part of the abdomen, but serves as two glands in one, a digestive exocrine gland and a hormone-producing endocrine gland. Working as an exocrine gland, the pancreas secretes enzymes to digest the lipids, proteins and carbohydrates in food (Olubummo, 2010). It weighs between 70 g and 110 g (Gromada *et al.*, 2007; Nobukawa, 2007). The pancreas is attached to the

duodenum, the first part of the small intestine via the common pancreatic bile duct. The gland is also extended towards the spleen (Figure 2.1.),

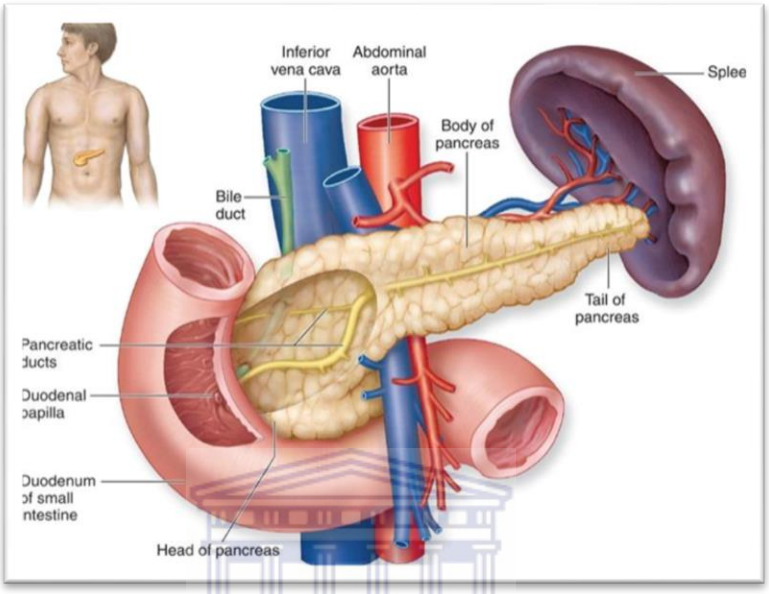


Figure 2.1. Diagram showing the front view of the pancreas (From Nobukawa, 2007).

The Langerhans islets (Figure 2.2.) consist of insulin releasing β-cells (65-90 %) forming the core of the islet, glucagon-releasing alpha-cells (15-20 %) (Zhou et al., 2011), pancreatic polypeptide cells producing PP-cells (1 %) and somatostatin-producing delta-cells (3-10 %) (Elayat et al., 1995). β-cells, which couple nutrients metabolism with electrical activity to modulate the produce and release of insulin, have been studied most frequently. The islets of Langerhans play an important role in glucose metabolism and organize of blood glucose concentration (Jo et al., 2007).

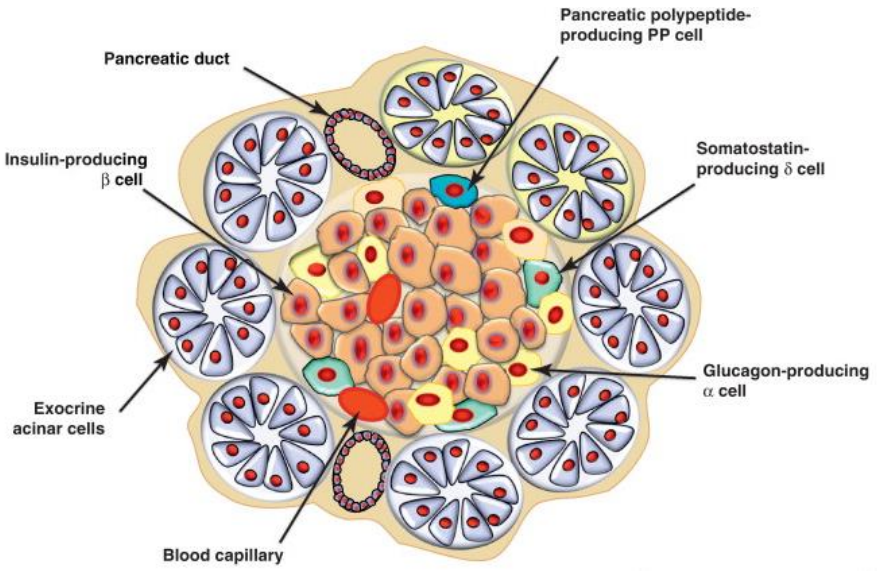


Figure 2.2. Diagram showing the schematic representation of islets of Langerhans embedded in the exocrine parenchyma (From Jo *et al*., 2007).

2.1.1 Pancreatic hormones

2.1.1.1 Insulin

The effective action of insulin is to decrease blood glucose level in response to elevate blood glucose after a meal. β- cells can respond quickly to spikes in blood glucose levels by excreting some of its stored insulin while simultaneously generating more of the hormone (Wilcox, 2005).

2.1.1.1.1 Insulin structure

The structure of human insulin, as shown in Figure 2.3, consists of 51 amino acids forming two polypeptide chains (A and B) (Lotfy, 2012). The smaller A-chain consists of 21 amino acids while the B-chain consists of 30 amino acids. The two chains are attached by two disulphide links between cysteine amino acids. Similar disulphide links are found between cysteine amino acids within the A-chain. The

purpose of these disulphide links is to give the insulin molecule its three-dimensional structure which guarantees the precise physiological function of insulin (Weise *et al.*, 2009).

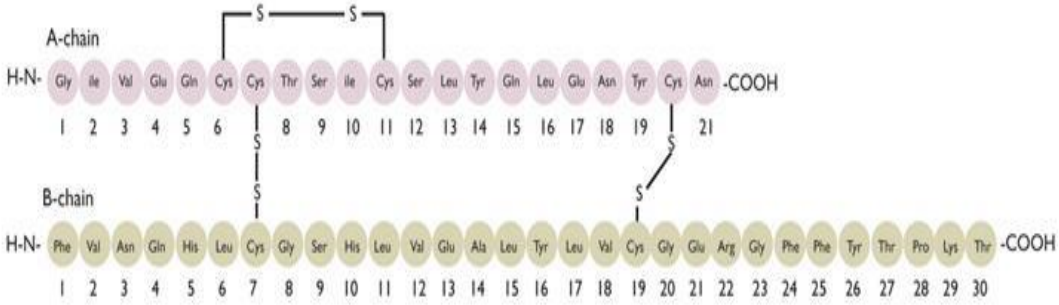


Figure 2.3. Diagram showing the structure of human insulin A-chain and B-chain (From Lotfy, 2012)**.**

2.1.1.1.2 Insulin synthesis

The two polypeptide chains, A and B, are not created as a single polypeptide chain but are formed by proteolytic processing of a bigger precursor, proinsulin (Steiner, 2004). Transcription of the insulin gene results in a mRNA which is translated in the ribosome to produce a pre-proinsulin molecule. Removal of its signal peptide during insertion into the ER generates proinsulin. This molecule then undergoes chain folding and disulphide bond formation, to form an enclosed microvesicle which is transported to the Golgi apparatus where it is packaged into storage granules (Wicksteed *et al*., 2001). The proinsulin in the Golgi apparatus undergoes proteolysis to form the mature insulin. Insulin and free C peptide, packaged in the Golgi apparatus into secretory granules, accumulate in the cytoplasm (Xu et al., 2003). These secretory granules include mainly mature insulin (and C peptide) with minor quantity of unconverted proinsulin (Wicksteed et al., 2001; Wilcox, 2005).

The prime signal in insulin synthesis by β-cells is the blood glucose concentration. Hypoglycemia, (2-4 mM glucose), reduces the synthesis of proinsulin, while blood glucose concentration of 4-6 mM enhances the synthesis of proinsulin (Steiner et al., 2009; Brandenburg, 2008).

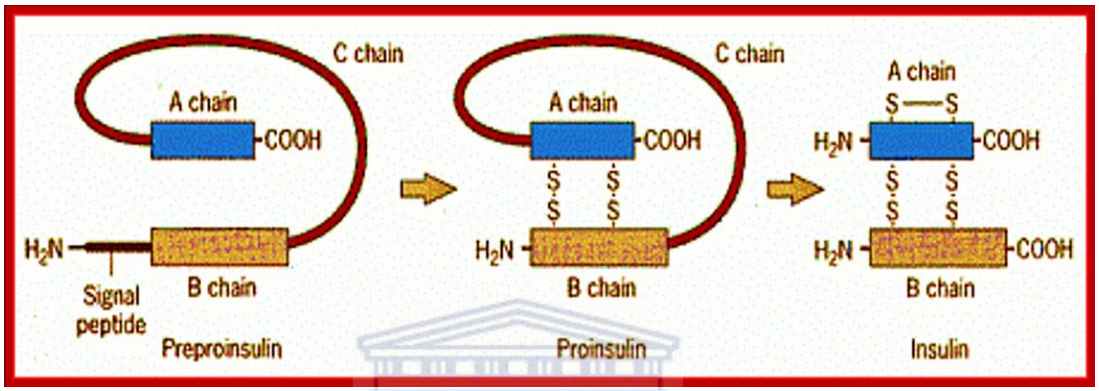


Figure 2.4. Diagram showing the conversion of proinsulin to signal peptide, insulin,C-peptide, and four basic amino acids and four basic amino acids (From Lotfy, 2012).

2.1.1.1.3 Insulin secretion

When the beta cell is appropriately stimulated, insulin is secreted from the cell by exocytosis. C peptide is also secreted into the blood but has no biological activity. Insulin secretion can be divided into two phases; the first phase of insulin releases the K_{ATP} channel dependent insulin secretion (Figure2.5). In humans the glucose is taken up into b-cell via glucose transporter one (GLUT1) but in mice by glucose transporter two (GLUT2) (Hiriart and Aguilar-Bryan, 2008). Glucose is then metabolised by glycolysis and the tricarboxylic acid (TCA) cycle (Herman and Kahn, 2006). Both pathways provide substrates (FADH2 and NADH) for the respiratory chain within mitochondria to produce ATP. ATP is moved into the cytoplasm, where it binds to the Kir6.2 subunit of the KATP – channel (Klingenberg, 2008). The binding of ATP causes channel closure, resulting in the depolarisation of the plasma membrane. The

depolarisation triggers the opening of the L-type voltage-gated Ca^{+} channels, which leads to the influx of Ca^{2+} ions. Calcium triggers the exocytosis of insulin secretory vesicles and results in the release of insulin from beta cells (Straub and Sharp, 2002). As Ca^{+} uptake and insulin release are dependent on potassium channel closure by ATP, this corridor is called the KATP channel dependent insulin secretion pathway.

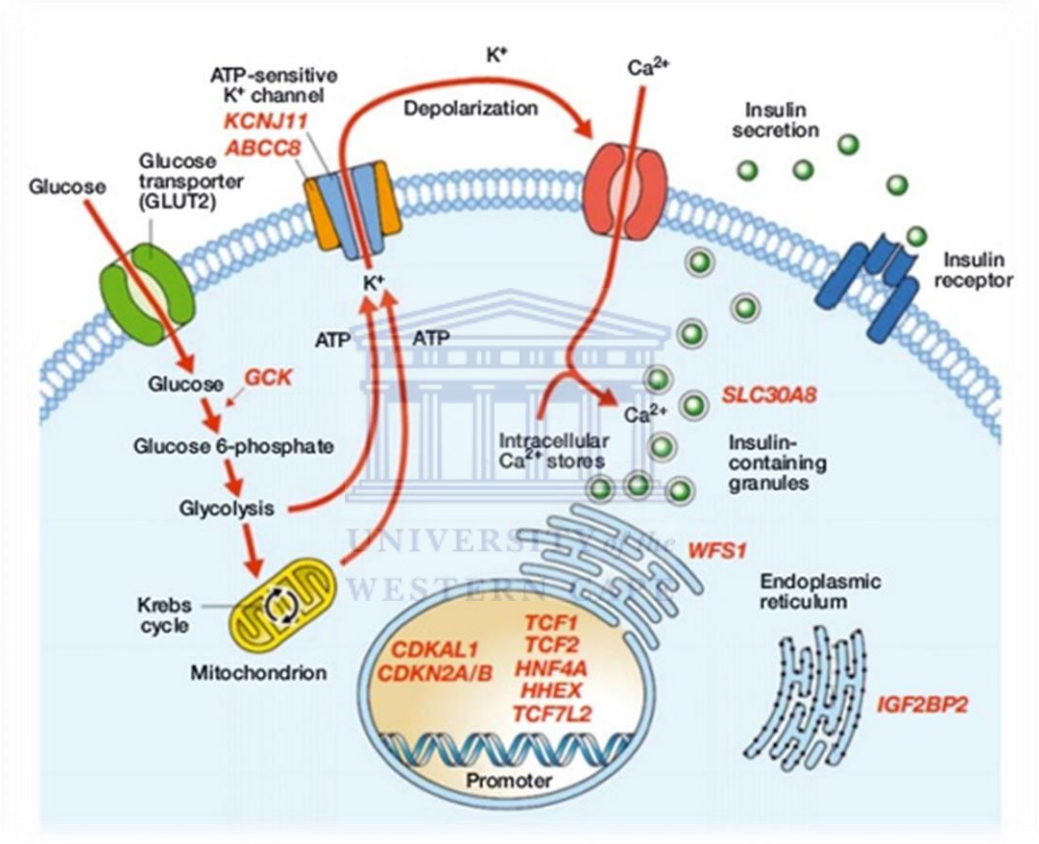


Figure 2.5. Diagram showing the glucose stimulated first-phase insulin release the beta cells (From Maechler &Wollheim, 2000).

.

The second phase of insulin secretion is called KATP -channel independent insulin secretion, as insulin secretion occurs despite the potassium channel being open or otherwise dysfunctional (Tengholm and Gylfe, 2009). The second phase involves augmented insulin release in response to elevated intracellular calcium levels. This

mechanisms are still unknown but are thought to involve changes in actions and concentrations of cAMP, phospholipase C and plasma membrane phosphoinositides (Straub and Sharp, 2002; Tengholm and Gylfe, 2009). Poorly defined metabolic coupling pathways such as generation of mitochondrial NADH, malonyl-CoA and cytosolic long chain-CoA esters are also required (Prentki *et al*., 1997).

2.1.1.2 Glucagon

Glucagon is a polypeptide hormone consisting of a single polypeptide containing 29 amino acids. It is secreted by the alpha cells on the islets of Langerhans.The cells surround a core of insulin secreting beta cells (see Figure 2.2), reflecting a close relationship between the hormones insulin and glucagon. Its function is to maintain and increase blood glucose levels when needed. This is achieved by stimulating glycogenolysis in the liver, promoting gluconeogenesis and by decreasing glucose utilisation by the liver, so glucose can be secreted into the bloodstream (Nonogaki, 2000).

2.1.1.2.1 The interaction between insulin and glucagon

Insulin and glucagon have antagonistic actions on substrate fluctuations, which are controlled by the insulin-glucagon ratio. The normal molar ratio is 2:1. It is converted to 1:2 when there is a demand for mobilization of endogenous substrates (Adkins-Marshall *et al.,* 1990). A decrease in insulin release and the increase in glucagon release can lead to glycogenolysis and gluconeogenesis in the liver and lipolysis in adipose tissue. After a meal, the insulin-glucagon ratio is elevated to about 10:1, mainly due to enhanced insulin release. This leads to activation of glucose uptake associated with glucose oxidation and conversion of glucose to glycogen in either liver or muscle (Lotfy, 2012). The pancreatic endocrine action is controlled through many factors including glucose. This control involves the parasympathetic nervous

system that enhances insulin and glucagon release, while the sympathetic pathway enhances glucagon uptake but reduces insulin release (Nonogaki, 2000).

2.1.1.3 Somatostatin

Pancreatic somatostatin is a neuropeptide consisting of 14 amino acids (SS14) (Figure 2.6). Somatostatin is manufactured in the form of prosomatostatin by δ-cells of islets of Langerhans, but is also synthesized by intestinal cells. Somatostatin (SS14) strongly decreases the secretion of glucagon, insulin and pancreatic polypeptide (Muroyama *et al.*, 2004). Somatostatin secretion is encouraged by insulin when blood glucose levels are high. Somatostatin inhibits insulin synthesis and secretion, thus insulin can prevent its own release by stimulating somatostatin secretion (Lotfy, 2012).

Generally, it appears that there is a negative feedback relationship of somatostatin with either glucagon or insulin (Muroyama *et al.*, 2004). Somatostatin may be an additional treatment of diabetes mellitus through its suppressing effect on glucagon release (Muroyama *et al.*, 2004).

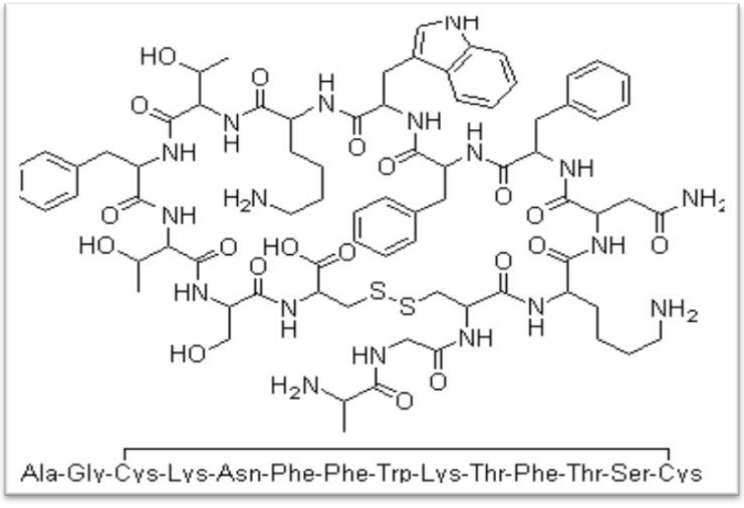


Figure 2.6. Diagram showing the structural features of somatostatin (From Conlon *et al*., 1988).

2.1.1.4 Pancreatic polypeptide (PP)

Pancreatic polypeptide (PP) is a peptide consisting of 36 amino acids secreted and produced by PP cells of the pancreas which are primarily located in the islets of Langerhans, which is part of a family of peptides that also includes neuropeptide Y (NPY) and peptide YY (Lotfy, 2012). The essential function of PP is to reduce the release of pancreatic bicarbonate and proteins (Clark *et al.*, 1984; Tatemoto *et al.*, 1982). After a meal, the secretion of PP is stimulated by the decrease in blood glucose level (Lotfy, 2012). The PP release is stimulated by the vagal cholinergic reflex. Accordingly, PP may induce vagal stimulation of the pancreas and many organs in the gut. The PP secretion after a meal leads to reduction of food ingestion via decreasing the rate of stomach-emptying (Lotfy, 2012).

2.2 Diabetes Mellitus (DM)

Diabetes Mellitus is one of the pancreatic diseases defined as a chronic disease that occurs when the pancreas does not produce sufficient amount of insulin, or when the body does not effectively use the insulin it produces (i.e. when the cells become resistant to insulin, insulin resistance). Insulin mresistance (IR) may occur at the level of the insulin receptor or at the level of downstream signalling (Wilcox, 2005). Hyperglycemia is a common sign of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels (Sugihara *et al.*, 2008).

Diabetic prevalence for all age-groups across the world has been found to be 2.8 % in 2000 and an estimated to increase to 4.4 % in 2030 (Wild *et al.*, 2004). This is further supported by a study finding which speculate that the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Deshpande *et al*., 2008). Men have a higher diabetic prevalence compared to women. There are however more women with diabetes than there are men (Wild *et*

al., 2004). In 1980, the World Health Organisation (WHO 2003) classified diabetes into type 1 diabetes mellitus (T1DM), an auto-immune disease resulting in the destruction of pancreatic β-cells and type 2 diabetes mellitus (T2DM). A strong association exists with obesity occurs as a result of failing pancreatic β-cell function, responsible for the production of insulin, often alongside insulin resistance.

Butler et al (2003) predicted that a 50% decrease in β-cell function in the presence of IR may causes hyperglycaemia. When hyperglyceamia develops, glucose itself leads to loss of glucose stimulate-insulin release and impaired glucose utilisation (glucose toxicity), which results in an exacerbation of the disease.

Persistent high blood glucose levels could result in the glycosylation of proteins which can lead to the irreversible damage as present in diabetes, of tiny blood vessels and nerve endings especially in the areas of from the heart, retina and renal glomerulus (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. Around 75 % of macrovascular diabetic mortality is due to coronary heart disease (Deshpande *et al*., 2008).

2.3 Carbohydrate digestion

Carbohydrates are organic compounds, including starches and sugars that serve as a major energy source for the body (Huber *et al.,* 2005; Zhang *et al.,* 2010). Carbohydrate digestion starts in the oral cavity with salivary amylase and then continues in other parts of the gastrointestinal tract, particularly in the small intestines (Roberfroid, 2004; Huber *et al.*, 2005).

Most of the digestion enzymes are hydrolases which are secreted by the salivary glands and gastric glands. The digestive enzymes act similar to the lysosomal

enzymes, but at different pH optima (Thornhill *et al.,* 2008). Lysosomal enzymes are generally active at acidic pH while the digestive enzymes, except pepsins, have their activity optima at a pH of 6.5 to 7.5. Many digestive enzymes have trivial, but functional names, such as trypsin and pepsin which were used before the systematic terminologies were developed. Table 2.1 shows the sources, activators, substrates, actions and end products of the enzymes of digestion (Deguara *et al.,* 2003; Gawlicka *et al.,* 2000).

Table 2.1. The sources, activators, substrates, actions and end products of the enzymes of digestion reproduced from (Arthur & Gruner, 2007).

Source	Enzyme	Activator	Substrate	Action	Products
Salivary glands	Salivary α-amylase (ptyalin)	Cl-	Starch	Hydrolyzes 1:4α linkages	α-Limit dextrins, maltoriose, and maltose
Lingual Glands	Lingual lipase		Triglycerides		Fatty acids and 1,2 diacylgliserols
Stomach	Pepsins pepsinogens	HCl	Proteins and polypeptides	Cleave peptide bonds adjacent to aromatic amino acids	Proteoses, peptons and polypeptides
	Gasrtic lipase		Triglyserides	Lipolysis	Fatty acids and glycerol
Pancreas	Endopptidases Trypsin (Trypsinogen)	Enterokinase and trypsin	Proteins and polypeptides	Cleaves peptide bonds adjacent to arginine or lysine	Polypeptides and amino acids
	Chymotrypsins (chymotrypsinogens)	Trypsin	Proteins and polypeptides	Cleaves peptide bonds adjacent to arginine or lysine	Polypeptides and amino acids
	Carboxypeptidase A (procarboxypeptidase A)	Trypsin	Proteins and polypeptides	Cleaves carboxyterminal amino acids that have aromatic or bramched aliphatic side chains	Polypeptides and amino acids
	Carboxypeptidase B (procarboxypeptidase B)	Trypsin	Proteins and polypeptides	Cleaves carboxyterminal amino acids that have basic side chains	Polypeptides and amino acids
	Colipase (procolipase)	Trypsin	Fat droplets	Facilitates exposure of active site of pancreatic lipase	
	Pancreatic lipase		Triglycerides	Lipolysis	Monoglycerides and fatty acids

	Cholesteryl ester Hydrolase		Cholesteryl esters		Cholesterol and fatty acids
	Pancreatic α-amylase	CL-	Starch	Hydrolyzes 1:4α linkages	α-Limit dextrins, maltoriose, and maltose
	Phospholipase A2 (Prophospholipase A2)	Trypsin	Phospholipids		Fatty acids, Lysopholipids
Intestinal mucosa	Dipeptidase		Dipeptides		Amino acids
	Maltase		Maltose, maltotriose		Glucose
	Lactase		Lactose		Galactose and glucose
	Sucrase		Sucrose		Fructose and glucose
	α-Limited dextrinase		A-Limit dextrins		Glucose
Cytoplasm of mucosal	Various peptidases		Di, tri, and tetrapeptides		Amino acids

2.4 The digestion enzymes

The digestive enzymes α- amylase and α-glucosidase assist in the breakdown of complex carbohydrates (oligosaccharides and starch) in the digestive tract, forming simple sugars such as glucose, sucrose and maltose (Dong *et al.,* 2012). One of the important treatments for high postprandial glucose levels is to delay the absorption of glucose by using using inhibitors of the hydrolysing enzymes α- amylase and α-glucosidase.

2.4.1 Alpha-amylase

Alpha-amylase (EC 3.2.1.1) is a digestive enzyme that hydrolyses alpha -1, 4 bonds of large polysaccharides such as starch and glycogen by converting them into smaller products which are glucose and maltose. Alpha-amylase is widespread among living organisms (Maurus *et al.,* 2005). The primary sequences of α-amylases in different species show poor serial homology, although the three-dimensional structures of the enzymes are found to be conserved in organisms ranging from microbial to mammalian (Janeček *et al.,* 2014; Kandra, 2003). Striking amongst the structural similarities is the existence of three domains in the enzyme structure. Domain A consists of an α/β keg and contains the active site, domain B protrudes from the side of domain A and consist of the calcium binding site, and domain C forms a structurally independent antiparallel β-barrel (Maurus *et al.,* 2005; Kandra, 2003). The identical structural features of human pancreatic α-amylase are shown in Figure 2.7.

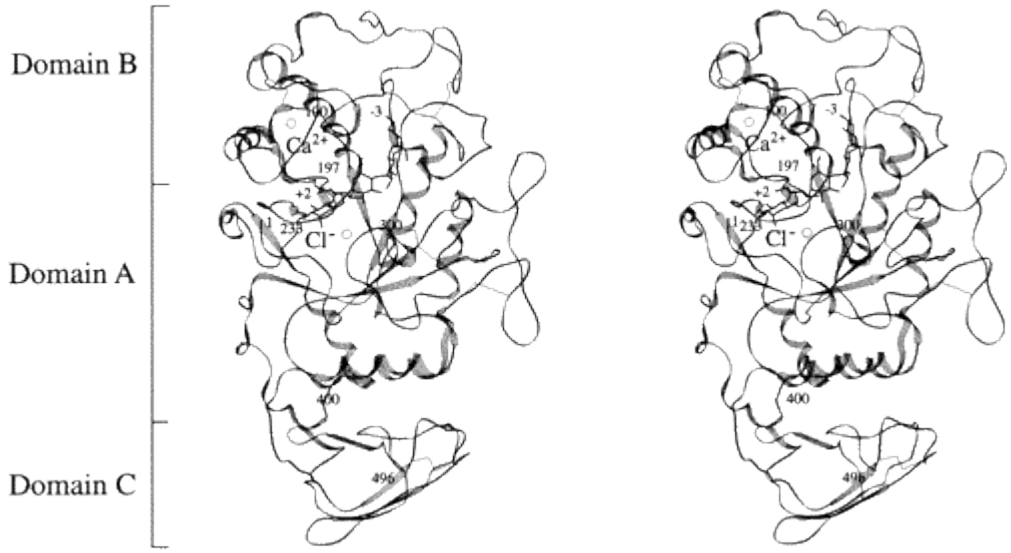


Figure 2.7. Diagram showing the structural features of human pancreatic α-amylase (From Maurus *et al.,* 2005).

Alpha-amylase begins the process of starch digestion. It takes starch chains and breaks them into short saccharides with two or three glucose units. Two similar types of amylase are made in the human body. Salivary amylase is synthesized in the serous acinar cells of the saliva glands. It is then stored in secretory granules inside the cells and then released from the salivary cells, especially with an increase in response to taste or chewing motions of the jaw (Maurus *et al.,* 2005). It starts the breakdown of starch into smaller pieces when food is chewed. The second amylase and pancreatic amylase, are secreted by the pancreas, completes the digestion of carbohydrates. The saccharides are broken into individual glucose units by a collection of enzymes that are tethered to the walls of the intestine (Yilmazer-Musa *et al.,* 2012; Lordan *et al.,* 2013).

2.4.2 Alpha-Glucosidases Enzyme

Alpha-glucosidase is an enzyme that breaks down starch and disaccharides to glucose and maltose (Lu & Sharkey, 2006), and is also called alpha-1, 4-glucosidase EC 3.2.1.20 (Figure 2.8) (Woo & Wynne, 2011) which is located in the brush border of the small intestine and acts upon 1,4-alpha bonds (Sun & Henson, 1990).

Alpha-Glucosidase is involved in carbohydrate breakdown. It plays a crucial role in diabetes and also in viral infections and cancer. Alpha-glucosidase delays the hydrolysis of carbohydrates and alleviate postprandial hyperglycemia (Hemmerich, 2001; Kumar *et al.*, 2011)

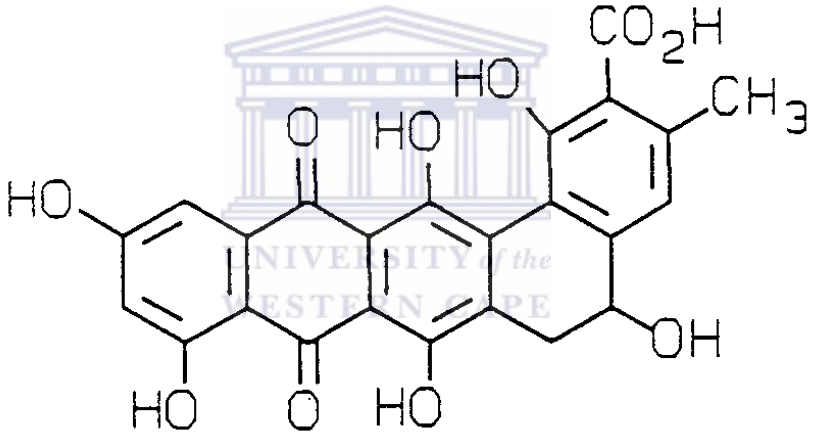


Figure 2.8. Diagram showing the alpha-glucosidase chemical structural (From Woo & Wynne, 2011).

2.5 Glucose metabolism

Once glucose has been transported into the beta cells it enters glycolysis in the cytoplasm where it is metabolised to pyruvate (Herman and Kahn, 2006). Pyruvate enters mitochondrial matrix where it acts as a substrate for pyruvate carboxylase, which converts pyruvate to oxaloacetate, and for pyruvate dehydrogenase, which converts it to acetyl-CoA (Herman and Kahn, 2006). Both oxaloacetate and acetyl-

CoA are substrates for the TCA cycle that provides NADH and FADH2 for the mitochondrial respiratory chain (Wollheim and Maechler, 2002).

2.6 Mitochondrial function in beta cells

Mitochondrion is double-membrane organelles with multiple essential cellular functions. The main function of this to produce energy in the form of ATP from acetyl CoA derived from, fats, carbohydrates and proteins (Dimmer *et al*., 2002). ATP is the universal currency of energy in the cell. It is an energy-rich molecule because it contains two phosphoanhydride bonds. Energy is released when these bonds are broken. ATP can be synthesized from adenosine diphosphate (ADP) by two processes, which are substrate-level phosphorylation and oxidative phosphorylation. Substrate level phosphorylation is the process by which ATP is formed from the direct phosphorylation of ADP (Klingenberg, 2008). All of these processes related to cell energy production and utility, therefore, mitochondrion is the power plant of cells. The primary or secondary alterations in mitochondria related signalling pathways could be explained multiplicity of organelle functions and variability in the pathophysiology (Dimmer *et al*., 2002; Klingenberg, 2008). Around 90% of cellular ATP is generated by oxidative phosphorylation, a process via which ATP is formed as electrons are transferred from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) to molecular oxygen, by a series of electron carriers that make up the electron transport chain (ETC) (Wollheim and Maechler, 2002). Carbohydrates are converted to pyruvate through glycolysis, which is held in the cytosol. Pyruvate is subsequently actively transported across the inner mitochondrial membrane, and into the mitochondrial matrix where it is oxidised and changed it to acetyl CoA by pyruvate dehydrogenase. It is well established that mitochondrial function is required for normal glucose-stimulated insulin secretion from pancreatic b cells. In addition, maternally inherited deformities in mitochondrial DNA that disturb mitochondrial function are known to cause an insulin-deficient form of diabetes like T1DM (Lowell and Shulman, 2005).

2.7 Medicinal plants used to control blood glucose level

Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, the search for newer drugs continues because the existing synthetic drugs have several limitations. Traditional treatments are prescribed by practitioners of alternative medicine or sometimes taken by patients as supplements to conventional therapy. Hypoglycemic action of some traditional treatments has been confirmed in animal models and T2DM (Arumugam *et al.,* 2013).

Herbal medicine plants having anti diabetic properties could be a useful source for the development of safer and effective oral hypoglycemic products. More than 400 traditional plants are used in the treatment of diabetes mellitus. The herbal drugs with antidiabetic activity are yet to be commercially formulated as modern medications; moreover, they have been acclaimed for their therapeutic medicinal properties in the traditional systems (Arumugam *et al.,* 2013). Only a small number of these have received the scientific and medical evaluation to estimate their efficacy. Traditional treatments may provide valuable clues for the development of new oral hypoglycemic agents and simple dietary adjuncts (Arumugam *et al.,* 2013). Table 2.2 shows the most important medicinal plants used for the control of blood glucose level (Kavishankar, 2011).

Table 2.2. Most important medicinal plants having antidiabetic activity. Reproduced from (Arumugam *et al.*, 2013).

Plant name	Family	Parts used	Type of extract	Activity
Alangiumlamarckii	Alangiaceae	Leaves	Alcoholic	Antidiabetic
Albiziaodoratissima	Mimosaceae	Bark	Methanol	Antidiabetic
Axonopuscompressus	Poaceae	Leaves	Methanol	Antidiabetic
Berberis vulgaris	Berberidaceae	Root	Aqueous	Hypoglycaemic
Brassssicajuncea	Cruciferae	Seed	Aqueous	Hypoglycemic
Caesalpiniadigyna	Fabaceae	Root	Methanol	Antidiabetic
Catharanthusroseus	Apocynaceae	Leaf	Methanol	Hypoglycemic
Centauriumerythrea	Gentianaceae	Leaf	Aqueous	Antidiabetic
Chaenomelessinensis	Rosaceae	Fruits	ethyl acetate	Antidiabetic
Cocosnucifera	Arecaceae	Leaf	hydro-methanol	Antihyperglycemic
Costusspeciosus	Costaceae	rhizome	hexane	Antidiabetic
Cyclocaryapaliurus	Cyclocaryaceae	Bark	Aqueous, PE, chloroform, ethyl acetate &n-butanol	Hypoglycemic
Dilleniaindica	Dilleniaceae	Leaves	Methanolic	Antidiabetic
Embeliaribes	Myrsinaceae	Berries	Hexane	Antidiabetic
Hybanthusenneaspermus	Violaceae	Whole plant	Alcholic	Antidiabetic
Lippanodiflora	Verbenaceae	Whole plant	Methanol	Antidiabetic and Hypolipidemic
Lithocarpuspolystachyus	Fagaceae	Leaves	Ethanol & Aqueous	Hypoglycemic
Marrubiumvulgare	Lamiaceae	Aerial part	Methanol	Hyperglycmia And dyslipidemia
Ocimum sanctum	Lamiaceae	Aerial	part	HydroalcholicAntidiabetic

Opuntiastreptacantha	Cactaceae	Leaves	Ethanol	Antihyperglycemia
Psidiumguajava	Myrtaceae	Fruits	Ethanol	Antihyperglycemic
Semecarpusanacardium	Anacardiaceae	nut	Milk	Antidiabetic
Prosopisglandulosa	Fabaceae	Whole plant	Gelatine/Jelly	Antidiabetic
Ophiopogonjaponicus	Asparagaceae	Root	Ethanol	Hypoglycemic
Setariaitalica	Poaceae	Seed	Aqueous	Antihyperglycemic
Solanumtorvum	Solanaceae	Fruits	Methanol	Antihyperglycemic
Cassia auriculata	Caesalpiniacae	Leaves	Aqueous	Antihyperglycemic
Zygophyllum album	Zygophyllaceae	Whole plant	Ethanol	Antidiabetic
Vitexnegundo	Lamiaceae	Leaves	Methanol	Antihyperglycemic
Viscumschimperi	Viscaceae	aerial parts	Methanolic	Antihyperglycemic &Hypolipidaemic
Symplocoscochinchinensis	Symplocaceae	Leaves	Hexane	Antidiabetic
Enicostemmalittorale	Gentianaceae	Whole plant	aqueous	Antidiabetic
Vacciniumarctostaphylos	Ericaceae	Fruit	Ethanolic	antidiabetic
Solanumxanthocarpum	Solanaceae	Leaves	Aqueous and Methanol	Antihyperglycemic

2.8 Hypoxis hemerocallidea (*HH*)

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 2.9 (Nair, 2006; Nair & Kanfer, 2006).

Figure 2.9. Diagram showing the photograph of a Young Corm of *HH*, Flowers are symmetrical with 6 petals, which are bright yellow, giving the plant its common name Yellow Stars (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 corms (450 g) are at least three years old, whereas the large corms (800 g) are older(Nair, 2006). The fleshy corms of *HH* are yellow in colour on the inside (Figure 2.10), but soon turn to dark brown due to oxidation, when exposed to air (Nair, 2006). The uses of this plant can be attributed to a few of the medicinal compounds found in the plant of which hypoxoside, sitosterol, and its aglycone derivative rooperol are the most well-known compounds (Street & Prinsloo, 2012).

Figure 2.10. Diagram showing the photograph of the Transection of a Corm of *HH* (From Nair, 2006).

2.8.1 Vernacular names

The family name of *Hypoxis hemerocallidea* is Hypoxidaceae. The most common names for *HH* are Star Lily, Yellow Stars, Afrika-patat, Sterretjie, African Potato, Inkomfe and Ilabatheka (Street and Prinsloo, 2012).

2.8.2 Geographical distribution

In South Africa, the genus is distributed in five provinces, namely, Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga and Gauteng but it is also found in Lesotho, Swaziland, Mozambique, and Zimbabwe (Singh, 2007). There are reports of its existence in various other countries including Madagascar, New Zealand, Malawi, America, Mauritius and other Central Africa countries (Mogatle, 2008).

2.8.3 Botanical classification

The particular nomenclature for Hypoxis depends on leaf figurers, leaf venation, flower figurers and inflorescence (Singh, 2007). In Africa there are 30 types of Hypoxis. Most of these kinds tend to become common endemic to southern Africa. Hypoxia hemerocallidea found in the Eastern Cape region of South Africa (Mogatle, 2008). Most of the species are arranged under one of names are presented below:

- *Hypoxis angustifolia var buchananii.*
- *Hypoxis argenta var sericea.*
- *Hypoxia colchicifolia.*
- *Hypoxia floccose.*
- *Hypoxis hemerocallidea.*
- *Hypoxia longifolia.*
- *Hypoxis obtuse.*
- *Hypoxia ridicule.*
- *Hypoxis ridigula var pilosissima.*

2.8.4 Traditional forms

Hypoxis hemerocallidea have been used traditionally for a wide range of purposes by different tribes of Southern Africa. They have been used by the Zulu tribe as an

internal parasiticide, purgative and to treat delirium. The Maniyaka tribe applied the ash to treat wounds, whereas the Karanga tribe used it as a remedy for bilious vomiting, anorexia, abdominal pain and fever (Botha & Penrith, 2008).

The corm of the plant has been used in folk medicine to treat a variety of diseases which include the common cold, flu, hypertension, adult-onset diabetes, psoriasis, urinary tract infections, testicular tumours and prostate hypertrophy. *HH* is used to build up the immune system of patients suffering from HIV/AIDS, anticancer, antidiabetic, antimicrobial, antioxidant, and anti-inflammatory treatment (Mogatle, 2008). Table 2.3 shows the most important traditional uses of *HH*.

Table 2.3. Traditional uses of *HH* reproduced from (Mogatle, 2008).

Reported Use	Part of plant	Preparation	Area	Reference
Diabetes	Corms	Fresh corms boiled and taken orally	Eastern Cape South Africa	(Erasto *et al*., 2005)
Prostate Hypertrophy	NS*	NS*	Maputo Mozambique	(Mills *et al*., 2005)
Urinary or venereal diseases	NS*	NS*	KwaZulu Natal South Africa	(Louw *et al*., 2002)
Cancer	Corms	Pulverised corms are boiled And taken orally	Eastern Cape Southern Africa	(Koduru *et al*., 2007)
Wound management	Leaves corms	Dry powder sprinkled on wounds Fresh or dried material extracted and used as wash	Eastern Cape South Africa	(Grierson and Afolayan, 1999)
HIV/AIDS management	NS*	NS*	Johannesburg, South Africa	(Babb *et al*., 2007)

*NS – not state.

2.8.5 Aspects of the use, pharmacology and phytochemistry

HH contains many chemical constituents of which hypoxoside, rooperol, and the phytosterol, β-sitosterol are the main ones (Muwanga, 2006; Boukes and van de Venter 2011; Sathekge, 2011). These chemical agents contribute to the wide array of pharmaceutical properties that extracts of *HH* provides (Owira and Ojewole, 2009).

Hypoxoside is the trivia name for (E)-1, 5 bis (4′-B-D-glucopyranossyloxy-3′-hydroxyphenyl) pent-1-en-4-yne, a norlignan diglucoside from the rootstock of the family Hypoxidaceae. Phytosterols such as $\beta\beta$-sitosterols is effective against benign prostate hypertrophy, and a decoction of the rootstocks is also used as a laxative (Mills, Cooper, *et al.*, 2005; Steenkamp, 2003). Rooperol could be acquired by treating hypoxoside with a $\beta\beta$-glucosidase to remove the attached glucose groups. In addition to hypoxoside and rooperol the rootstocks also consists of $\beta\beta$-sitosterols, monterpene glycosides, stigmastanols, and stanols (Street & Prinsloo, 2012). The chemical analysis of *HH* showed that the species have various classes of secondary metabolites namely saponnins, glycosides, polyphenols, steroids and tannins. The rootstock of *HH* has produced three cytokinins identified as zeatinriboside, zeatinglucoside and zeatin (Street & Prinsloo, 2012).

2.8.6 Toxicity

A lethal dose (LD50) in mice was found to be 1948 ± 57 mg/kg of *HH* aqueous extract after oral administration (Ojewole, 2006). A lower dose (≤ 1600 mg/kg) of *HH* aqueous extracts is safe while relatively higher doses were toxic and/or lethal to the mice (Ojewole, 2006). A clinical trial was conducted involving 24 cancer patients at Karl Bremer Hospital, Bellville, South Africa (Smit *et al.*, 1995), where the toxicity of hypoxoside, which was administered in the form of a hypoxis plant extract, was assessed. The patients were given 1200 - 3200 mg standardised hypoxis plant extract (50-55% hypoxoside content) /day, in three divided doses. Based on

haematological and biochemical tests it was concluded that the dose administrated was not toxic (Mogatle, 2008).

2.8.7 Registered Patents

Several patents of Hypoxis, hypoxoside, rooperi and rooperol have been registered in the Europe and the USA. A patent registered under the title (method of treating viral infections) was registered by Liebenberg (Mogatle, 2008). That related to bulbous of the family of hypoxidaceae in preparation of medicament to treat viral infection by cutting the rate of reduction of T lymphocytes 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 Equipment

3.1.1.1 Lonza group Brighton, UK supply:

- RPMI (Roswell park-memorial institute) 1640 media (BE12-702F).
- RPMI1640 phenol Red free media (BE12918F).
- Glucose (BE02-040E).
- Trypsin EDTA (BE17-161F).
- Penicillin-Streptomycin (BE17-161F).
- L-Phosphate Buffered Saline (PBS) (17-516F).
- glutamine (BE17-60SE).

3.1.1.2 Medical Research Council (MRC) supply:

- RIN5F cells passage 18.

3.1.1.3 SPL Life sciences supply:

- 12- and 96-well plates.
- Tissue culture flasks (25 cm^2 and 75 cm^2).
- Test tubes (15 ml and 50 ml).
- Eppendorf vials.
- Pipettes (2 ml and 10 ml).

3.1.1.4 Gibco Invitrogen, Karlsruhe supply:

- Foetal Bovine Serum (FBS).

3.1.1.5 Kimix chemicals supply:

- Ethanol.

3.1.1.6 Lasec supply:

- Whatman's filter paper number 4 and 1 (240 μm thick).

3.1.1.7 Sigma-Aldrich supply:

- Triton X-100.
- Crystal violet (c3886).
- Rat Insulin ELISA Kit (090B0743)

3.1.1.8 Thermo Scientific supply:

- M-PER Mammalian Protein Extraction Reagent prod # (78501).
- BCA Protein Assay Kit (PG205949).

3.1.1.9 Merck supply:

- 25 % Gluteraldehyde.

3.1.1.10 Scharlau Scientific supply:

- Dimethylsulphoxide (DMSO) SU0155.

3.1.1.11 Biovision supply:

- α- Amylase Activity Colorimetric Assay Kit (K711-100).
- α- Glucosidase Activity Colorimetric Assay Kit (K690-100).

3.1.1.12 Roche supply:

- XTT kit [2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt].

3.1.1.13 Vacuum system:

Brock crumbtion, (BS2208).

3.1.1.14 Centrifuges:

Lasec, Hermle (Z160M).

3.1.1.15 Microscopes:

Zeiss, Oberkochen, (195-42424 ICM405).

3.1.1.16 Plate shaker

MINI Rocker, BOECO, 96-well flat bottom.

3.1.1.17 Thermo Scientific supply:

- 96-well ELISA (Enzyme-linked immunosorbant assay).
- 5 % CO_2 Cell culture incubator

3.1.1.18 Spectrophotometer

- Beckman, Model (DU 640).

3.2 Methods

3.2.1 General cell culture procedures

The RIN-5F cell line is a clone derived from the RIN-m rat pancreatic islet. The cells produce and secrete insulin as well as L-dopa-decarboxylase, a marker for cells

having amine precursor uptake and decarboxylation (APUD) (Bhathena *et al.,* 1982; Bhathena *et al.,* 1984).

The cells were maintained in 25 cm² and 75 cm² flasks in RPMI 1640 medium containing 0.5 µM of glucose, 100 u/ml penicillin and 100 pg/ml streptomycin and 16 % foetal bovine serum (FBS). The cells were maintained at 37 °C, 5 % CO_2 and 95 % humidity. Cells were allowed to grow for 72 hours before the media was removed and replaced with fresh growth medium.

3.2.2 Passaging cells

Once the cells culture in flasks reached 70 -80 % confluencey the culture medium was aspirated and the cells were washed using 1 ml of PBS. The PBS was aspirated and 1 ml of 0.25 % trypsin was added to the flask and incubated at 37 °C until the cells began to detach, after a period of 3-5 minutes. The flasks were tapped lightly to allow cells to detatch from the surface of the flasks. Equal amounts of medium were added to inhibit the action of trypsin. The cell suspension was then centrifuged for 5 minutes at 2000 rpm. Following this, the supernatant was aspirated and 2 ml of culture medium was used to re-suspend the pellet. Every 1 ml of the suspension was added to 4 ml of culture medium in tissue culture flasks, then used in experiments or incubated again at 37 °C and 5 % CO_2.

3.2.3 Cryo-preservation

Cryo-preservation followed the same procedure as mentioned in the section 3.2.2. After the cells had been centrifuged it was re-suspended in “freeze medium”. Freeze medium is composed of 10 % DMSO, 20 % FBS and 70 % culture medium in a ratio of 1: 2:7. The cell suspension was then added to a cryo-vial and frozen at - 80 °C.

3.2.4 Cell counts

Cryo-preserved cells were thawed and added to 4 ml of culture medium in a centrifuge tube and centrifuged for 5 minutes at 2000 rpm. Following this, the supernatant was aspirated and the cells were re-suspended in 2 ml culture medium.

Cell proliferation and cell density was determined using a haemocytometer. A ratio of 1:3 (trypan blue: cell suspension) was used to determine the amount of cells. A volume of 10 µl trypan blue was added to 10 µl of the cell suspension in an Eppendorf tube. A 10 µl aliquot of this solution was then placed on each side of the slide and counted. Dead cells took up the dye and were therefore stained blue, whilst the viable cells did not take up the dye, so they remained clear and were counted. The number of cells per ml was determined by the following calculation:

Equation 1

$$\text{Cells/ml} = \text{Average number of cells} \times \text{Dilution factor} \times 10^3.$$

The cell suspension was then diluted with medium to give the required cell number and was seeded into the appropriate culture plates.

3.3 Plant material

Crushed powder corms of *Hypoxis hemerocallidea* (*HH*) (Family: Hypoxidaceae) were purchased from Global fusion trading store in Hout Bay – Cape Town, South Africa.

3.3.1 Preparation of *HH* corm aqueous extract

Boiling distilled water (1.5 L) was added to 50 g of *HH* cruched powder and left at room temperature for 3 hours, after which it was filtered through cheese cloth and

Whitman's filter paper number 4 and 1 consecutively. The filtrating process was completed using a vacuum system. Liquid nitrogen was used to freeze it quickly and the filtrate was freeze dried for 4-5 days.

The extract obtained was concentrated to dryness under reduced pressure at 30 ± 1 ^{o}C. The resulting crude extract obtained was freeze-dried, finally giving (8 g) *Hypoxis hemerocallidea* powder (*HHP*). Without any further purification, aliquot portions of the crude extract residue, were weighed and dissolved in RPMI1640 media for use on each day of the experiment.

3.4 Experimental techniques

3.4.1 Determination of cell proliferation of RIN-5F cell by crystal violet staining

Crystal violet is a method used to determine cell numbers by staining the DNA (Skehan *et al.*, 1990). Crystal violet was used to quantify cell number in monolayer cultures as a function of the absorbance of dye taken up by the cells. The cell numbers were expressed as a percentage of the cells propagated in growth medium. The fixed dye measured photometrically after solubilisation, correlates with the nuclear DNA content and thus with cell number (Basu *et al.,* 1968). Crystal violet belongs to a class of intensely colored organic compounds called triphenylmethane dyes. The chemical structure (Figure 3.1) and color of crystal violet depend on the pH, making it a valuable acid−base indicator as well as an excellent dye. The major structural form of crystal violet is the monovalent cation that can rapidly permeate the cell wall (Basu *et al.,* 1968).

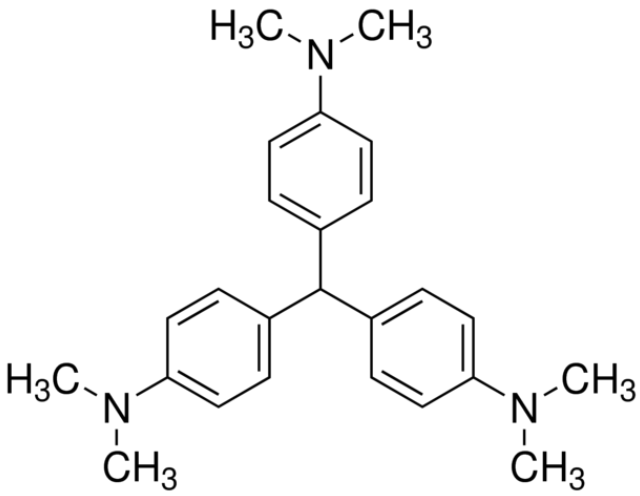


Figure 3.1. Chemical structure of crystal violet (Basu *et al.*, 1968)**.**

Crystal violet stain was prepared by dissolving 2 g of crystal violet in 20 ml of 95 % ethyl alcohol and 0.8 g ammonium citrate monohydrate and was dissolved in 80 µl deionized water. Subsequently, the two mixtures were added together and filtered as crystal violet working solution.

Cell proliferation and cytotoxicity assays can be used for drug screening and cytotoxicity tests of chemicals. They are based on different cell functions such as enzyme activity, cell adherence, cell membrane permeability, co-enzyme production, ATP production and nucleotide uptake activity (Ishiyama, 1997; Tominaga *et al.*, 1999).

3.4.1.1 The effect of glucose on cell proliferation

The cells were seeded at approximately $5x10^3$ cells per well in 96-well plates. The plates were incubated at 37 ^oC and 5 % CO_2 for 24 hours to allow for attachment. The medium was discarded. Untreated cells served as control, and the other cells were treated with different concentrations of glucose (5, 10, 20, 37.5, 50, 55, 74 and 92.3 mM) for 1, 3, 6 or 24 hours.

3.4.1.2 The effect of *HH* on cell proliferation

The cells were seeded at approximately $5x10^3$ cells per well in 96-well plates. The plates were incubated at 37 ^oC and 5 % CO_2 for 24 hours to allow for attachment. The medium was discarded. Untreated cells served as controls, and the other cells were treated with different concentrations of *HH* (50, 100, 150, 200 and 500 µl/mol) for 1, 3, 6 or 24 hours. After the cells were exposed and the medium was discarded from each well, 100 µl of gluteraldehyde (1 %) was added, followed by incubation at room temperature (RT) for 15 minutes. Next, the gluteraldehyde was removed and crystal violet working solution was added to each of the wells and then incubated at room temperature for 30 minutes. Thereafter, the plates were immersed in running tap water for 15 minutes and then dried by placing on paper towel. The plates were then left overnight.

The next day 200 µl (0.2 %) of Triton X-100 were added to each well and incubated at room temperature for 30 minutes. Subsequently, 100 ml of the solution in the wells were transferred to new 96-well microplates and the absorbance read at 570 nm using a 96 wells microplate reader. The experiment was repeated 3 different times. The viability was calculated by the formula below (Equation 2):

Equation 2

$$\text{cell proliferation (\%)} = \frac{(\text{OD})\text{of treated cells}}{(\text{OD})\text{of untreated cells}} \text{ X } 100$$

3.4.2 Cell viability assessment by XTT assay (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

The XTT cell viability assay was described in 1988 by Scudiero *et al*. (Scudiero *et al.,* 1988) as an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colourless or slightly yellow compound that when reduced

becomes brightly orange. This color change is accomplished by breaking apart the positively-charged quaternary tetrazole ring (Berridge *et al.*, 2005). The formazan product of XTT reduction is soluble and can be used in a real-time assay. XTT is thought to be excluded from entering cells by its net negative charge (Berridge *et al.*, 2005). Considerable evidence suggests that XTT dye reduction occurs at the cell surface facilitated by trans-plasma membrane electron transport (Berridge *et al.*, 2005). Mitochondrial oxidoreductases are thought to contribute substantially to the XTT response with their reductants being transferred to the plasma membrane. It has been proposed that the XTT assays actually measure the pyridine nucleotide redox status of cells (Scudiero *et al.*, 1988; Berridge *et al.*, 2005).

PMS (N-methyl dibenzopyrazine methyl sulfate), an intermediate electron acceptor is the activation reagent in the XTT cell viability assay kit. PMS mediates XTT reduction by picking up electrons at the cell surface, or at a site in the plasma membrane that is readily accessible and forms a reactive intermediate that then reduces XTT to its highly pigmented formazan product (Figure 3.2) (Berridge *et al.*, 2005).

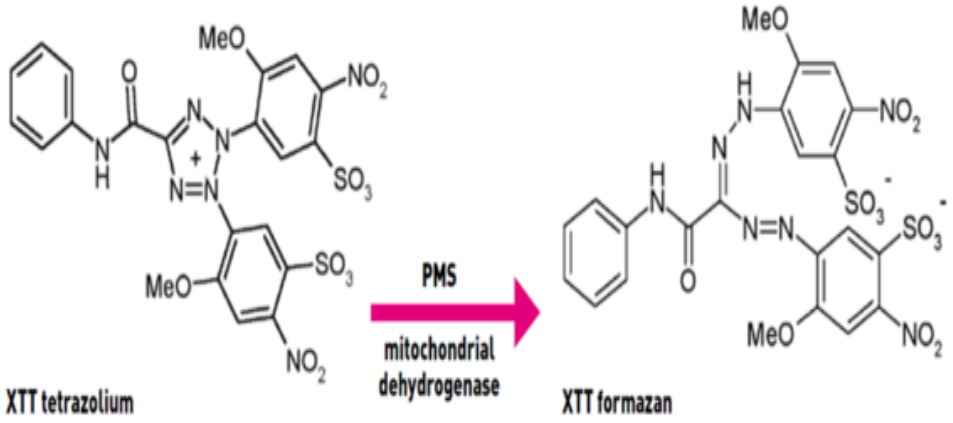


Figure 3.2. Cell viability assay XTT (Berridge *et al.*, 2005).

The XTT assay is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity. Quantification of viable cells in a population gives an indication of cell viability. Warm XTT labelling mixture (provided in the kit and prepared according to manufacturer's instructions) was added to cell culture wells. The plate was incubated at 37 ^{o}C, 5 % CO_2 and 95% humidity for 3-4 hours. Metabolically active cells convert the XTT into insoluble formazan crystal that can be directly quantified using a spectrophotometric plate reader. The absorbance was measured at 450 nm and results recorded.

Kit contents:

- XTT labelling reagent (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).
- Electron-coupling reagent.

To prepare XTT labelling mixture, the XTT labelling reagent was mixed with electron-coupling reagent prior to use.

3.4.2.1 The effect of glucose on cell viability

The cells were seeded at approximately $5x10^3$ cells per well in 96-well plates. The plates were incubated at 37 ^oC and 5 % CO_2 for 24 hours to allow for attachment. The medium was discarded. Untreated cells served as controls and the other samples were treated with different concentrations of glucose (5, 10, 20, 37.5, 50, 55, 74 and 92.3 mM). The 50µl XTT labelling mixture was added to each well and the plate read at 405nm at time 0 minute. The plate was incubated for 1 h at 37 °C and 5 % CO_2 and read again at 405 nm to get the actual data. To obtain the final result the 0 time reading was subtracted from the 1hr reading.

3.4.2.2 The effect of *HH* on cell viability

The cells were seeded at approximately $5x10^3$ cells per well in 96-well plates, and plates were incubated at 37 ^oC and 5 % CO_2 for 24 hours to allow for attachment. The medium was discarded. Untreated cells served as control, and the other cells were treated with different concentrations of *HH* (50, 100, 150, 200 and 500 µl/mol) for 1, 3, 6 or 24 hours. A volume of 50 µl of XTT labelling mixture was added to each well and the plate read at 405 nm at time 0. The plate was then incubated for 1 hour at 37 °C and 5 % CO_2 and read again at 405 nm to get the actual data. The absorbance of the samples was determined using a microplate (ELISA) reader. The experiments were repeated 3 times. The cell viability was calculated by the formula below (Equation 3):

Equation 3

$$\text{cell viability } (\%) = (\text{ODT1} - \text{ODT0}) \text{ X } 100$$

3.4.3 Insulin Secretion Assays for RIN5-F cells

Equal numbers of cells were seeded in 12 well tissue culture plates containing phenol red free RPMI1640 with 0.5 µM of glucose, supplemented with 340 µL-glutamine and 5 % FPS. The cells were incubated at 37 ºC for 24 hours to allow for attachment. Cells were washed twice with PBS and exposed to *HH* (100 µg/ml or 500 µg/ml) and/or 2 mM or 50 mM glucose for 30 minutes to stimulate insulin secretion. The medium was removed from each well and discarded. The cells were washed twice with PBS. Fresh medium containing same treatment of glucose and *HH* was added to each well (2 ml/well). Untreated cells served as controls.

The plates were incubated for 30 minutes and then 1 ml of the supernatant was removed, placed in Eppendorf tubes, and stored at -80 °C. The plate were re-incubated for 90 minutes, then 1ml of supernatant was placed in an Eppendorf and stored at (-80 °C). All samples were performed in triplicate and each experiment was repeated three times. Insulin content was determined by an ELISA.

3.4.3.1 Determination of insulin secretion by RIN 5F cells

Insulin was measured in the cell culture supernatants using a commercially available kit (RAB0904 Rat Insulin ELISA Kit). This assay employs an antibody specific for human insulin coated on a 96-well plate. Standards and samples were pipetted into the wells and the insulin present in a sample was bound to the wells by the immobilised antibody. The wells were washed and biotinylated anti-human insulin antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. The wells were again washed, a TMB substrate solution was added to the wells and colour developed in proportion to the amount of insulin bound. The stop solution changed the colour from blue to yellow, and the intensity of the colour was measured at 450 nm.

3.4.3.2 Determination of protein

3.4.3.2.1 Procedure for lysis of adherent mammalian cells

To determine the protein concentration of each sample the culture medium was carefully removed from the adherent cells. The cells were washed once in PBS. A volume of 50 µl of M-PER® Reagent was added to each well and the plate was shaken gently for 5 minutes. The lysate was collected and transferred into a new Eppendorf and centrifuged at 14,000 x g for 8 minutes to pellet the cell debris. The supernatant was transferred to new Eppendorf tubes. An aliquot of 25 µl of supernatant was transferred to each well to prepare it for the protein determination assay. The protein content was measured by the BCA Protein Assay Kit (PG205949) according to instructions.

3.4.3.2.2 Protein kit

A volume of 25 µl of each protein standard (25 µg/ml – 2000 µg/ml) and the samples were prepared and added to the wells. The working reagent (WR) was prepared by mixing 5 ml of Reagent A (500 ml containing sodium carbonate, 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 per well was added. 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.

3.4.4 Alpha amylase and alpha glucosidase inhibition activity

3.4.4.1 Alpha Amylase

Alpha amylase inhibition of the *HH* aqueous extract was measured using a commercially available Amylase Activity Colorimetric Assay. The assay used ethylidene-pNP-G7 as the substrate. Once the substrate had been specifically cleaved by α-amylase, the smaller fragments produced were acted upon by α-glucosidase, which caused the ultimate release of the chromophore that was measured at 405 nm. Different concentrations of *HH* (5 and 10 µg/ml) were prepared in α- amylase Assay Buffer **p**H 6.9 and used as a negative control. The dose dependency of the *HH* extract on alpha amylase was measured using same negative control concentrations (5 and 10 µg/ml). A volume of 2 µl alpha amylase activity solution was added to the positive control and test samples. A volume of 100 µl of the reaction mixture was added into each well and mixed. Optical density was measured immediately (To) at 405 nm to get OD To. The reaction was incubated at 25 °C for various times (5, 10, 15, 50, 25, 30 and 35) minutes (T_1). Optical density was measured again at 405 nm to get OD T_1. The reaction kinetics was observed and the linear range for To-T_1 was chosen.

Equation 4

$$\text{AmylaseActivity} = \frac{B}{\text{T} \times \text{V}}$$

B is the nitrophenol amount obtained from Standard Curve (nmol).

T is the time between T0 and T1 (min).

V is the pre-treated sample volume added to the reaction well (ml).

3.4.4.2 Alpha Glucosidase

Alpha Glucosidase inhibition of the *HH* aqueous extract was measured using a commercially available Glucosidase Activity Colorimetric Assay. The α-Glucosidase hydrolysed the substrate mix to release the p-nitrophenol that were measured calorimetrically (OD = 410 nm). This kit measured 0.1-10 mU of α-glucosidase activity in the samples. Different concentrations of *HH* (5 and 10 µg/ml) were prepared in α-Glucosidase Assay buffer pH 6.9 and used as a negative control. The dose dependency of the *HH* extract on alpha glucosidase was measured using same negative control concentrations (5 and 10 µg/ml). A volume of 2 µl Alpha glucosidase activity solutions was added to the positive control and test samples. A volume of 50 µl of the reaction mixture was added into each well and mixed. Optical density was measured immediately (To) at 410 nm to get ODTo. The reaction was incubated at 25 °C for different time period (5, 10, 15, 20, 25, 30 and 35) minutes (T_1). Optical density was measured again at 410 nm to get ODT$_1$. The reaction kinetics was observed and the linear range for To-T$_1$ was choosing.

Equation 5

$$\boldsymbol{\alpha - \text{glucosidase Activity}} = \frac{Sa/Ss}{V}$$

Sa = the slope of the enzyme activity (OD/min) in the sample well.

Ss= slope of Standard Curve (OD/nmol)

V = sample volume added to the well (µl)

3.5 Data analysis

All data are reported as means ± SEM or median. Statistical differences among groups were analysed by analyses of Two-way ANOVA (non-parametric test) followed by Kruskal-Wallis test. Graphs and calculation were generated using Excel. $P < 0.05$ was considered as significant and data analysis was done using Graph Pad Prism version 5.1 for Windows (GraphPad Software, San Diego, CA, USA).

CHAPTER IV

4 Results

The pancreatic islet tumour cell line (RIN-5F) was cultured in RPMI 1640 medium in 25 cm² and 75 cm² culture flasks at 37 ^{o}C under a 5 % CO_2 humidified atmosphere and supplemented with 16 % foetal bovine serum (FBS).

The RIN-5F cells were exposed to different concentrations of glucose (5, 10, 20, 37.5, 50, 55, 74, and 92.3 mM) at different time periods of 1, 3, 6 and 24 hours. The RIN-5F cells were also exposed to different concentrations of *HH* (50, 100, 150, 200 and 500 µ/ml) at different time periods of 1, 3, 6 and 24 hours. Cell proliferation was evaluated using crystal violet staining procedure and cell viability was evaluated using XTT assay.

4.1 Cell proliferation of RIN-5F cell using crystal violet staining

The cell proliferation of RIN-5F cells was tested by means of the crystal violet staining procedure after exposure to different concentrations of glucose or *HH*.

4.1.1 Effect of Glucose on cell proliferation

The results of the effect of different concentration of glucose (5, 10, 20, 37.5, 50, 55, 74 and 92.3 mM) on RIN-5F cell proliferation at different exposure time of 1, 3, 6 and 24 hours are shown in Figure 4.1 and in Table 4.1. Statistically the cell numbers in 5, 10, 20, 37.5, 55, 74 and 92.3 mM glucose were not significantly different ($P > 0.05$) from the control group after 1, 3 and 6 hours. The cell numbers in 50 mM glucose after 1 hour (134±13%; $P \leq 0.001$) and 3 hours (128±19.2%; $P \leq 0.01$) were significantly higher than the control group.

When cells were exposed for 24 hours, statically the cell numbers in 5 mM (79±2.4%; $P \leq 0.05$), 10 mM (78±2.8%; $P \leq 0.05$), 20 mM (79±2.45%; $P \leq 0.05$), 37.5 mM (75±4.6%; $P \leq 0.01$), 50 mM, (73±4.6%; $P \leq 0.01$), 55 mM, (70±2.6%; $P \leq 0.001$), 74 mM (70±4.3%; $P \leq 0.001$) and 92.3 mM (66±2%; $P \leq 0.001$) thus all glucose concentrations were significantly lower than the control group.

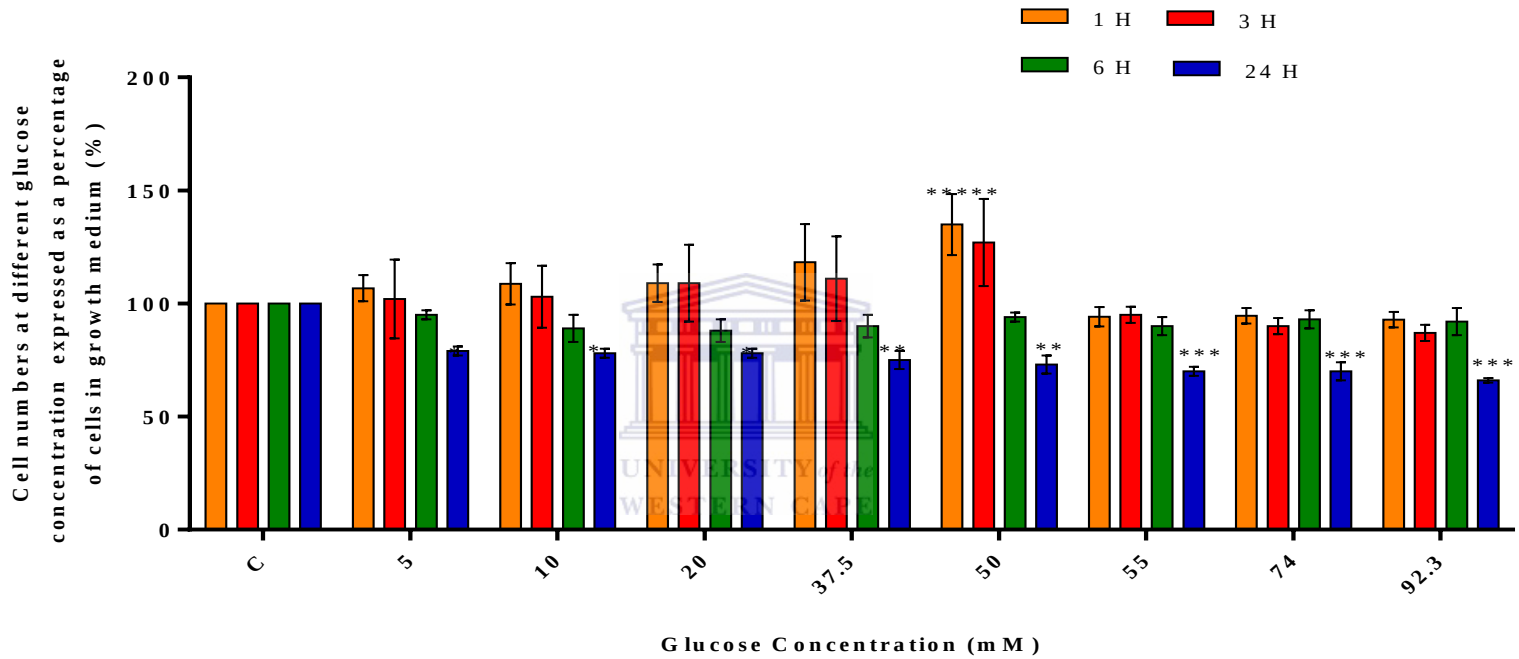


Figure 4.1. The effect of 1, 3, 6 and 24 hours exposure to glucose (5, 10, 20, 37, 5, 50, 55, 74 and 92.3 mM) on RIN-5F cell proliferation using crystal violet staining.

Values are indicated (mean ± SD) as a percentage of the respective control groups.The values are given in Table 4.1.

Table 4.1. The effect of 1, 3, 6 and 24 hours exposure to glucose (5, 10, 20, 37, 5, 50, 55, 74 and 92.3 mM) on RIN-5F cell proliferation using crystal violet staining.

	1 H	3 H	6 H	24 H
C	100±0	100±0	100±0	100±0
5	106±5.8	102±17	96±2.8	79±2.4*
10	108±9.2	103±13.7	89±6.4	78±2.8*
20	108±8.3	109±17	88±5.2	79±2.4*
37.5	118±16.9	111±18.7	90±5.8	75±4.6**
50	134±13.4***	128±19.2**	94±2.8	73±4.6**
55	94±4.2	95±3.5	90±4.7	70±2.6***
74	94.5±3.4	90±3.5	93±4.7	70±4.3***
92.3	93±3.4	87±3.5	92±6.3	66±2***

Values are expressed as the mean ± SD. Cell numbers are expressed as a percentage of the respective control groups, Significant differences between the glucose (5, 10, 20, 37, 5, 50, 55, 74 and 92.3 mM) groups and the control groups using (2 way ANOVA) are indicated; *P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

4.1.2 Effect of *HH* on cell proliferation

The results of the effects of *HH* on RIN-5F cells proliferation after exposure to different concentrations (50, 100, 150, 200 and 500 µg/ml) of *HH* for 1, 3, 6 and 24 hours are shown in Figure 4.2 and in Table 4.2. Statistically the cell numbers in 50, 100 and 150 µg/ml of *HH* were not significantly different ($P > 0.05$) from the control group after 1, 3, 6 and 24 hours.

When cells were exposed to 200 µg/ml *HH* for 1 hour (116±4%; $P \leq 0.01$), 3 hours (119±3%; $P \leq 0.01$) and 6 hours (120±13.1%; $P \leq 0.001$) cell proliferation was significantly higher than the control group. The cell numbers in 500 µg/ml *HH* treated samples after 1 hour (117±7%; $P \leq 0.01$), 3 hours (120±6.9%; $P \leq 0.001$) and 6 hours (135±10.6%; $P \leq 0.001$) were also significantly higher than the control group. Cells exposed for 24 hours were not significantly different ($P > 0.05$) from the control group.

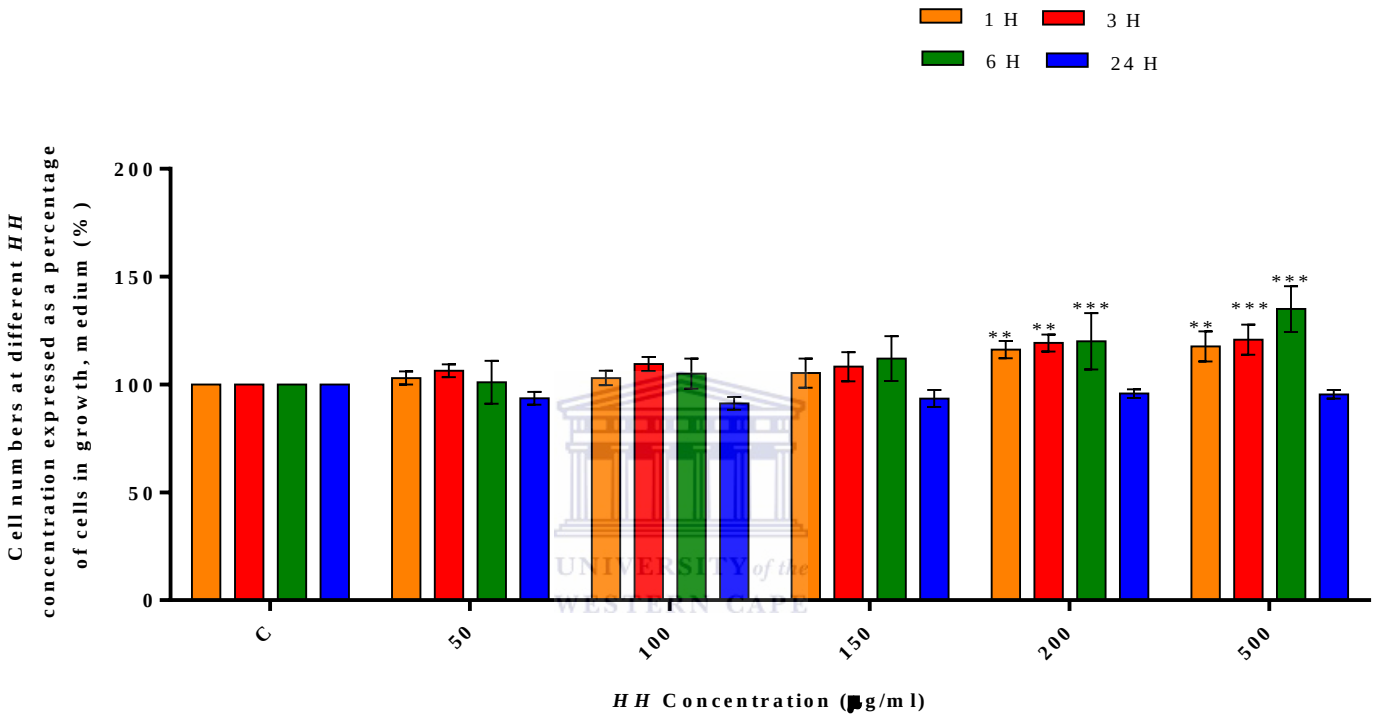


Figure 4.2. The effect of 1, 3, 6 and 24 hours exposure to *HH* (50, 100, 150, 200 and 500 µg/ml) on RIN-5F cell proliferation using crystal violet staining.
Values are indicated (mean ± SD) as a percentage of the respective control groups.The actual values are given in Table 4.2.

Table 4.2. The effect of 1, 3, 6 and 24 hours exposure to *HH* (50, 100, 150, 200 and 500 µg/ml) on RIN-5F cell proliferation using crystal violet staining.

	1 H	3 H	6 H	24 H
C	100±0	100±0	100±0	100±0
50	103±3.0	106±3.02	101±9.9	93±3.11
100	103±3.3	109±3.3	105±7	91±3.7
150	105±6.7	108±6.7	112±10.3	93±4
200	116±4**	119±3.0**	120±13.1***	95±2.7
500	117±7**	120±6.9***	135±10.6***	95±2.9

Values are expressed as the mean ± SD. Cell numbers are expressed as a percentage of the respective control groups. Significant differences between the *HH* (50, 100, 150, 200 and 500 µg/ml) groups and the control groups using (2 way ANOVA) are indicated; ** $P \leq 0.01$, *** $P \leq 0$.

4.2 Viability of RIN-5F cell using XTT assay

The cell viability or bioreductive capacity of RIN-5F cells was tested by mean of the XTT assay after exposure to different concentrations of glucose or *HH*.

4.2.1 Effect of Glucose on cell viability

The results of the effect of glucose on RIN-5F cell viability after exposure to different concentrations (5,10, 20, 37.5, 50, 55, 74 and 92.3 mM) of glucose for 1, 3, 6 and 24 hours are presented in Figure 4.3 and in Table 4.3. Statistically the cell viability in 5, 10 and 20 mM glucose were not significantly different ($P > 0.05$) from the control group after 1, 3 and 6 hours. When the cells were exposed to 5mM glucose (79±2.5%; $P \leq 0.05$%), 10 Mm glucose (78±1.7%; $P \leq 0.05$%) and 20 mM glucose (79±2.5%; $P \leq 0.05$%) for 24 hours the viability was significantly lower than the control group.

When the cells were exposed to 37.5 mM glucose for 3 hours the cell viability were not significantly different ($P > 0.05$) from the control group, while the cell numbers after 6 hours (79±8.1%; $P \leq 0.05$) and 24 hours (76±3.8%; $P \leq 0.01$) were significantly lower than the control group, and the cell viability after 1 hour (119±9.8%; $P \leq 0.05$) was significantly higher than the control group.

When cells were exposed to 50 mM glucose for 1 hour and 3 hours the viability of the cells was significantly higher than the control group (133±12.6%; $P \leq 0.001$ and 128±16%; $P \leq 0.001$ respectively). Cell viability after 6 hours (80±10.4%; $P \leq 0.05$) and 24 hours (75±4.2%; $P \leq 0.01$) was significantly lower than the control group. When cells were exposed to 55 mM glucose the cell viability after 1 and 3 hours was not significantly different ($P > 0.05$) from the control group, while, the cell viability after 6 hours (81±6.7%; $P \leq 0.05$) and 24 hours (69±2.4%; $P \leq 0.001$) was significantly lower than the control group.

When cells were exposed to 74 mM glucose the cell viability after 1, 3 and 6 hours was not significantly different ($P > 0.05$) from the control group, the viability of the cell after 24 hours (74±5.9%; $P \leq 0.01$) was significantly lower than the control group. When exposed to 92.3 mM glucose, cell viability after 1 and 3 hours was not significantly different ($P > 0.05$) from the control group, while the cell viability after 6 hours (81±6.8%; $P \leq 0.05$) and 24 hours (70±6.5%; $P \leq 0.001$) was significantly lower than the control group.

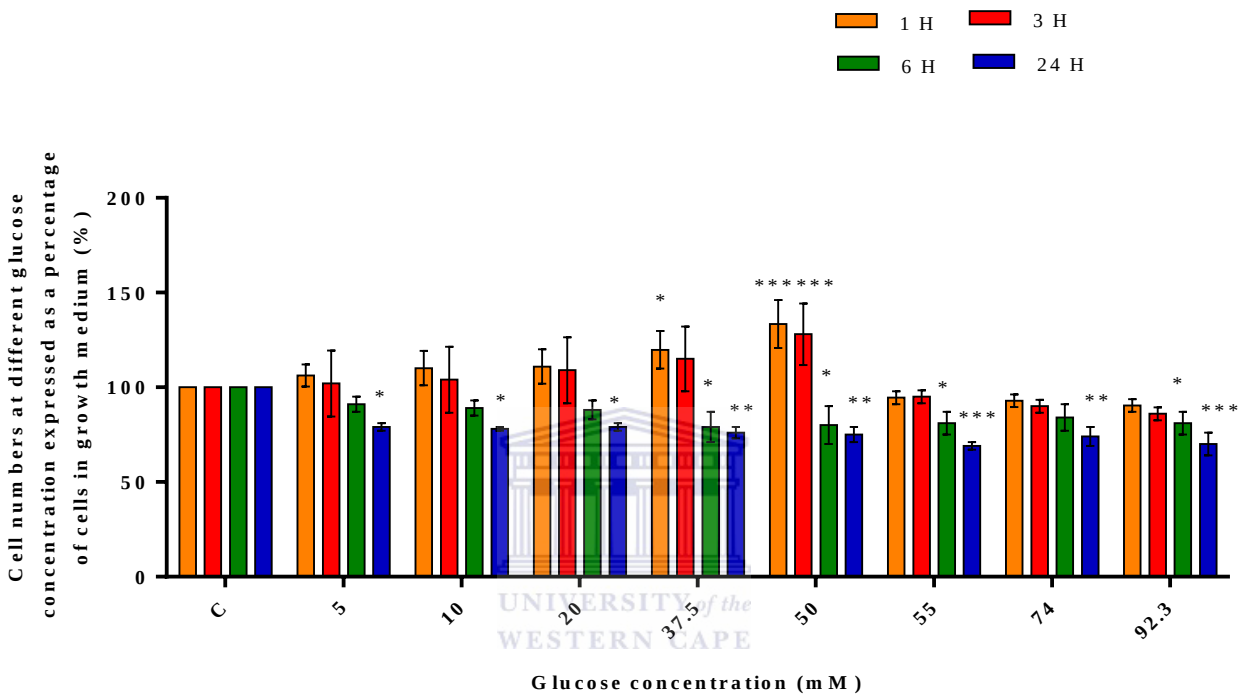


Figure 4.3. The effect of 1, 3,, 6 and 24 hours exposure to glucose (5, 10, 20, 37, 5, 50, 55, 74 and 92.3 mM) on RIN-5F cell viability using the XTT assay.

Values are indicated (mean ± SD) as a percentage of the respective control groups. The actual values are given in Table 4.3.

Table 4.3. The effect of 1, 3, 6 and 24 hour's exposure to glucose (5, 10, 20, 37, 5, 50, 55, 74 and 92.3 mM) on RIN-5F cell viability using the XTT assay.

	1 H	3 H	6 H	24 H
C	100±0	100±0	100±0	100±0
5	106±5.7	102±17.4	91±4.8	79±2.5*
10	110±9.1	104±17.4	89±4.2	78±1.7*
20	111±9.1	109±17.3	88±5.6	79±2.5*
37.5	119±9.8*	115±17	79±8.1*	76±3.8**
50	133±12.6***	128±16***	80±10.4*	75±4.2**
55	95±3.4	95±3.3	81±6.7*	69±2.4***
74	93±3.4	90±3.3	84±7.2	74±5.9**
92.3	90±3.4	86±3.3	81±6.8*	70±6.5***

Values are expressed as the mean ± SD. Cell numbers are expressed as a percentage of the respective control groups. Significant differences between the glucose (5, 10, 20, 37.5, 50, 55, 74 and 92.3 mM) groups and the control groups using (2 way ANOVA) are indicated; *P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

4.2.2 Effect of *HH* on cell viability

The results for the effect of *HH* on RIN-5F cell viability after exposure to different concentrations (50, 100, 150, 200 and 500 µg/ml) of *HH* for periods of 1, 3, 6 and 24 hours are shown in Figure 4.4 and in Table 4.4. Statistically the cell viability in 50, 100 and 150 µg/ml *HH* was not significantly different ($P > 0.05$) from the control group after 1, 3, 6 and 24 hours.

When cells were exposed to 200 µg/ml *HH* the viability of the cell after 1 hour (117±3.6%; $P \leq 0.05$), 3 hours (120±3.6%; $P \leq 0.01$) and 6 hours (119±14%; $P \leq 0.05$) was significantly higher than the control group, while the cell viability after 24 hours was not significantly different ($P > 0.05$) from the control group.

When cells were exposed to 500 µg/ml *HH* the cell viability after 1 hour (118±3.3%; $P \leq 0.01$), 3 hours (121±3.3%; $P \leq 0.01$) and 6 hours (130±12%; $P \leq 0.001$) was significantly higher than the control group, but the viability of the cells after 24 hours was not significantly different ($P > 0.05$) from the control group.

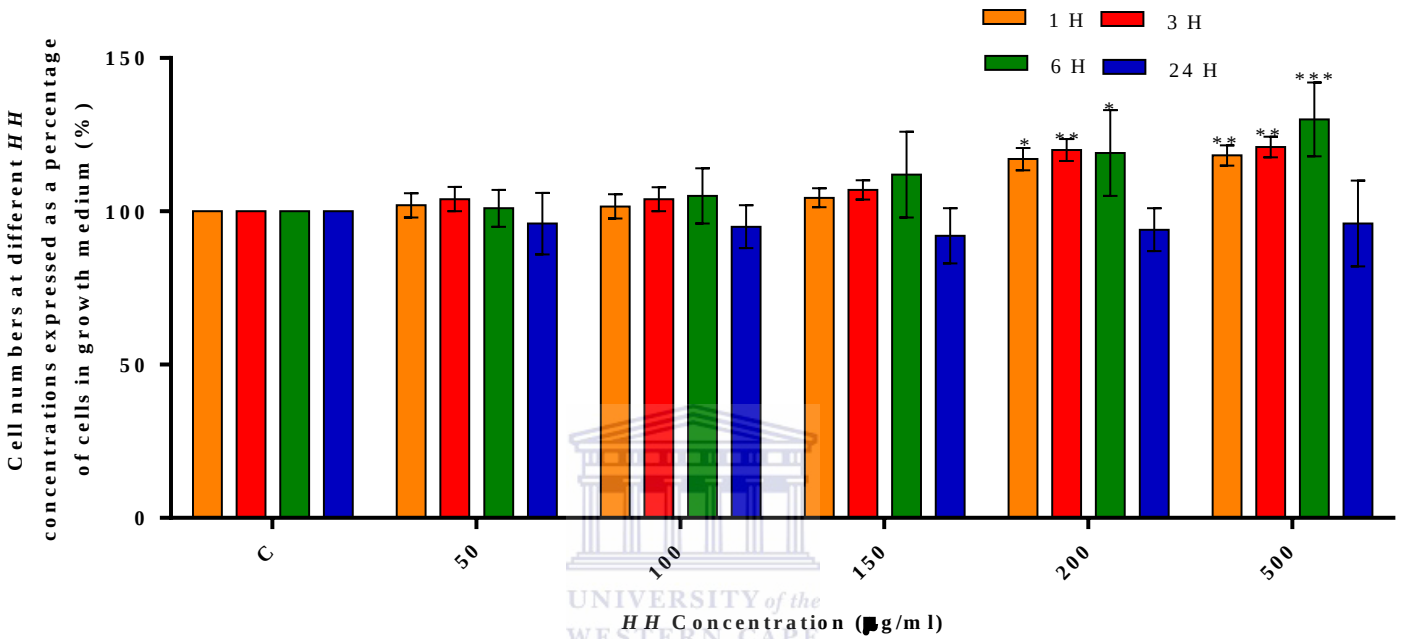


Figure 4.4. The effect of 1, 3, 6 and 24 hours exposure to *HH* (50, 100, 150, 200 and 500 µg/ml) on RIN-5F cell viability using the XTT assay.
Values are indicated as the mean ± SD as a percentage of the respective control groups. The actual values are given in Table 4.4.

Table 4.4. The effect of 1, 3, 6 and 24 hours exposure to *HH* (50, 100, 150, 200 and 500 µg/ml) on RIN-5F cell viability using the XTT assay.

	1 H	3 H	6 H	24 H
C	100±0	100±0	100±0	100±0
50	101±3.0	104±3.9	101±6.9	96±10.4
100	101±3.1	104±3.9	105±9.2	95±7.2
150	104±3.1	107±3.1	112±14	92±9.7
200	117±3.6*	120±3.6**	119±14*	94±7.4
500	118±3.3**	121±3.3**	130±12***	96±14.6

Values are expressed as the mean ± SD. Cell numbers are expressed as a percentage of the respective control groups. Significant differences between the *HH* (50, 100, 150, 200 and 500 µg/ml) groups and the control groups using (2 way ANOVA) are indicated; *P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

4.3 Insulin secretion studies

The effect of glucose and *HH* on RIN-5F cell insulin secretion was evaluated. The cells were exposed to two concentrations of *HH*, 100 µg/ml and 500 µg/ml. They were also exposed to two concentrations of glucose, 2 mM (low glucose) and 50 mM (high glucose). The exposure time was 30 minutes and 90 minutes.

4.3.1 Insulin secretion after 30 minutes

The insulin secretion data for RIN-5F cells which were exposed to different concentrations of glucose, 2 mM and 50 mM, are presented in Figure 4.5. Statistically the insulin secretion in 2 mM glucose (1.31±0.23 mIU/mg protein; $P \leq 0.05$) and 50 mM glucose (1.51±0.14 mIU/mg protein; $P \leq 0.01$) was significantly higher than the control group (0.97±0.12 mIU/mg protein). The Insulin secretion in 2 mM glucose was not significantly different ($P > 0.05$) from that in 50 mM glucose.

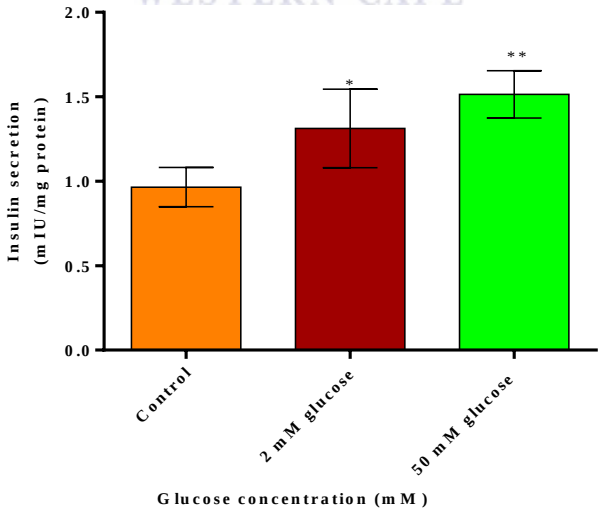


Figure 4.5. Insulin secretion by RIN-5F cells exposed to different glucose concentrations for 30 min.
Values are the mean ± SD. *P ≤ 0.05, ** P ≤ 0.01, compared to control group.

The results of RIN-5F cells which were exposed to different concentrations of *HH*, 100 µg/ml and 500 µg/ml for 30 minutes are presented in Figure 4.6. Statistically the insulin secretion in 100 µg/ml *HH* (2.17±0.13 mIU/mg protein; $P \leq 0.01$) and the insulin secretion in 500 µg/ml *HH* (3.9±0.11 mIU/mg protein; $P \leq 0.001$) was significantly higher than the control group (0.97±0.12 mIU/mg protein). The insulin secretion in the 500 µg/ml treated samples was significantly higher ($P \leq 0.001$) than the 100 µg/ml *HH* treated group.

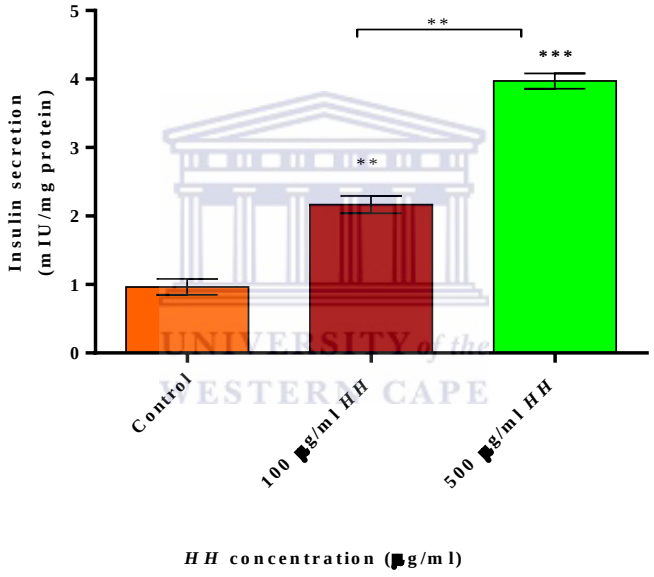


Figure 4.6. Insulin secretion by RIN-5F cells exposed to different *HH* concentrations for 30 min.
Values are the mean ± SD. **P ≤ 0.01, *** P ≤ 0.001, compared to control group.

Insulin secretion by RIN-5F cells exposed to 2mM (low glucose) in combination with either 100 µg/ml *HH* or 500 µg/ml *HH* for 30 minutes are shown in Figure 4.7. Statistically the insulin secretion in 2 mM glucose +100 µg/ml *HH* (2.37±0.15 mIU/mg protein; $P \leq 0.001$) was significantly higher than the 2 mM glucose (1.31±0.23 mIU/mg protein). Insulin secretion in 2 mM glucose +500 µg/ml *HH* (1.30±0.14 mIU/mg protein; $P > 0.05$) was not significantly different from the 2 mM glucose (1.31±0.23 mIU/mg protein). The insulin secretion by 2 mM glucose + 500 µg/ml *HH* treated samples was significantly lower ($P > 0.001$) than 2 mM glucose +100 µg/ml *HH* treated group.

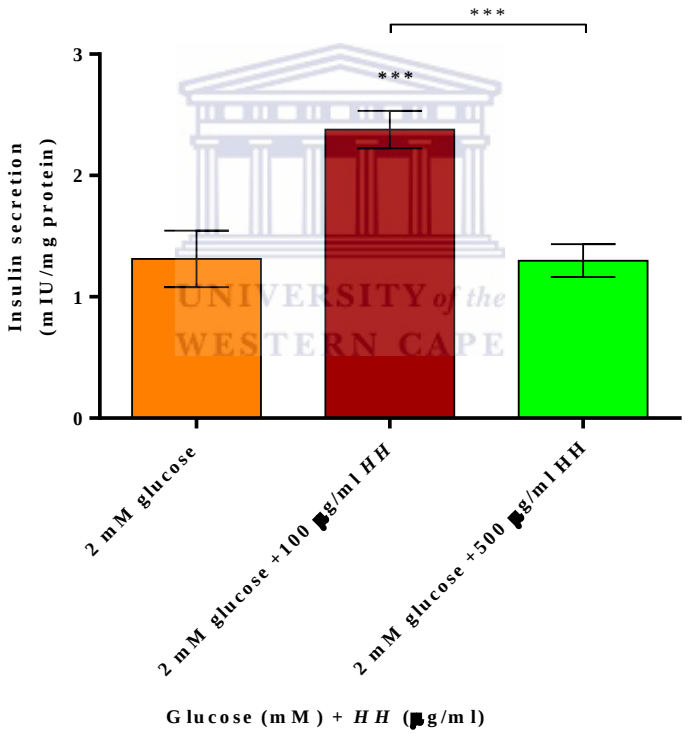


Figure 4.7. Insulin Secretion by RIN-5F cell exposed to 2Mm glucose and different *HH* concentrations for 30 min.
Values are the mean ± SD. *** $P \leq 0.001$, compared to 2 mM glucose group.

The RIN-5F cells exposed to 50 mM (high) glucose in combination with either 100 µg/ml *HH* or 500 µg/ml *HH* for 30 minutes are shown in Figure 4.8. Statistically the insulin secretion in 50 mM glucose + 100 µg/ml *HH* (0.56±0.08 mIU/mg protein; $P \leq 0.001$) and 50 mM glucose + 500 µg/ml *HH* (0.34±0.03 mIU/mg protein; $P \leq 0.001$) was significantly lower than 50 mM glucose only (1.51±0.14 mIU/mg protein). The insulin secretion by 50 mM glucose + 500 µg/ml *HH* was not significantly different ($P > 0.05$) from the 50 mM glucose + 100 µg/ml *HH* treated group.

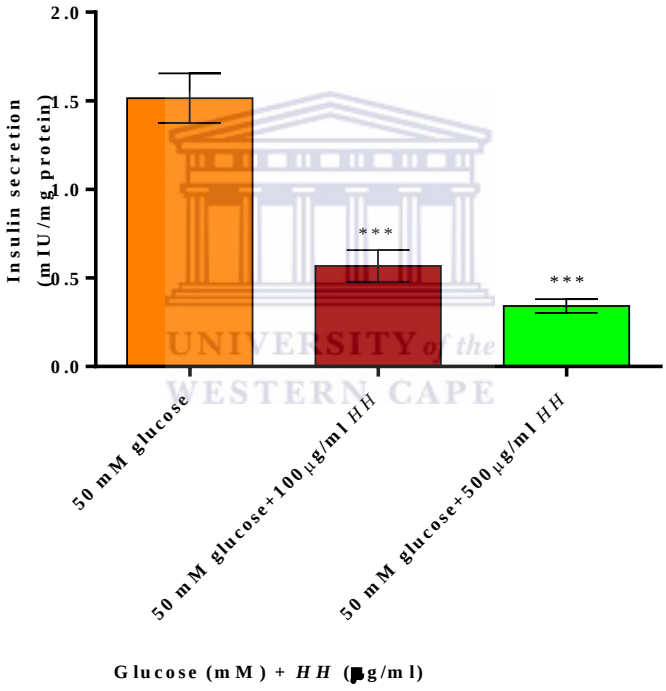


Figure 4.8. Insulin Secretion by RIN-5F cell exposed to 50mM glucose and different *HH* concentrations for 30 min.
Values are the mean ± SD. *** $P \leq 0.001$, compared to 50 mM glucose group.

4.3.2 Insulin secretion after 90 minutes

The insulin secretion data for the RIN-5F cells exposed to different concentrations of glucose, 2 mM and 50 mM for 90 minutes are presented in Figure 4.9. Statistically the insulin secretion in 2 mM glucose (2.27±0.17 mIU/mg protein; P>0.05) was not significantly different from the control group (1.81±0.17 mIU/mg protein). The insulin secretion in 50 mM glucose (2.9±0.17 mIU/mg protein; $P \leq 0.001$) was significantly higher than the control group (1.81±0.17 mIU/mg protein). The Insulin secretion in 2 mM glucose was not significantly different ($P > 0.05$) from the 50 mM glucose treated samples.

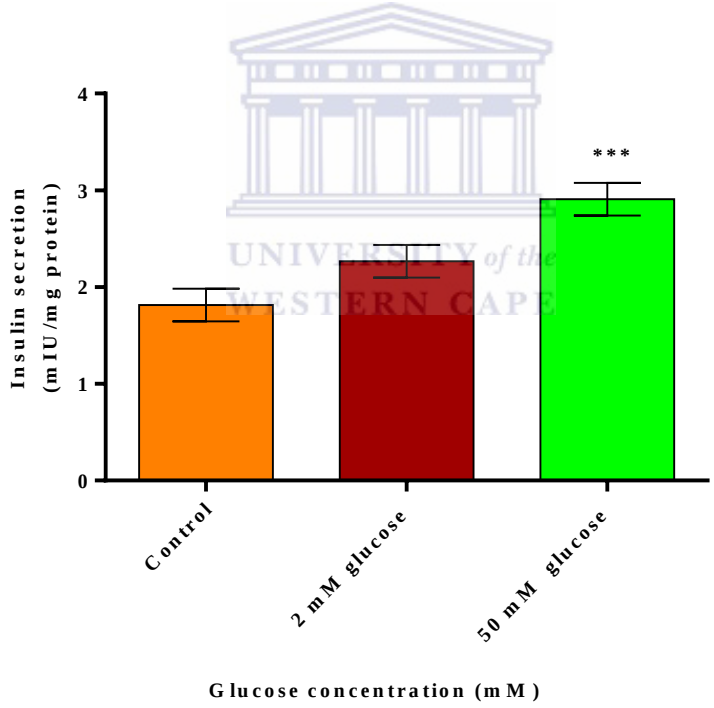


Figure 4.9. Insulin secretion by RIN-5F cell exposed to different glucose concentrations for 90 min.
Values are the mean ± SD. *** $P \leq 0.001$, compared to control group.

The insulin secretion results of the RIN-5F cells which were exposed to different concentrations of *HH*, 100 µg/ml and 500 µg/ml, are presented in Figure 4.10. Statistically the insulin secretion in 100 µg/ml *HH* (4.3±0.17 mIU/mg protein; $P \leq 0.01$) and 500 µg/ml *HH* (7.87±0.17 mIU/mg protein; $P \leq 0.001$) were significantly higher than the control group (1.81±0.17 mIU/mg protein). The insulin secretion by cells exposed to 500 µg/ml *HH* was significantly higher ($P \leq 0.01$) than the cells exposed to 100 µg/ml *HH* group.

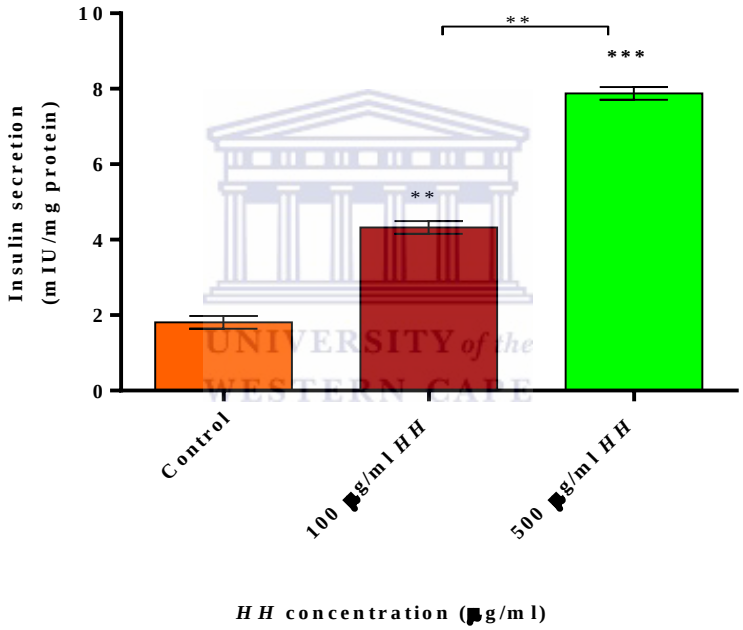


Figure 4.10. Insulin Secretion by RIN-5F cells exposed to different *HH* concentrations for 90 min.
Values are the mean ± SD. **P ≤ 0.01, *** P ≤ 0.001, compared to control group.

Insulin secretion by RIN-5F cells exposed to 2 mM (low) glucose in combination with either 100 µg/ml *HH* or 500 µg/ml *HH* are shown in Figure 4.11.Statistically the insulin secretion by 2 mM glucose + 100 µg/ml *HH* (4.69±0.16 mIU/mg protein; $P \leq 0.001$) was significantly higher than 2 mM glucose only (2.27±0.17 mIU/mg protein),

while the insulin secretion by 2 mM glucose + 500 µg/ml *HH* (2.56±0.17 mIU/mg protein; P > 0.05) was not significantly different from that in 2 mM glucose treated cells (2.27±0.17 mIU/mg protein). The insulin secretion by 2 mM glucose + 500 µg/ml *HH* was significantly lower (P ≤ 0.001) than 2 mM glucose +100 µg/ml *HH* treated group.

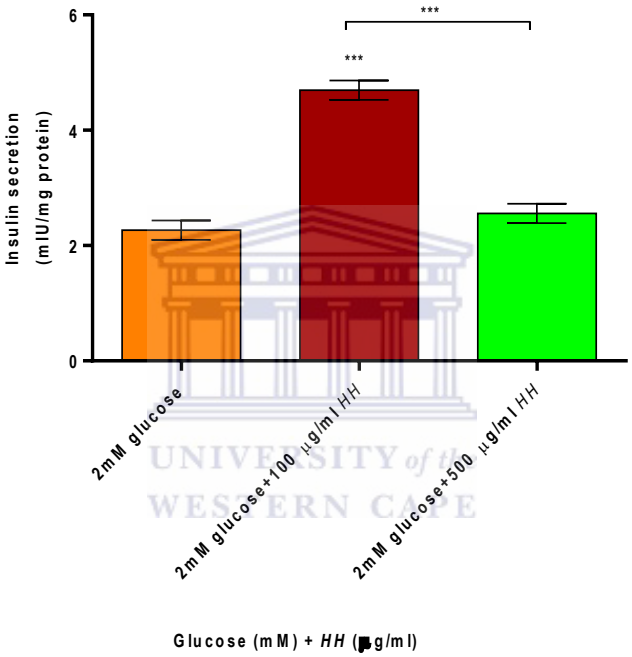


Figure 4.11. Insulin Secretion by RIN-5F cells exposed to 2mM glucose and different *HH* concentrations for 90 min.
Values are the mean ± SD. ** P ≤ 0.01, *** P ≤ 0.001, compared to 2 mM glucose group.

Insulin secretion by cells exposed to 50 mM (high) glucose and either (100 µg/ml *HH* or 500 µg/ml *HH*) are shown in Figure 4.12. Statistically the insulin secretion of cells exposed to 50 mM glucose + 100 µg/ml *HH* (1.09±0.19 mIU/mg protein; P ≤ 0.001) and 50 mM glucose + 500 µg/ml *HH* (0.59±0.18 mIU/mg protein; P ≤ 0.001) was significantly lower than that of cells exposed to 50 mM glucose (2.91±0.17 mIU/mg

protein). The insulin secretion in 50 mM glucose + 500 µg/ml *HH* was not significantly different ($P \leq 0.05$) from the 50 mM glucose + 100 µg/ml *HH* group.

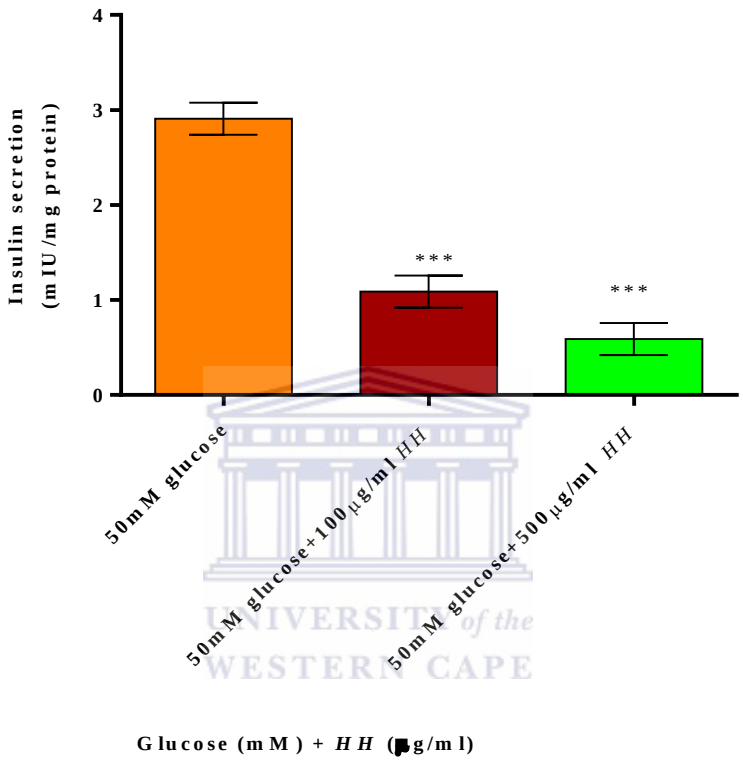


Figure 4.12. . Insulin Secretion by RIN-5F cells exposed to 50mM glucose and *HH* different concentrations for 90 min.
Values are the mean ± SD. *** $P \leq 0.001$, compared to the 50 mM glucose group.

4.4 Effect of *HH* on alpha amylase and alpha glucosidase activity

4.4.1 Effect of *HH* on alpha amylase activity

Figure 4.13 shows the standard curve generated for the determination of alpha amylase activity.

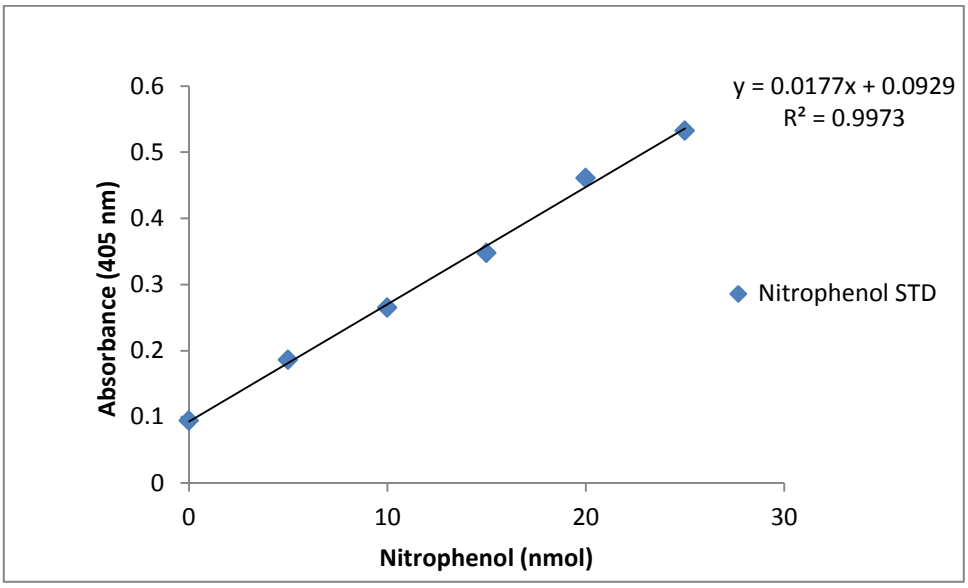
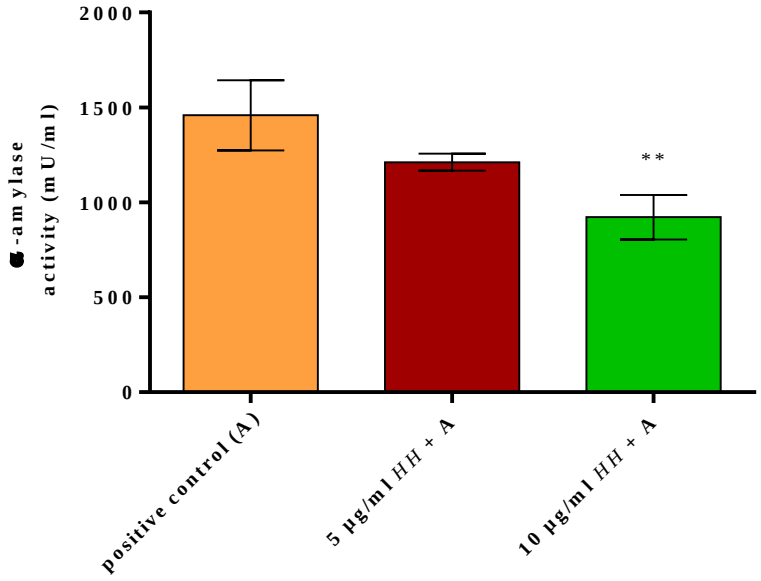


Figure 4.13. Nitrophenol standard curve used for determining α-amylase activity.

The standard curve shows a linear relationship between the nitrophenol concentrations and the absorbance.

Figure. 4.14. shows the effect of the different concentrations of *HH* (5 µg/ml and 10 µg/ml) on alpha amylase activity. In the presence of 5 µg/ml *HH*, amylase activity was not significantly different from the α-amylase positive control (1459±185 U/ml vs 1212±109 U/ml; $P > 0.05$). In the presence of 10 µg/ml *HH*, alpha amylase activity was significantly lower (922±117 U/ml vs 1459±185 U/ml; $P \leq 0.01$) than that of the positive control. The results were expressed as the mean± SEM value of alpha amylase activity, in 3 different experiments.



.

Figure 4.14. Alpha amylase activity in the presence of two concentrations of *HH*. Values are the mean ± SEM. *HH*: *Hypoxis hemerocallidea*, A: Alpha amylase. ** P ≤ 0.01, compared to positive control.

4.4.2 Effect of *HH* on alpha glucosidase activity

Figure.4.15. shows the standard curve generated for determination of alpha glucosidase activity.

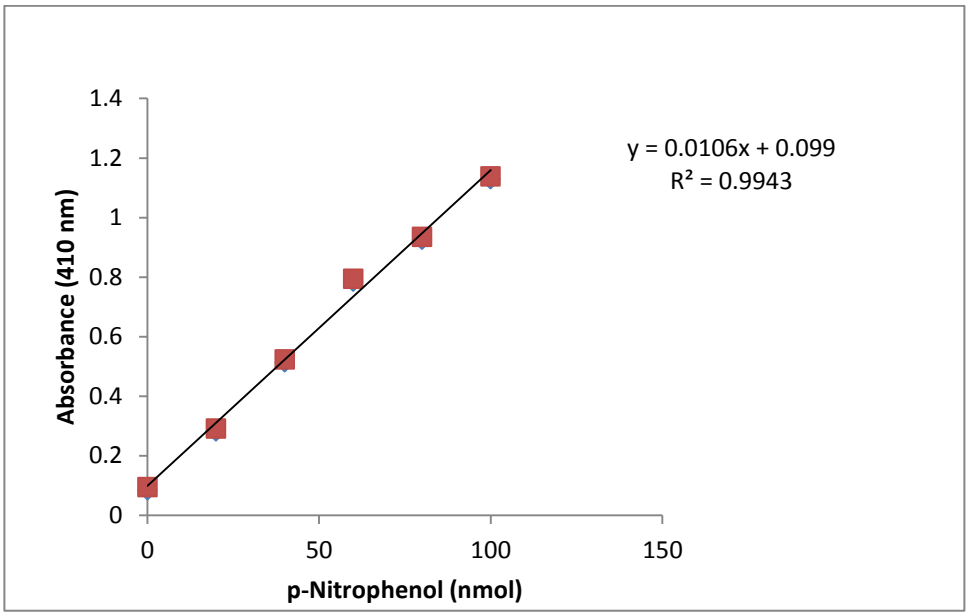


Figure 4.15. p- Nitrophenol standard curve used for determining α-glucosidase activity

The standard curve shows a linear relationship between the nitrophenol concentrations and the absorbance.

Figure. 4.15. shows the effect of the different concentrations of *HH* (5 µg/ml and 10 µg/ml) on alpha glucosidase activity. *HH* significantly decreased the activity of the α-glucosidase positive control (1.84± 0.12 U/ml vs 0.12± 0.3 U/ml; $P \leq 0.001$) in the presence of 5 µg/ml *HH*, and to 0.13± 0.3 U/ml ($P \leq 0.001$) in the presence of 10 µg/ml *HH*. The results were expressed as the mean± SEM value of alpha amylase activity, in 3 different experiments.

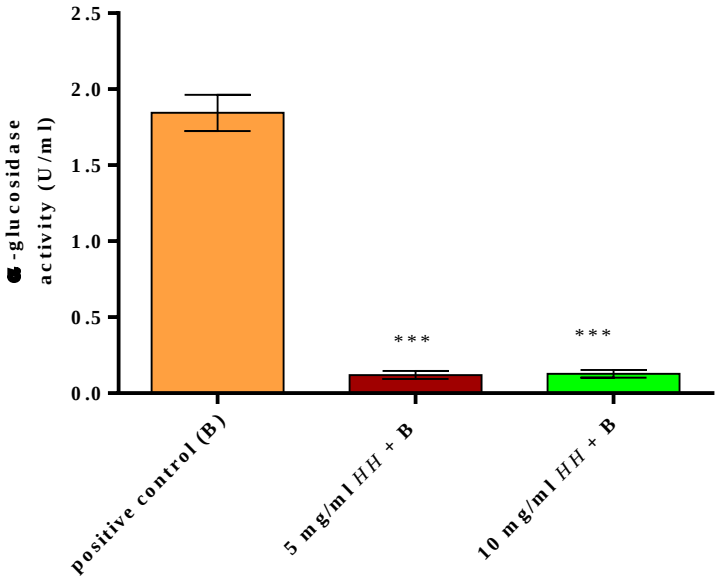


Figure 4.16. . Alpha glucosidase activity in the presence of two concentration of *HH*. Values are the mean ± SEM. *HH*: *Hypoxis hemerocallidea*, G: Alpha glucosidase. *** P ≤ 0.001, compared to the positive control.

CHAPTER V

5 Discussion

The main aim of this research was to study the possible hypoglycemic effect using RIN-5 F pancreatic tumor cells. To accomplish this, the study design was divided into three parts: (1) to test whether exposure of RIN-5F cells to glucose or the *HH* aqueous extract will effect cell proliferation and cell viability, (2) to test whether the *HH* extract have an effect on insulin secretion and (3) to test whether the *HH* extract has an effect on alpha amylase and alpha glucosiadase enzyme activity. The *HH* aqueous extract caused significant reductions in the blood glucose levels of the fasted normal and STZ-treated diabetic rats (Mahomed & Ojewole, 2003). Erlwanger & Cooper (2008) found significant antidiabetic effects starting at 100 mg/kg in both normoglycemic and hypoglycemic rats. A previous study also indicated that the *HH* has anti diabetic properties and suggested that the effect is due to *HH* having antioxidant properties (Katerere & Eloff, 2008). While it is likely that the hypoglycemic effect of the plant extract is largely due to its sterolin content and/or phytosterols, the exact mechanism of its hypoglycemic action is still obscure and will have to await further studies (Mahomed & Ojewole, 2003).

5.1 Effect of glucose and *HH* on cell proliferation and cell viability

5.1.1 Proliferation studies

The present study was to clarify whether prolonged *in vitro* exposure of RIN-5F cells to glucose and *HH* impairs mitosis and growth of these cells. The energy to perform cell division, growth, and replacement of damaged effected cells, is derived from the aerobic metabolism of glucose. The energy required for nuclear division appears to be

fully or partly derived from the anaerobic metabolism of glucose (Holt, 1980; Greiner *et al.*, 1994).

The glucose stimulates cell cycle progression, which implies that an increase in glucose could stimulate cell proliferation and division (Salpeter *et al.*, 2010). Figure 4.1. and Table 4.1 showed mostly a decrease in cell proliferation especially after the 24 hour exposure time period where a significant decrease was observed. The only significant increase in cell proliferation was observed in the cells exposed to 50 mM glucose for 1 hour and 3 hours. This would imply that an increase in glucose could inhibit cell proliferation and division when the cells are exposed for at least 24 hours. Salpeter *et al* (2010) reported that β-cells are quick to regain their capacity of re-entering the cell cycle post-mitosis and implicate glucose control of cyclin D_2 expression in the regulation of cell cycle progression. In previous studies, glucose appears to be beneficial for maintaining cell metabolism and proliferation *in vitro*. A recent study showed that the culture expansion under low glucose conditions protect the desired characteristics for tissue formation (Heywood *et al.*, 2014). Cell proliferation is modulated in accordance with its metabolic status, and this will interfere in the cell's biology and function, since the signal transduction, genetic expression, and metabolism depend on protein transformation processes (Fock *et al.*, 2010).

Figure 4.2. and Table 4.2 show proliferation differences of *HH* on RIN5-F cells which may play an important role in the administration of *HH* in traditional medicine for the treatment of disease. An increase in cell proliferation was observed at concentrations of 200 mg/ml *HH* higher for 1, 3 and 6 hours. Thus would imply adaptation to the *HH* extract and survival of these cells. When the cells were exposed to the *HH* extract over 24 hours, a slight but not significant decrease in cell proliferation was seen. The decrease could be due to cytotoxic effects since cytotoxicity on MDA-MB-231 human breast cancer cells are shown to be due to the presence of β-sitosterol or other unknown active compounds in the chloroform *HH*

extracts (Awad & Fink, 2000). Boukes & van de Venter (2011) demonstrated that the mechanism by which *HH* induced cytotoxicity in cancer cells could be due to apoptosis and cell cycle arrest. This is the first time that cytotoxicity of *HH* is studied on RIN-5F cells and the mechanisms of possible cytotoxic effects must be elucidated.

5.1.2 Viability studies

Mitochondrial dehydrogenase activity is often used to measure cell viability, including β-cell viability (Maechler & Wollheim, 2001). β-cell mitochondria serve as fuel sensors, generating factors that couple nutrient metabolism to the exocytosis of insulin-containing vesicles (Maechler & Wollheim, 2001). This study was to clarify whether prolonged *in vitro* exposure of RIN-5F cells to glucose and *HH* concentration impairs the viability of these cells by affecting mitochondrial dehydrogenase activity. Findings displayed in Figure 4.3 and Table 4.3 show that cell viability was decreased significantly by glucose after 6 hours and 24 hours exposure. An increase in cell viability was observed at concentrations below 50 mM glucose, in the cells exposed for 3 hours or less. Although slight, the increase in viability observed at 37.5 and 50 mM glucose was significant, and most probably due to an increase in mitochondrial dehydrogenase activity (Berridge *et al.,* 1996). A significant decrease was seen in cells exposed to glucose for 24 hours at all concentrations tested and cells exposed to glucose for 6 hours at concentrations above 37.5 mM glucose since glucose can influence cyclin D_2 expression in cell growth control (Salpeter *et al.,* 2010). This decrease of cell viability after 24 hours could be due to down regulation of cyclin D_2 that prevent replicated β-cells from returning to the pool of dividing cells (Salpeter *et al.,* 2010).

Figure 4.4 and Table 4.4 show the cell viability of *HH* on RIN5-F cells which may play an important role in the administration of *HH* in patients. An increase in cell viability was observed at concentrations of 200 µg/ml and 500 µg/ml if cells were exposed for 6 hours or less. This increase in cell viability could be indicative of a

marked increase in the activity of the mitochondrial dehydrogenase and electron carriers which indicates that this plant extract may target the mitochondria of the pancreatic cell (Scaduto & Grotyohann, 1999). When the cells were exposed to the *HH* extract over 24 hours, *HH* did not affected cell viability significantly and only a slight but insignificant decrease in cell viability was observed. This could ascribed to cellular stress, that leads to a decrease in mitochondrial dehydrogenase activity (Berridge *et al.,* 1996). Cyclin D_2 together with cyclin Dependent kinase 4 (cdk4) is required for cell cycle progression. Inhibition of the cyclin D_2 and (cdk4) complex leads to the induction of a cell cycle block that can influence cell proliferation and ultimately cell proliferation.

Compared to the cell proliferation, the cell viability seems here to be slightly more sensitive in detecting the metabolic activity of the RIN-5F cells. Using the XTT assay, cells exposed to glucose showed a possible significant increase in the activity of the mitochondrial dehydrogenases by 37.5 mM and 50 mM glucose (Table 4.3), while the crystal violet assay showed a significant increase in cell proliferation starting from 50 mM glucose (Table 4.1). However, this difference in the sensitivity of both assays may be also a result of different passage of cells used. Using the XTT assay results in Table 4.4, cells exposed to *HH* extract showed a significant increase in the activity of the mitochondrial dehydrogenases after exposure to 200µg/ml *HH* and 500 µg/ml after 3 and 6 hours.

These results show a marked increase in the cell viability and cell proliferation when exposed to high glucose concentrations and high dose of *HH* extract for short time period 1, 3 and 6 hours. Alteration of cyclin abundance is sufficient to change the rate of cell cycle progression, since over expression of G1 cyclins such as cyclin D accelerates cells through G1. Conversely, inhibition of their function can prevent entry into the S phase (Musqrove *et al.,* 1988). It is possible what an initial increase in cyclin D_2 levels by the higher glucose concentrations stimulated cell cycle progression, while continues exposure (24 hours) induced a depletion in cyclin D_2

and mitochondrial dehydrogenase causing a decrease in cell proliferation and cell viability.

5.2 Effect of *HH* on insulin secretion

The purpose of the study was also to evaluate the effect of *HH* on RIN-5F cell insulin secretion. When the demand for insulin is increased by physiological or pathological changes, β-cells can adapt by enhancing insulin secretion via increased β-cell function and/or increased β-cell mass (Andrali *et al.,* 2008; Leibiger *et al.,* 2008). 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). The RIN-5F cells are the most important cells in secretion of insulin and secretion of small amounts of glucagon and somatostatin (Poitout *et al.,* 1996).

Glucose stimulated insulin secretion in low (2 mM) and high (50 mM) concentration for 30 minutes (Figure 4.5). Also shown (Figure 4.9) is increased insulin secretion from RIN5F cells after exposed to high glucose (50 mM) after 90 minutes, while, insulin secretion after exposed to low glucose (2 mM) was no significant difference from the control group. Interestingly, there was not significantly different between high glucose (50 mM) and low glucose (2 mM) after exposed of RIN-5F cells after 30 minutes and 90 minutes. 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 different concentration, (5.5 mM) and (16.7 mM) of glucose when comparing to the control group.

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 result in stimulation 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). The antihyperglycemic activity of *HH* achieved through insulin secretory potentiation may be due to the presence of the β- sitosterol (Farmer *et al.*, 2012).

The present study has also shown the beneficial use of *HH* in inducing insulin secretion from pancreatic cells. Findings displayed in Figure 4.6 show that insulin secretion was increased from RIN5F cells after exposure to low *HH* (100 µg/ml) or high *HH* (500 µg/ml) for 30 minutes. Exposure of RIN5-F cells to *HH* for 90 minutes caused a further increase in insulin secretion from (4.3±0.17 mIU/mg protein; $P \leq 0.01$) in 100 µg/ml, to (7.87±0.17 mIU/mg protein; $P \leq 0.001$) in 500 µg/ml (Figure 4.10). At both 30 minutes and 90 minutes, insulin secretion was significantly higher when cells where exposed to 500 µg/ml *HH* compared to 100 µg/ml *HH* (Figure 4.6 and Figure 4.10). *HH* significantly increased glucoses stimulated insulin secretion by RIN-5F cells. The stimulation of insulin secretion and potential glucose absorption by RIN-5F was dependent on the dose and severity of hyperglycemia. Steroids might stimulate the islets to secrete insulin and might increase sensitivity to insulin, thereby enhancing glucose uptake. Similarly, Achrekar *et al* (1991) reported that an aqueous extract of the pulp of *Eugenia jambolana* stimulated the release of insulin both in vitro and in vivo.

Figure 4.7 illustrates that in the presence of the low *HH* concentration (100 µg/ml, 30 minutes) insulin secretion is higher than at the 2 mM glucose concentration, while, at the high *HH* concentration (500µg/ml, 30 minutes) insulin secretion was not significantly different from the 2mM glucose. It is important to note that at the low *HH* concentration (100µg/ml) insulin secretion is higher than at the high *HH* concentration (500µg/ml). Similar results are obtained after 90 minutes finding displayed in Figure 4.11. We do not know whether the rate of insulin secretion

remained constant over the 90 minutes. It is possible that the β-cells became either desensitized or exhausted. There are two clear concepts for glucose-induced desensitizations, the first is a consequence of functional changes in the β-cell that impair glucose-recognition. The second, a consequence increased secretory activity in the β-cell leads to a depletion of releasable insulin (Rustenbeck, 2002). Increase insulin secretion from β-cells occurs by two various mechanisms. The first, under high-glucose conditions, proceeded by a classical secretory pathway involving KATP 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).

In the presence of high-glucose (50 mM), at both 30 minutes and 90 minutes, insulin secretion was significantly decreased when cells where exposed to low concentration of *HH* (100 µg/ml) and high concentration of *HH* (500 µg/ml) (Figure 4.8 and Figure 4.12), however, there was no significant difference between low *HH* (100 µg/ml) and high *HH* (500 µg/ml). The significant decrease in insulin secretion in the presence of high-glucose (50 mM) for a long period caused cell dysfunction and may induce cell apoptosis in type 2 diabetes. High glucose has many implications, initially resulting in "glucose hypersensitization" and later apoptosis, by different mechanisms. High glucose does not induce or activate IL-1, NF-B, or inducible nitric oxide synthase in rat or human cells. This will have to be tested in RIN-5F cells, especially in the presence of *HH* (Miriam, cnop *et al.*, 2005).

5.3 Effect of *HH* on α-amylase and α-glucosidase activity

The inhibition of α-amylase activity and α-glucosidase activity is considered to be an effective strategy for the control of blood glucose levels by slowing the digestion of glucose (Tundis *et al.*, 2010). As shown in Figure 4.14 the *HH* extract inhibited α-amylase enzyme activity. The maximum inhibition was 37 % at a concentration of 10 mg/ml, while 5 mg/ml *HH* failed to produce significant inhibition. Moreover, from

Figure 4.16 It is clear that the *in vitro* α-glucosidase inhibitory studies demonstrated that *HH* extract had significant α- glucosidase inhibitory activity. The maximum inhibition was 93.5 % at a concentration of 10 mg/ml while 5 mg/ml inhabit α-glucosidase activity by 93 %. Mcdougall *et al.,* (2005) showed that strawberry and raspberry extracts were more effective as α-amylase inhibitors than blueberry, blackcurrant, or red cabbage. Conversely, α-glucosidase was more readily inhibited by blueberry and blackcurrant extracts.

The extent of inhibition of α- glucosidase was related to their anthocyanin components. Different polyphenolic components of these fruits may influence different steps in starch digestion in a synergistic manner (Honda & Hara, 1993). Some medicinal plants such as *Horsfieldia amygdalina*, has been reported to have anti-diabetic activity when assessed using presently available experimental techniques (Honda & Hara, 1993; Grover *et al.,* 2002; Latha *et al.,* 2004; Mai *et al.,* 2007). Sweet potato, berry, and polyphenols from tea have a significant antioxidant activity since various phenolic compounds have been generally accepted as anti-oxidant agents, it has been shown that the activity of α-glucosidase and α-amylase is effectively inhibited by anti-oxidant agents. A previous study also indicated that the *HH* have anti diabetic properties and it also suggests that the effect is due to *HH* having antioxidant properties (Katerere and Eloff, 2008). Therefore, these findings indicate that the differences in the inhibitory effects of *HH* on the activity of α-glucosidase and α-amylase could be due to the levels of anti-oxidant compounds in their extracts.

Mahomed & Ojewole (2003) reported that the antidiabetic activities of *HH* using 800 mg/kg aqueous extract causes 20- 30% and 48-54 % reductions in the blood glucose level of fasted normal and STZ-treated diabetic rats. Both the aqueous and ethanolic extracts of *HH* are able to scavenge hydroxyl radicals through their anti-oxidant property by the presence of hypoxidae (Nair *et al* (2007).

5.4 Conclusions

Our objectives were:

- To determine the toxicity of *Hypoxis hemerocallidea* and glucose on pancreatic RIN-5 F cell viability
- To determine whether *Hypoxis hemerocallidea* can increase insulin secretion from the pancreatic RIN-5 F cells.
- To determine whether *Hypoxis hemerocallidea* can inhibit the activity of α-amylase and α -glucosidase.

(Evaluation of the ability to plant extract to increase glucose uptake by cells falls outside the scope of this thesis)

A marked increase in the cell viability and cell proliferation was found when RIN-5F cells were exposed to high glucose concentrations and high dose of *HH* extract for a short time period (1, 3 and 6 hours). The *HH* aqueous extract has good potential for the management of blood glucose levels due to its ability to inhibit α-amylase activity and α-glucosidase activity. The *HH* aqueous extract increased insulin secretion under our basic experimental conditions and in the presence of low glucose levels, but not at high (50 mM) glucose concentrations. Insulin secretion in the presence of different glucose concentrations, in the presence of *HH*, needs further investigation. It would be particularly important to test the effect of *HH* on insulin secretion when the concentration of glucose in the medium is between 10 and 25 mM, levels more likely to be found in uncontrolled diabetes.

REFERENCES

Adkins-Marshall, B.A., Myers, S.R., Hendrick, G.K., Williams, P.E., Triebwasser, K., Floyd, B., Cherrington, A.D., 1990. Interaction Between Insulin and Glucose-Delivery Route in Regulation of Net Hepatic Glucose Uptake in Conscious Dogs. Diabetes 39, 87–95.

Ammälä, C., Ashcroft, F.M., Rorsman, P., 1993. Calcium-independent potentiation of insulin release by cyclic AMP in single beta-cells. Nature 363, 356–358.

Andrali, S.S., Sampley, M.L., Vanderford, N.L., Ozcan, S., 2008. Glucose regulation of insulin gene expression in pancreatic beta-cells. Biochem. J. 415, 1–10.

Arciero, P.J., Vukovich, M.D., Holloszy, J.O., Racette, S.B., Kohrt, W.M., 1999. Comparison of short-term diet and exercise on insulin action in individuals with abnormal glucose tolerance. J. Appl. Physiol. 86, 1930–1935.

Aronoff, S.L., Berkowitz, K., Shreiner, B., Want, L., 2004. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectr. 17, 183–190.

Arthur, R., Gruner, T., 2007. Digestion and enzymes. J. Complement. Med. 6, 54–59.

Arumugam, G., Manjula, P., Paari, N., 2013. A review: Anti diabetic medicinal plants used for diabetes mellitus. J. Acute Dis. 2, 196–200.

Awad, a B., Fink, C.S., 2000. Phytosterols as anticancer dietary components: evidence and mechanism of action. J. Nutr. 130, 2127–2130.

Babb, D.A., Pemba, L., Seatlanyane, P., Charalambous, S., Churchyard, G.J., Grant, A.D., 2007. Use of traditional medicine by HIV-infected individuals in South Africa in the era of antiretroviral therapy. Psychol. Health Med. 12, 314–320.

Basu, P.S., Biswas, B.B., Pal, M.K., 1968. Extraction of crystal violet-iodine complex from gram positive bacteria by different solvents and its implication on gram differentiation. Histochemie 14, 221–229.

Berridge, M., Tan, A., McCoy, K., Wang, R., 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica 4–9.

Berridge, M. V, Herst, P.M., Tan, A.S., 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. 11, 127–152.

Bhathena, S.J., Awoke, S., Voyles, N.R., Wilkins, S.D., Recant, L., Oie, H.K., Gazdar, A.F., 1984. Insulin, glucagon, and somatostatin secretion by cultured rat islet cell tumor and its clones. Proc. Soc. Exp. Biol. Med. 175, 35–38.

Bhathena, S.J., Oie, H.K., Gazdar, A.F., Voyles, N.R., Wilkins, S.D., Recant, L., 1982. Insulin, glucagon, and somatostatin receptors on cultured cells and clones from rat islet cell tumor. Diabetes 31, 521–531.

Botha, C.J., Penrith, M.L., 2008. Poisonous plants of veterinary and human importance in southern Africa. J. Ethnopharmacol. 119, 549–558.

Boukes, G.J., van de Venter, M., 2011. Cytotoxicity and mechanism(S) of action of Hypoxis spp. (African Potato) against HeLa, HT-29 and MCF-7 cancer cell lines. J. Med. Plants Res. 5, 2766–2774.

Boukes, G.J., Venter, M. Van De, Oosthuizen, V., 2008. Quantitative and qualitative analysis of sterols / sterolins and hypoxoside contents of three Hypoxis (African potato) spp . Aferican J. Biotechnol. 7, 1624–1629.

Brandenburg, D., 2008. History and diagnostic significance of C-peptide. Exp. Diabetes Res. 2008, 57-62.

Brown, L., Heyneke, O., Brown, D., van Wyk, J.P.H., Hamman, J.H., 2008. Impact of traditional medicinal plant extracts on antiretroviral drug absorption. J. Ethnopharmacol. 119, 588–592.

Cnop, M., Welsh, N., Jonas, J.-C., Jörns, A., Lenzen, S., Eizirik, D.L., 2005. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 54 Suppl 2, S97–S107.

Colberg, S., Colberg, S.R., 2009. Diabetic Athlete's Handbook. Available at: https://books.google.com/books?hl=ar&lr=&id=9SmabycfsI8C&pgis=1 [Accessed July 20, 2015].

Conlon, J.M., Deacon, C.F., Hazon, N., Henderson, I.W., Thim, L., 1988. Somatostatin-related and glucagon-related peptides with unusual structural features from the European eel (Anguilla anguilla). Gen. Comp. Endocrinol. 72, 181–189.

Chiasson, J.-L., Aris-Jilwan, N., Belanger, R., Bertrand, S., Beauregard, H., Ekoe, J.-M., Fournier, H., Havrankova, J., 2003. Diagnosis and treatment of diabetic ketoacidosis and the hyperglycemic hyperosmolar state. Can. Med. Assoc. J. 168, 859–866.

Deguara, S., Jauncey, K., Agius, C., 2003. Enzyme activities and pH variations in the digestive tract of gilthead sea bream. J. Fish Biol. 62, 1033–1043.

Deshpande, A.D., Harris-Hayes, M., Schootman, M., 2008. Epidemiology of diabetes and diabetes-related complications. Phys. Ther. 88, 1254–64.

Dimmer, K.S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., Westermann, B., 2002. Genetic basis of mitochondrial function and morphology in Saccharomyces cerevisiae. Mol. Biol. Cell 13, 847–53.

Dong, H.-Q., Li, M., Zhu, F., Liu, F.-L., Huang, J.-B., 2012. Inhibitory potential of trilobatin from Lithocarpus polystachyus Rehd against α-glucosidase and α-amylase linked to type 2 diabetes. Food Chem. 130, 261–266.

Elayat,A, El-Naggar, M.M., Tahir, M., 1995. An immunocytochemical and morphometric study of the rat pancreatic islets. J. Anat. 186, 629–37.

Erasto, P., Adebola, P.O., Grierson, D.S., Afolayan, A.J., 2005. An ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province , South Africa. J. Biotechnol. 4, 1458–1460.

Erlwanger, K.H., Cooper, R.G., 2008. The effects of orally administered crude

alcohol and aqueous extracts of African potato (Hypoxis hemerocallidea) corm on the morphometry of viscera of suckling rats. Food Chem. Toxicol. 46, 136–139.

Fock, R.A., Blatt, S.L., Beutler, B., Pereira, J., Tsujita, M., de Barros, F.E. V, Borelli, P., 2010. Study of lymphocyte subpopulations in bone marrow in a model of protein-energy malnutrition. Nutrition 26, 1021–1028.

Gawlicka, A., Parent, B., Horn, M.H., Ross, N., Opstad, I., Torrissen, O.J., 2000. Activity of digestive enzymes in yolk-sac larvae of Atlantic halibut (Hippoglossus hippoglossus): Indication of readiness for first feeding. Aquaculture 184, 303–314.

Gray, A.M., Flatt, P.R., 2007. Insulin-releasing and insulin-like activity of the traditional anti-diabetic plant Coriandrum sativum (coriander). Br. J. Nutr. 81, 203–209.

Greiner, E.F., Guppy, M., Brand, K., 1994. Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. J. Biol. Chem. 269, 31484–31490.

Grierson, D.S., Afolayan, A.J., 1999. An ethnobotanical study of plants used for the treatment of wounds in the Eastern Cape, South Africa. J. Ethnopharmacol. 67, 327–332.

Gromada, J., Franklin, I., Wollheim, C.B., 2007. Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. Endocr. Rev. 28, 84–116.

Grover, J.K., Yadav, S., Vats, V., 2002. Medicinal plants of India with anti-diabetic potential. J. Ethnopharmacol. 81, 81–100.

Haidar, B., Denis, M., Marcil, M., Krimbou, L., Genest, J., 2004. Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter. J. Biol. Chem. 279, 9963–9981.

Hemmerich, S., 2001. Carbohydrate sulfotransferases: Novel therapeutic targets for

inflammation, viral infection and cancer. Drug Discov. Today. 6 (1), 27–35.

Herman, M.A., Kahn, B.B., 2006. Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. J. Clin. Invest. 116, 1767–75.

Henquin, J.-C., 2004. Pathways in Beta-Cell Stimulus-Secretion Coupling as Targets for Therapeutic Insulin Secretagogues. Diabetes 53, S48–S58.

Heywood, H.K., Nalesso, G., Lee, D. a, Dell'accio, F., 2014. Culture expansion in low-glucose conditions preserves chondrocyte differentiation and enhances their subsequent capacity to form cartilage tissue in three-dimensional culture. Biores. Open Access 3, 9–18.

Hiriart, M., Aguilar-Bryan, L., 2008. Channel regulation of glucose sensing in the pancreatic beta-cell. Am. J. Physiol. Endocrinol. Metab. 295, E1298–306.

Holt, J.A., 1980. The extra nuclear control of mitosis & cell function. A theory of cellular organisation. Med. Hypotheses 6, 145–192.

Honda, M., Hara, Y., 1993. Inhibition of rat small intestinal sucrase and α-glucosidase activities by tea polyphenols. Biosci. Biotechnol. Biochem. 57, 123–124.

Huber, G.W., Chheda, J.N., Barrett, C.J., Dumesic, J.A., 2005. Production of liquid alkanes by aqueous-phase processing of biomass-derived carbohydrates. Science 308, 1446–1450.

Ishiyama, M., 1997. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44, 1299–1305.

Janeček, Š., Svensson, B., MacGregor, E.A., 2014. α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. Cell. Mol. Life Sci. CMLS, 71(7), 1149–1170.

Jo, J., Choi, M.Y., Koh, D.-S., 2007. Size distribution of mouse Langerhans islets. Biophys. J. 93, 2655–2666.

Kahn, S.E., Hull, R.L., Utzschneider, K.M., 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444, 840–846.

Kandra, L., 2003. α-Amylases of medical and industrial importance. In: Journal of Molecular Structure: theochem. pp. 487–498.

Katerere, D.R., Eloff, J.N., 2008. Anti-bacterial and anti-oxidant activity of Hypoxis hemerocallidea (Hypoxidaceae): Can leaves be substituted for corms as a conservation strategy? South African J. Bot. 74, 613–616.

Klingenberg, M., 2008. The ADP and ATP transport in mitochondria and its carrier. Biochim. Biophys. Acta 1778, 1978–2021.

Knip, M., Veijola, R., Virtanen, S.M., Hyöty, H., Vaarala, O., Åkerblom, H.K., 2005. Environmental triggers and determinants of type 1 diabetes. Diabetes 54. (SUPPL. 2), S125–S136.

Koduru, S., Grierson, D.S., Afolayan, A.J., 2007. Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province , South Africa. Curr. Sci. 92, 906–908.

Kumar, V., Prakash, O., Kumar, S., Narwal, S., 2011. α-glucosidase inhibitors from plants: A natural approach to treat diabetes. Pharmacogn *Reviews*. 5 (9), p.19

Kuznetsov, B.G., 1978. "Entero-insular axis" and regulation of blood sugar and insulin levels following oral glucose loading. Fiziol. Zh. SSSR Im. I. M. Sechenova 64, 1626–1631.

Latha, M., Pari, L., Sitasawad, S., Bhonde, R., 2004. Scoparia dulcis, a traditional antidiabetic plant, protects against streptozotocin induced oxidative stress and apoptosis in vitro and in vivo. J. Biochem. Mol. Toxicol. 18, 261–72.

Lee, Y.Y., Park, K.S., Pak, Y.K., Lee, H.K., 2005. The role of mitochondrial DNA in the development of type 2 diabetes caused by fetal malnutrition. J. Nutr. Biochem. 16, 195–204.

Leibiger, I.B., Leibiger, B., Berggren, P.-O., 2008. Insulin signaling in the pancreatic

beta-cell. Annu. Rev. Nutr. 28, 233–251.

Lordan, S., Smyth, T.J., Soler-Vila, A., Stanton, C., Paul Ross, R., 2013. The α-amylase and α-glucosidase inhibitory effects of Irish seaweed extracts. Food Chem. 141, 2170–2176.

Lotfy, M.I., 2012. Physiological, morphological and molecular biological studies of the effect of glucagon-like peptide-1 and exenatide in the diabetic rat pancreas. School of Forensic and Investigative Science. University of Central Lancashire.

Louw, C.A.M., Regnier, T.J.C., Korsten, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. J. Ethnopharmacol. 82, 147–154.

Lowell, B.B., Shulman, G.I., 2005. Mitochondrial dysfunction and type 2 diabetes. Science 307, 384–7.

Lu, Y., Sharkey, T.D., 2006. The importance of maltose in transitory starch breakdown. Plant, Cell Environ. 29 (3), 353–366.

MacDonald, P.E., Eliasson, L., Rorsman, P., 2005. Calcium increases endocytotic vesicle size and accelerates membrane fission in insulin-secreting INS-1 cells. J. Cell Sci. 118, 5911–20.

Maechler, P., Li, N., Casimir, M., Vetterli, L., Frigerio, F., Brun, T., 2010. Role of mitochondria in beta-cell function and dysfunction. Adv. Exp. Med. Biol. 654, 193–216.

Maechler, P., Wollheim, C.B., 2001. Mitochondrial function in normal and diabetic beta-cells. Nature 414, 807–12.

Mahomed, I.M., Ojewole, J.A.O., 2003. Hypoglycemic effect of Hypoxis hemerocallidea corm (African potato) aqueous extract in rats. Methods Find. Exp. Clin. Pharmacol. 25, 617–623.

Mai, T.T., Thu, N.N., Tien, P.G., Van Chuyen, N., 2007. Alpha-glucosidase inhibitory and antioxidant activities of Vietnamese edible plants and their

relationships with polyphenol contents. J. Nutr. Sci. Vitaminol. (Tokyo). 53, 267–276.

Maurus, R., Begum, A., Kuo, H.-H., Racaza, A., Numao, S., Andersen, C., Tams, J.W., Vind, J., Overall, C.M., Withers, S.G., Brayer, G.D., 2005. Structural and mechanistic studies of chloride induced activation of human pancreatic alpha-amylase. Protein Sci. 14, 743–755.

McDougall, G.J., Shpiro, F., Dobson, P., Smith, P., Blake, A., Stewart, D., 2005. Different polyphenolic components of soft fruits inhibit alpha-amylase and alpha-glucosidase. J. Agric. Food Chem. 53, 2760–2766.

Mills, E., Cooper, C., Seely, D., Kanfer, I., 2005. African herbal medicines in the treatment of HIV: Hypoxis and Sutherlandia. An overview of evidence and pharmacology. Nutr. J. 4, 19.

Mogatle, S., 2008. African traditional medicines-antiretroviral drug interactions: the effect of african potato *(Hypoxis hemerocallidea)* on the pharmacokinetics of efavirenz in humans. Rhodes University, South Africa.

Muroyama, A., Uehara, S., Yatsushiro, S., Echigo, N., Morimoto, R., Morita, M., Hayashi, M., Yamamoto, A., Koh, D.-S., Moriyama, Y., 2004. A Novel Variant of Ionotropic Glutamate Receptor Regulates Somatostatin Secretion From -Cells of Islets of Langerhans. Diabetes 53, 1743–1753.

Nair, V.D.P., Dairam, A., Agbonon, A., Arnason, J.T., Foster, B.C., Kanfer, I., 2007. Investigation of the antioxidant activity of African potato (Hypoxis hemerocallidea). J. Agric. Food Chem. 55, 1707–1711.

Nair, V.D.P., Kanfer, I., 2006. High-performance liquid chromatographic method for the quantitative determination of hypoxoside in African potato (Hypoxis hemerocallidea) and in commercial products containing the plant material and/or its extracts. J. Agric. Food Chem. 54, 2816–2821.

Nair, V.P., 2006. Pharmaceutical analysis and drug interaction studies: African Potato (Hypoxis hemerocallidea). Rhodes University, South Africa.

Nobukawa MD PhD, B., 2007. Vascular anatomy of the pancreas. In: Pancreas - Pathological Practice and Research. S. Karger AG. 8–11.

Nonogaki, K., 2000. New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia 43, 533–49.

Ojewole, J.A.O., 2006. Antinociceptive, anti-inflammatory and antidiabetic properties of Hypoxis hemerocallidea Fisch. & C.A. Mey. (Hypoxidaceae) corm [’African Potato'] aqueous extract in mice and rats. J. Ethnopharmacol. 103, 126–134.

Olubummo, A., 2010. Human Anatomy and Physiology: Study Notes. Available at: https://books.google.com/books?hl=ar&lr=&id=xL0D9MIJ3H8C&pgis=1 [Accessed July 20, 2015].

Poitout, V., Olson, L.K., Robertson, R.P., 1996. Insulin-secreting cell lines: classification, characteristics and potential applications. Diabetes Metab. 22, 7–14.

Prentki, M., Tornheim, K., Corkey, B.E., 1997. Signal transduction mechanisms in nutrient-induced insulin secretion. Diabetologia 40, S32–S41.

Purrello, F., Vetri, M., Gatta, C., Gullo, D., Vigneri, R., 1989. Effects of High Glucose on Insulin Secretion by Isolated Rat Islets and Purified -Cells and Possible Role of Glycosylation. Diabetes 38, 1417–1422.

Quesada, I., Tudurí, E., Ripoll, C., Nadal, A., 2008. Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. J. Endocrinol. 199, 5–19.

Reddy, H., 2012. Association of Estrogen Receptor Gene Polymorphism & the Lipid Profile Changes in Type 2 Diabetic Indian Women. SSRN Electron. J. p,63.

Roberfroid, M., 2004. Inulin-Type Fructans: Functional Food Ingredients. CRC Press.235-264.

Rustenbeck, I., 2002. Desensitization of insulin secretion. Biochem. Pharmacol. 63, 1921–1935.

Salpeter, S.J., Klein, A.M., Huangfu, D., Grimsby, J., Dor, Y., 2010. Glucose and aging control the quiescence period that follows pancreatic beta cell replication. Development 137, 3205–3213.

Scaduto, R.C., Grotyohann, L.W., 1999. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys. J. 76, 469–477.

Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., Boyd, M.R., 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 48, 4827–4833.

Singh, Y., 2007. Hypoxis (Hypoxidaceae) in southern Africa: Taxonomic notes. South African J. Bot. 73, 360–365.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. JNCI J. Natl. Cancer Inst. 82, 1107–1112.

Slaaby, R., Schäffer, L., Lautrup-Larsen, I., Andersen, A.S., Shaw, A.C., Mathiasen, I.S., Brandt, J., 2006. Hybrid receptors formed by insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) have low insulin and high IGF-1 affinity irrespective of the IR splice variant. J. Biol. Chem. 281, 25869–25874.

Smit, B.J., Albrecht, C.F., Liebenberg, R.W., Kruger, P.B., Freestone, M., Gouws, L., Thero, E., Bouic, P.J.D., Etsebeth, S., Van Jaarsveld, P.P., 1995. A phase I trial of hypoxoside as an oral prodrug for cancer therapy - Absence of toxicity. stellenbosch university, South Africa.

Steenkamp, V., 2003. Phytomedicines for the prostate. Fitoterapia. 74 (6), pp.545–552.

Steiner, D., 2004. The proinsulin C-peptide—a multirole model. Exp. Diabetes Res. 2004.

Steiner, D.F., Park, S.-Y., Støy, J., Philipson, L.H., Bell, G.I., 2009. A brief perspective on insulin production. Diabetes. Obes. Metab. 11 Suppl 4, 189–96.

Straub, S.G., Sharp, G.W.G., 2002. Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes. Metab. Res. Rev. 18, 451–63.

Street, R., Prinsloo, G., 2012. Commercially important medicinal plants of South Africa: A review. J. Chem. 2013, 1–16.

Sugihara, S., Sasaki, N., Amemiya, S., Kohno, H., Tanaka, T., Matsuura, N., 2008. Analysis of weight at birth and at diagnosis of childhood-onset type 2 diabetes mellitus in Japan. Pediatr. Diabetes 9, 285–290.

Sun, Z., Henson, C.A., 1990. Degradation of Native Starch Granules by Barley alpha-Glucosidases. Plant Physiol. 94, 320–327.

Szkudelski, T., 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol. Res. 50 (6), 537–546.

Tengholm, A., Gylfe, E., 2009. Oscillatory control of insulin secretion. Mol. Cell. Endocrinol. 297, 58–72.

Thornhill, A.H., Harper, I.S., Hallamz, N.D., 2008. The development of the digestive glands and enzymes in the pitchers of three Nepenthes species: N. alata, N. tobaica, and N. ventricosa (Nepenthaceae). Int. J. Plant Sci. 169, 615–624.

Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., Watanabe, M., 1999. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal. Commun. 36, 47–50.

Tundis, R., Loizzo, M.R., Menichini, F., 2010. Natural Products as α-Amylase and α-Glucosidase Inhibitors and their Hypoglycaemic Potential in the Treatment of Diabetes: An Update. Mini Rev. Med. Chem. 10 (4), 315–331.

Van Op den Bosch, J., Adriaensen, D., Van Nassauw, L., Timmermans, J.-P., 2009. The role(s) of somatostatin, structurally related peptides and somatostatin receptors in the gastrointestinal tract: a review. Regul. Pept. 156, 1–8.

Weise, W.J., Sivanandy, M.S., Block, C.A., Comi, R.J., 2009. Exenatide-associated ischemic renal failure. Diabetes Care. 32, e22–3.

Wicksteed, B., Herbert, T.P., Alarcon, C., Lingohr, M.K., Moss, L.G., Rhodes, C.J., 2001. Cooperativity between the preproinsulin mRNA untranslated regions is necessary for glucose-stimulated translation. J. Biol. Chem. 276, 22553–22558.

Wilcox, G., 2005. Insulin and insulin resistance. Clin. Biochem. Rev. 26, 19–39.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care 27, 1047–1053.

Wollheim, C.B., Maechler, P., 2002. B-Cell Mitochondria and Insulin Secretion: Messenger Role of Nucleotides and Metabolites. Diabetes 51, S37–S42.

Woo, T.M., Wynne, A.L., 2011. Pharmacotherapeutics for Nurse Practitioner Prescribers. F.A. Davis. 524-581

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., Chen, H., 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J. Clin. Invest. 112, 1821–1830.

Yilmazer-Musa, M., Griffith, A.M., Michels, A.J., Schneider, E., Frei, B., 2012. Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of α-amylase and α-glucosidase activity. In: Journal of Agricultural and Food Chemistry. pp. 8924–8929.

Young, J.W., 1977. Gluconeogenesis in cattle: significance and methodology. J. Dairy Sci. 60, 1–15.

Zhang, Y., Li, P., Cheng, L., 2010. Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in “Honeycrisp” apple flesh. Food Chem. 123, 1013–1018.

Zhou, J.-Y., Dann, G.P., Liew, C.W., Smith, R.D., Kulkarni, R.N., Qian, W.-J., 2011. Unraveling pancreatic islet biology by quantitative proteomics. Expert Rev. Proteomics 8, 495–504.