Anti-diabetic activity of extracts and a bioactive compound isolated from *Hypoxis hemerocallidea* (Hypoxidaceae) in a murine model of spontaneous diabetes

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Faculty of Veterinary Science

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I declare that the thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD). In the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science.

These studies are the result of my own investigation, except where the work of others is acknowledged, and has not been submitted in any other form to another University.

DEDICATION

To my family: Valdes and Sean

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LIST OF ABBREVIATION

1D	1-dimentional
2D	2-dimentional
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BEA	Benzene, ethyl acetate, ammonia
CEF	Chloroform, ethyl acetate, formic acid
DPPH	1,1 diphenyl-2-picrylhydroazyl
DCM	Dichloromethane
DM	Diabetic Mellitus
EC	Effective concentration
ER	Endoplasmic reticulum
EMW	Ethyl acetate, methanol, water
DMSO	Dimethyl sulfoxide
FADH	Flavin adenine dinucleotide
GLUT	Glucose transport receptors
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PRCD	β- sitosterol
HMIT	H+-coupled myo-inositol transporter
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
IP3	Inositol 1,4,5-trisphosphate
IRS	Insulin responsive substrates
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
NADH	Nicotinamide adenine dinucleotide
PKC	Protein kinase C

RAS	Retrovirus-associated DNA sequences
pH	Potential of hydrogen
LC	Lethal concentration
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SGLT	Na^+ - coupled carrier system
Cu	Copper
Mn	Manganese
Fe	Iron
Cr	Chromium
Ni	Nickel
Pb	Lead
Zn	Zinc
ICP-MS	Inductive Couple Plasma Mass Spectrophotometer

ABSTRACT

A previous study investigated and demonstrated that *Hypoxis hemerocallidea* (Fisch and Mey) (Hypoxidaceae) corm extracts used traditionally, stimulated insulin secretion from pancreatic β cells maintained under *ex vivo* conditions. The effect was also related to the antioxidant activity of extracts. In order to find leads with unique chemical structures which may exert a hitherto unexploited mode of action, phytochemical investigation on extracts of the plant was carried out. This involved the following steps: authentication and extraction of the plant material, separation and isolation of the constituents of interest, characterization of the isolated compounds and quantitative evaluation. Furthermore, the following study also followed the normal sequence of principles of replacement, in that all necessary non-animal and *ex vivo* animal models were first to optimise evaluation of the product, before testing using a mouse model.

With *H. hemerocallidea* being traditionally used as wild bulbs, we firstly ascertained the risk of this tradional use with specific focus on heavy metals. This is important as certain soil minerals are known to increase antioxidant capacity in plants, and associated extracts, as a stress response to soil heavy metal content. However, the use of these wild plants may pose a safety concern to the person consuming the plant as a result of their potential heavy metal content. Corms collected from the wild from five different geographical regions of South Africa, were evaluated for their concentration of metal concentrations and associated antioxidant activity. Among the trace metals investigated, iron was the highest, for the corm collected from Ga-rankuwa. No link was found between the corm's antioxidant activity and environmental conditions.

We further investigated the corm from Ga-rankuwa, since it had the highest antioxidant activity for its anti-diabetic activity. Assays included glucose uptake in C_2C_{12} myocytes, 3T3-L1 preadipocytes and insulin secretion in rat insulinoma RiNm5F cells in total five different fractions and three compounds isolated. Only the methanol crude extract, fraction III & V and isolated β-sitosterol significantly increased insulin release and lowered blood glucose levels both in C_2C_{12} myocytes and 3T3-L1 preadipocytes to a degree. All the crude extracts, fractions and tested compounds had relatively low cytotoxicity against all the diabetic cell lines used.

In the final study we determined the effect of *H. hemerocallidea* and its isolated compound, β-sitosterol in a murine mouse model of spotaneous diabetes. The β-sitosterol induced slight weight loss in the mice and stabilised blood glucose concentration after the 4 weeks treatment course. No changes in the haematology were evident while clinical chemistry evaluations showed slight treatment-related changes in triglycerides and total cholesterol. The study concludes that *H. Hemerocallidae* was not a suitable as a sole treatment agent in the management of diabetes. However it holds promise as an add-on treatment to lifestyle intervention for the management of type-II diabetes.

PAPERS PREPARED FROM THIS THESIS

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Chapter 1:

General Introduction

1.1 Background and purpose

The word diabetes is borrowed from the Greek word *siphon* as the affected individuals experience clinical signs of polyuria and with loss of water from the body viz. a person with diabetes appears to be siphoned by the urinary system (American Diabetes Association, 2009; Bradshaw et al., 2006; Afolayan and Sunmonu, 2010). Diabetes could either be characterised as mellitus or insipidus (American Diabetes Association, 2012; Afolayan and Sunmonu, 2010). Diabetes mellitus is characterized by chronic hyperglycaemia and abnormal fat and protein metabolism caused by defects in insulin production or action (Khan et al., 2009; Ahmed and Goldestein, 2006), while diabetes insipidus in contrast, is characterized by excessive thirst and production of an excess volume of severely diluted urine.

Of the two forms, diabetes mellitus is the most prominent form in the world. Diabetes mellitus continues to cause greater mortality in human population than any other non-communicable disease, and is responsible for about 5% of death globally (International Diabetes Federation, 2017; World Health Organization, 2010; Gabrielsson and Weiner, 2006). The most recent predictions from the International Diabetes Federation shows that there are 382 million people with diabetes globally, with the number expected to increase to almost 600 million affected persons by 2035 (Moller and Flier, 1991; Zimmet et al., 2001). Although several forms of diabetes mellitus are known on the basis of their aetiology and clinical presentation, diabetes mellitus is best classified into two groups. Type I diabetes mellitus resulting from failure

of pancreatic β–cells to produce insulin usually form the autoimmune destruction of pancreatic β–cells, and the more common type II diabetes mellitus which is the heterogenous disorder characterised by a decreased sensitivity of target cells to insulin at first (Davidson, 1991) and/or a decrease in functionality of the β-cell (Cerasi, 2000). The β-cell dysfunction includes abnormalities in pulsatility and kinetics of insulin secretion, quantitative and qualitative abnormalities of insulin produced and progressive β-cell death (Cerasi, 2000).

Once the amount of insulin produced is too low to facilitate glucose uptake, or if the insulin being produced fails to work effectively the result is glucose accumulation in the blood, i.e. hyperglycaemia develops when rates of glucose entry into the circulation exceed the rate of tissue uptake (Gerich, 2000). As the disease progresses tissue or vascular damage ensues leading to severe diabetic complications such as retinopathy, nephropathy, cardiovascular complications, neuropathy and ulceration. All as the result of the excess glucose inducing protein glycosylation (Hove et al., 2004; Seki et al., 2004).

The control measures and the problems associated with diabetes mellitus to human population are well documented (Dormandy et al., 2005; Zimmet et al., 2001; World Health Organization, 2010; Parejo et al., 2002; Anderson et al., 2003). Exogenous insulin forms the mainstay of treatment for patient with type 1 diabetes mellitus, while life style intervention and oral hypoglycaemic agents are considered the cornerstone for the treatment and management of type 2 diabetes (Shai et al., 2010; Rotchford and Rotchford, 2002; Yin et al., 2008; Alarcon-Aguilara et al., 1998). The oral hypoglycaemic agents include alpha glucosidase inhibitors (e.g. acarbose), sulphonylureas (e.g. tolbutamide and glyburide), meglitinide analogue and

thiazolidenediones (e.g. troglitazone, rosiglitazone and pioglitazone) (Kumar and Clark, 2002; DeFronzo, 1999; Davidson, 1991).

Despite the numerous treatment modalities available, treatment is palliative, with the result that diabetes is the largest non-communicable disease in the world (World Health Organization, 2010; Moller and Flier, 1991). In addition to the treatments being palliative, they are also expensive. As a result, there is need for alternative treatments and herbal remedies appears to be a sound option (Ojewole, 2006). Plant-based medicines have also been given prominence by the South African government. This increase in interest on plants is prompted by the understanding that plant-based medicines may have fewer side effects compared to allopathic medicine as well as the widespread use of the plants in the country (Obomsawin, 2008). In South Africa, 32 plant species have been identified for the treatment of diabetes based on the major ethnobotanical surveys document by Erosto et al. (2005). Possible mechanisms include mimicking insulin action during glucose disposal into peripheral tissue and/or enhancing insulin secretion from pancreatic ß-cells (Gray and Flatt, 1997; Erosto et al., 2005; Bhandari et al., 2008). Moreover, plants may have numerous or substantial amounts of antioxidants and their action is an important property of plant medicines associated with diabetes pathophysiology (Mc Cune and Johns, 2002). *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) is a one such plant (Deutschländer et al., 2009; van der Vender, 2008; Afolayan and Sunmonu, 2010; Boaduo et al., 2014).

Very little information is available on the anecdotal claim of the antidiabetic property of *H*. *hemerocallidea.* The Phytomedicine Programme of the University of Pretoria has investigated the potential anti-diabetic activity of selected plants. In one study survey information indicated the potential value of *Hypoxis hemerocallidea* in the treatment of

diabetes, which was supported by insulin secretory activity ex vivo and excellent antioxidant activity (Boaduo et al., 2014). This supported work by Zibula and Ojewole 2000; Ojewole, 2006; and Mahomed and Ojewole, 2003, who were able to demonstrate the ability of the plant aqueous extracts to significantly reduce blood glucose concentration in streptozotocin induced *diabetic* rats. Other than these studies, the active component or the effect of plant extracts in cases of natural diabetes remains unknown.

H. hemerocallidea is a herbaceous perennial plant; also known as *Hypoxis rooperi* and commonly known as the African potato, is a genus of the family Hypoxidaceae (Singh, 2007; Deutschländer et al., 2009). It usually grows in meanders, grassland and mountainous areas of South Africa, South America, Australia and coastal regions of Asia. The corm of the *H*. *hemerocallidea* has been used in folk medicine to treat a variety of diseases which include common cold, flu, hypertension, adult-onset diabetes, psoriasis, urinary infections, testicular tumours, prostate hypertrophy, HIV/AIDS, some central nervous system disorders, cancer, microbial infections and inflammation treatment (Ojewole, 2006; Van wyk, 2008, Bhandari et al., 2008; Street and Prinsloo, 2012; Laporta et al., 2007). For this study we focused on *H. hemerocallidea* as a promising candidate for discovering antidiabetic lead molecules.

1.1.1 Research question

Can *H. hemerocallidea* extracts or a constituent be a promising candidate as an antidiabetic lead molecule?

1.1.2 Objectives

The objectives of this study are:

Objective 1: To evaluate if the antioxidant activity of *H. hemerocallidea* extracts are influence by plant stressors like, soil heavy metal concentrations, organic matter content and pH.

Objective 2: To investigate the potential antioxidant activity of *H*. *hemerocallidea* extracts in relation with their intrinsic total phenolic content.

Objective 3: To isolate the anti-oxidant constituents of interest, and to characterize these isolated compounds.

Objective 4: To determine the effect of the *H. hemerocallidea*, selected plant corm crude extract, fractions and compounds on the glucose uptake of preadipocytes (Fat 3T3-L1) and myocytes (C_2C_{12}) and also on insulin sensitizing (RIN-m5F rat insulinoma).

Objective 5: To determine the cytotoxicity of *H. hemerocallidea* plant corm crude extract, fractions and isolated compounds on diabetic cell lines.

Objective 6: To *In vivo* determine the antidiabetic activity of the *H. hemerocallidea*, selected plant corm crude extract and compound/s on spontaneous diabetic mice.

1.1.3 Expected outcomes of the study

- This study may provide pharmacological support to the reported folkloric and anecdotal uses of the plant's corm (African potato) in the treatment and /or management of diabetes mellitus in some parts of South Africa.

- It may lead to the development of plant-derived products, which may be effective, with fewer side effects and easily accessible to the poor people of South Africa.
- The study may lead to the isolation of active compound which, in turn, will give insights into their action on the human body and possible chemical synthesis.
- The awarded of a PhD degree.

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Chapter 2:

A review on the interaction between Type II diabetes mellitus, polyphenolics and oxidative stress

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2.1 Abstract

Diabetes was until very recently seriously underrated as a threat to global public health. However, the situation is now changing, and the world can no longer ignore the inexorable rise in prevalence of, in particular type 2 diabetes. In this review the physiological significance of glucose transport in homeostasis, glucose transport pathways, the functionality of the glucose transport (GLUT) receptors on insulin synthesis, secretion and binding is discussed. Special attention is given to insulin resistance and its association on diseases of metabolic disorders (type 2 diabetes mellitus). This will focus on the mechanisms and contexts, physiological and pathological, of type 2 diabetes mellitus. The polyphenolic compounds, antioxidant defence system and their protection mechanism role on type 2 diabetes mellitus are also discussed.

Keywords: Type II diabetes mellitus, polyphenolics, cell culture and oxidative stress

2.1.1 Introduction

Diabetes mellitus, a chronic disorder characterized by high blood glucose (hyperglycaemia), results from an absolute deficiency insulin or insulin resistance (poor tissue insulin sensitivity) or both (World Health Organization, 2006). While numerous forms of the disease are described the most important is type 2 diabetes, a heterogenous disorder caused by an initial decreased sensitivity of target cells to insulin (Burcelin et al., 1999; Freychet et al., 1971) and later by a decrease in functionality of the β-cell. Type 2 diabetes was first suggested as a disease in the 1970s, on discovery of the insulin plasma membrane receptor (Freychet et al., 1971), was later shown to be associated with increased oxidative stress in the development and progression of disease (Baynes, 1991). Type 2 diabetes mellitus is also usually accompanied by increased production of free radicals or impaired antioxidant defences (Halliwell and Gutteridge, 1990). At present, type II diabetes has a high global prevalence with an estimated 8% of the adults being diabetic. Despite the importance of this non-communicable disease, currently available therapeutics are mainly palliative with no cure available. As a result, much research is focused on finding newer remedies that could lead to a cure.

With oxidation being a key feature of the disease, current studies are focused on the ability of plant polyphenolic compounds in antioxidant defence system or their protection mechanism role on type 2 diabetes mellitus. In this review, we provide an overview of the physiological significance of glucose transport in homeostasis, the physiology and pathology of type 2 diabetes mellitus and finally the value of polyphenolic compounds in type 2 diabetes mellitus.

2.1.2 Glucose homeostasis

Several laboratory studies have been undertaken over the last two decades to determine the physiological significance of glucose transport in homeostasis. In 1992, Kahn showed that glucose homeostasis was maintained by the coordinated regulation of three processes (Kahn, 2000):

- Glucose absorption via the small intestine: whereby the digested (via mechanical and chemical digestion) nutrients (e.g. monosaccharides the carbohydrates which are being broken down into glucose by maltases, sucrases and lactases enzymes) pass into the blood vessels in the wall of the epithelium intestine through a process of diffusion. Hence, glucose and amino acid co-transport; and fructose are absorbed by facilitated diffusion.
- Glucose production in the liver: the liver supplies glucose by turning glycogen into glucose in a process called glycogenolysis**.** The liver also can manufacture necessary glucose by harvesting amino acids, waste products and fat byproducts. This process is called gluconeogenesis (see section 2.1.3.3)**.** However, it should be noted that the liver both store and manufacture glucose depending upon the body's need. The need to store or release glucose is primarily signaled by the hormones insulin and glucagon.
- Consumption of glucose by the tissues: method of glucose uptake differs throughout tissues depending on two factors; the metabolic needs of the tissue and availability of glucose. The two ways in which glucose uptake can take place are facilitated diffusion (a passive process) and secondary active transport (an active process which indirectly requires the hydrolysis of ATP).

2.1.2.1 Glucose transport pathways

Once glucose is present in the blood, it needs to enter the cell through facilitated diffusion. Owing to glucose's hydrophilic nature, diffusion across the lipid bilayered plasma membrane is very slow and insufficient to meet the energy needs of the cell. Therefore, to meet the cell's energy needs, glucose entry into the cell needs to be facilitated via transporters (Tiwari and Rao, 2002; Daily et al., 1998). Glucose transporters are membrane associated carrier proteins which are regulated in the steady-state concentration by insulin that promotes their synthesis (Mueckler, 1990; Cushman and Wardzala, 1980). Many scientists have indicated that there are two distinct families of glucose transporters, namely: a Na^+ - coupled carrier system (SGLT) and the facilitative glucose transporters (GLUT) (Bouche et al., 2004; Rodnick et al., 1992; Wright, 2001 and Carruthers, 1990).

The SGLT family uses the electrochemical sodium gradient to transport glucose against concentration gradients and are prominent in intestine and kidney (Shepherd and Kahn, 1999). At least eleven SGLT transporters have been identified since 1987 (Wood and Trayhurm, 2003; Wright, 2001; Wright, 2011). SGLT1 is responsible for the dietary uptake of glucose from the small intestine lumen whereas SGLT2 plays a major role in glucose reabsorption from proximal renal tubules (Bouche et al., 2004; Wright, 2001).

Members of the facilitative GLUT family act as the main regulators of glucose homeostasis. Prior to 1990, six different transporter proteins of the facilitative diffusion type, encoded by distinct genes, were identified in mammalian tissues indicated as GLUT1 through GLUT5 and GLUT7 according to their chronological identification by molecular cloning (Bell et al., 1990; Mueckler, 1990; Waddell et al., 1991). Currently, 13 members of the GLUT family have been identified (Wright, 2011). According to

Joost et al., (2002) following a naming convention agreement, the thirteen members were named GLUT1– 12 and HMIT (H+-coupled myo-inositol transporter). Based on a dendrogram (Figure 1) of Wood and Trayhurn, (2003) which is created on various transporters exhibit different substrate specificities, kinetic properties and tissue expression profiles, as modified, there are three obvious subclasses, Class I comprise of GLUT1, 2, 3, 4, class II which contain GLUT 5, 7, 9, 11 and the last class III which contain GLUT 6, 8, 10, 12 and HMIT. These three subclasses (I–III) share common sequence motifs.

Irrespective of their class, these transporters facilitate diffusion of glucose, under a procedure that is not energy reliant and that follows Michaelis–Menton kinetics (Gottesman et al., 1984). The high-affinity transporters i.e. GLUT 1, 3, 4 have a Michaelis–Menton constant (Km) lower than the normal range of blood glucose concentrations and are capable of providing glucose transport under basal conditions for many cells. GLUT1 mediates basal glucose transport in skeletal muscle and adipose tissue, whereas GLUT3 is the major neuronal transporter (lowest Km) and GLUT4 is present in skeletal muscle, heart and adipose tissues (Tordjman et al., 1989). Low-affinity ligand binding involves less intermolecular force between the ligand and its receptor. The low-affinity carriers (GLUT2) are on ß-cells and in tissues exposed to large glucose fluctuations, such as intestine, liver and kidney (Gottesman et al., 1984; Bouche et al., 2004). Moreover, it is a bidirectional transporter, allowing glucose to flow in 2 directions, since it is required in liver cells to take up glucose for glycolysis, and to release glucose during gluconeogenesis. Therefore, all three monosaccharides (glucose, galactose and fructose) are transported from the intestinal mucosal cell into the portal circulation by GLUT2. A summary of the GLUT receptors are presented in Table 1.

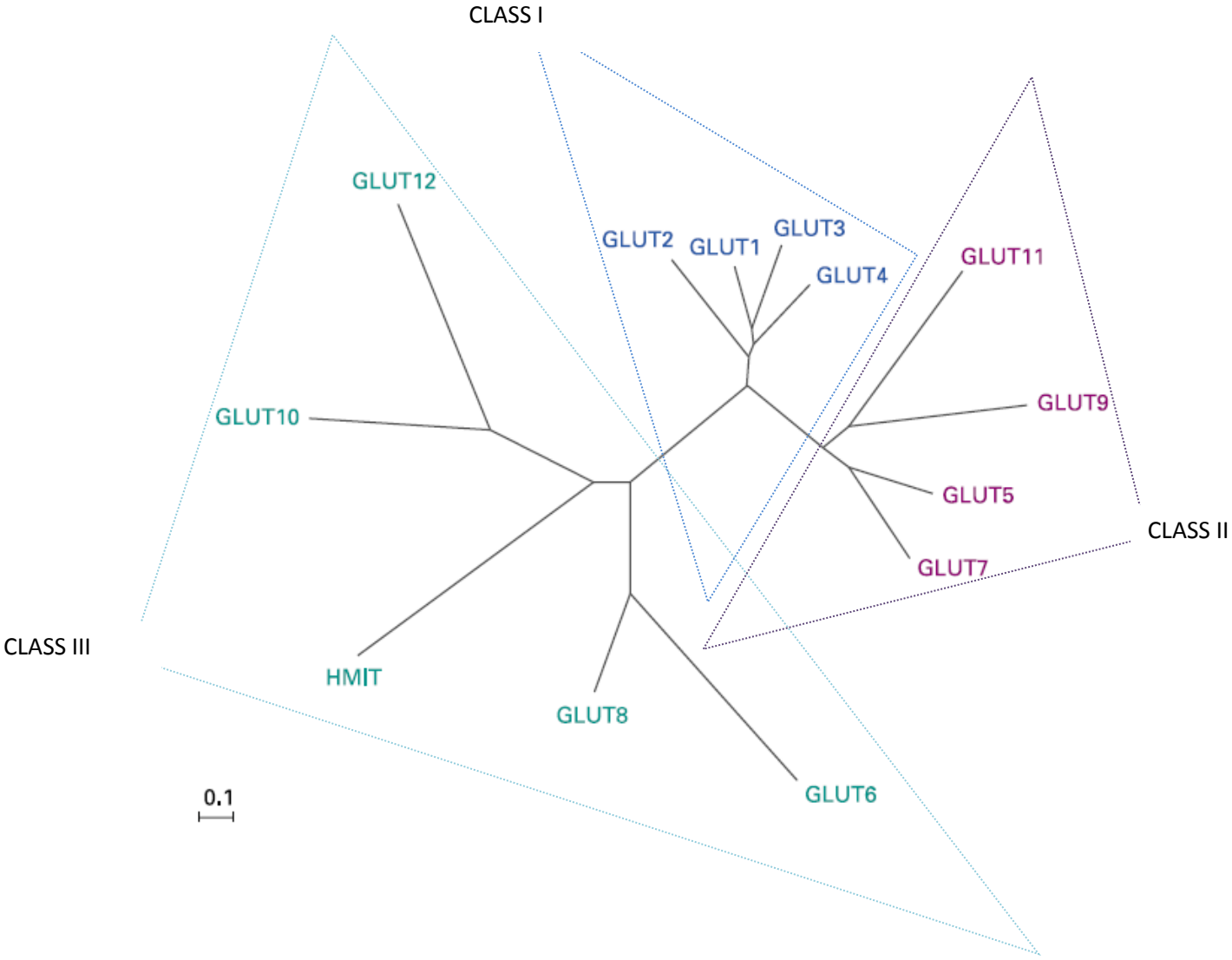


Figure 1: Dendrogram of the 13 members of human facilitated-diffusion glucose transporters (GLUTs) (Felsenstein, 1989).

An unrooted radial phylogram was drawn from a multiple sequence alignment of the thirteen members of the human GLUT family. These three subclasses (I–III) also share common sequence motifs. As mentioned by Wood and Trayhurn, (2003), the tree was created using neighbour-joining analysis of a distance matrix produced with PHYLIP software (Felsenstein, 1989). However, we further modified the tree. The scale bar symbolizes 0·1 substitutions per amino acid position. HMIT, H+-coupled myo-inositol transporter.

Table 1: Facilitated-diffusion glucose transporters (GLUTs).

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
GLUT 1 (Class I)	None	Red blood cell membranes, blood-brain, blood-tissue barriers: renal cells, ovarian cells, pancreatic cells, oral cells, prostate cells and all in all it is ubiquitous.	Insulin-independent	Glucose-high affinity	Present	Present	Ebeling et al., 1998; Wang et al., 2000; Gould et al., 1991; Mueckler et al., 1985.

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
GLUT 2 (Class I)	None	Pancreatic islets, liver, kidney and small intestine	Insulin-independent	Glucose and Fructose-low affinity	Not present	Not present	Kellett and Brot-Laroche 2005; Fukumoto et al., 1988; Gould et al., 1991.
GLUT 3 (Class I)	None	Blood cells in brain	Insulin-independent	Glucose-high affinity	Not present	Present***	Mantych et al., 1992; Gould et al., 1991; Kayano et al., 1988.
GLUT 4 (Class I)	None	White adipose tissue, brown adipose tissue, brain, heart and muscles	Insulin dependent	Glucose-high affinity	Present	Present	James et al., 1989; Fukumoto et al., 1988; Joost and Thorens 2001; Wood and Trayhurn 2003;

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
							Shepherd and Kahn, 1999.
GLUT5 (Class II)	None	Testis, enterocytes in the small intestine, kidney, liver skeletal muscles.	Insulin-independent	Fructose and Glucose- at very low affinity	Present	Present	Hundal et al., 1998, Barone et al., 2009, Kayano et al., 1990; Davidson et al 1992
GLUT 6 (class III)	GLUT 9	Brian, spleen, leucocytes	Insulin-independent	Glucose- low affinity	Not present	Not determine	Waddell et al., 1992; Bell et al., 1990; Lisinski et al., 2001; Doege et al., 2000.
GLUT 7 (class II)	Not existing	Intestine, colon, testis and prostate	Not determine	Glucose and Fructose- high affinity	Not determine	Not determine	Manolescu et al., 2005; Joost and Thorens 2001;

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
							Scheepers et al., 2004.
GLUT 8 (class III)	GLUTX1	Testis, brain, adipocytes and all in all it is ubiquitous.	Only in blastocytes	Glucose-high affinity	Present	Present	Scheepers et al., 2004; Carayannopoulos et al., 2000; Doege et al., 2000; Ibberson et al., 2000; Lisinski et al., 2001.
GLUT 9 (class II)	GLUTX	Liver, kidney	Not determine	Not determine	Not determine	Not determine	Scheepers et al., 2004; Phay et al., 2000;

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
GLUT 10 (class III)	Not existing	Liver, pancreas	Insulin-independent	Glucose-very high affinity	Present***	Not determine	Scheepers et al., 2004; Dawson et al., 2001; Mc Vie-Wylie et al., 2001
GLUT 11 (class II)	GLUT1D	Heart, muscle	Insulin-independent	Fructose-long form, Glucose- low affinity	Present	Not present	Doege et al., 2001; Scheepers et al., 2004; Wu et al., 2002; Sasaki et al., 2001
GLUT 12 (class III)	GLUT8	Protista, heart, muscle, small intestine and white adipose tissue	Insulin-dependent	Not determine	Present	Present	Scheepers et al., 2004; Rogers et al., 2002.

Isoform and class	Previous name	Expression and intracellular localization	Insulin sensitive	Functional characteristics in terms of transportation	Present in skeletal muscle	Present in the white adipose tissues	References
HMIT (class III)		Brian	Not determined	H^+-myo-inositol	Not present***	Present***	Scheepers et al., 2004; Uldry et al., 2001.

KEY: ***: mRNA only; HMIT: H^+-coupled myo-inositol transporter; Low affinity glucose transport: means it occurs in a high capacity glucose transporter; High affinity glucose transport: means it occurs in low capacity glucose transporter; Insulin-dependent: means insulin sensitive (it does rely on insulin for facilitated diffusion of glucose); Non-insulin dependent: means not insulin sensitive (it does not rely on insulin for facilitated diffusion of glucose).

2.1.3 Insulin secretion and synthesis

An important part in the functionality of the GLUT receptors is their physiological control. Glucose entry into the cell may be passive by diffusion or more active via specific hormonal control. Therefore, insulin mediates its actions through binding to insulin receptors. However, as mentioned above some GLUT receptors are insulin or non-insulin dependent, for example, most brain cells, having GLUT 1 as the principal transporter protein, are able to move glucose intracellularly at very low blood glucose blood concentrations without the need for insulin. While, adipose cells and muscle cells have GLUT 4 as the major glucose transporter protein, which requires insulin for its action (Burks and White, 2001).

2.1.3.1 Insulin synthesis

Insulin is a peptide hormone produced by beta cells in the pancreas from a single chain precursor of 110 amino acids known as preproinsulin (Krall and Beaser, 1988). Preproinsulin contains a sequence of 24 primarily hydrophobic amino acid residues attached to the amino terminus of the B chain. After translocation through the membrane of the rough endoplasmic reticulum, these 24 amino acid sequences are rapidly cleaved by a proteolytic enzyme to yield proinsulin (Patzelt et al., 1978; David and Granner, 1996). An amino-terminal B chain, a carboxyl-terminal A chain and a connecting peptide in the middle known as the C peptide are the three domains of proinsulin (Bowen, 1999).

Hereafter, the proinsulin undergoes folding with the three disulfide bonds being formed (Figure 2). Subsequently, the folded proinsulin is carried from the endoplasmic reticulum (ER) to the Golgi apparatus and is packed into secretory vesicles with the enzymes which are accountable for cleaving it into yield insulin and C-peptide. The insulin and C-peptide are at that point stored in secretory granules with islet amyloid

polypeptide and other products which are less abundant β-cell secretary products (Nishi et al., 1990; Patzelt et al., 1978; Kanner, 2003).

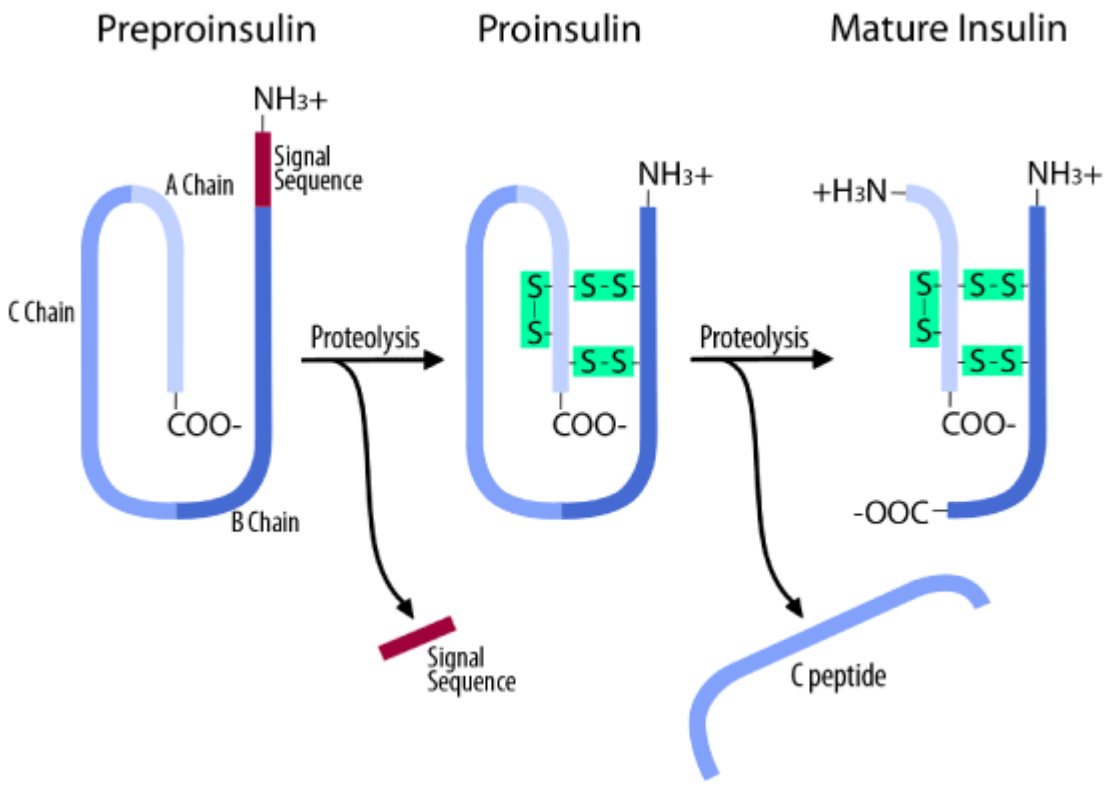


Figure 2: Steps in the formation of insulin from preproinsulin.
The signal peptide sequence (23 amino acids) is removed from the N-terminus of preproinsulin by proteases. This forms three disulphide bonds producing proinsulin. Further proteolytic cleavage of proinsulin removes the C-peptide producing mature insulin (Nishi et al., 1990; Patzelt et al., 1978; Kanner, 2003).

2.1.3.2 Insulin secretion

a). Insulin dependent glucose flux

While insulin secretion may be linked to amino acids, fatty acids and ketone body determinants, glucose uptake into the blood is the most important physiological incident that stimulates insulin release or gene transcription and mRNA translation (Poitour et al., 2006; Krall and Beaser, 1988) (Figure 3). Following normal post-prandiol increases in glucose, insulin is secreted in two phases: The first phase is the rapid and involves the release of the stored pool of insulin in the islet cells (accounts for about 5% of islet insulin). This phase is very rapid and happens within 10 minutes after the signal is received (Krall and Beaser, 1988; Newsholme and Leech, 1992; Guyton and Hall, 2000; Nelson and Cox, 2005; Saltiel and Kalm, 2001). The physiological steps are as follow:

Firstly, the increased glucose in the plasma is able to enter the β-cells via the low affinity GLUT 2 receptors. Once within the cell, the transported glucose enters the process of glycolysis and the Krebs cycle, where multiple, high-energy ATP molecules are formed by oxidation, increasing the ATP: ADP ratio within the cell. This inhibits potassium ions (K^+) from exiting the cell by facilitated diffusion by inhibiting ATP-sensitive SUR1/Kir6.2. The increased intracellular potassium depolarization the cell surface membrane, resulting in the opening of voltage-gated calcium ion ($Ca2^+$) channels open allow calcium ions to enter the cells. At the same time, cAMPGEFII/Epac2 which is abundant in the brain, neuroendocrine and endocrine tissues including pituitary, adrenal and pancreatic islets (Ozaki et al., 2000). It acts through Rap to stimulate the activation of phospholipase C, which cleaves the membrane phospholipid phosphatidyl inositol 4, 5-bisphosphate into inositol 1, 4, 5-trisphosphate and diacylglycerol. The resultant inositol 1, 4, 5-trisphosphate (IP3)

binds to receptor proteins in the plasma membrane of the endoplasmic reticulum, allowing for the release of stored Ca_2^+ ions from the ER via IP3-gated channels which further raises the intracellular concentration of calcium ions to a level that are able to stimulate the release of stored within secretory vesicles. The second stage is a reserve pool, the release of which requires ATP-dependent mobilization of insulin-containing granules into the rapidly releasable pool for subsequent exocytosis. ATP it is one of the end products of photophosphorylation, aerobic respiration and fermentation. It is used by enzymes and structural proteins in many cellular processes, including biosynthetic reactions, motility and cell division (Krall and Beaser, 1988; Newsholme and Leech, 1992; Guyton and Hall, 2000; Nelson and Cox, 2005; Saltiel and Kalm, 2001).

Phase 2 is more complex than releasing stored insulin. It also involves signalling of the nucleus of the β-cells which stimulates the insulin gene which in turn on the transcription of mRNA which sends the signal to the part of the cell that is necessary to stimulate the production of insulin (Gerich, 2000; Krall and Beaser, 1988).

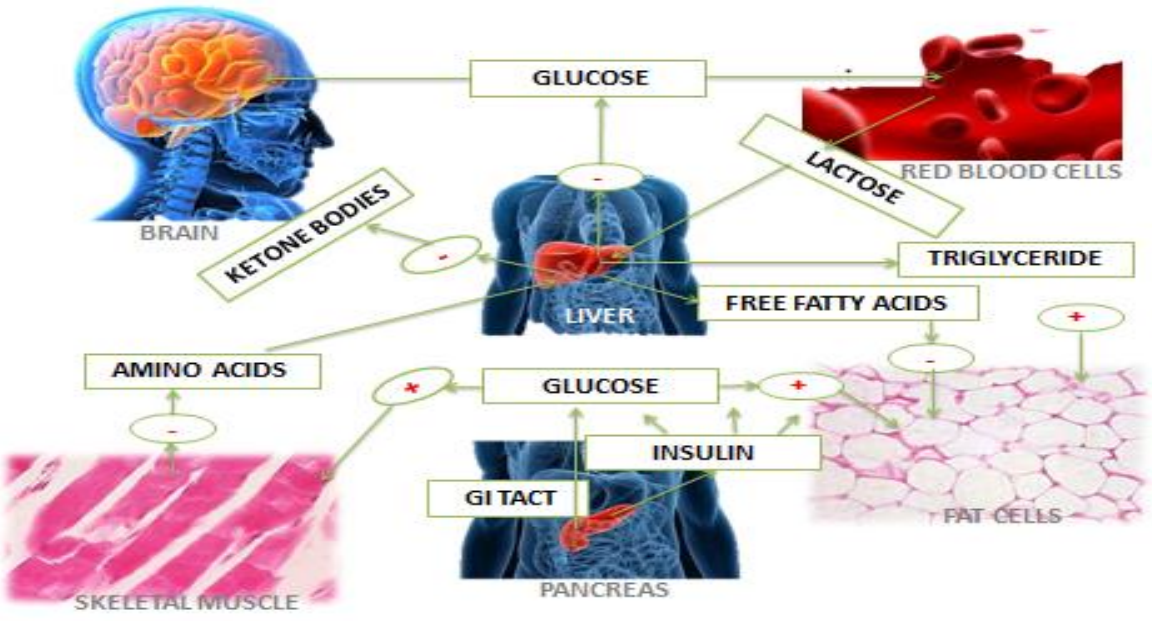


Figure 3: A presentation of how insulin acts as the principal regulator of energy metabolism.

When glucose or other nutrients are absorbed from the gastrointestinal tract, this elicits insulin secretion. Insulin regulates the metabolism of multiple fuels. Selected actions of insulin are indicated (+, activation; -, inhibition). Insulin activates transport of glucose into muscle and adipose tissue and promotes synthesis of glycogen and triglycerides. Insulin also inhibits hepatic glucose production by inhibiting both glycogenolysis and glucogenesis. Insulin does not directly regulate the metabolism of red blood cells, which uses glycolysis to provide energy. Although the brain uses glucose in the fed state, it can also use ketone bodies when levels rise high enough (e.g. during fasting) (Taylor, 1999).

2.1.3.3 Insulin binding

Once insulin is released it binds to a specific insulin receptor which belongs to the large class of tyrosine kinase receptors (which are the high-affinity cell surface receptors for many polypeptide growths factors, cytokines, and hormones) leading to the activation of numerous cascades (Figure 4). The number of insulin receptors on individual cells varies from as few as 40 per cell on erythrocytes to 30,000 on hepatocytes and adipocytes and are related to the physiological needs of the cell (David and Granner, 1996). This includes systemic glucose metabolism, including the translocation of Glut-4, Glut-1 or Glut- 2 transporters from intracellular pools to the

plasma membrane in various tissues, depending on the cell type and inflow of glucose, glycogenesis, glycolysis, gluconeogenesis and glycogenolysis (Noguchi et al., 2013, Pessin and Saltiel, 2000; Whiteman et al., 2002). At present the process by which insulin selectively regulates glycolysis, gluconeogenesis and glycogenesis remains unknown. The stimulation of GLUT4 translocation is, however, better understood (Bjornholm and Zierath, 2005).

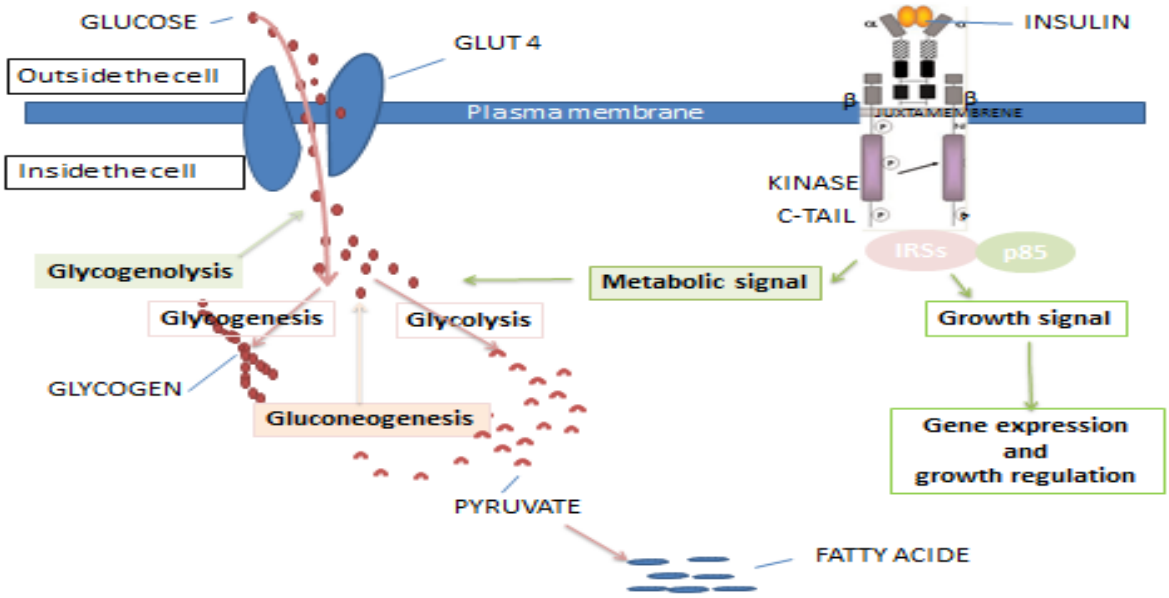


Figure 4: Illustration of insulin binding to its receptor which consists of 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits with tyrosine kinase activity.
Glycogenesis: is the process of glycogen biosynthesis from glucose, whereby glucose molecules are added to chains of glycogen for storage in the liver and muscles (Bollen et al., 1998; Roach et al., 2001). Glycolysis: Glycolysis is the initial metabolic pathway through which glucose is broken down into ATP (Pilkis et al., 1983; Okar et al., 2001). Gluconeogenesis: is essentially the reverse of glycolysis, involving the synthesis of glucose from two pyruvate molecules (Nordlie et al., 1999). Glycogenolysis: it is a process where glycogen can be rapidly broken down into molecules of glucose and is stimulated by low glucose concentrations in the cell (Nordlie et al., 1999; Petersen et al., 1996).

Once released insulin is transported within the blood stream to its target tissues, at which it binds to the *α*2-*β*2-heterotetrameric insulin receptor situated on the cell surface (David and Granner, 1996). The human insulin receptor is a disulphide-linked α2-β2-heterotetrameric bifunctional complex, comprising of 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits with tyrosine kinase activity. Insulin

binding to α subunit prompts the transphosphorylation of one β subunit with resultant increased intracellular catalytic activity of the kinase (Watson et al., 2004; Saltiel and Pessin, 2003; Schaffer, 1994). Moreover, the receptor likewise experiences autophosphorylation at other tyrosine residues in the juxta-membrane regions and intracellular tail (Figure 4). In addition to insulin binding to the extracellular α subunit, it leads to another conformational change that enables the binding of ATP to the intracellular component of the β subunit (Wolever, 1990). ATP binding triggers phosphorylation of the β subunit conferring tyrosine kinase activity. This facilitates tyrosine phosphorylation of intracellular substrate proteins named insulin responsive substrates (IRS). The insulin responsive substrates can then bind other signalling molecules which mediate further cellular actions of insulin (Kido et al., 2001).

There are four types of insulin responsive substrates proteins:

- IRS 1 is phosphorylated by both the insulin receptor and insulin-like growth factor 1 receptor (linked to the functioning of the growth hormone), which facilitates the mitogenic effects of insulin. IRS 1 is proposed to be the major IRS in skeletal muscle.
- IRS 2 is suggested to be the main IRS in liver and facilitates peripheral actions of insulin and growth of pancreatic β cells (Kido et al., 2001).
- IRS 3 is found only in adipose tissue, β cells and liver, while
- IRS 4 is present in the thymus, brain and kidney (Burks and White, 2001).

Phosphorylated IRS proteins bind specific src-homology-2 domain proteins, which include significant enzymes like phosphatidylinositol 3-kinase and phosphotyrosine phosphatase SHPTP2 (or Syp), and other proteins that lack enzymatic activity, but which link IRS-1 with intracellular signalling systems (Kido et al., 2001; Withers and

White, 2000). Phosphatidynositol 3-kinase encourages the translocation of glucose transporter proteins such as GLUT4; glycogen, lipid and protein synthesis; anti-lipolysis; and the control of hepatic gluconeogenesis (Burks and White, 2001). Phosphatidynositol 3-kinase acts via serine and threonine kinases such as Akt/protein kinase B, protein kinase C and PI dependent protein kinases 1 and 2. The retrovirus-associated DNA sequences (RAS) pathway triggers transcription factors and stimulates the growth supporting actions of insulin. Therefore, phosphatidynositol 3-kinase largely facilitates insulin's metabolic effects, for an example, cellular glucose uptake, while RAS significantly mediates insulin's mitogenic effects (Kido et al., 2001), together with other less well described actions (Kido et al., 2001; Burks and White, 2001; Pessin and Saltiel, 2000).

b). Non-insulin dependent glucose transport

Exercise has also been shown to play a role in glucose transportation, which is insulin independent. In one study obese Zucker rats, with known severe faults in insulin-stimulated glucose uptake and insulin-dependent GLUT-4 translocation albeit normal levels of total muscle GLUT-4 protein (King et al., 1992), had normal increases in glucose uptake and GLUT-4 translocation following intense electrical stimulation of muscle contractions (Brozinick et al., 1992). While the mechanism behind exercise-stimulated GLUT-4 translocation is yet to be elucidated, an increase in sarcolemmal and T-tubular glucose transport is essential for the contraction-induced increase in skeletal muscle glucose uptake during exercise (Antonescu et al., 2011).

2.1.4 Diseases of metabolic disorders

Diabetes mellitus is a chronic metabolic disorder of multiple aetiologies characterized by high blood glucose level (hyperglycaemia) resulting from absolute deficiency of insulin or insulin resistance (poor tissue insulin sensitivity) or both (World health organization, 2006). The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Moreover, numerous pathogenic routes are involved in the development of diabetes. These can start from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action (American Diabetes Association, 2012).

2.1.4.1 Classification of diabetic mellitus

Diabetes mellitus can be best classified into four major categories namely, of which Type II diabetes, which accounts for 90–95% of those with diabetes (World health organization, 2015, American Diabetes Association, 2012):

- **Type I diabetes:** Also known as insulin-dependent or childhood-onset diabetes mellitus affects mostly younger children. It is due to beta-cell (β-cell) destruction, usually resulting to complete insulin deficiency.
- **Type II diabetes:** Also known as non-insulin-dependent or maturity-on set diabetes mellitus. It is due to a combination of resistance to insulin action, where tissue lose sensitivity to insulin and progressive insulin secretory defect.
- **Type III (Gestational diabetes mellitus):** Diabetes identified in the second or third trimester of pregnancy that is not clearly explicit diabetes (Bottalico, 2007).
- **Secondary diabetes:** Specific types of diabetes due to other causes, e.g., diseases of the exocrine pancreas (such as cystic fibrosis), monogenic diabetes

syndromes (such as neonatal diabetes and maturity-onset diabetes of the young child) and drug- or chemical-induced diabetes (such as in the treatment of HIV/AIDS or after organ transplantation) (Davidson, 1991).

a) Type II diabetes

According to Ahmed and Goldstein, (2006), type II diabetes mellitus is characterized by progressive deterioration of normal β-cell function due to peripheral insulin resistance. For the latter a person generally has adequate or higher than normal levels of insulin in the blood, and yet still have elevated glucose plasma concentrations, due to poor responsiveness of cells to insulin. More importantly, despite the peripheral non-responsiveness to insulin, the higher glucose concentrations continue to stimulate the β-cell to produce insulin. Eventually due to the heavy burden of constant insulin production, the β-cells become exhausted and finally undergo apoptosis (Butler et al., 2004; Rosak, 2001). In terms of clinical recognition, this form of diabetes may be undiagnosed for several years. The hyperglycaemia in the earlier stages it is frequently not severe enough for the patient to notice any of the classic symptoms (details on symptoms are listed in the diabetic association of South Africa, 2001) of diabetes (American Diabetes Association, 2012). Van Tilburg et al., 2008; Jain and Saraf, 2008 mentioned that the main risk factors of developing this form of diabetes increases with age, gender, obesity, racial/ethnic background and lack of physical activity. According to the World Health Organization, (2015) it was estimated worldwide that 9% of adults at the age of 18 years and older had diabetes in the year 2014. Moreover, in 2012, an estimated 1.5 million people died from diabetes.

b) Aetiology of Type II diabetes mellitus

Abnormal islet cell function is a key and requisite feature of type 2 diabetes. Pathophysiology of Type II diabetes mellitus may be caused by a combination of genetic factors related to impaired insulin secretion, ethnic factors and environmental factors (Figure 5).

Genetic factors: this factor includes insulin receptor, insulin receptor substrate (IRS)-1and 2 gene polymorphisms that directly affect insulin signals and also polymorphisms of thrifty genes for an example: β3 adrenergic receptor gene and the uncoupling protein gene which is related with visceral obesity and promote insulin resistance (Cheng and Funtus, 2005).

- **Insulin resistance:** is a pathological condition in which cells fail to react to the standard actions of the hormone insulin (Chiu et al., 2007), with the result that glucose, amino acids and fatty acids can no longer optimally enter the cell. The net result is an increase in circulating plasma insulin, blood glucose concentrations (Bjornholm and Zierath, 2005). Insulin resistance in muscle and fat cells is characterized by faults at several levels namely; phosphorylation of insulin receptor substrate-1 and 2 (IR-1 and IR-2), decrease in kinase activity, activity of intra-cellular enzymes and glucose transporter (Glut-4) translocation (Cheng and Funtus, 2005). Insulin resistance in liver cells results in decreased glycogen synthesis and storage and also to a failure to overpower glucose production and release into the blood (Bryant et al., 2002; Chiu et al., 2007). This kind of insulin resistance in liver is characterized by faults also in numerous levels, namely; glucose-6-phosphate, increase in glucagon concentration, phosphoenolpyruvate carboxykinase activity, acetyl-CoA, hepatic fatty acid

oxidation and concentration of plasma free fatty acids (Cheng and Funtus, 2005; Kohei, 2010). The most common type of insulin resistance is related to overweight and obesity in a disorder known as the metabolic syndrome. It is generally agreed that insulin resistance has a major genetic component (Kahn et al., 1996; Chiu et al., 2007; Ghosh and Shock, 1996; McGarry, 2002; Todd, 1996), but efforts to unravel predisposing genes have largely been ineffective.

Impaired insulin secretion; this relates to an impaired glucose tolerance which is induced by a reduction in glucose responsive of the initial-stage insulin secretion and a reduction in additional insulin secretion after meals that may cause postprandial hyperglycaemia (Kohei, 2010). Impaired insulin secretion is generally progressive, and its development includes glucose toxicity and lipotoxicity (Kohei, 2010). However, it is not known if this dysfunction is genetic and/or attained.

Environmental factors can be divided into internal environmental factors and external environmental factors.

a) **Internal environmental factors**. This includes inflammatory mechanisms, adipocytokines and hepatocyte factors.

- **Inflammatory mechanisms;** Low-level inflammation is a key factor in the pathogenesis of type 2 diabetes (Schulze et al., 2005). The inflammation is activated by mediators including white blood cell, C-reactive protein, interleukin (IL)-6 and plasminogen activator inhibitor-1, etc. (Cheng and Funtus, 2005; Xu et al., 2011; Li et al., 2009).
- **Adipocytokines** has also been described as an additional key factor in the pathogenesis of type 2 diabetes. Tumour Necrosis Factor Alpha (TNF-α) is

produced mainly by monocytes, lymphocytes, adipose tissue (Ouchi et al., 2011). TNF-α increases the discharge of free fatty acids in adipocytes; blocks the production of adiponectin which plays a role in insulin-sensitizing activity in adipose tissue; and hinders the activity of tyrosine-residue phosphorylation (Lastra et al., 2006; Sánchez et al., 2005). Another small serine protease, adipsin that is secreted by adipocytes, also seems to be related with insulin resistance, dyslipidemia and adiposity (Pyrzak et al., 2010).

- **Hepatocyte factors:** Fetuin-A is a multifunctional glycoprotein which is completely secreted from hepatocytes in human. Epidemiological studies show that serum fetuin-A is associated with metabolic syndrome, microalbuminuria, and insulin resistance (Ix et al., 2006).

b). External environmental factors: environmental endocrine disrupters, also called as environmental oestrogen, are chemicals that hinder the endocrine or hormone system in animals and people. *In vivo*, environmental endocrine disrupters (e.g. Bisphenol A [BPA, 2, 2-bis(4-hydroxyphenyl) propane], act as metabolic disturber to cause adverse human health effects by destroying the liver function, disrupting pancreatic β cell function, disturbing thyroid hormone and encouraging obesity (Wang et al., 2012). It is also known to induce insulin resistance (Alonso-Magdalena et al., 2006).

Ethnic factors. Certain ethnic groups including African-Americans, Hispanic Americans, Native Americans, and Asian Americans are more likely to develop type 2 diabetes. The explanations for ethnic differences in the risk of type 2 diabetes are not entirely understood. For example, Asian Indians (people from India, Pakistan, and Bangladesh) have a high prevalence of type 2 diabetes compared to Caucasians although, the incidence of obesity, an important risk factor in the increase of type 2

diabetes, is significantly lower in Asian Indians compared to Caucasians. Hence, one important factor contributing to increased type 2 diabetes in Asian Indians is higher insulin resistance compared to Caucasians. This difference in the degree of insulin resistance may be due to either an environmental or a genetic factor or by combination of both (Figure 5) (Abate and Chandalia, 2001).

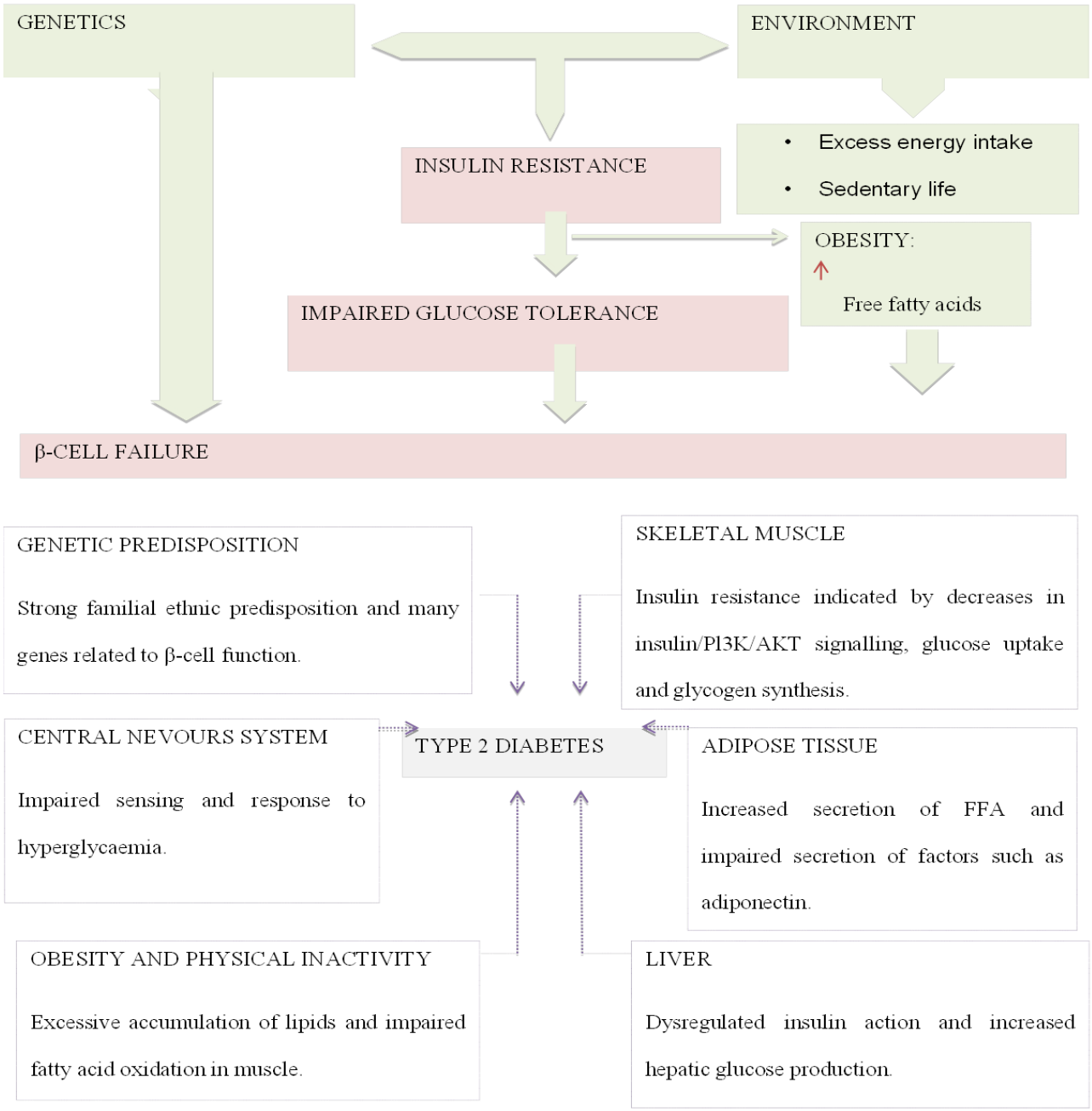


Figure 5: Overview of the pathogenesis of Type 2 Diabetes mellitus.
Current concepts on Type 2 Diabetes mellitus include a defect in insulin-mediated glucose uptake in skeletal muscle, a disruption of secretory function of adipocytes, a dysfunction of pancreatic β-cells, impaired sensing and response to hyperglycaemia in the central nervous system, an excessive of lipids, and impaired fatty acid oxidation due to obesity, physical inactivity and genetic predisposition (Lin and Sun, 2010).

2.1.5 Pathology of Diabetes Mellitus Type II

As mentioned in the section 2.1.4., diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia resulting from defects of insulin action, insulin secretion or both (American Diabetes Association, 2009; Astrup and Finer, 2000). The

hyperglycaemia that results in the course of diabetes usually leads to a number of secondary complications, including: ketoacidosis, eye problems, foot problems, peripheral artery disease, hypertension, high cholesterol and neuropathy. The injurious effects of hyperglycaemia can be further divided into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Michael and Fowler, 2008; Shepherd and Kahn, 1999).

2.1.6 Pathophysiology of Diabetes Mellitus

Due to the importance of the disease, much focus has also been placed on the pathophysiology of Diabetes Mellitus (DM). Current speculation, indicate that oxidative stress plays an important role in the pathogenesis of diabetes mellitus. Oxidative stress can be defined as a state in which reactive oxygen species (ROS) and reactive nitrogen species (RNS) reach excessive levels, either by excess production or insufficient removal (Brownlee, 2001). ROS and RNS can be due to free radicals (such as superoxide ($\bullet O_2^-$), hydroxyl ($\bullet OH$)), peroxyl ($\bullet RO_2$), hydroperoxyl ($\bullet HRO_2^-$), nitric oxide ($\bullet NO$) and nitrogen dioxide ($\bullet NO_2^-$) and non-radicals (such as hydrogen peroxide (H_2O_2), hydrochlorous acid (HOCl), peroxynitrite ($ONOO^-$), nitrous oxide (HNO_2), and alkyl peroxynitrates (RONOO)) (Johansen et al., 2005). With ROS and RNS being highly reactive molecules, the pathological consequence of an excess in ROS and RNS concentrations is damage to proteins, lipids and DNA (Johansen et al., 2005). This leads to physiological dysfunction, cell death, pathologies such as diabetes and cancer and aging (Ceriello, 2006). There are several signalling molecular pathways involved in ROS formation and ROS induced damage which are related to oxidative stress in diabetes. Most of them are related to glucose and/or lipid metabolism: glucose oxidation and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the

polyol pathway, hexosamine pathway, diacylglycerol formation and Protein kinase C (PKC) activation, glyceraldehyde autoxidation, advanced glycation end-products (AGEs) and stress-sensitive signalling pathways (Buse, 2006).

2.1.7 Signalling molecular pathways involved in ROS formation and damage

There are several signalling molecular pathways involved in ROS formation and ROS induced damage which are related to oxidative stress in diabetes (Figure 6).

a) Glucose oxidation and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Usually, electron transfer via complexes I, III, and IV extrudes protons into the intermembrane space, producing a proton gradient that drives ATP synthase (complex V) as protons moves back via the inner membrane into the matrix (Figure 7) (Nelson and Cox, 2005).

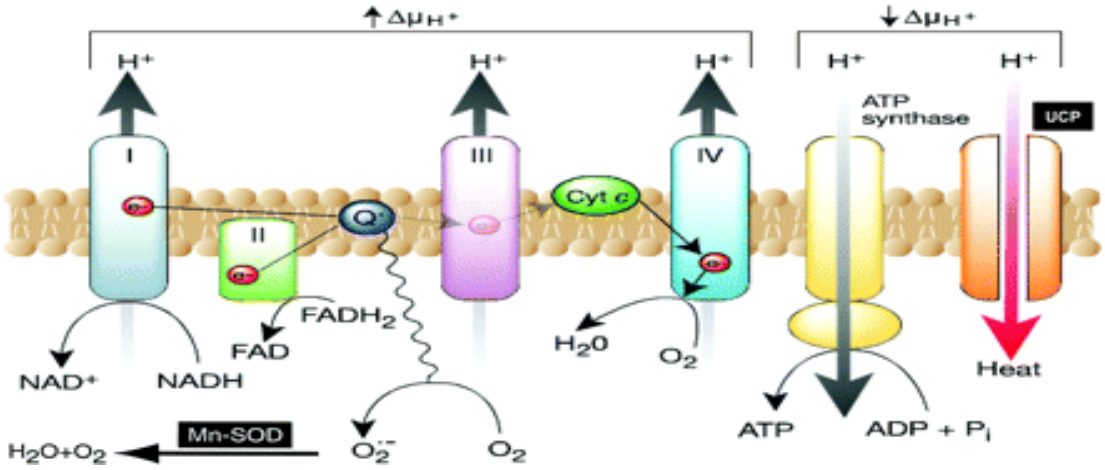


Figure 6: Formation of ROS by the mitochondrial electron transport chain.
The electron donors NADH and $FADH_2$ are produced by the oxidation of glucose-derived pyruvate (see section 2.1.6: glycolysis metabolic pathways). The flow of the donated electrons (e^-) via the electron transport chain in the inner mitochondrial membrane pumps H^+ ions into the intermembrane space. Once the voltage gradient increases because of increased flux of electron donors, hence additional superoxide is generated. H^+ ions can pass back across the inner membrane along their concentration gradient, either via ATP synthase in order to produce ATP, which wastes the energy of the proton gradient as heat. **Key:** Cyt c: indicates cytochrome *c*. (Adapted from Brownlee, 2005).

However, in contrast, in diabetic cells with elevated intracellular glucose concentration, there is more glucose-derived pyruvate being oxidized in the citric acid cycle rising the flux of electron donors (NADH and $FADH_2$) into the electron transport chain. Hereafter, the voltage gradient across the mitochondrial membrane rises until a serious threshold is reached resulting in electron transfer into the complex III being blocked (Trumpower, 1990; Nelson and Cox, 2005; Reeves et al., 1974). This results in electrons being backed up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thus generating superoxide (Figure 6). The following process has been demonstrated to occur in primary arterial endothelial cells (Korshunov et al., 1997). It has been suggested that hyperglycaemia-induced mitochondrial superoxide production activates harmful pathways by inhibiting the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lane et al., 2009; Berg et al., 2002), which is responsible for phosphorylating the two-final product of the pentose phosphate

pathway. With high ROS production, one gets resultant DNA damage and activation of the enzyme poly-ADP-ribose polymerase-1 (PARP-1) which is responsible for DNA repair (Brownlee 2005; Brownlee, 2001; Maechler and Wallheim, 2001; Garrett and Grisham, 2010).

b) The polyol pathway

The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol, which is then converted to fructose. This mechanism does not create an increase ROS, but rather results a redox imbalance that favours oxidative damage. Under normal circumstances the aldo-keto reductases have a low affinity for glucose and play a very small role in total glucose metabolism. However, under hyperglycaemic conditions, the elevated intracellular glucose results in elevated enzymatic transformation to the polyalcohol sorbitol, with subsequent reductions in NADPH (Brownlee, 2005; Brownlee, 2001). With NADPH being a cofactor required for the regeneration of reduced glutathione, an antioxidant mechanism, the net result is an exacerbation of intracellular oxidative stress (Brownlee, 2005; Brownlee, 2001). Another implication of the increase in the production of sorbitol in cells like the retina. It can also cause complications in the GI tract, including bleeding, perforated colonic ulcers, ischemic colitis and colonic necrosis, particularly in patients with uremia (Islam and Sakaguchi, 2006).

c) Hexosamine biosynthesis pathway

Hexosamine biosynthetic pathway is a glucose metabolic pathway, usually accounting for only 2–5% of total glucose metabolism that has been associated with post-translational protein modification by glycosylation and the synthesis of glycolipids, proteoglycans, and glycosylphosphatidylinositol anchors (Vosseller et al., 2002). It is

also responsible for synthesizing amino sugars physiologically. The Glutamine fructose-6-phosphate amidotransferase (GFAT) enzyme changes fructose 6-phosphate to glucosamine 6-phosphate, which is then changed to UDP-N acetylglucosamine, which is important for synthesizing the glycosyl chains of proteins and lipids. Specific O-Glucosamine-N-Acetyl transferases utilizes this metabolite for post-translational modification of specific serine and threonine residues on cytoplasmic and nuclear proteins (Buse, 2006). The hexosamine biosynthetic pathway has been hypothesized to be involved in the development of insulin resistance and diabetic vascular complications (Schleicher and Weigert, 2000). Oxidative stress has also been associated in the effects of glucosamine which may also induce pancreatic β-cell dysfunction (Kaneto et al., 2001).

d) Diacylglycerol formation resulting into protein kinase C activation

Diacylglycerol (DAG) is a glyceride consisting of two fatty acid chains covalently bound to a glycerol molecule through ester linkages. DAG has unique functions as a basic component of membranes, an intermediate in lipid metabolism and a key element in lipid-mediated signalling. Phosphatidyl serine, calcium and DAG are required for activation of the Protein Kinase C family which consists of at least eleven isoforms of serine/threonine kinases, which contribute in signalling pathways. DAG levels are raised chronically in the diabetic or hyperglycaemic environment due to an increase in the glycolytic intermediate dihydroxyacetone phosphate. This intermediate is metabolised to glycerol-3-phosphate, which successively increases *de novo* synthesis of DAG. Protein kinase C (PKC) isoforms have several kinds of cellular signals, including activation of NADPH oxidase, resulting in excessive ROS generation (Brownlee, 2001).

e). Glyceraldehyde autoxidation

As mentioned above in section 2.1.6, glyceraldehyde 3-phosphate is a phosphorylation product formed from glucose during anaerobic glycolysis. The related compound, dihydroxyacetone phosphate, also adds to intracellular glyceraldehyde concentrations via enzymatic conversion by triose-phosphate isomerase. Subsequently, glyceraldehyde 3-phosphate is oxidized by glyceraldehyde-phosphate dehydrogenase (GAPDH). Continuation of glycolysis yields pyruvate, which come into the mitochondria where it is oxidized to acetyl-CoA, and the processes of the tricarboxylic acid cycle and oxidative phosphorylation begin (Wolff and Dean, 1987). Therefore, accumulation of glyceraldehyde 3-phosphate, besides activating the advanced glycation end-products (AGE) formation and the PKC pathway, can oxidize itself. This autoxidation generates hydrogen peroxide (H_2O_2), which further contributes to oxidative stress (Morales-González et al., 2010).

f) Advanced glycation end-products (AGEs)

In order for intracellular and extracellular AGEs to be formed, intracellular hyperglycaemia is the primary initiating event which must take place. Reactive intracellular dicarbonyl glyoxal, methylglyoxal and 3-deoxyglucosone react with amino groups of intracellular and extracellular proteins to form AGEs (Degenhardt et al., 1998). There are many general mechanisms which can harm the cells when intracellular formation of AGE precursors is formed, for example when plasma proteins are altered by AGE precursors which bind to AGE receptors (such as RAGE and AGE-R1,2 and 3) on cells like vascular endothelial cells and vascular smooth muscle cells.

As a result, AGE receptors binding prompts the formation of ROS, which in turn activates PKC (Brownlee, 2001; Morales-González et al., 2010).

g) Stress-sensitive signalling pathways

The most broadly studied intracellular pathway that is a target of hyperglycaemia, ROS, and oxidative stress is the transcription factor NF-κB (Kyriakis and Avruch., 1996). p38 MAPK pathway and c-Jun Terminal kinase (JNK) is also known as stress-activated protein kinase (SAPK) participate in cellular responses to stress due to osmotic shock, cytokines and UV light, playing a role in cellular proliferation, apoptosis, and inflammatory responses. NF-κB plays a serious role in facilitating immune and inflammatory responses and apoptosis. NF-κB helps to control the expression of a many genes, including several of those connected to the complications of diabetes (Rains and Jain, 2011).

Moreover, oxidative stress is also involved in activation of several stress-sensitive signalling pathways (for example a transcription factor NF-κB, which together with PARP acts as a transcriptional coactivator of inflammation molecules such as iNOS), which can result in inflammation, cytokine release, and even apoptosis (Gomperts et al., 2003; Brownlee, 2003; Robertson et al., 2003).

All in all, oxidative stress has a fundamental role in the development of diabetes complications, both at the micro- and macrovascular levels.

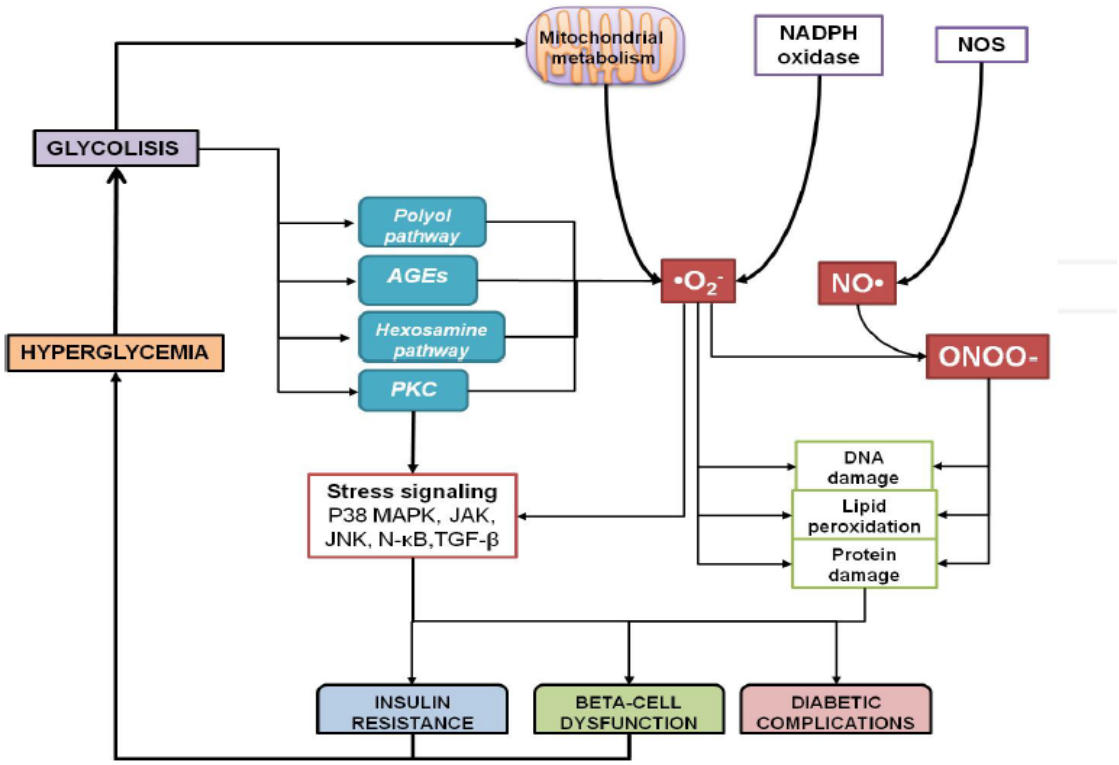


Figure 7: Oxidative pathways in diabetes mellitus (Brownlee, 2003; Johansen et al., 2005).

2.1.8 Treatment and management of Type II diabetes mellitus

The main goal in the management of diabetes mellitus is to return blood glucose concentrations to physiological levels both post and pre-prandiol (Bailey, 2000). Lifestyle intervention is the mainstay with pharmacological therapy used only when the hyperglycaemia becomes severe (Anderson et al., 2003; Klein et al., 2004). During this intervention, patients receive standardized general diabetes education with a detailed focus on dietary interventions and the significance of increasing physical activity. Those with moderate hyperglycaemia or in whom lifestyle changes are anticipated to be unsuccessful are usually promptly started on an antihyperglycaemic agent at diagnosis, which can later be modified or possibly discontinued if lifestyle changes are successful. The different antihyperglycaemic agents available, their

cellular mechanism, primary physiological action, advantages and disadvantages are presented in Table 2. A brief discussion of the different classes are listed below (Dormandy et al., 2005; Chiasson 2003; Turner, 1998a; UKPDS Group 1991; Holman et al., 2008; Turner, 1998b; Stratton et al., 2000).

- **Binguanides** (e.g**.** Metformin) work by preventing the liver from converting fats and amino-acids into glucose. They also activate an enzyme (AMPK) which helps cells to respond more effectively to insulin and take in glucose from the blood.
- **Sulfonylureas** (e.g. Glyburide/ glienclamide) block ATP sensitive potassium channels in Beta cells of the islets and reduce the potassium permeability of Beta cells. This causes depolarization of the cells, calcium entry into the cell, which causes increased insulin secretion. The insulin released reduces plasma glucose concentrations. They also increase insulin responsiveness by preventing phosphorylation
- **Meglitinides (glinides)** (e.g. Repaglinide) bind to an ATP-dependent K^+ (K_{ATP}) channel on the cell membrane of pancreatic beta cells in a similar manner to sulfonylureas but have a weaker binding affinity and faster dissociation from the SUR1 binding site. This increases the concentration of intracellular potassium, which causes the electric potential over the membrane to become more positive. This depolarization opens voltage-gated Ca^{2+} channels. The rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of (pro) insulin.
- **Thiazolidinediones** (e.g. Rosiglitazone) act by activating PPARs (peroxisome proliferator-activated receptors), a group of nuclear receptors, with greatest specificity for *PPARγ* (gamma). The endogenous ligands for these receptors

are free fatty acids (FFAs) and eicosanoids. When activated, the receptor binds to DNA in complex with the retinoid X receptor (RXR), another nuclear receptor, increasing transcription of a number of specific genes and decreasing transcription of others. The main effect of expression and repression of specific genes is an increase in the storage of fatty acids in adipocytes, thereby decreasing the amount of fatty acids present in circulation. As a result, cells become more dependent on the oxidation of carbohydrates, more specifically glucose, in order to yield energy for other cellular processes.

- **Alpha-glucosidase inhibitors** (e.g. Acarbose) work by preventing the digestion of carbohydrates (such as starch and table sugar). Carbohydrates are normally converted into simple sugars (monosaccharides), which can be absorbed through the intestine. Hence, alpha-glucosidase inhibitors reduce the impact of carbohydrates on blood sugar.
- **Dipeptidyl peptidase 4 (DPP-4) inhibitors** (e.g. Vildagliptin) work by blocking the action of DPP-4, an enzyme which destroys the hormone incretin. Incretins help the body produce more insulin only when it is needed and reduce the amount of glucose being produced by the liver when it is not needed.
- **Bile acid sequestrants** (e.g. Colesevelam) bind with cholesterol-containing bile acids in the intestines and are then eliminated in the stool. The usual effect of bile acid sequestrants is to lower LDL cholesterol by about 10%-20%. Small doses of sequestrants can produce useful reductions in LDL cholesterol.
- **Dopamine-2 agonists** (e.g. Bromocriptine) are thought to act on the circadian neuronal activities in the hypothalamus, to reset an abnormally elevated hypothalamic drive for increased plasma glucose, free fatty acids, and triglycerides in insulin-resistant patients.

- **Glucagon-like peptide-1 (GLP-1) receptor agonists** (e.g. Liraglutide) is widely distributed in pancreatic islets, brain, heart, kidney, and the gastrointestinal tract including the stomach. Its function is not known for all these locations and numerous attempts have been made to identify alternative GLP-1R.
- **Amylinomimetics** (e.g. Pramlintide) are synthetic drugs that act like a naturally occurring hormone called amylin. Amylinomimetics work with insulin to control blood sugars after meals. This medicine slows down food moving through your stomach and slows down sugars moving into your blood.
- **Insulins** (e.g. Human Neutral Protamine Hagedorn) are used to take the place of insulin that is normally produced by the body, by helping to move sugar from the blood into other body tissues where it is used for energy. It also stops the liver from producing more sugar.

Table 2: Antihyperglycemic agents prescribed for use in people

Class	**Compound (s)**	**Cellular mechanism**	**Primary physiology action(s)**	**Advantages**	**Disadvantages**	**Cost**
Biguanides	-Metformin	Activates AMP-kinase	- Decrease Hepatic glucose production^{RED}	-Extensive experience -No weight gain -No hypoglycaemia -Cardiovascular complications events^{RED}	-Gastrointestinal side effects -Lactic acidosis -Vitamin B12 deficiency -Multiple contraindications i. serum creatinine ≥1.5 mg/dL in males or ≥1.4 mg/dL in females ii. Abnormal creatinine clearance from any cause, including shock, acute myocardial infarction, or	Low

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
					septicaemia iii. Acute or chronic metabolic acidosis with or without coma, including diabetic ketoacidosis	
Sulfonylureas	-Glyburide/ glienclamide -Glipizide -Gliclazide[b] -Glimepiride	Closes K_{ATP} channels on β-cell plasma membranes	Decreased Insulin secretion^{INC}	-Extensive experience -Microvascular diseases (such as hypertension and hyperlipidaemia)^{RED}	-Hypoglycaemia -Weight gain -Blunts myocardial ischemic preconditioning (adaptive mechanism by which a brief period of reversible ischaemia increases the heart's tolerance to a subsequent	Low

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
					longer period of ischaemia).	
Meglitinides (glinides)	-Repaglinide -Nateglinide	Closes K_{ATP} channels in β-cell plasma membranes	-Decreases Insulin secretion [INC]	-Postprandial glucose excursions (is the change in glucose concentration after a meal and the incremental glucose area) [RED] -Dosing flexibility	-Hypoglycaemia -Weight gain -Blunts myocardial ischemic -Frequent dosing schedule	High
Thiazolidinediones	-Pioglitazone -Rosiglitazone[c]	Activates the nuclear transcription factor; peroxisome proliferators-activated receptor (PPAR) -y	-Increases Insulin sensitivity [INC]	-No hypoglycaemia -Durability -HDL-C[INC] -Triglycerides[RED]	-Weight gain -Oedema/heart failure -Bone fractures - low-density lipoprotein cholesterol(rosiglitazone) [INC]	High[c]

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
				-Cardiovascular complications events[RED]	- myocardial infarction mortality in meta-analysis, rosiglitazone [INC] -Bladder cancer (pioglitazone) [INC]	
α-Glucosidase inhibitors	-Acarbose -Miglitol -Voglibose[b,d]	Inhibits intestinal α-glucosidase	-Slows intestinal carbohydrate digestion/ absorption	-No hypoglycaemia - Postprandial glucose excursions [RED] -Cardiovascular complications events [RED] -Nor systemic	-Generally modest Haemoglobin (HB)A_{1c} Efficacy -Gastrointestinal side effects -Frequent dosing schedule	Moderate
DPP-4 inhibitors	-Sitaglitin -Vildagliptin[a] -Saxagliptin	Inhibits dipeptidyl peptidase (DPP)-4 activity, increasing	- Insulin secretion [INC] (glucose-dependent)	--No hypoglycaemia -Well tolerated	- Generally modest HBA_{1c} Efficacy -Urticarial/	High

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
	-Linagliptin Alogliptin[b,d]	postprandial active incretin (GLP-1,GIP) concentrations	-Glucagon secretion[RED] (glucose-dependent)		Angioedema -Pancreatitis	
Bile acid sequestrants	-Colesevelam	Binds bile acids in intestinal tract, increasing hepatic bile acid production; activation of farnesoid X receptor (FXR) in liver	-Unknown -Hepatic glucose production[RED] -Incretin levels[INC]	-No hypoglycaemia -LDL-C[RED]	-Generally modest HBA_{1c} efficacy -Constipation -Triglycerides[INC] -[RED]absorption of other medications	High
Dopamine-2 agonists	-Bromocriptine (quick-release)[d]	Activates dopaminergic receptors	-Modulates hypothalamic regulation of metabolism	-No hypoglycaemia -CVC events[RED]	-Generally modest Haemoglobin (HB)A_{1c} efficacy -Dizziness/ syncope -Nausea	High

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
			-Insulin sensitivity^{INC}		-Fatigue -Rhinitis	
Glucagon-like peptide-1 (GLP-1) receptor agonists	-Exenatide extended release -Liraglutide	Activates GLP-1 receptors	- Insulin secretion^{INC} (glucose-dependent) -Glucagon secretion^{RED} (glucose-dependent) -Slows gastric emptying -Satiety^{INC}	-No hypoglycaemia -Weight reduction -Potential for improved β-cell mass/function -Cardiovascular protective actions	-Nausea/ Vomiting -Acute pancreatitis -C-cell hyperplasia/ medullary thyroid tumors in animals -Injectable -Training requirements	High
Amylinmimetics	-Pramlintide[d]	Activates amylin receptors	-Glucagon secretion^{RED} -Slows gastric empting -Satiety^{INC}	-Weight reduction -Postprandial glucose excursions^{RED}	-Generally modest HbA_{1c} efficacy -Nausea/ Vomiting -Hypoglycemia unless insulin dose is	High

Class	Compound (s)	Cellular mechanism	Primary physiology action(s)	Advantages	Disadvantages	Cost
					simultaneously reduced -Injectable -Frequent dosing schedule	
Insulins	-Human Neutral Protamine Hagedorn -Human regular -Lispro -Aspart -Glulisine	Activates insulin receptors	-Glucose disposal^{INC} -Hepatic glucose production	-Universally effective -Theoretically unlimited efficacy -Microvascular risk^{RED}	-Hypoglycaemia -Weight gain -Mitogenic effects -Injectable -Training requirements	Variable[f]

KEY: [a]Limited use in the U.S./Europe. [b]Not licensed in the U.S. [c]prescribing highly restricted in the U.S.; withdrawn in Europe. [d]Not licensed in Europe. [e]Was available as a generic product in 2012. [f]Depends on type (analogs >human insulin) and dosage. **INC**: increased; **RED**: reduction; **CKD**: chronic kidney disease; **DVD**: cardiovascular disease; **DPP-4**: dipeptidyl peptide 4; **GIP**: glucose-dependent insulinotropic peptide; **GLP-1**: glucagon-like peptide 1; **HDL-C**: HDL-cholesterol; **LDL-C**: LDL-cholesterol; **PPAR**: peroxisome proliferator-activated receptor; **ProACTIVE**: Prospective Piaglitazone Clinical Trial Macrovascular Events; **STOP-NIDDM**, Study to prevent Non-Insulin-Dependent Diabetes Mellitus; **UKPDS**, UK Prospective Diabetes Study (Dormandy et al., 2005; Chiasson 2003; Turner, 1998a; UKPDS Group 1991;Holman et al., 2008; Turner, 1998b; Stratton et al., 2000).

2.1.9 Role of antioxidant defence systems and their protection mechanism on diabetes mellitus

In recent years, several studies have confirmed the roles of the body's antioxidants system with the pathology of processes and their potential therapeutic implications (Mats et al., 1999). Antioxidant defence mechanisms include both enzymatic (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) and non-enzymatic processes (including vitamins A, C, and E, glutathione, α-lipoic acid, vitamins B1, B2, B6, and B12, mixed carotenoids, several bioflavonoids, cofactors like folic acid, uric acid, albumin and antioxidant minerals like copper, zinc, manganese and selenium). They work in synergy with each other and against different types of free radicals (Feher et al., 1987; Maritim et al., 2003). As mentioned above in section 2.1.8. ROS have been linked to diabetic complications, antioxidants have shown promise as a possible therapy for the prevention and treatment of these diseases, especially given the exciting evidence from experimental, epidemiological and clinical studies (Yusuf et al*.,* 2000; Kritharides and Stocker, 2002; Hodis et al., 2009; Shargorodsky et al., 2010 and Johansen et al., 2005). These studies have confirmed the usefulness of antioxidants which might consequently be helpful for treating diabetes and its complications. The antioxidants are the molecules which are able to slow or avoid the oxidation process of other molecules (Agnieszka et al., 2011). There are two different class of antioxidants:

Class I: These natural antioxidants are known as the chain breaking antioxidants which protect the body from lipid free radicals by converting them into stable end products: (a) Vitamins, which are vital for metabolic activities e.g. vitamin B and ascorbic acid; (b) Mineral antioxidants, which act as cofactors of important enzymatic antioxidants and play dynamic role in metabolism of several macromolecules e.g.

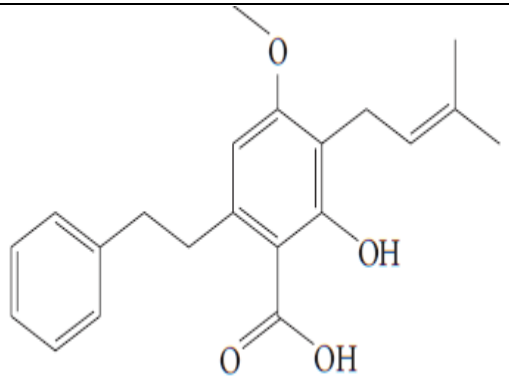
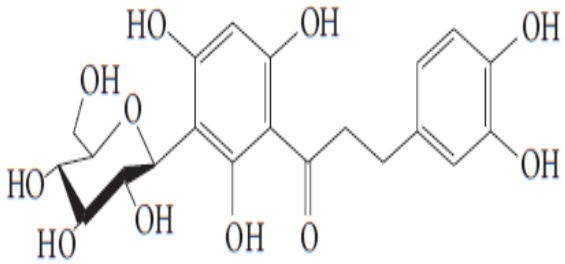
copper, iron, zinc and manganese; and (c) Phytochemicals: e.g. flavonoids which are the phenolic complexes, carotenoids which are fat soluble pigment, catechins which are the major bio-active antioxidants, beta carotene which are present abundantly in carrot and the lycopenes which are the red pigments that are vital phyto-constituents of fruit and vegetables (Agnieszka et al., 2011).

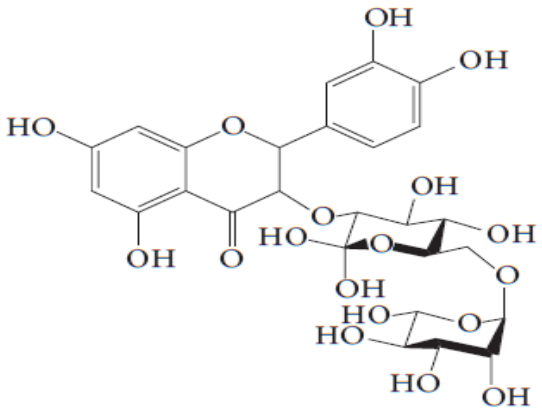
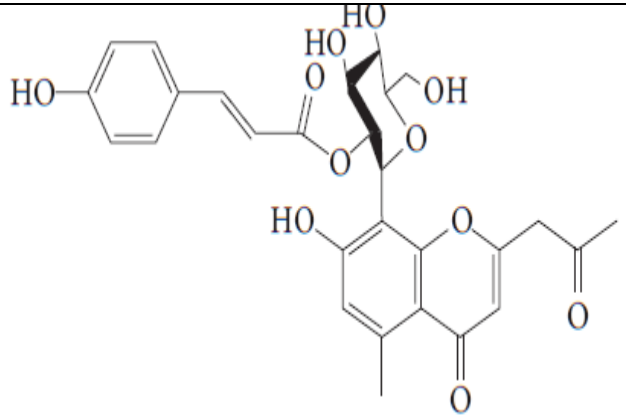
Class II: synthetic antioxidants are known to belong into phenolic group of compounds. Synthetic phenolic antioxidants are *p*-substituted, whereas the natural phenolic compounds are mostly *o*-substituted. Synthetic phenolic antioxidants are always substituted with alkyl groups to improve their solubility in fats and oils and to reduce their toxicity. They achieve the vital function of binding free radicals, destroying oxidative stress and inhibiting the chain reactions through various biological actions for example, butylated hydroxyl toluene and esters of gallic acid (Wolff, 1993; Agnieszka et al., 2011).

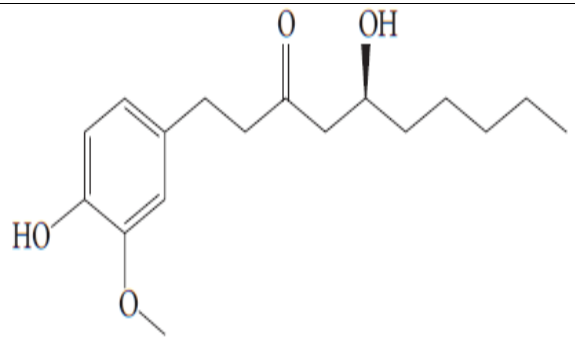
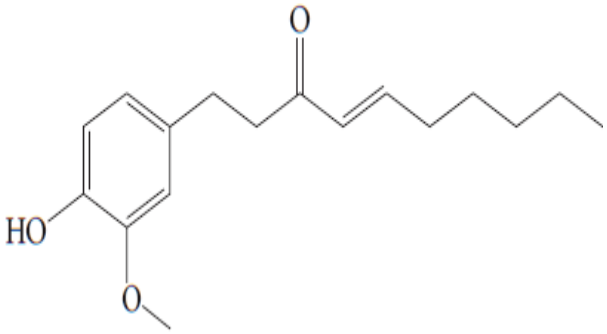
2.1.10 Plants and their active ingredients against diabetes mellitus

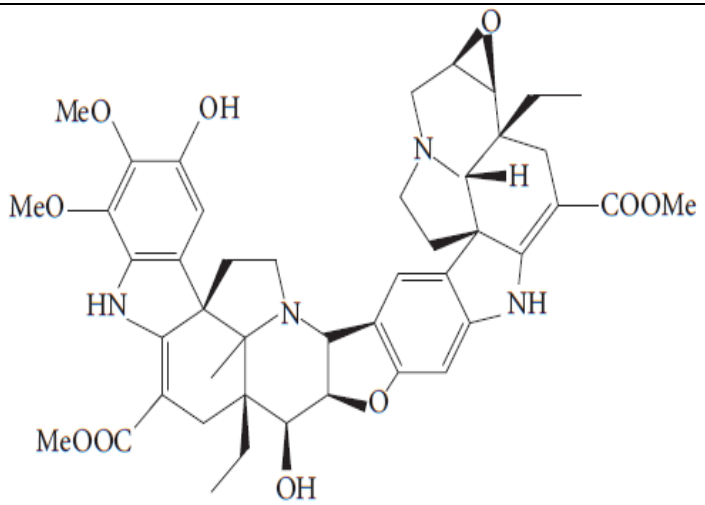
Despite the numerous treatment modalities available, the management of diabetes mellitus with no side effects remains a challenge. As a result, much focus is placed on new therapies, with the investigation of natural anti-diabetic products from plants is one such a field. The ethnobotanical statistics list approximately 800 plants that may possess antidiabetic potential (Alarcon-Aguilara et al., 1998). The chemical and antidiabetic actions of some of the compounds from plants, discussed in this section are summarized in Table 3.

Table 3: The chemical and anti-diabetic actions of some of the compounds from plants.

Plant name and Compound name	**Chemical structure**	**Antidiabetic action (s)**	**References**
Glycyrrhiza uralensis Amorfrutin 1		Regulate insulin resistance	Weidner et al., 2012
Aspalathus linearis: Aspalathin		Regulate two or more pathways (insulin tolerance, β-cell function, and inhibition of α-glucosidase)	Kawano et al., 2009; Park et al., 2010

Plant name and Compound name	Chemical structure	Antidiabetic action (s)	References
Rutin (quercetin-3-O-rutinoside)			
Aloe vera: Aloeresin A		Regulate two or more pathways (suppression of α-glucosidase activity (gut glucose absorption) and insulin resistance)	Jong-Anurakkun et al., 2008

Plant name and Compound name	Chemical structure	Antidiabetic action (s)	References
Zingiber officinale: Gingerol		Regulate two or more pathways (islet cell protection and increased insulin receptor signalling)	Chakraborty et al., 2012
Shogaol		Can elevate glucose uptake in response to insulin in muscles and adipose cells	

Plant name and Compound name	Chemical structure	Antidiabetic action (s)	References
Tabernaemontana divaricate and *Epipactis microphylla*: Conophylline		Regulate β-cell function	Kawakami et al., 2010

2.1.11 Role of polyphenolics as anti-oxidant and anti-diabetic compounds

Polyphenols are products of the secondary metabolism of plants and they are derived from two key synthetic pathways, namely; the acetate pathway and the shikimate pathway which both arise from glucose metabolism within the plants (Wollgast and Anklam, 2000). The main categories of polyphenolics are namely; flavonoids and phenolic acids, phenolic alcohols and lignans (Abbas et al., 2017). Research on flavonoids and other polyphenols, their antioxidant properties, and their effects in disease prevention only began after 1995 (Scalbert, 2005). Polyphenolic are important in the plant for normal growth development and defence against infection and injury (Liu et al., 2005; Miyamoto et al., 1998; Rassi et al., 2002; Shen et al., 2011). The antioxidant activity of phenolics is mostly due to their redox potential, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They also have a metal chelation potential (Rice-Evans et al., 1995). Moreover, norlignan derivatives (i.e., diphenylpentane compounds) like hypoxoside, and its aglycone rooperol, dehydroxyrooperol I and II and bis-dehydroxyrooperol which can be derived from all the common *Hypoxis* species, also exhibit antioxidant activity (Laporta et al., 2007). However, *Hypoxis* species are best known for their high concentration of phytosterols (mainly stigmasterol, β-sitosterol, brassicasterol and ergosterol) which are acknowledged for their cholesterol-lowering actions and antidiabetic activities (Bouic, 2001; Betto et al., 1992).

Current evidence strongly supports a contribution of polyphenols to the role in the prevention of diabetes mellitus (Johnston et al., 2005). However, the physiological mechanisms involved in the advantageous effects of polyphenols continue to be poorly understood. Polyphenols are well known for their antihyperglycaemic effects, safety and non-side-effects. Potential efficacy of polyphenols on glucose homeostasis and

carbohydrate metabolism has been well investigated in *in vitro*, animal models and some clinical trials (Hanhineva et al., 2010). Figure 8 summarized the advantageous effects of polyphenols on management of blood glucose in diabetes. The hypoglycaemic effects of polyphenols are mainly ascribed to decreasing intestinal absorption of dietary carbohydrate, modulation of the enzymes involved in glucose metabolism, development of β-cell function and insulin action, stimulation of insulin secretion and the anti-oxidative and anti-inflammatory possessions of these components (Bahadoran et al., 2013).

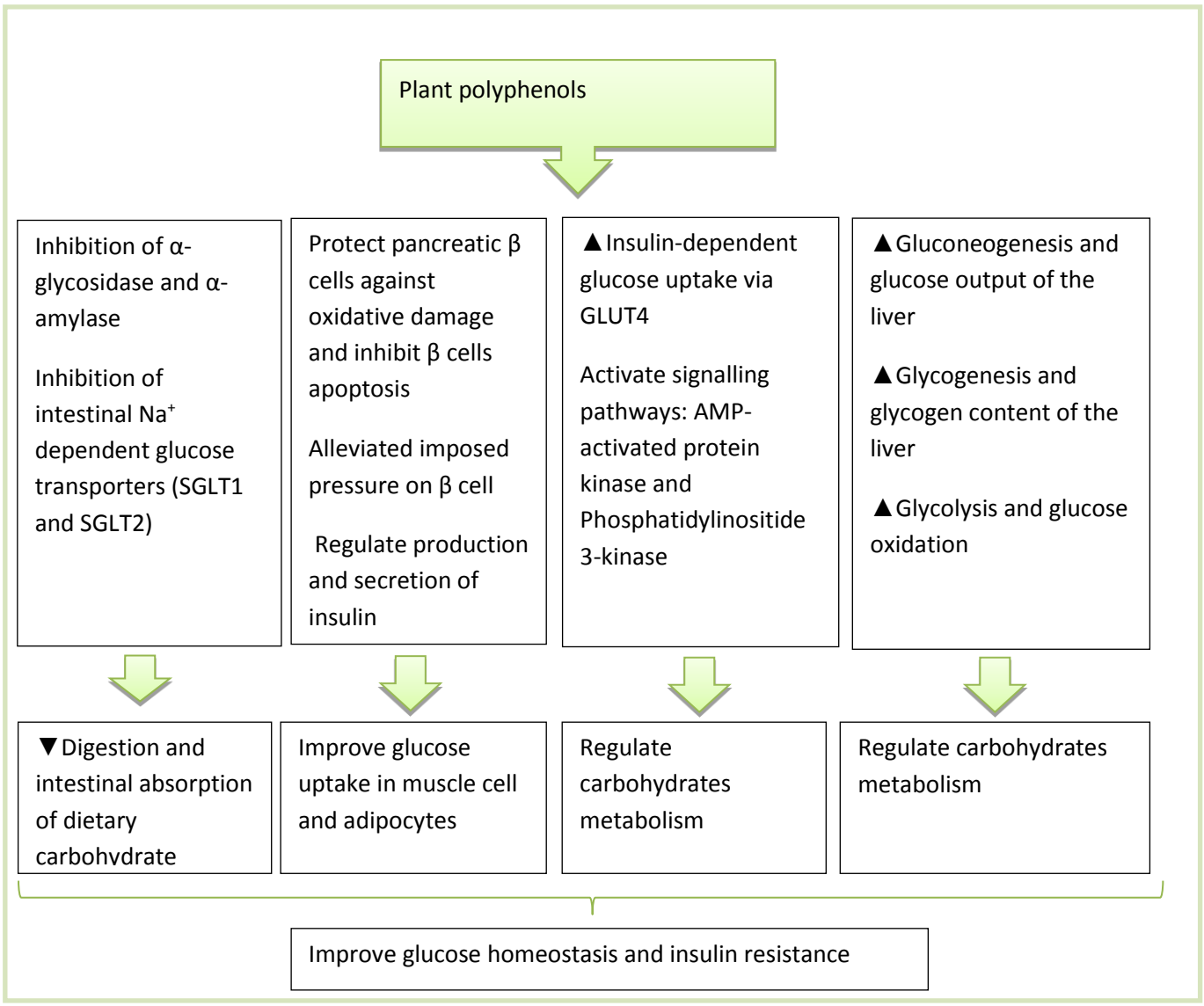


Figure 8: The advantageous effects of polyphenols on management of blood glucose in diabetes ▲Increases; ▼Decreases

2.1.12 Concluding remarks

Diabetes is a slow killer with unknown curable treatments. Hence, it is essential to keep the blood glucose levels of patients under strict control to avoid the complications. One of the problems with tight control of glucose levels in the blood is that such efforts may lead to hypoglycaemia that generates much severe complications than an increased level of blood glucose and/or drug induced side effects. It’s therefore important for alternative methods in diabetes treatment to be found.

2.1.13 References

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Chapter 3:

Soil parameters from different geographical areas in South Africa affects the antioxidant activity of *Hypoxis hemerocallidea* extracts

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3.1 Abstract

Hypoxis hemerocallidea is an African herbal medicine that is wild harvested, and widely used due *inter alia* to its strong antioxidant activity. Anti-oxidant activity is linked to plant stressors like soil heavy metal concentrations, organic matter content and pH. Certain minerals, are known to increase antioxidant capacity. If the high anti-oxidant activity is caused by heavy metals stressing the plant, the plant parts may not be completely safe. For this study we determined the relationship between the concentration of selected soil metal concentrations, the *H. hemerocallidea* corm metal concentrations and corm antioxidant activity. *H. hemerocallidea* corms were collected from five different geographical regions of South Africa. The soil and corm metal concentrations varied between sites. In general, the highest corm and soil metal concentrations were Fe, Mn, and Cr. Among the trace metals investigated Fe was the highest, particularly for corms collected from Ga-rankuwa 83.7 ± 0.03 µg g^{-1}. The soil and corm crude samples from Ga-rankuwa with high levels of metals (e.g. Fe, Cr, Ni, Pb) had greater antioxidant activity (EC_{50} of 1.68±0.49 µg/ml). This indicates that the plant has the ability to bio-accumulate heavy metals. We were, however, unable to link the corm's antioxidant activity to environmental conditions. The results highlight the potential danger of using naturally harvested bulbs growing in undefined soils.

Key words: Anti-oxidant activity, heavy metal, polyphenolic content, organic matter and pH.

3.2 Introduction

Throughout the ages, humans have relied on nature for their basic needs such as the production of food stuffs, shelter, flavours, fragrances and not the least medicine, with as many as 80% of the world's population relying on herbal remedies (Gurib-Fakim, 2006). With plants forming the basis of traditional medicine system for thousands of years, it is not surprising that they continue to provide mankind with new remedies. More so together with substantial research in the field, with some communities still being wholly reliant (Hu and Kitts, 2000; Gurib-Fakim, 2006. While numerous reasons are contributory to the prominence of herbal remedies in an age of "modern medicine", one area of interest come from the belief that plant-based medicines may have fewer side effects when compared with orthodox drugs, which may be valid in chronic disease management like diabetes mellitus. Another concern is the cost of the treatment, for which the herbal remedies may serve as a cheaper alternative.

Diabetes mellitus is a chronic metabolic disorder of multiple aetiologies characterized by high blood glucose level (hyperglycaemia) resulting from absolute deficiency insulin or insulin resistance (poor tissue insulin sensitivity) or both (Afolayan and Sunmonu, 2010). The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2012). Diabetes mellitus can be best classified into four major categories namely, of which Type II diabetes, which accounts for 90–95% of those with diabetes (American Diabetes Association, 2012). Type II diabetes, also known as non-insulin-dependent or maturity-on set diabetes mellitus,

results from a combination of resistance to insulin action where tissue lose sensitivity to insulin and progressive insulin secretory defect.

The main goal in the management of diabetes mellitus is to return blood glucose concentrations to physiological levels both post and pre-prandial (Anderson et al., 2003). Lifestyle intervention is the mainstay with pharmacological therapy used only when the hyperglycaemia became severe (Anderson et al., 2003). The current available commercial treatments included the oral hypoglycaemic agents such as alpha glucosidase inhibitors (e.g. acarbose), sulphonylureas (e.g. tolbutamide and glyburide), meglitinide analogue and thiazolidenediones (e.g. troglitazone, rosiglitazone and pioglitazone) (Shai et al., 2010; Kumar and Clark, 2002; DeFronzo, 1999; Davidson, 1991). Numerous herbal remedies are also used for the management of diabetes, with one such plant in use being the African potato (*Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall.) (Ojewole, 2006).

H. hemerocallidea is a herbaceous perennial plant; also known as *Hypoxis rooperi*, that grows in meanders grassland and mountainous areas of South Africa, South America, Australia and coastal regions of Asia (Van wyk, 2008). Extracts of the corm have been ingested by man for a diversity of ailments (Nair and Kanfer, 2008) in addition to its use in the treatment of diabetes. In a study on streptozotocin (STZ) induced diabetic rats, Zibula and Ojewole (2000), were able to demonstrate that an aqueous extract of the plant, was able to show reductions in the blood glucose concentration of treated animals. Most recently *in vitro* studies, by Boaduo et al., 2014 have shown that *H. hemerocallidea* acetone corm extract may lead to a direct stimulation of insulin release by the islet cells.

While the active compound in the plant is yet to be identified, it has been suggested that the plant may be active due to its high phenolic content, which from other studies are compound known to have multiple biological effects including antioxidant activity (Packer et al., 1999; Mc Cune and Johns, 2002; Joshi and Kaul, 2001). Non-enzymatic antioxidants as well as antioxidant enzymes are known to counteract the effect of reactive oxygen species and reactive nitrogen species. Aside from the enzymatic antioxidants such as superoxide dismutase, peroxidase and catalase, the formation of reactive oxygen species is also prevented by a non-enzymatic antioxidant system (the low molecular mass compounds such as glutathione, ascorbic acid, α-tocopherol, carotenoids and phenolic compounds) (Rice-Evans and Miller, 1996; Gough and Cotter, 2011). Moreover, in the presence of the metal ion cofactors such as copper (Cu), zinc (Zn), or manganese (Mn), superoxide dismutase located in the cytosol and mitochondria, can catalytically convert the $O_2^{\cdot -}$ into oxygen and H_2O_2 (Gough and Cotter, 2011).

In the plant, the polyphenolic compounds are important for normal growth development and defence against infection and injury (Miyamoto et al., 1998; Rassi et al., 2002; Shen et al., 2011). They also have a metal chelation potential (Rice-Evans and Miller, 1996). With the phenolic compounds being protective to the plant, there is evidence that the induction of phenolic metabolism in plants in general may be a response to heavy metal stress and organic matter (Morgan et al., 1997; Ajasa et al., 2004). With the plant being used medicinally for its high phenolic content and if heavy metals cause the high antioxidant activity, there is concern that the use of the plant pose a threat to human health due to heavy metal accumulation. Previous studies by Chang, 1995 and Steenkamp et al., 2002; Street, 2012, have also raised concerns that that the heavy

metals present in plants used medicinally in South Africa could pose a risk to human health. For this chapter, we investigated if the antioxidant and polyphenol content of the African Potato could be due to the heavy metal concentration within the corm and the soil it grows in, in an attempt to evaluate the safety of this commonly used herbal plant.

3.3 Materials and Methods

3.3.1 Site collection of plants and soil

H. hemerocallidea (Fisch and Mey) (Hypoxidaceae), were collected in January, from five sites in different South African geographical locations (Eastern cape province (Mthatha), KwaZulu-Natal province (Ndwedwe), Gauteng province (Ga-rankuwa), Mpumalanga province (Volkstrust) and Limpopo province (Polokwane) (Appendix A). Sites were selected from the reported previous reported sites for the natural occurrence of the plants. Only five mature corms of approximately 10-15 cm in diameter to the total of 0.5 kg in wet weight were harvested. *H. hemerocallidea* were verified by SANBI (South African National Institute) at the National Herbarium in Pretoria. All the plant samples were washed to remove soil particles and dried at room temperature. The plant samples were grinded into fine powder (to a size of less than 1 mm) for extraction (Jankel and Kunkel grinder).

At each location of sampling, soil five samples were also collected from each of the above mentioned five locations from 0 to 30 cm depth with the aid of an auger of 7.0 cm in diameter. After sampling at each site, the auger was thoroughly washed using deionized water so as to avoid mixing of soils. The soil sample from Volkstrust was collected from a mountainous area and was best described as dark vertisols. The sample from Ga-Rankuwa collected from a hill slope region was best described as rusty-red plinthosols.

The sample from Polokwane collected from a level terrain was best described as yellowish-brown cambisols. The samples from Mthatha and Ndwedwe were both on a hill side and best described as dark brown leptosols.

3.3.2 Trace metal analysis of the soil and plant samples

In order to determine trace metals content in soil, the ground soil samples were further sieved to pass through a mesh < 60µm. The total metal content of the 0.5 g plant and 5 g soil samples were determined by digesting the samples with a mixture of HNO_3 (10 ml and 12 ml for plant and soil samples, respectively) and HCl (3 ml and 5 ml for plant and soil samples, respectively) (65% Merck supra pure). The resulting solutions were analyzed for trace element content of copper (Cu), zinc (Zn), manganese (Mg), iron (Fe), chromium (Cr), lead (Pb), nickel (Ni) using Inductive Couple Plasma Mass Spectrophotometer (ICP-MS) (High Resolution ICP-MS operated at RF power: 1350 w , Plasma gas flow rate: 15 L/min., Carrier gas flow: 0.94 L/min., Make up gas: 0.15 L/min., Sample take rate: 100 µL/min., Sample depth: 6.0 mm, Detector mode: on).

3.3.3 Organic matter content and pH of the soil samples

Organic matter contents of the soil samples were determined on loss-on-ignition at 550^{o}C for at least 30 min. After cooling in the desiccator to ambient temperature, weighing to the nearest 1 mg, (*ma)* was done. Weighing into the crucible 0.5 g to 5 g of the dried sludge to the nearest 1 mg, (*mb)* was also done, which then was heated in the furnace at 550^{o}C for at least 60 min. The mass of the residue on ignition and thus the loss on ignition was regarded as constant when the mass obtained after a further half-hour period of ignition at 550^{o}C in the preheated furnace, (*mc - ma),* differs max. 0.5% of the previous

value or 2 mg, whichever was the greater. For the drive of quality reassurance this procedure was repeated twice, and blanks were prepared separately for the soil materials.

The soil pH was determined in 0.01M $CaCl_2$ (1:2 soil solution ratio) and in distilled water using a pH meter fitted with glass electrode (Jen Wal Model 3015 digital). It was off importance to make soil slurry of both solutions since they may provide different pH values. For most acid soils, a buffer of pH 7.0 and another of pH 4.0 was used to calibrate the pH meter. For alkaline soils pH 10.0 and pH 6.0 buffers were required.

3.3.4 Preparation of *H. hemerocallidea* corm extracts

Five grams of each grounded sample of *H. hemerocallidea* corms were extracted with 50 ml of methanol and were placed on a labotec shaker for 30 minutes and then centrifuged at 3000 rpm (704 x g) for 10 minutes. The resulting extracts were filtered using Whatman No. 1 filter paper to remove plant debris, and the filtrates were allowed to dry under a stream of air at room temperature. The extracts were subsequently used for *in vitro* experiments.

3.3.5 Qualitative and quantitative evaluation of antioxidant activity of *H. hemerocallidea* corm extracts.

The extracts prepared above were dissolved in methanol (10 µl of 10 mg/ml) and were visualised on Merck TLC E_{254} 10 X 20cm after elution in four solvent systems: Ethylacetate : Methanol : Water (E:M:W) (10:1,35:1 v/v), Benzene : Ethanol : Ammonia hydroxide (B:E:A) (18:2:0.2), Chloroform : Ethylacetate : Formic acid (C:E:F) (10:8:2) and

Ethyl acetate: Butanone:Water:Formic acid (BUTANONE) (5:3:1:1). Plates were visualized under a UV fluorescent light or after reaction with vanillin. Free radical scavenging bands were visualised by spraying plates with 0.2% of 1,1 diphenyl-2-picrylhydroazyl (DPPH) in methanol as descried by Deby and Margotteaux (1970); Parejo et al., (2002). Free radical scavenging potential of the extracts were also quantified using the DPPH method. In short, the methods relied on a potential antioxidants ability to react with DPPH and convert it to 1-1-diphenyl -2-picryl hydrazine, as measured by change in the absorbance produced at 517nm, after one hour at room temperature. The extent of DPPH radical scavenging (mechanism) at different concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98 and 0.49 µg/ml) of *H. hemerocallidea* extracts was measured, with Trolox and L-ascorbic acid as positive control. All experiments were performed in triplicate.

3.3.6 Determination of total polyphenolic content

Total polyphenolic content of the corm extracts from each site were determined according to the method of Ragazzi and Veronese (1973). Twenty µL of each extract (125 µg/mL) was added to 200 µL distilled water and 40 µL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min and then 40 µL of 20% sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Gallic acid was used as a standard for the calibration curve. Total polyphenol content was calibrated using the linear equation based on the calibration curve. All analysis was done in triplicates. Total polyphenolic content was expressed as mg gallic acid equivalents (GAE)/g.

3.4 Statistics

The concentration of heavy metals per site in the soil and corm were analysed through descriptive statistics. The difference between locations were compared by either the student t-test (for dichotomous variables) or the analysis of variance, while the association between the soil and corm concentrations were evaluated by linear regression. Multiple regression was used to rule out any interactions on corm and heavy metal concentrations. For the free radical scavenging activity, antioxidant activity and total phenolic content of the extracts, the results were presented as the means ± standard deviation. EC_{50} was computed from the resulting dose-response curves.

3.5 Results

3.5.1 Trace metal analysis of the soil and corm samples

The concentration of metals measured in the soil and corm of the various areas of collection are presented in Table 1. In general, the soil and corm metal concentrations between the elements varied by site (Figure 1). Trends were however present, with the highest concentrations for all the elements being Fe, Mn and Cr. Iron concentrations were in general high for all sites, with the exception of Polokwane samples which had a mean value of 0.09 ±0.03 µg g^{-1} (range to 293.9-0.09 µg g^{-}1/soil) for the soil sample and 33.8±0.17 µg g^{-1} (range 83.7-33.82 03 µg g^{-1}) for corm samples. The Ga-rankuwa corm samples had the highest concentration of Fe. The ratio of Mn to Fe was highest for collections in Mthatha, Volksrust, Ndwedwe corm samples vase in Ga-rankuwa and Polokwane corm samples. While the ratio of Mn to Fe was the lowest for all sites soil samples. No relationship was present between a particular heavy metals concentration in the soil versus the concentration within the corm trace metal, irrespective of the site of sampling.

Table 1: Mean concentration (± SD) of elements from the soil and corm samples from the different study area at depth of 0-15 cm in µg g^{-1}.
Key: Highlighted red: Maximum not allowable limits of heavy metal

MINERALS (µg g-1)	MTHATHA			VOLKSRUST			NDWEDWE			GA-RANKUWA			POLOKWANE		
	soil	corm	Ratio	soil	corm	Ratio	soil	corm	Ratio	soil	corm	Ratio	soil	corm	Ratio
Cu	0.18±0.01	0.32±0.07	1:2	6.13±0.02	0.49±0.02	13:1	2.07±0.02	0.16±0.02	1:13	42.06±0.50	4.49±0.01	9:1	0.64±0.04	6.7±0.01	11:1
Zn	88.46±0.13	25.4±0.35	5:1	17.29±0.02	13.2±0.07	1.3:1	33.73±0.02	0.33±0.01	51:0.5	20.56±0.06	0.47±0.02	44:1	4.86±0.02	32.7±0.06	1:7
Mn	89.08±0.55	86.1±0.01	1.04:1	88.98±0.12	67.3±0.02	1.3:1	104.6±0.55	80.2±0.01	1.3:1	115.3±0.12	19.05±0.02	6:1	0.252±0.01	0.8±0.10	3.2:1
Fe	130.1±0.03	34.1±0.02	4:1	293.9±7.81	63.5±0.02	5:1	284±0.01	57.7±0.15	5:1	255.4±0.36	83.7±0.03	3:1	0.09±0.03	33.8±0.17	0.01:4
Cr	0.02±0.01	0.11±0.02	1:6	0.08±0.01	0.12±0.01	1:2	0.04±0.01	0.1±0.02	3:1	41.71±0.01	0.13±0.01	16:0.05	239.7±0.10	0.13±0.01	92:0.05
Ni	1.85±0.10	0.15±0.03	12:1	0.91±0.04	0.15±0.01	6:1	0.82±0.03	0.16±0.02	5:1	40.12±0.02	0.17±0.03	12:0.05	22.15±0.01	0.15±0.01	73:0.5
Pb	23.7±0.57	2.98±0.01	8:1	27.33±0.01	2.75±0.08	10:1	28.23±0.06	5.21±0.02	5:1	30.5±0.20	5.01±0.01	6:1	20.91±0.08	3.01±0.01	7:1

Metal trend in concentrations

Corm samples

	Greater than (>) highest						Smaller than (<) lowest
MTHATHA	Fe	Mn	Zn	Pb	Ni	Cu	Cr
VOLKSRUST	Fe	Mn	Pb	Zn	Cu	Ni	Cr
NDWEDWE	Fe	Mn	Zn	Pb	Cu	Ni	Cr
GA-RANKUWA	Fe	Mn	Cu	Cr	Ni	Pb	Zn
POLOKWANE	Cr	Ni	Pb	Zn	Cu	Mn	Fe

Soil samples

	Greater than (>) highest						Smaller than (<) lowest
MTHATHA	Mn	Fe	Zn	Pb	Cu	Ni	Cr
VOLKSRUST	Mn	Fe	Zn	Pb	Cu	Ni	Cr
NDWEDWE	Mn	Fe	Pb	Zn	Cu	Ni	Cr
GA-RANKUWA	Fe	Mn	Pb	Cu	Zn	Ni	Cr
POLOKWANE	Cr	Ni	Pb	Zn	Cu	Mn	Fe

Figure 1: The trend in element concentrations (µg g^{-1}) for soil and corm samples at different sites.

3.5.2 Organic matter content and pH of the soil samples

The mean concentration for soil organic matter content from all the study areas ranged from 0.04% to 0.67%. The lowest concentration of organic matter content was observed from Ga-rankuwa soil sample (Table 2). Moreover, there seemed to be an increase in DPPH activities as organic matter content decreases. While, the pH of the soils from the study sites differed from 4.95 to 6.51 ($CaCl_2$) and 5.79 to 6.60 (H_2O) (details shown in Table 2), there were significant differences in the value determined for the pH from the sites ($p < 0.05$).

3.5.3 Qualitative and quantitative evaluation of antioxidant activity of *Hypoxis hemerocallidea* corm extracts.

There was variation in antioxidant activities for the extracts collected from different sites, with the Ga-rankuwa corm extract showing more bands with free radical scavenging (Figure 2).

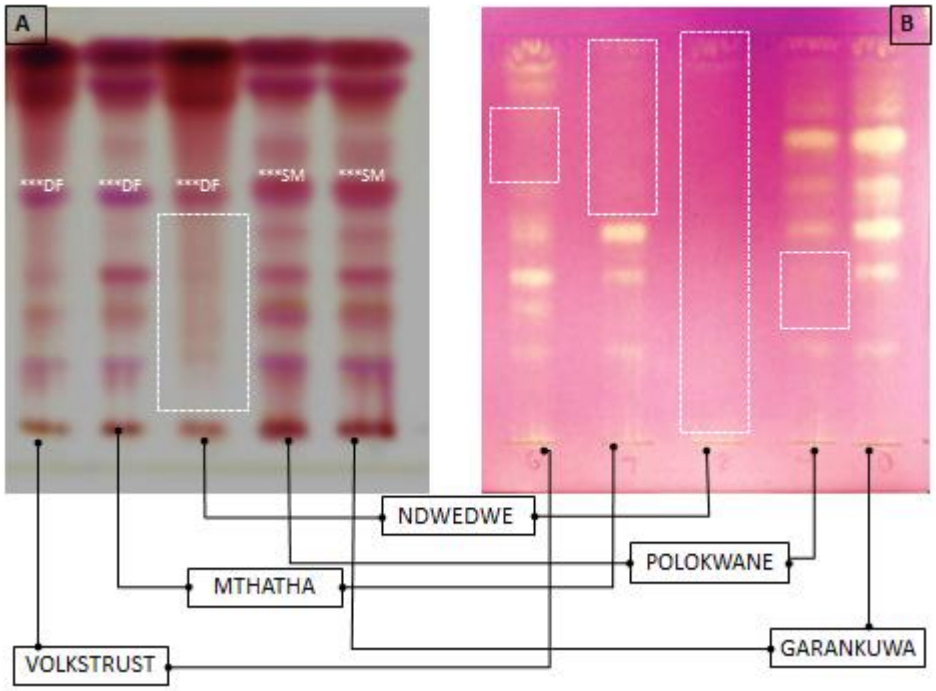
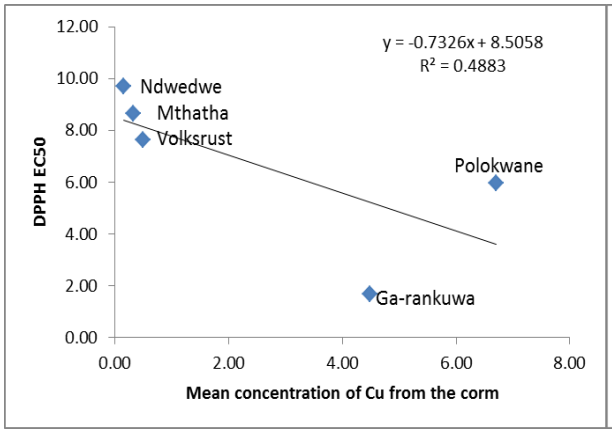
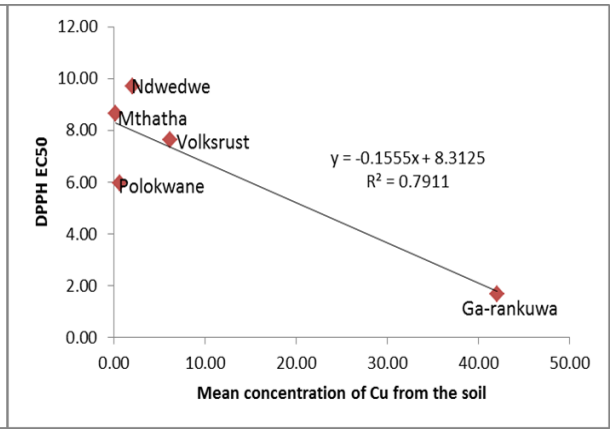


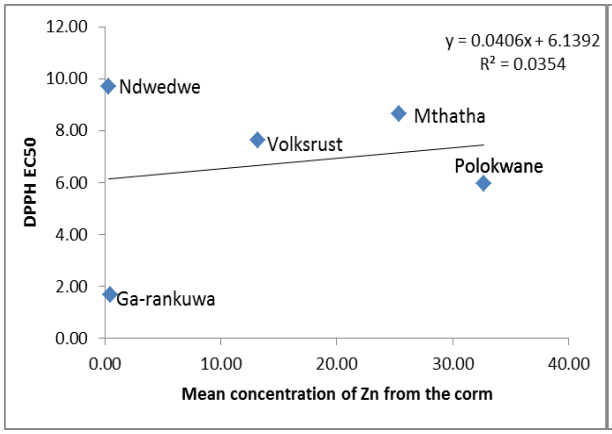
Figure 2: BUTANONE eluent (a) TLC chromatogram for methanol extracts from different areas in South Africa ***DF indicates different compounds from ***SM (same compounds) compounds. (b) Antioxidant TLC chromatogram for methanol extracts from different sites in South Africa. Rectangular dotted lines: indication of not a better resolution of compounds.

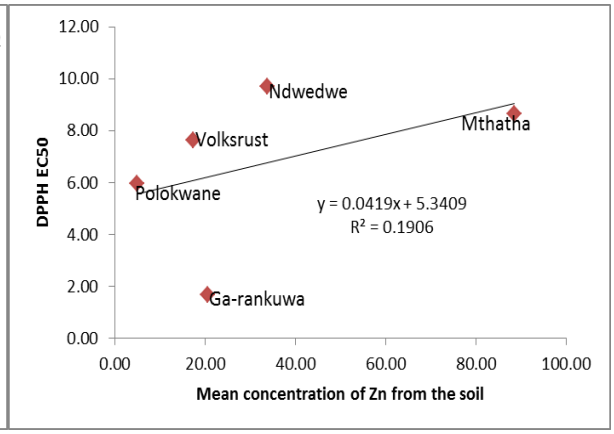
The radical scavenging effect was found to generally increase for both the tested samples and standards in a dose dependent manner. Table 2 shows the scavenging effect of crude extract from all the five mentioned different sites on DPPH radicals. EC_{50} analysis showed that the DPPH radical scavenging activity of the Ga-rankuwa corm crude extract

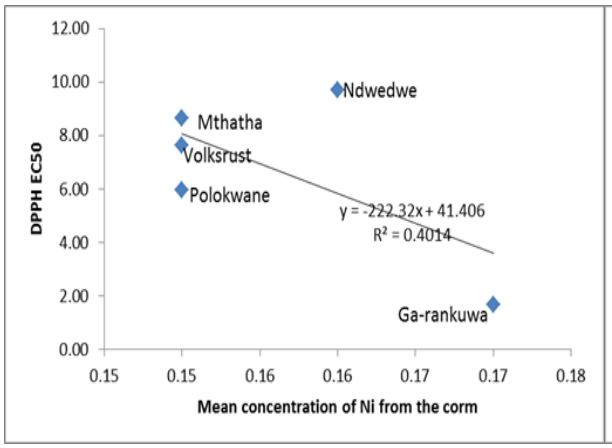
(1.68±0.49 µg/ml) was found to be significantly higher ($p < 0.05$) followed by Polokwane corm crude extract (5.95±1.53 µg/ml) when compared to other crude extract of different sites. The EC_{50} value for Ga-rankuwa corm crude extract was not close to that for L-ascorbic acid (2.14±0.86) and Trolox (3.04±0.44) which were the positive controls (Table 2). In general, soil and corm crude samples (e.g. from Ga-rankuwa) with high levels of metals (e.g. Fe, Cr, Ni, Pb) yielded greater antioxidant activity (Figure 3).

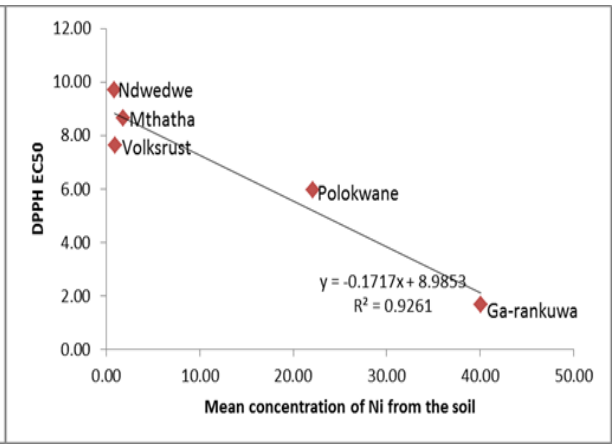

y = -0.7326x + 8.5058
R² = 0.4883
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Cu from the corm

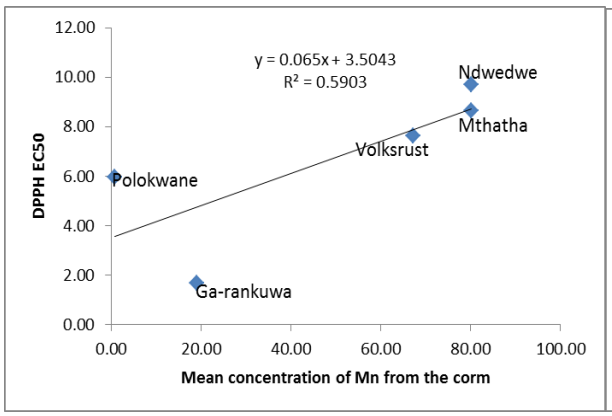

y = -0.1555x + 8.3125
R² = 0.7911
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Cu from the soil

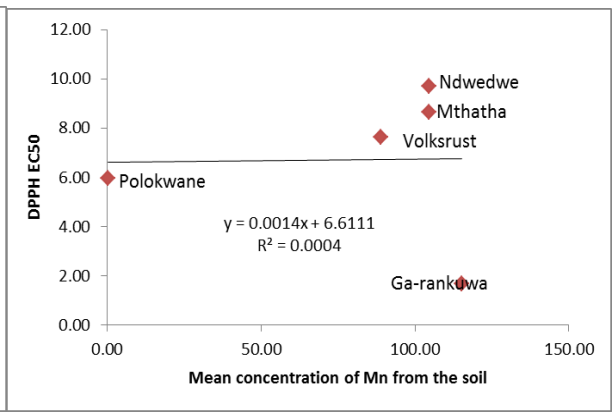

y = 0.0406x + 6.1392
R² = 0.0354
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Zn from the corm


y = 0.0419x + 5.3409
R² = 0.1906
Ndwedwe
Volksrust
Mthatha
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Zn from the soil


y = -222.32x + 41.406
R² = 0.4014
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Ni from the corm


y = -0.1717x + 8.9853
R² = 0.9261
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Ni from the soil


y = 0.065x + 3.5043
R² = 0.5903
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Mn from the corm


y = 0.0014x + 6.6111
R² = 0.0004
Ndwedwe
Mthatha
Volksrust
Polokwane
Ga-rankuwa
DPPH EC50
Mean concentration of Mn from the soil

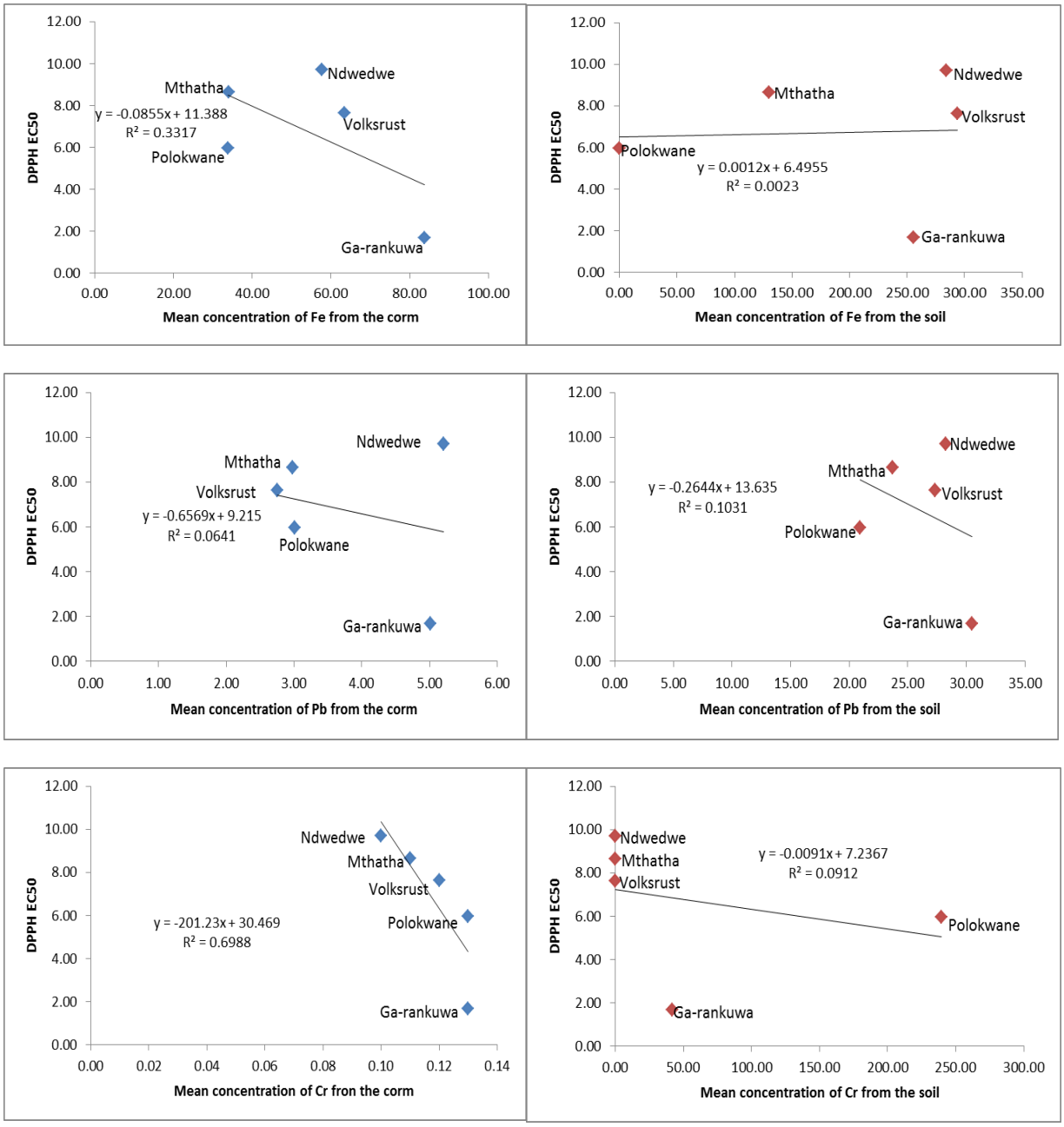


Figure 3: Regression lines showing relationship between the mean concentration of the metals from the soil or corm crude samples and corm DPPH EC_{50} from different sites

3.5.4 Determination of total phenolic contents

Total phenolic content of the extracts was calculated from the regression equation of calibration curve (Y = 26.093x; R^2 = 0.998) and expressed as mg gallic acid equivalents (GE) per gram of sample in dry weight (mg/g). The absorbance values obtained at different concentrations of gallic acid were used for the construction of calibration curve.

The total phenolic content (determined as gallic acid equivalents or GAE) of the corm crude extract from different sites are shown in Table 2. The total phenolic content ranged between 42.01 mg and 56.53 mg GAE/g dry weight of crude. The Ga-rankuwa corm extract showed the highest total phenolic content (56.53 mg GAE/g crude) (as compared to other sites $p< 0.05$), whereas the phenolic content of Ndwedwe corm extract was lower (42.01 mg GAE/g).Through correlation analysis for phytochemical contents with EC_{50} values of radical scavenging activities of various corm extracts from different sites, the content of phenolic and EC_{50} of scavenging ability on DPPH radicals was found to be significant with positive correlation.

Table 2: EC_{50} values for antioxidant activity, total polyphenolic content of corm crude extract of *H. hemerocallidea* and the pH values, organic matter content of the soil from different sites.

Samples µg/ml	**DPPH Scavenging ability**	**Total phenolic content**	**Soil pH**	**Soil Organic matter (%)**
	(Mean ± SD)	**(Mean ± SD)**		
	Corm extract from different sites		**$CaCl_2$:H_2O**	
Polokwane	5.95±1.53 SDK	49.78±6.04 SDD	6.51:6.60	0.49%
Mthatha	8.65±0.44 SDK	42.12±7.53 SDD	5.82:5.98	0.58%
Volksrust	7.64±1.46 SDK	53.08±7.98 SDD	5.79:6.01	0.60%
Ga-rankuwa	1.68±0.46 SDK	56.53±8.94 SDD	4.95:5.79	0.04%
Ndwedwe	9.70±2.68 SDK	42.01±6.81 SDD	5.96:6.26	0.67%
L-ascorbic acid	1.57±0.93 SDK			
Trolox	3.59±1.70 SDK			

Key for DPPH radicals or Total phenolic content: same letter = means significantly different at $p < 0.05$ from each site. While different letter =means no significantly different at $p > 0.05$ from each site.

3.6 Discussion

The use of herbal remedies is an important practice in South Africa, to such an extent the government has recently brought control measures to protect consumers. This control unfortunately does not include herbal remedy practitioners who prepare more patient specific medicines. This raise concerns as some plants may be dangerous due to factors such as their heavy metal concentrations of wild harvested plant material. Cases of heavy metal poisoning from exposure to high levels of arsenic (As), chromium (Cr) and magnesium (Mg) have reported from the use of *Bulbine natalensis* and *Alepidea amatymbica* (Steenkamp et al., 2002; Mtunzi et al., 2012; Ernst 2002). For this study we investigated if a commonly used herbal medicine could pose health risks, from the accumulation of selected heavy metals. We also investigated if selecting crops based on efficacy does not lead to higher potential heavy metal exposure (Chang, 1995). We found that plants accumulated high concentrations of Fe, particular for the corms collected from Ga-rankuwa and Mthatha. This corroborates previous findings that *H. hemerocallidea* has the capacity to take up and accumulate high amounts of Fe (Jonnalagadda et al., 2008).

Another important finding was that the high anti-oxidant activity, free radical scavenging activity and the high phenolic content from the Ga-rankuwa corms was associated with the plant growing in soils with the lowest concentration of organic matter and lowest pH as compared to corms collected from other sites. From a physiological point, the anti-oxidant and free radical scavenging activity can be linked to the plants polyphenolic content. Within the plant, these polyphenols play a vital role as an antioxidant in living

systems due to the presence of hydroxyl groups in ortho and para- positions (Lapornik et al., 2005; Scalbert et al., 2005; Nair et al., 2008). Furthermore, the use of *H. hemerocallidea* is favoured because of its high anti-oxidant potential, especially in type II diabetes. However, the physiological mechanisms involved in the advantageous effects of polyphenols is poorly understood. The hypoglycaemic effects of polyphenols are thought to decreasing intestinal absorption of dietary carbohydrate, modulation of the enzymes involved in glucose metabolism, development of β-cell function and insulin action and stimulation of insulin secretion (Bahadoran et al., 2013). Moreover, some of the elements, for example, Zn, Mn, Cu and Fe determined in this study are known also to play beneficial role as co-enzymes in antioxidant processes and deficiency in any of these essential elements may impair the overall function of the oxidation systems (Lemberkovics et al., 2002; Hashmi et al., 2007; Langille and MacLean, 1976; Khan et al., 2007). However, extremely high levels of these essential elements can be toxic (Sandstead, 1995). This illustrates the dangers of selecting plants by their beneficial activity without taking into consideration other factors such as potential toxicity.

Iron, Ni and Cr measured concentrations did not exceed the maximum not allowable limits of heavy metal in soil and plant established by standard regulatory bodies such as World Health Organization (WHO), Food and Agricultural Organization (FAO) and Ewers U, Standard Guidelines in Europe (Fe soil 50000 µg/g and plant 425.00 µg/g; Ni soil 50 µg/g and plant 67.00 µg/g; Cr soil 100 µg/g; Pb soil 100 µg/g and plant 0.30 µg/g). From a pathological aspect, high intake of Fe, Ni and Cr may be toxic, causing severe damage in the stomach or haematemesis leading to gastric discomfort, nausea, vomiting and diarrhoea. It may also lead to necrosis of mucosal cells and perforation of the gut wall.

Nonetheless while the levels were not at toxic levels, this does not preclude a more insidious chronic effect, as these metals can accumulate in the body and food chain (Langille and MacLean, 1976). When trying to ascertain the link between plant and soil concentrations, Olowoyo et al., 2011 indicated that while Fe, Ni and Cr levels in the plant may also be reliant on the concentrations of Fe, Ni and Cr in soil. Which may not be the case in our present study, since no relationship was present between a particular heavy metals concentration in the soil versus the concentration within the corm trace metal, irrespective of the site of sampling when multiple regression model was used to rule out any interactions on corm and heavy metal concentrations. One reason for this may be the link between metal uptake by plants being dependent on the bioavailability of the metal in the water phase, which in turn depends on the retention time of the metal, as well as the interaction with other elements and substances in the water. In addition, many researchers for example, Fritioff and Greger, 2003, Kondakova et al., 2009; Wang and Lin, 2000; Iqbal and Bhanger, 2006, have demonstrated that the uptake of Fe, Ni and Cr in the plant can be governed by factors such as soil pH and organic matter content of the soil. Moreover, according to Lee et al. (1996) the common micronutrients such as Fe, Zn and Ni are more accessible within a soil pH range of 5 to 7. Which was in line with the soil pH range and the organic matter levels obtained in this study.

3.7 Conclusion

We were unable to show a clear link between the corm's antioxidant activity and environmental conditions. The latter was a general concern, that the selection of plants based on only their activity could be inadvertently result in exposure of people to heavy metals. Nonetheless, the high levels of metal concentrations found in some of the

samples do support general concerns that the safety and quality assurance in South African herbal medicines wild harvested requires screening for heavy metals. This may also indicate that the cultivation of valued medicinal plants may be necessary to avoid heavy metal accumulation as well as to enable reliability in terms of quality and efficacy.

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Chapter 4:

Anti-diabetic activity associated with glucose uptake and insulin secretion of *Hypoxis hemerocallidea* extracts and isolated compounds

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4.1 Abstract

Hypoxis hemerocallidea extracts have been used to treat diabetes. The aim of this study was to confirm the activity and to isolate the compound(s) in *H. hemerocallidea* responsible for antidiabetic activity. To also evaluate the cytotoxicity of the crude extract, fractions and isolated compounds from the corm of *Hypoxis hemerocallidea.* Five different fractions were prepared from a methanol extract of *H. hemerocallidea* corm and three compounds were isolated. The quantity isolated of two of these compounds were not high enough to elucidate the structures. The third compound was characterised as β-sitosterol by NMR spectroscopy. There was a mild dose related decrease in cell viability as concentration was increased from 0.031 to 0.5 mg/ml when the crude extract, fractions and isolated compounds from the corm of *Hypoxis hemerocallidea* were used. Moreover, the glucose uptake in C_2C_{12} myocytes and 3T3-L1 pre-adipocytes and the insulin secretion in rat insulinoma RiNm5F cells of the fractions and compounds were determined. All the fractions and crude methanol extract lowered glucose levels both in C_2C_{12} myocytes and 3T3-L1 preadipocytes to a degree. Similar results were obtained for the three isolated compounds. β-sitosterol had a higher activity in the glucose uptake in 3T3-L1 pre-adipocytes and C_2C_{12} myocytes. Exposure of rat insulinoma RiNm5F cells to the methanol crude extract, fractions or compounds stimulated insulin secretion. Only the methanol crude extract, fraction III & V, β-sitosterol compound and glibenglamide (positive control) led to a significant increase in the release of insulin. It appears that β-sitosterol may be the one of the compounds responsible for the promising *in vitro* anti-diabetic activity. Since, β-sitosterol occurs so widely in plants it may be responsible for the protection of plant-rich diets against diabetes.

Keywords: *Hypoxis hemerocallidea,* glucose utilisation, insulin secretion and β-sitosterol.

4.2 Introduction

Diabetes mellitus is known and recognised since ancient times. Despite considerable progress in scientific studies on Type 2 Diabetes Mellitus and research and development towards new antidiabetic therapy, newer remedies are still in great demand due to the limited efficacy and undesirable side effects of some currently available therapeutic products. One avenue of continued research focuses on known herbal remedies already used by many people. This is not surprising as systematic scientific investigations carried out on medicinal plants led to identifying a growing number of active constituents for different indications (Chang et al., 2013). Some of the well-known examples are acarbose, reserpine, atropine, metformin, vincamine, vinblastine, vincristine and morphine (Hamburger and Hostettmann, 1991).

Furthermore, diabetes as a disease is well-documented in traditional systems since the first known diabetes symptoms was mentioned in 1552 B.C., when Hesy-Ra, an Egyptian physician, recognised frequent urination as a symptom of a mysterious disease that also caused leanness. Centuries later, people known as "water tasters" made diagnosis of diabetes by tasting people's urine that were suspected to have diabetes. To recognise this feature, in 1675 the word "mellitus," which mean honey, was added to the name "diabetes," meaning siphon. It wasn't until the 1800s that scientists developed chemical tests to detect the presence of sugar in the urine (Nori Janosz et al., 2009; Park et al., 2011; Chang et al., 2013; Ojewole, 2006). Since 1965 the World Health Organisation published several guidelines to diagnose diabetes (Alberti et al., 2007; World Health Organization, 2006). Both diagnosis and classification were reviewed in 1999 and were published as the guidelines for the

definition, diagnosis and classification of diabetes mellitus (World Health Organization, 1999).

A number of studies have evaluated herbal remedies used to treat diabetes and many plant constituents have the ability to modulate glycemic index by influencing serum glucose concentration, Glut-4 translocation, glucose utilisation, glycogen synthesis and/or insulin secretion, under both *in vivo* and *in vitro* conditions (Chang et al., 2013; Nori Janosz et al., 2009; Park et al., 2011; Ojewole, 2006; Mohiuddin et al., 2009). One commonly used herbal remedy is derived from *Hypoxis hemerocallidea Fisch. & C.A. Mey. (Hypoxidaceae)* (*Hypoxis*). It is a plant with a bitter taste used traditionally for a wide variety of conditions including diabetes mellitus, haemorrhage and prostate hypertrophy (Ojewole, 2006; Msonthi and Magombo, 1983).

As a result of its common use, this plant received substantial attention to investigate the rational of its use. The orally administered aqueous corm extract to diabetic rats significantly reduce blood glucose concentration by 30.2% to 48.5% (Zibula and Ojewole, 2000; Ojewole, 2006; Mahomed and Ojewole, 2003). The mechanism of the plant's action has been described by Boaduo (2010), who demonstrated that the acetone extract increases insulin secretory activity by the pancreatic beta cells. With plant extracts having beneficial effect in animal studies, its active component could potentially serve as a new therapeutic molecule. Because the active compound is unknown, this study was designed to isolate the plant's active compound(s) using bioassay-guided fractionation with *in vitro* glucose utilisation and insulin secretion assays as selection parameters.

4.3 Material and Methods

4.3.1 Preparation of *H. hemerocallidea* methanol crude extract

Corms of *H. hemerocallidea*, approximately 10-15 cm in diameter, were collected in Ga-Rankuwa in Gauteng province. The identity of the plant was confirmed by taxonomists at the National Herbarium of the South African National Biodiversity Institute in Pretoria. Corms were cut into smaller pieces and were dried at room temperature before being ground into a powder.

Corm powder (650 g) was extracted with 5.0 L of methanol (5.0 L) on a Labotec shaker for 24 hours. The resulting extract was filtered using Whatman No. 1 filter paper to remove plant debris, and the filtrate dried under a stream of air at room temperature. The method of the National Cancer Institute in the USA (Suffness and Douros, 1979 as cited and applied by Martini and Eloff, 1998) was used. The plant extract was dissolved in 1:1 mixture of chloroform ($CHCl_3$) and distilled water (H_2O) and the two phases were separated in a separatory funnel. The water fraction was mixed with an equal volume of n-butanol (BuOH) in a separating funnel to yield the water and n-butanol fractions. The chloroform fraction was dried in a rotary evaporator under reduced pressure and extracted with an equal volume of hexane and 10% water in methanol mixture. The water in methanol was mixed with chloroform to yield the chloroform and 35% water in methanol fractions. This afforded five solvent-solvent fractions of the extract.

The fractions were separated by TLC using three solvent systems: ethylacetate: methanol: water (E: M: W) (10:1, 35:1 v/v), chloroform: ethylacetate: formic acid (CEF) (10:8:2) and ethyl acetate: butanone: water: formic acid (EBWF) (5:3:1:1). The

chromatograms were subsequently sprayed with DPPH (1,1-Diphenyl-2-picrylhydrazyl) and vanillin sulphuric acid (1% solution of vanillin in concentrated sulfuric acid).

4.3.2 Isolation and identification of the compound from n-butanol fraction

The n-butanol fraction collected from solvent-solvent fractionation (section 4.3.1), was subjected to column chromatography using silica gel (60-120 mesh size). Firstly, 30 g of the dried n-butanol fraction was dissolved in methanol and adsorbed in silica gel (40 g). The mixture was allowed to dry under a stream of air and packed on top of a glass column (100 x 2.5 cm) containing 270g of silica gel. The column was eluted with hexane, followed by a 10% increasing gradient of ethyl acetate in hexane, and then by a 10% increasing gradient of methanol in ethyl acetate up to 100% methanol. The fractions collected were analyzed by thin layer chromatography for homogeneity and the fractions with the similar chemical composition, were pooled together. Five main fractions were obtained and the yields of fraction F (I), F (II), F (III), F (VI) and F (V) were 136 mg, 310 mg, 1280 mg, 290 mg and 360 mg, respectively. The anti-diabetic activity (glucose uptake and insulin secretion assay) of the fractions F (I), F (II), F (III), F (VI) and F (V) were determined (Table 1 and 2). Fraction (I), (III) and (V) were chromatographed over a Sephadex column (35 x 2.0 cm) eluted with dichloromethane (100%) followed with an increasing gradient (5%-100%) of ethyl acetate in dichloromethane followed with 10% to 20% of methanol in ethyl acetate and yielded the compounds used in this study for anti-diabetic activity. The structure of the other two compounds from fraction (I) and (V) could not be elucidated because insufficient material was isolated. The structure of the isolated compound was determined by one dimensioned (^{1}H and ^{13}C) and two-dimensional NMR spectroscopy. ^{1}H NMR spectra

were measured at 399 MHz and the ^{13}C NMR were measured at 100MHz. All NMR experiments were conducted at a constant temperature of 26°C with a Varian 400MHz spectrometer. Chemical shifts (δ) were measured in parts per millions (ppm) from internal standard tetramethylsilane (TMS).

4.3.3 Biological assays

4.3.3.1 Maintenance of cell lines

The 3T3-L1 pre-adipocyte (CL-173), C_2C_{12} myocyte cell lines (CRL-1772) and rat insulinoma RINm5F (CRL-11065) were obtained from the American Type Culture Collection (ATCC) through Highveld Biological (Johannesburg, South Africa). The 3T3-L1 pre-adipocyte and C_2C_{12} myocyte cell lines were routinely maintained in DMEM culture medium (1.5 g/L $NaHCO_3$), supplemented with 10% bovine calf serum and 4 mM glutamine (Sigma, South Africa). The RINm5F rat insulinoma cell line was routinely maintained in RPMI-1640, supplemented with 10% foetal bovine serum, 10 mM HEPES and 1 mM sodium pyruvate.

Proliferating pre-adipocytes and myocytes were supplemented with fresh growth medium every 2-3 days, until the monolayers required trypsinisation for sub-culturing. Trypsinisation was performed using 0.25% trypsin-EDTA (3 ml) in PBSA for 5 - 10 min, then centrifuged at 1500 for 2 min, prior to suspension in fresh growth medium (2 ml) for counting.

4.3.3.2 Glucose uptake assay experimental procedures in C_2C_{12} myocytes and 3T3-L1 pre-adipocytes

The method described by Olaokun et al. (2014) was used with some modification to determine the glucose utilisation activities of 3T3-L1 adipocytes and C_2C_{12} myotubes. To prepare cells for assay, cells in monolayer culture (at <70% confluence) were

dislodged with Trypsin, counted, and re-suspended in fresh complete DMEM. The cell suspension (200 µl) was seeded at a density of c. 30,000 cells/ml for 3T3-L1 pre-adipocytes and 25,000 cells/ml for C_2C_{12} myocytes into each well of 96-well plates. The seeded plates were incubated at 37◦C for 2 days to allow cells to confluent.

Once confluent, differentiation was induced by changing the medium for C_2C_{12} cells to DMEM supplemented with 2% BCS while those of 3T3-L1 cells was changed to DMEM supplemented with 10% FBS, 5 µg/ml insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine and 10 µmol/l dexamethasone.

Prior to glucose utilisation assay, *H. hemerocallidea* crude methanol extracts/fractions/compounds prepared by reconstituting in phosphate-buffered saline (PBS) to a concentration of 100 mg/ml, which was further diluted with appropriate growth medium before using it to determine the activity. On day five after differentiation, the medium was removed by aspiration and immediately replaced with 100 µl of incubated medium containing the plant crude methanol extract, fractions or compounds (different concentrations of 0.5, 0.25, 0.125, 0.063, 0.031 mg/ml), which were added to the appropriate wells, while the control wells contained incubation medium only. For the positive control (insulin), 0.01, 0.1, 1, 10, 100 µM (0.058, 0.58. 5.8. 58 and 580 µg/ml) concentrations were used. After 60 min of incubation, 20 µl of each reaction mixture was removed and placed into a new 96-well plate. The glucose concentration of the medium was determined by the glucose oxidase method, using a commercial test kit (Sigma GAGO 20), according to the manufacturer's instructions. Tests were carried out in triplicate and each experiment was repeated three times. The absorbance of the mixture in the wells was measured at 540 nm using a Multiscan MS microtiter plate reader (Labsystems).

4.3.3.3 Insulin sensitizing effect of Rat insulinoma RiNm5F cells

Insulin secretion of RINm5F insulinoma cells was determined by the method described by Olaokun et al., (2014). Briefly RINm5F cells were cultured in complete medium of RPMI-1640 supplemented with 10% fetal bovine serum, 10 mM HEPES and 1 mM sodium pyruvate. The cell suspensions were prepared from 70%-80% confluent monolayer cultures seeded in complete medium (200 µl) at a density of 100,000 cells/ml into each well of 96-well plates and incubated for 48 h. After this, the medium was removed by aspiration and replaced with freshly prepared Krebs-Ringer bicarbonate buffer with no glucose (135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$), supplemented with 10 mM HEPES and 1 mg/ml bovine serum albumin, 0.5% pH 7.4 and incubated for a further 2 h. Following this, the medium in the test wells (column 3-11) was removed and replaced with 100 µl of glucose free Krebs-Ringer bicarbonate buffer containing plant crude extract/fractions/compounds at different concentrations of 0.5, 0.25, 0.125, 0.063, 0.03 mg/ml. Glibenclamide (0.01, 0.1, 1, 10, 100 µM) was used as positive control and column 2 test wells were replaced with plain medium (untreated cells control). The cells were incubated at 37^{o}C in a 5% CO_2 incubator for 1 h. The insulin content of supernatants was determined using DRG diagnostic Insulin (Rat) ELISA kit, according to the manufacturer's instructions. The percentage insulin secreted was calculated using the following calculation.

$$\% \text{ Insulin secreted} = \left[\left(\frac{\Delta \text{ A sample (treated cells)}}{\Delta \text{ A control (untreated cells)}} \right) \times 100 \right] - 100$$

4.3.3.4 Cytotoxicity assay

The extract of *H. hemerocallidea* corm, fractions and compounds were tested for *in vitro* cytotoxicity, using the above-mentioned cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay Mosmann, 1983. Briefly, 100 µL of media (RMPI 1640/ DMEM) was added into each of the 96-well plates from row B to row G (triplicate). Then, 100 µL of diluted (with phosphate-buffered saline) plant extract, fractions and compound/s were added in row A and row B. Starting from row B the 200 µL of solution (100 µL drug + 100 µL media) were mixed and 100 µl from row B were added into next row (row C) by using micropipette and a serial dilution was done up to row G. The cultured cells were harvested by trypsinization, pooled in a 50 mL vial. Then, the cells were seeded at a density of 1×106 cells/mL cells/well (100 µL) into 96-well micro-titer plates from row B to row G. Finally, 200 µL of cells were added in row H as a control. Each sample was replicated 3 times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/mL) was added into each well and the cells incubated for another 2-4 h until purple precipitate was clearly visible under a microscope. Subsequently, the medium together with MTT (190 µL) was aspirated off the wells, DMSO (100 µL) was added and the plates shaken for 5 min. The absorbance for each well was measured at 540 nm in a micro-titre plate reader (Mosmann, 1983) and the percentage cell viability (CV) was calculated manually using the formula:

$$CV = \frac{\text{Average abs of duplicate drug wells}}{\text{Average abs of control wells}} \times 100$$

A dose-response curve was plotted to enable the calculation of the concentrations that kill 50% of the cells (IC_{50}). All data are expressed as Means ± SE. Differences between

control and treated cells were analysed using the student's t-test. A p value<0.05 was considered significant.

4.4 Statistical analysis

The results are expressed as mean ± S.E.M (standard error of the mean). Statistical significance between the groups was evaluated by one-way variance (ANOVA) analysis for both glucose utilisation assays and insulin secretion. A *p*-value < 0.05 value was considered as statistically significant.

4.5 RESULTS

4.5.1 Solvent fractions of corm crude extract developed in three solvent systems

The TLC chromatograms (Figures 1, 2, 3), clearly show that the water and n-butanol fractions contained the most active antioxidant compounds. There were more antioxidant compounds in the n-butanol fraction (Figure 1, 2, 3). The best separations were obtained using the relatively polar eluents EBWF and EMW. The most antioxidant compounds as expected, were present in the polar fractions. The vanillin sulphuric acid spray reagent led to interesting different compounds being separated.

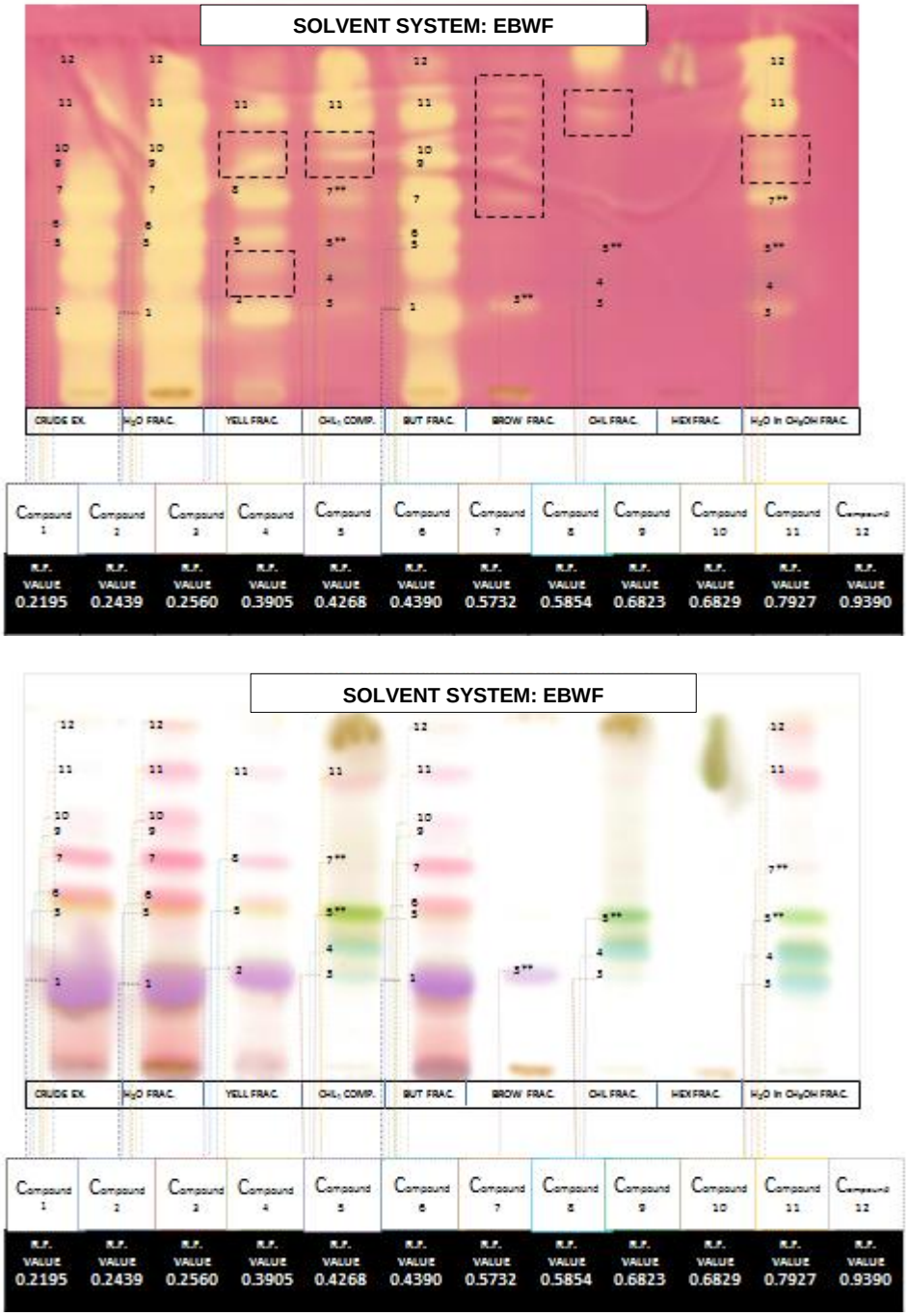

Figure 1: Thin layer chromatography and anti-oxidant qualitative analysis of solvent fractionations using EBWF solvent system (A) sprayed with DPPH solution, (B) sprayed with vanillin in sulphuric acid. KEY: The dotted circled lines represent the compounds which were not visible on the TLC fingerprint sprayed with sprayed with vanillin in sulphuric acid; Rf: retention factor; ** : same Rf value; Ex: extract; Yell: yellow in colour; CHL: chloroform; BUT: Butanol; HEX: Hexane; Frac: fraction.

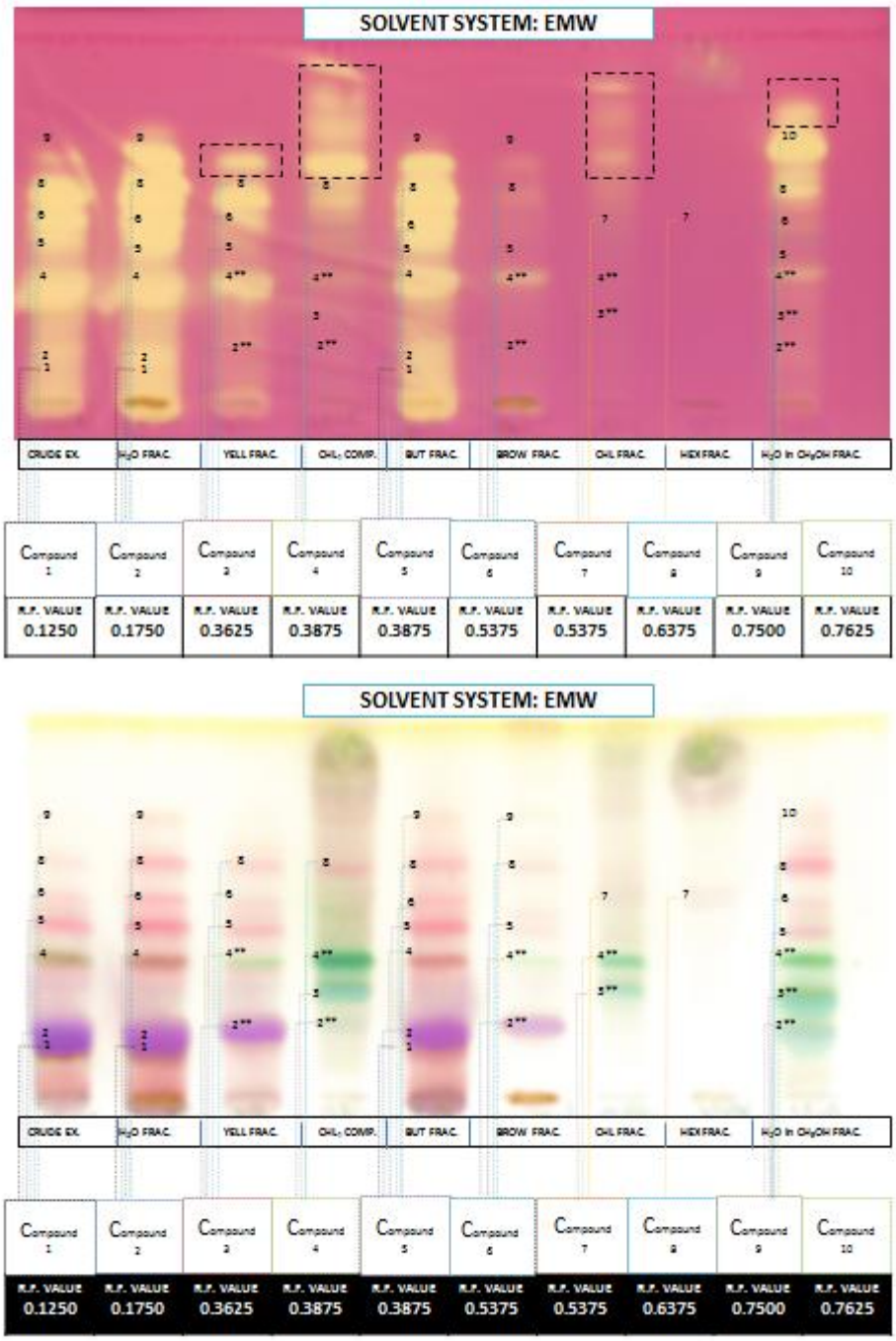

Figure 2: Thin layer chromatography and anti-oxidant qualitative analysis of solvent fractionations using EMW solvent system (A) sprayed with DPPH solution, (B) sprayed with vanillin in sulphuric acid. KEY: The dotted circled lines represent the compounds which were not visible on the TLC fingerprint sprayed with sprayed with vanillin in sulphuric acid; Rf: retention factor; ** : same Rf value; Ex: extract; Yell: yellow in colour; CHL: chloroform; BUT: Butanol; HEX: Hexane; Frac: fraction.

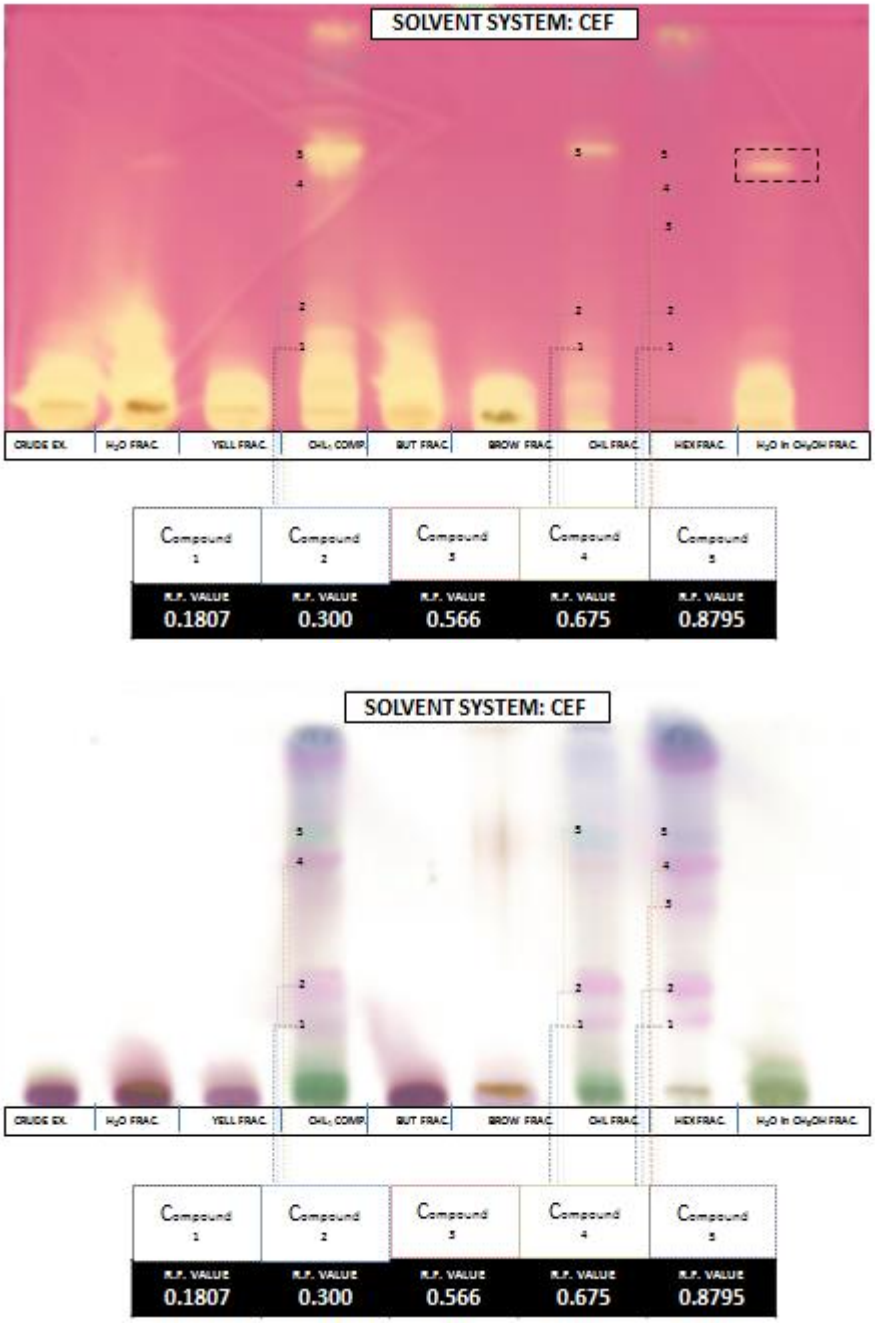

Figure 3: Thin layer chromatography and anti-oxidant qualitative analysis of solvent fractionations using CEF solvent system (A) sprayed with DPPH solution, (B) sprayed with vanillin in sulphuric acid. KEY: The dotted circled lines represent the compounds which were not visible on the TLC fingerprint sprayed with vanillin in sulphuric acid; Rf: retention factor; **: same Rf value; Ex: extract; Yell: yellow in colour; CHL: chloroform; BUT: Butanol; HEX: Hexane; Frac: fraction.

4.5.2 Isolation and identification of the compound from *H. hemerocallidea* n-butanol extracts

Fraction (I), (III) and (V) were selected based on their high anti-diabetic activities (Table 1 and 2). Fraction (I), (III) and (V) were fractionated in a Sephadex column eluted with DCM and methanol to afford three compounds coded A (0.8 mg), B (0.91 mg) and C (16.02 mg) (Figure 4). The structure of white powder compound coded as C was confirmed by 1H-1H COSY, HMBC and HMQC spectra (Appendix B1 to B5) as β-sitosterol (Figure 5).

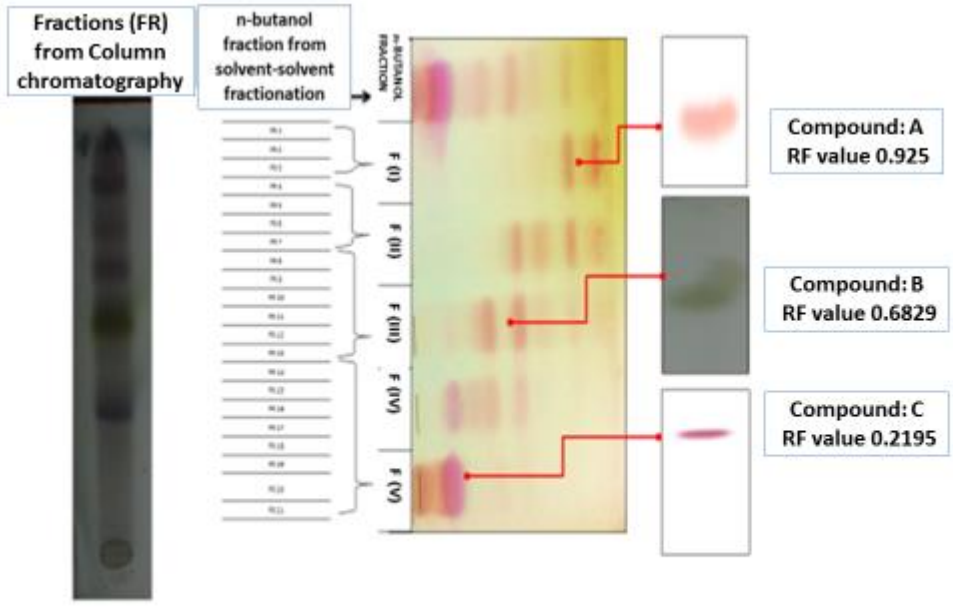

Figure 4: Illustration indicating the isolation of the compounds from *H. hemerocallidea*. KEY: F meaning fractions from column chromatography.

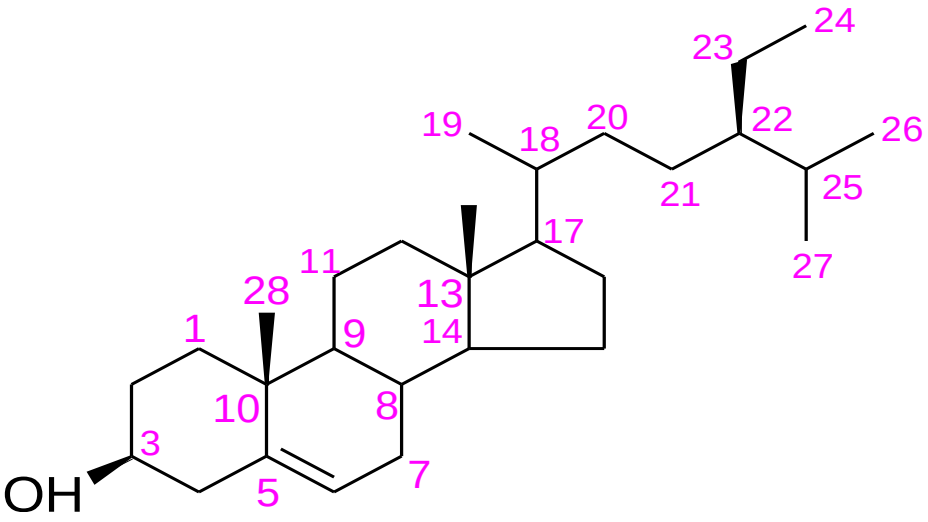

Figure 5: Structure of β-sitosterol.

The ^{13}C NMR and Distortion less Enhancement by Polarisation Transfer (DEPT) (Appendix B2) spectra displayed signals at δ 140.99: C, 121.93: CH and 72.03: CH ppm, corresponding respectively to carbons C-5, C-6 and C-3 characteristic for a β-sitosterol. This was established by the occurrence of signals on its ^{1}H NMR spectrum at δ 5.361 (^{1}H, brd, 4.0 Hz, H-6), 3.524 (^{1}H, m, H-3), corresponding to protons H-6 and H-3, respectively. Moreover, the ^{1}H NMR spectrum had signals at δ 1.01 (^{3}H, d, 8.0 Hz, H-29) and 0.682 (^{3}H, s, H-18) assignable to six methyl groups. Furthermore, on its ^{13}C NMR spectrum the presence of total number of 30 carbons with signals due to six angular methyl groups was appropriate for this compound to be a phytosterol (Appendix B1 to B5).

The structures of two other brownish or clear compounds could not be elucidated by NMR due to the low quantities isolated and impurities despite appearing relatively pure on the TLC chromatograms (Figure 4).

4.5.3. Glucose utilisation activity of C_2C_{12} myotubes and 3T3-L1 adipocytes

The results of the methanol crude extract, fractions and compounds on glucose utilisation of C_2C_{12} myotubes and 3T3-L1 adipocytes are presented in Table 1. In general, exposure of C_2C_{12} myotubes and 3T3-L1 adipocytes to methanol crude extract, fractions or compounds resulted in a dose related lowering of glucose concentrations in the surrounding tissue fluid. The results showed that all the fractions and crude methanol extract had some potential in lowering glucose levels ($p<0.001$), both in C_2C_{12} myotubes (F(I); 61.23 ± 0.41%, F(II); 71.92 ± 0.34%, F(III); 70.78±0.74%, F(IV); 68.81 ± 0.72% and F(V); 61.84 ± 0.72%; crude; 75.14 ± 0.67%) and 3T3-L1 adipocytes (F(I); 54.25 ± 1.90%, F(II); 65.86 ± 1.59%, F(III); 66.86 ± 0.19%, F(IV) 68.39 ± 0.93%; and F(V); 65.05 ± 0.73%) at a concentration of 0.125 mg/ml.

The *in vitro* glucose utilisation of C_2C_{12} myotubes was enhanced by β-sitosterol indicating that this was the active compound (69.32 ± 0.77%) in lowering glucose concentrations ($p<0.001$) at a concentration of 0.125 mg/ml with compound A and B, being marginally less active (68.38 ± 1.58%; 54.41 ± 1.22%, respectively) at a concentration of 0.125 mg/ml. Similar results were obtained for *in vitro* glucose utilisation activity of 3T3-L1 adipocytes enhanced by the β-sitosterol compound (68.60 ± 0.48%), compound A and B (61.56 ± 0.98%; 50.54 ± 0.63%, respectively) at a concentration of 0.125 mg/ml. The activity of β-sitosterol was similar to the crude methanol extract (71.64 ± 0.23%), also at 0.125 mg/ml. The activities seen were also similar to that for insulin although at a different concentration, which enhanced glucose utilisation of 3T3-L1 adipocytes and C_2C_{12} myotubes by 68.45 ± 0.35% and 60.90 ± 1.02% respectively, albeit at a lower concentration of 1μM.

4.5.4 Insulin release effect of Rat insulinoma RINm5F cells

The insulin sensitising effect of the methanol crude extract, fractions and compounds on RINm5F rat insulinoma cells are presented in Table 2. Exposure of RINm5F rat insulinoma cells to the methanol crude extract, fractions or compounds resulted in a dose related increase in insulin release. The methanol crude extract (3.31 ± 0.01 to 8.18 ± 0.12 µg/L) caused a significant ($p<0.001$) increase in the insulin release at a concentration of 0.03125 mg/ml to 0.5 mg/ml. The fractions of the crude methanol extract also enhanced insulin release. Fraction III (5.28 ± 0.04 to 8.04 ± 0.03 µg/L) had a higher release compared to the other fractions. β-sitosterol (3.38 ± 0.04 to 8.06 ± 0.01 µg/L), compound A (3.14 ± 0.05 to 5.55 ± 0.03 µg/L), compound B (2.97 ± 0.02 to 5.58 ± 0.04 µg/L) and glibenclamide (5.74 ± 0.11 to 8.26 ± 0.03 µg/L) (positive control) also led to a dose related increase in insulin release. Effects of β-sitosterol and glibenclamide different highly significantly ($p<0.001$) from the negative control.

Table 1: Effect of methanol crude extract, fractions and compounds from *H. hemerocallidea* on glucose uptake of C_2C_{12} myocytes and 3T3-L1 preadipocytes.

Concentration	SAMPLES									
	Glucose uptake % for C_2C_{12} myocytes Mean ± Standard error (n = 9)									
	Crude extract	**Compound A**	**Compound B**	**Compound C: β- sitosterol**	**F (I)**	**F(II)**	**F(III)**	**F(IV)**	**F(V)**	**Insulin**
0.5 mg/ml	81.23±0.14	70.75±0.22	57.54±1.53	72.14±0.31	67.24±0.42	73.74±0.86	74.88±0.47	73.88±0.43	67.80±0.69	70.42±1.54
0.25 mg/ml	79.52±0.45	70.06±0.78	55.86±1.55	70.51±0.20	62.45±0.72	74.09±0.55	72.72±0.19	72.17±0.23	64.82±0.68	65.24±0.71
0.125 mg/ml	75.14±0.67	68.38±1.58	54.41±1.22	69.32±0.77	61.23±0.41	71.92±0.34	70.78±0.74	68.81±0.72	61.84±0.72	60.90±1.02
0.063 mg/ml	68.79±0.46	66.09±1.14	53.13±2.37	67.08±0.90	60.70±0.35	69.22±0.24	67.34±0.35	66.78±0.96	57.95±1.82	59.78±1.05
0.03 mg/ml	58.87±0.77	63.00±2.41	50.40±0.60	64.94±0.75	58.02±0.03	64.96±0.82	64.12±0.65	63.73±1.34	55.88±2.01	58.91±0.42
	Glucose uptake % for 3T3-L1 preadipocytes Mean ± Standard error (n = 9)									
0.5 mg/ml	77.56±1.63	65.73±1.64	52.17±0.67	74.17±0.41	57.36±1.41	72.93±1.60	72.30±0.36	73.18±0.33	74.54±0.85	76.43±0.14
0.25 mg/ml	74.30±1.83	64.61±1.95	52.33±0.54	69.26±0.46	57.83±1.06	69.32±0.41	70.28±0.62	69.82±0.35	69.90±0.24	73.44±0.24
0.125 mg/ml	71.64±0.23	61.56±0.98	50.54±0.63	68.60±0.48	54.25±1.90	65.86±1.59	66.86±0.19	68.39±0.93	65.05±0.73	68.45±0.35
0.063 mg/ml	67.21±1.03	59.78±1.13	47.79±0.97	63.04±0.19	53.21±1.12	64.50±1.11	64.41±0.32	66.19±0.86	63.08±0.47	56.31±1.32
0.03 mg/ml	61.70±0.82	58.14±0.21	45.87±0.68	53.81±0.56	51.69±0.23	63.20±1.33	58.05±0.70	62.04±1.08	61.91±1.24	55.00±1.80

Key: - Different coloured numbers means that, different concentration was used of insulin from the highest to lowest (100, 10, 1, 0.1, 0.01 µM)

- Compounds coded A (unknown); B (β- sitosterol); C (unknown)
- Fractions coded F(I); F(II); F(III); F(IV); F(V)

Table 2: Insulin sensitizing effect of the methanol crude extract, fractions and compounds from *H. hemerocallidea* on rat insulinoma RiNm5F cells*.*

Concentration	SAMPLES **Insulin sensitizing effect (µg/L) for rat insulinoma RiNm5F cells** Mean ± Standard error (n = 6)									
	Crude extract	**Compound A**	**Compound B**	**Compound C: β- sitosterol**	**F (I)**	**F(II)**	**F(III)**	**F(IV)**	**F(V)**	**Glibenglamide**
0.5 mg/ml	8.18±0.12	5.55±0.03	5.58±0.04	8.06±0.01	7.84±0.02	7.86±0.06	8.04±0.03	7.85±0.05	8.18±0.04	8.26±0.03
0.25 mg/ml	7.30±0.38	5.31±0.23	3.22±0.04	8.05±0.02	5.49±0.04	5.45±0.03	8.03±0.04	5.43±0.01	8.02±0.01	8.17±0.07
0.125 mg/ml	6.09±0.46	5.53±0.01	3.08±0.04	5.66±0.01	5.43±0.05	5.38±0.06	5.63±0.05	5.40±0.05	5.75±0.02	7.65±0.09
0.063 mg/ml	5.30±0.30	3.56±0.38	2.99±0.03	5.59±0.01	5.28±0.07	2.97±0.01	5.46±0.03	5.28±0.03	5.73±0.04	7.42±0.34
0.03 mg/ml	3.31±0.01	3.14±0.05	2.97±0.02	3.38±0.04	3.22±0.38	2.96±0.01	5.28±0.04	2.89±0.04	3.34±0.02	5.74±0.11

Key: - Different coloured numbers means that, different concentration was used of glibenglamide from the highest to lowest (100, 10, 1, 0.1, 0.01 µM)

- Compounds coded A (unknown); B (β- sitosterol); C (unknown)
- Fractions coded F(I); F(II); F(III); F(IV); F(V)

4.5.5 Cytotoxicity assay

The crude extract, fractions and the isolated compounds from the corm of *Hypoxis hemerocallidea* were evaluated for their cytotoxicity against C_2C_{12} myocytes, 3T3-L1 preadipocytes and rat insulinoma RiNm5F cells in vitro using the MTT (3-(4,5 dimethylythiazol-2-yl)-2,5-diphenyl-2H-tetrazolium hydrobromide) assay and the results are obtainable in Table 3 and Figure 6, 7 and 8. The results showed that all the cell lines exposed to the crude extract, compounds and fractions had a mild dose related decrease in cell viability from 78.46% to 97.94%, 78.65% to 96.86%, 79.02% to 97.41% for C_2C_{12} myocytes, rat insulinoma RiNm5F cells and 3T3-L1 preadipocytes, respectively as the concentration increased from 0.031 to 0,5 mg/ml mg/ml (Figure 6, 7 and 8). Our samples were less toxic against C_2C_{12} myocytes, 3T3-L1 preadipocytes and rat insulinoma RiNm5F cells, respectively) (*p,* 0.05 vs. control: doxorubicin at 0.031–0.5 mg/mL).

Table 3: Cytotoxicity of the crude extract, fractions and compounds from *Hypoxis hemerocallidea* (LC_{50}±SE, mg/ml).

Cell Line	Crude extract	F (I)	F(II)	F(III)	F(IV)	F(V)	Compound C	Compound A	Compound B β- sitosterol	Doxorubicin
C2C12 myocytes	1.38±0.21	4.13±0.15	4.32±0.02	6.43±0.19	5.41±0.32	5.21±0.09	>0.5±0.69	>0.5±0.80	>0.5±0.59	3.22±1.21
3T3-L1 preadipocytes	6.57±0.86	5.24±0.11	7.31±0.40	5.92±1.17	5.57±0.46	7.12±0.92	>0.5±1.05	>0.5±0.19	>0.5±0.18	2.61±0.46
Rat insulinoma RiNm5F cells	9.08±0.42	3.32±0.12	7.70±0.68	5.48±0.57	6.15±0.41	5.23±0.76	4.28±0.54	>0.5±0.39	>0.5±0.68	3.68±0.58

Key: - Compounds coded A (unknown); B (β- sitosterol); C (unknown)
- Fractions coded F(I); F(II); F(III); F(IV); F(V)
- Doxorubicin with LC_{50} in µM

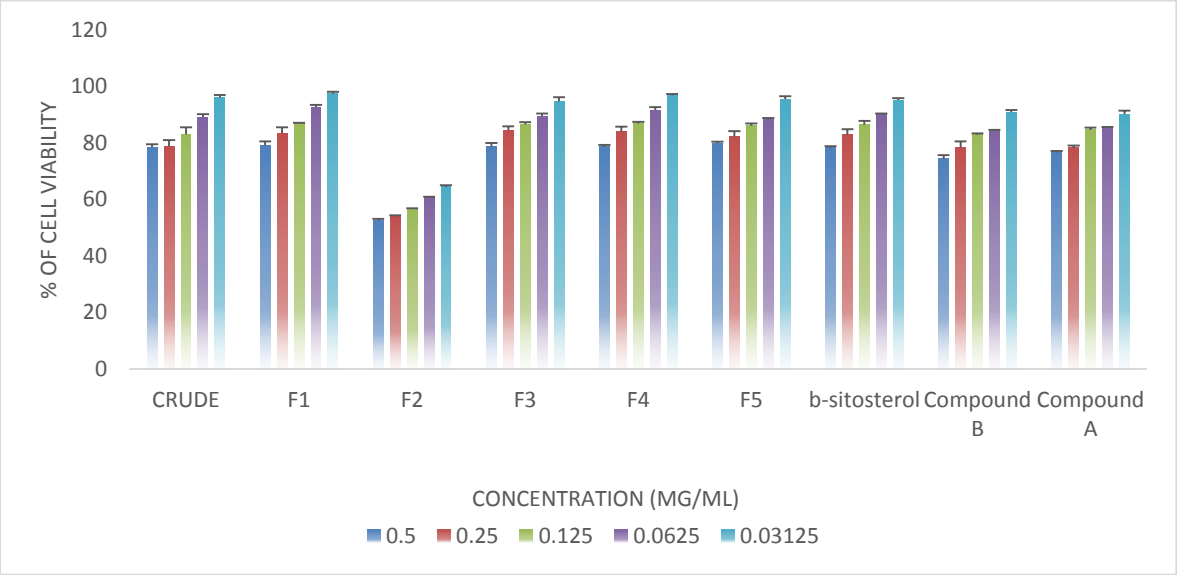

Figure 6: Dose-dependent cell viability of rat insulinoma RiNm5F cells exposed to crude extract, fractions and compounds from *Hypoxis hemerocallidea*. Each bar represents the mean± SE. Fractions coded Fractions coded F1; F2; F3; F4; F5.

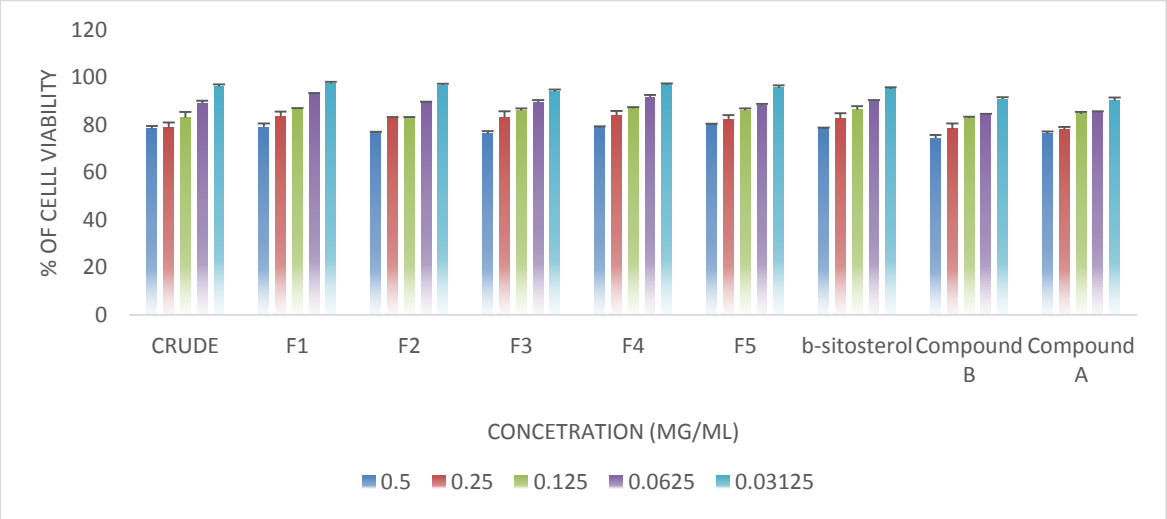

Figure 7: Dose-dependent cell viability of 3T3-L1 preadipocytes exposed to crude extract, fractions and compounds from *Hypoxis hemerocallidea.* Each bar represents the mean ± SE. Fractions coded Fractions coded F1; F2; F3; F4; F5.

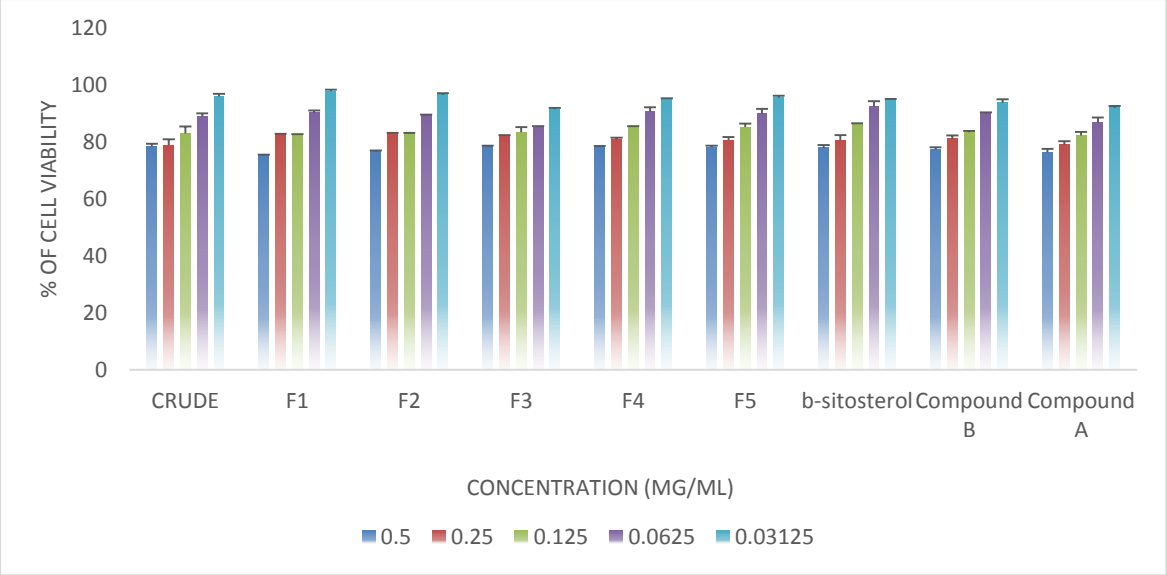

Figure 8: Dose-dependent cell viability of C_2C_{12} myocytes exposed to crude extract, fractions and compounds from *Hypoxis hemerocallidea.* Each bar represents the mean± SE. Fractions coded F1; F2; F3; F4; F5.

4.6 Discussion

For this study, chromatographic techniques were extensively used to characterise the *H. hemerocallidea* bioactive compounds, and to reveal β-sitosterol compound, an abundant phytosterol in plants (Zibula and Ojewole, 2000; Ojewole, 2006; Mahomed and Ojewole, 2003). This result was, however, not too surprising, as Mahomed and Ojewole, 2003, speculated that β-sitosterol could be the active compound responsible for the hypoglycaemic activity of plant extracts and abundant phytosterol concentrations.

It was confirmed, *in vivo,* that in normal and hyperglycaemic rats the oral supplementation of β-sitosterol increased fasting plasma insulin levels with corresponding decreased fasting glucose levels (Ivorra et al., 1988). This was attributed to increased insulin secretion (Ivorra et al., 1990). Similar to adipocytes, muscle cells are equally important in maintaining homeostasis of blood glucose levels (Marin et al., 1992). In an earlier report, Hwang et al., (2008), indicated that β-sitosterol induced glucose utilisation in a muscle cell line. It was reported that in 3T3-L1 cells, a mouse-derived adipocyte cell line, β-sitosterol inhibits growth and increases triglyceride accumulation (Awad et al., 2000). Our study also demonstrated that β-sitosterol increased the glucose utilisation activity of 3T3-L1 adipocytes and C_2C_{12} myotubes as well as improving insulin release from rat insulinoma RINm5F cells. This was also true to a lower extend for the two other unknown isolated compounds. The increasing glucose utilisation of 3T3-L1 adipocytes and C_2C_{12} myotubes as well as improving insulin release from rat insulinoma RINm5F by the crude methanol extract and some of the fractions (especially fraction III and V) were similar to that of β-sitosterol. However, their effects increased as the concentration increases for the

glucose utilisation of 3T3-L1 adipocytes and C_2C_{12} myotubes and the insulin release from rat insulinoma RINm5F.

Moreover, β-sitosterol has also been stated to provoke a multitude of bioactivities, which include, anticancer (Awad et al., 2007), antioxidant (Yoshida and Niki, 2003), angiogenic (Moon et al., 1999), anti-inflammatory (Backhouse et al., 2008), immunomodulatory activities (Bouic et al., 1996) and chemopreventive (Imanaka et al., 2008). Phytosterol mixtures that comprise of β-sitosterol were used as food additives in processed food for its nutraceutical benefits. For instance, studies revealed that β-sitosterol enriched margarine (Thompson and Grundy, 2005) and orange juice lower LDL cholesterol (Devaraj et al., 2004).

Information is lacking regarding safety and efficacy of β-sitosterol during pregnancy and its lactation. With the wide occurrence of β-sitosterol in food plants this is most likely not a problem, since no major adverse effects at recommended dose. Reduced carotenes and vitamin E absorption may occur (Wolters Kluwer Health, 2004). However, since plant sterols that consist of β-sitosterol reduce cholesterol absorption, it might also affect fat-soluble vitamins absorption. Hence, the effect of β-sitosterol on the blood levels of fat-soluble vitamins precursors is a debatable issue. Some studies have shown its consumption decreases levels of tocopherol (Hallikainen et al., 2000), carotenoids (Westrate and Meijer, 2008; Maki et al., 2001) and lycopene (Maki et al., 2001). Other studies reported that its consumption does not affect blood levels of carotenoids (Christiansen et al., 2001), tocopherol (Christiansen et al., 2001) and lycopene (Hendriks et al., 1999). However, for the positive controls used in this study namely insulin (which also induced glucose utilisation in 3T3-L1 adipocytes and C_2C_{12}

myotubes) and glibenclamide (which also improved insulin release from rat insulinoma RINm5F), epidemiological studies showed that the insulin use is associated with an increased risk of cardiovascular events and cancer (Currie et al., 2010). Moreover, large randomised controlled trials such as Action to Control Cardiovascular Risk in Diabetes (ACCORD) found no adverse safety signals associated with insulin use (Gerstein et al., 2008). On the other hand, glibenclamide can cause impaired renal function, low food intake, diarrhoea, alcohol intake and interaction with other drugs including serious, protracted and even fatal hypoglycaemic events (Asplund et al., 1983; Sonnenblick and Shilo, 1986). Not surprisingly the toxicity of *H. hemerocallidea* was not well documented, with one study indicating that Hypoxis extracts (45% hypoxoside) were not toxic (Laporta et al., 2007). In a later paper, the possible treatment of certain malignancies and HIV-infection with hypoxoside as a putative, nontoxic prodrug is also mentioned albeit without supportive information (Albrecht, 1995; Laporta et al., 2007). Nonetheless, the safety in persons with a degree of pancreatic pathology is not known.

We used the C_2C_{12} myocytes, 3T3-L1 preadipocytes and rat insulinoma RiNm5F cells cell lines as surrogates to investigate the potential for the plant extracts to be toxic, since these specific cell lines have been showed to be a reliable *in vitro* model for the study of drug used for anti-diabetic activity (Deutschländera et al., 2009). The reason we needed to establish the toxic potential of the plant was to ascertain how far the toxic and therapeutic effects were, as a general indicator of safety. From previous studies we have demonstrated that plant and its extracts exhibited an effect of 77.56% to 81.23% at on these cell lines when 0.5 mg/ml concentration was used, which indicates the potential value in the therapeutic management of diabetes. However, if

the toxic effect against the same cells is close to the effective concentration, then we would have to rule the plant extracts as a poor choice for diabetic treatment.

Based on our results all the cell lines exposed to the crude extract, compounds and fractions had a mild dose related decrease in cell viability. Further to this we were able to demonstrate that the β- sitosterol, the active suspected to be involved as be active ingredient especially for type II diabetes was also safe. This supports a long history of consumption, and it is generally recognized as safe without undesirable side effects (US Food and Drug Administration, 1997). Our results validate the reports by other researchers like Drewes et al., (1984) and Smit et al., (1995) who have also reported low toxicity values of *Hypoxis* since all their LC_{50} values were greater than 1 mg/ml.

4.7 Conclusion

Based on this study's results, the conclusion is that β-sitosterol is one of the compounds responsible for *H. hemerocallidea*, promising in vitro anti-diabetic activity. It is furthermore recommended that more studies must be undertaken to verify if indeed *H. hemerocallidea* and its associated compounds can target multiple metabolic pathways. More work is needed to characterize the two unknown compounds and also examine the possible synergistic effects of the isolated active compounds

4.8 References

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Chapter 5:

Antidiabetic activity of a *Hypoxis hemerocallidea* (Hypoxidaceae) corm extract, and isolated, β-sitosterol in spontaneous diabetic mice

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5. Abstract

Previous studies in our laboratory using, *ex vivo* cellular assays has demonstrated that acetone extracts of *H. hemerocallidea* functioned through increased insulin release from pancreatic beta cells. In this study we determined the efficacy of an *H. hemerocallidea* extract and the isolated active compound, β-sitosterol on spontaneously diabetic mice. Knockout strain C57 background strain mice were used as they carry a spectrum of genetic susceptibilities for obesity and impaired glucose tolerance. Abnormalities were evaluated in all mice, according to the procedures outlined by the OECD. The food was prepared for each treatment separately by mixing plant extract (250 mg) or positive control chlorpropamide (30 mg) to Epol pellets. Haematology, clinical chemistry, glucose tolerance, feed intake, faecal output and body weights were measured. Necropsy morphological and histological evaluations were recorded. There were no significant changes over time (4 weeks period) following β-sitosterol treatment. The animals on the plant extract and negative control (Epol diet) had a continuous increase in blood glucose level. The β-sitosterol and positive control also resulted in a slight decrease in the mouse weights over the study period, albeit with no significant changes in food intake and faecal output. Neither the *H. hemerocallidea* corm extract nor the standard mouse chow (Epol diet) reversed the weight gain of mice. There were no changes in the haematological parameters attributable to the administration of the tested treatments. There was slight treatment-related changes in triglycerides and total cholesterol relative to the control. There was a line of demarcation between the morphological changes in mice and the diet consumed on the amount of internal abdominal fat present. There were pathological signs in some of the mice particularly in myocardial fibres, renal tubular, glomerular, hepatocyte granularity and pancreas islets. Based on the results, none of the treatments could be considered highly effective for the management of type II diabetes as sole therapeutic intervention.

Key: *H. hemerocallidea*, spontaneously diabetic mice and Glucose tolerance test.

5.1 Introduction

Diabetes mellitus is one of the most important non-communicable diseases in the world. Not surprisingly numerous treatment options are available from allopathic remedies to complementary medicines based on more traditional methods of treatment. Of the available herbal remedies, *Hypoxis hemerocallidea* is a commonly used remedy locally. *H. hemerocallidea* corm has been reported to contain phytosterol glycosides, mainly β-sitosterol, rooperol (the aglycone of a hypoxoside, which is a 4, 4-diglucoside) and some sterolins (Drewes et al., 1984; Van Wyk et al., 2002). At present, the exact chemical compound in *H. hemerocallidea* corm that is responsible for the observed antidiabetic effects of the 'African Potato' aqueous extract is still not known. However, a number of investigators have shown that a host of secondary plant metabolites with diverse chemical structures possess the latter properties in various experimental animal models (Sugishta et al., 1982; Price et al., 1987; Singh et al., 1992; Akah and Okafor, 1992; Dongmo et al., 2003; Taesotiku et al., 2003; Adzu et al., 2003; Li et al., 2003). In all the current models, the plant has been investigated using streptozocin (STZ) induced diabetes in rodents. The hallmark of these models is the induction of diabetes by inducing damage to the pancreatic β-cells. While these types of models are helpful to determine if a plant or plant extract can decrease plasma glucose concentrations using a non-pancreatic method, it discriminates against those agents that enhance pancreatic activity. From previous studies in our laboratory, *ex vivo* models have shown that acetone extracts of *H. hemerocallidea* functions by increasing insulin released from pancreatic β-cells. As a result, we believe that the true effect of *H*. *hemerocallidea ex vivo* is yet to be established. For this study we used

spontaneously diabetic mice to evaluate determine the degree of efficacy of *H. hemerocallidea*. Knockout strain C57 background strain mice carry a spectrum of genetic susceptibilities for obesity and impaired glucose tolerance (IGT), both risk factors for the development of type 2 diabetes. A further benefit of using spontaneously diabetic animals, are that the animals also show the long-term pathology associated with the disease. With the plant containing β-sitosterol (a known natural anti-cholesterol compound), we also speculate these plant sterols would also be able to alleviate the defects seen in plasma cholesterol associated with diabetes mellitus.

5.2 Materials and Method

5.2.1 Animal material, Caging and Care

The spontaneous diabetic male mice (knockout strain C57 background; DBM heterozygous; 12 weeks; 29.0–42.0 g; N=45), were housed individually in type II individually ventilated cages (IVC) from Tecniplast in rooms maintained at 22±2°C, 40–60% relative humidity with a 12 h light-dark cycle. The mice were fed irradiated standard rodent chow pellets (Epol, South Africa), reverse osmosis water supplemented *ad libitum* and housed on cloth bedding material (Agrebe^{TM}). Cages were weekly changed. The animals were obtained from the central animal facility of Taconic Biosciences Inc, USA and Denmark (Vet import permit from Denmark, Appendix D). Autoclaved toilet rolls, egg containers, tissues and wooden sticks were provided as environmental enrichment. The cloth bedding was to allow for faecal collection without having to place the animals into metabolic cages. Prior, approval was obtained from the Institutional Animal Ethical Committee and the experiments were conducted as per the standard guidelines (CPCSEA, OECD guidelines no 420).

To allow acclimatisation, animals were maintained on the standard rodent chow for one week before being divided into four groups (n=11) for food treatment; Group A: Epol diet; Group B: Epol diet and β-sitosterol (100% pure compound obtained commercially from Sigma); Group C: Epol diet and plant methanol extract and Group D: Epol diet and positive control chlorpropamide. The feed of the test animals was supplemented with test material. The food was prepared for each treatment separately as plant extract (250 mg) or compound/ chlorpropamide (30 mg) to 1 kilogram of Epol pellets according to CPCSEA, OECD guidelines no 420 (Fixed Dose Procedure). Animals were maintained on their medicated feed for 4 weeks.

5.2.2 Glucose tolerance test

After a week on treatment, baseline blood glucose levels were measured using a hand-held glucose meter (OneTouch Ultra). Emla cream (lidocaine 2.5% and prilocaine 2.5%) was applied 2 hours before tail prick. For the glucose tolerance test (GTT), a sterile solution (dextrose 50%) at 2g of glucose/kg of body weight was injected intraperitoneally (ip). Glucose levels were subsequently evaluated at 30, 60, 90, and 120 mins. The entire procedure was repeated after a week and then again after another 2-week periods.

5.2.3 Feeds intake, faecal output and animal weights

Feed intake on a Monday and Friday every week was recorded. The amount of wasted feed at the bottom of the cage was also measured to get a proper indication of the feed intake. The faecal output and animal weights were also measured also on Monday and Friday of every week.

5.2.4 Clinical pathology

The animals were terminally anesthetized by isoflurane insufflation in a saturated bell jar and subjected to cardiac puncture. Blood samples were submitted on an automated analyser (IDEXX ProCyte Dx). The haematological parameters measured were: red blood cell count (RBC), white blood cell count (WBC), haemoglobin (Hb), haematocrit, mean corpuscular volume (MCV), neutrophils, eosinophils, basophiles, lymphocytes, monocytes and platelets count (PLT). Serum chemistry parameters included alkaline phosphatase activity (ALP), alanine aminotransferase (ALT), urea, plasma glucose, creatinine, total cholesterol, globulin, albumin and triglycerides.

5.2.5 Terminal pathology

During necropsy, gross morphological findings were recorded and specimens were collected from each individual in 10% buffered formalin. The following organ specimens were collected for histological examination: heart, liver, kidney, adrenal gland, pancreas and peripheral nerve. After fixation the organs were cut according to standard operating procedure using automated tissue processor an automated haematoxylin and eosin tissue staining. In addition to standard histopathological evaluation, the pancreatic islet size and diameter were quantified between the groups using the NIS-elements AR Imaging (Nikon, Japan) software program. All cuts were captured at 100x and 400x magnification, using 10x and 40x objectives. Normal islets of Langerhans were conceded to be around 50–500 µm in diameter, while less than 50 µm in diameter were considered to be small and more than 500 µm in diameter were considered to be enlarged (Mense and Rosol, 2018).

5.3 Data analysis

Data are expressed as mean ± standard deviation, and were analysed using Microsoft Office Excel 2007. Paired t-test was used to determine the significance of the difference among samples before and after (per time point) treatment per group with a significance level of 0.05. Chi-squared test for histological findings was used to determine whether there was a significant ($p<0.05$) difference between the expected frequencies and the observed frequencies in one or more categories.

5.4 Results

5.4.1 Monitoring of experimental animals

One death was observed (after one week on treatment) for the compound-treated group B. The appearances and behaviour of each mouse during the anti-diabetic study were observed with no abnormalities noted (Table 1). However, dark-coloured stools were observed in plant-extract group (group C). In addition, there was a lower significant differences in the body mass of the compound or positive control-treated groups (B and D groups) compared to the Epol diet (control)-treated or plant extract-treated groups (A and C groups) (Table1). However, these animals recovered within 14 days and gained weight.

Table 1. General appearance and behaviour of the mice during the anti-diabetic study.

Observations	GROUP A		GROUP B		GROUP C		GROUP D	
	On day 3	**On day 14 until day 44**	**On day 3**	**On day 14 until day 44**	**On day 3**	**On day 14 until day 44**	**On day 3**	**On 14 day until day 44**
Skin and fur	NT***		NT***	Normal	NT***		NT***	Normal
Eyes	Normal		NT***	Normal	Normal		NT***	Normal
Behavioural pattern	Normal	All Normal	Weak and shivering	Normal and active	Normal	All Normal	Weak and shivering	Normal and active
Stool-colour	Normal		Mixed with wasted food	Normal	Darker stools		Mixed with wasted food	Normal
Sleep	Normal		NT***	Normal	Normal		NT***	Normal
Diarrhoea	Not observed		Not observed	Not observed	Not observed		Not observed	Not observed

Group A: Standard pellet diet and water; Group B: Standard pellet diet, water and compound; Group C: Standard pellet diet, water and plant extract; Group D: Standard pellet diet, water and positive control. N.B: the animal food (25 g) was mixed with natural apple essence 30 ul (sugar free flavouring) to make the food more palatable. Since it was observed that groups B and D animals did not eat for the first 3 days post-added natural apple essence was supplemented. Key: NT*** not normal.

5.4.2 Blood glucose concentrations

The results of the GTT performed at week 4 are presented in Figures 1 and 2. All the animals were prediabetic at the start of treatment, with no significant changes evident over time (4 weeks period) for the compound and positive control, while the plant extract and control had continuously increasing blood glucose level. The blood glucose concentrations of all the animals did not return to baseline levels at the end of 120 minutes after 1, 2 and 4 weeks of treatment.

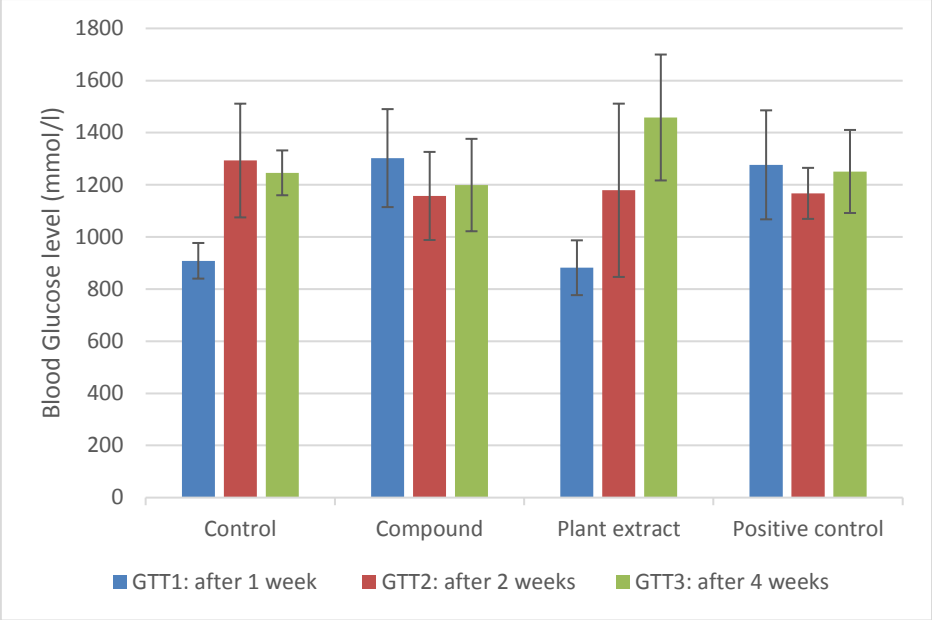


Figure 1: Basal glucose concentrations (mmol/l) (AUP) of different tested treatments on spontaneous diabetic mice after 1, 2 and 4 weeks of treatments.

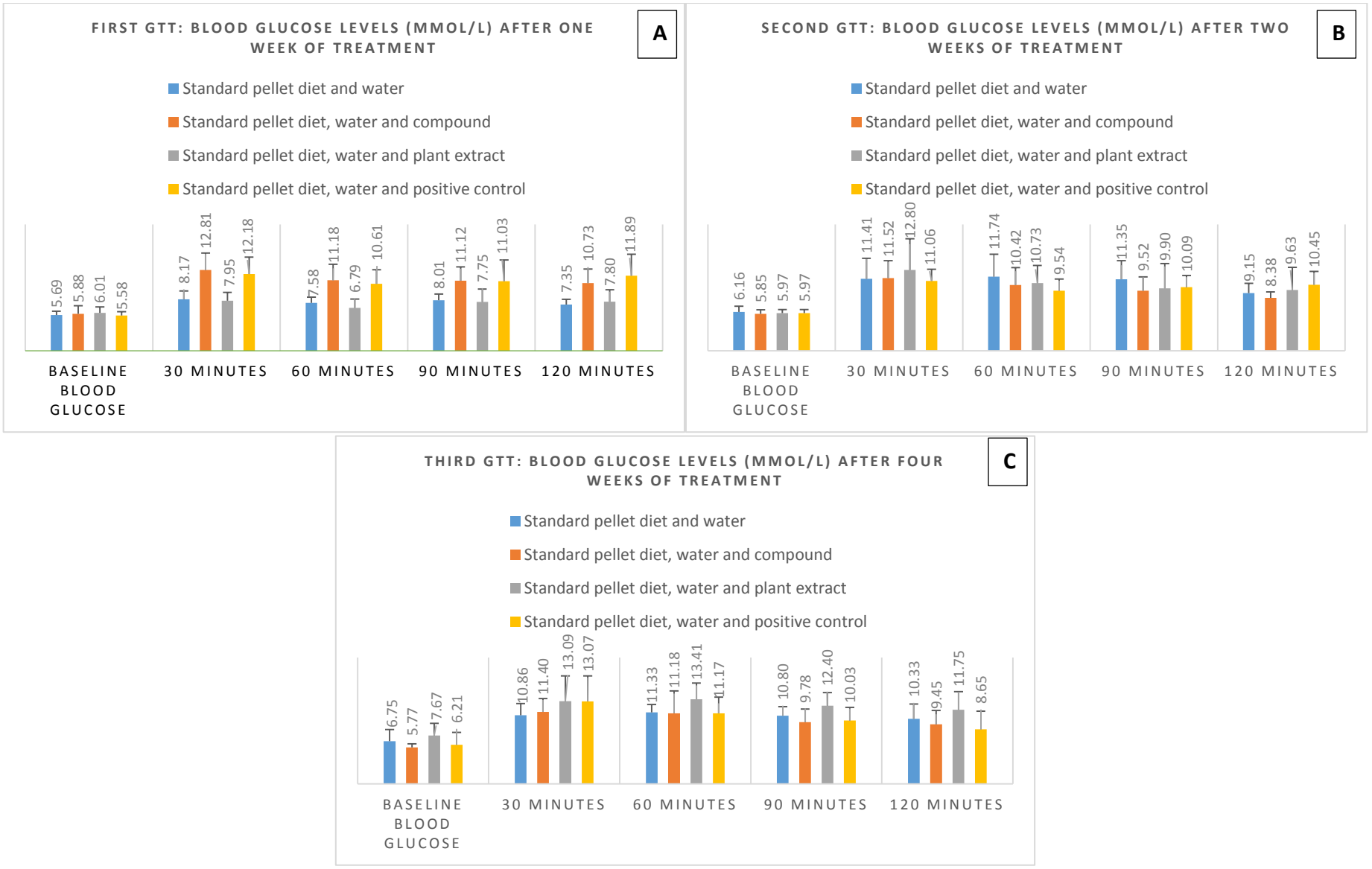
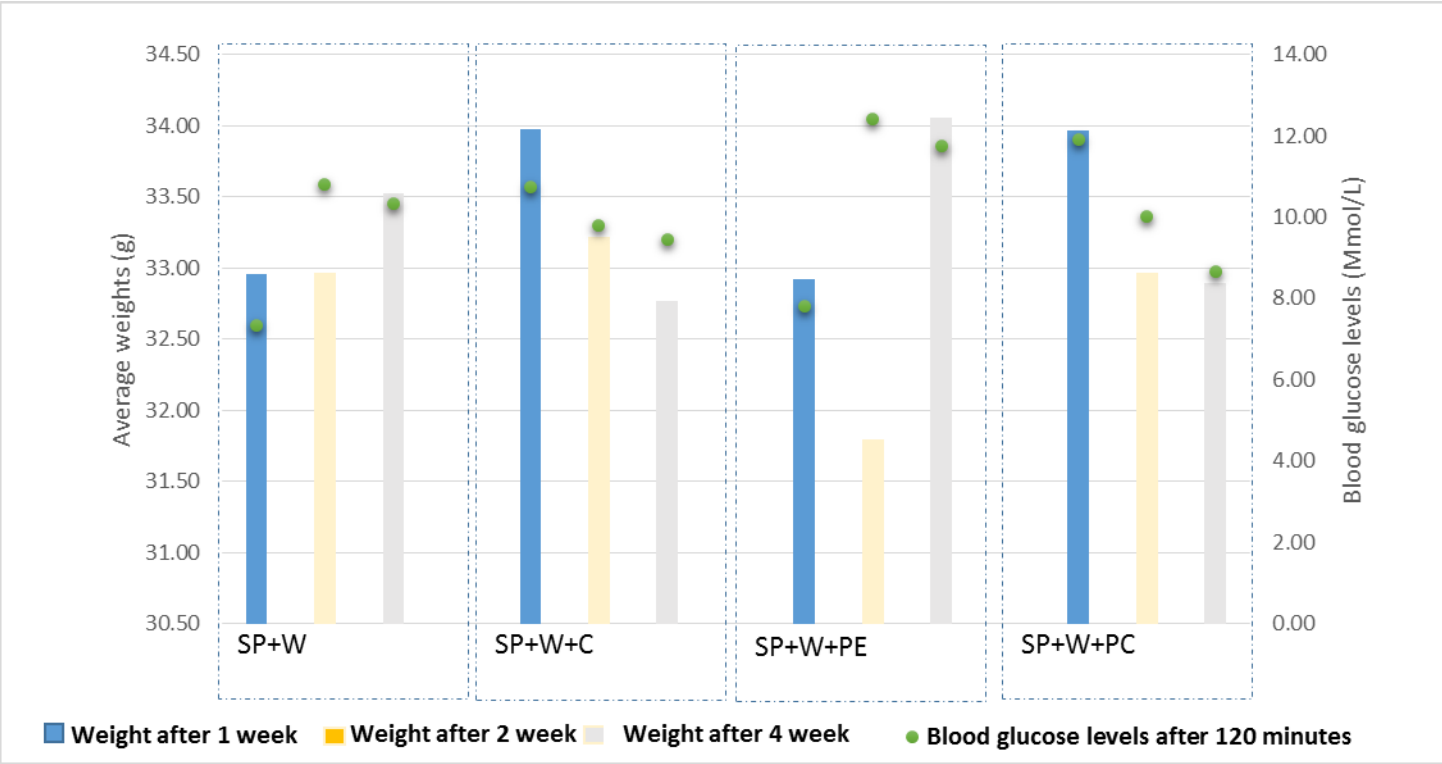


Figure 2: Blood glucose concentrations (mmol/l) of different tested treatments on spontaneous diabetic mice after 1 week (a), 2 weeks (b) and 4 weeks (c) of treatments.

5.4.3 Body and faecal weight and food Intake

Body weights of the mice after the first week treatments were 34.35 ± 2.12g for group A; 34.77 ± 2.28g for group B; 33.83 ± 4.68g for group C and 34.70 ± 1.95g for group D. After week 4 the body weights of the mice were 33,53 ± 1,83g for group A; 32.77 ± 2.61g for group B; 34.05± 3.44g for group C and 32.89± 2.02g for group D. A significant decrease difference was present ($P<0.05$) between the treatments (groups B or D) and the control (group A), which was evident as slight weight loss. Evident as a concurrent decrease in blood glucose concentrations was seen, between the blood glucose concentrations (mmol/l) and the weights (g) of spontaneous diabetic mice after 1, 2 and 4 weeks of treatments (groups B and D) (Figure 3).

Food intake of the mice a week after commencement of treatments were 22.16 ± 2.45g for group A, 15.58 ± 4.00g for group B, 20.82 ± 3.17g for group C, and 8.65 ± 4.05g for group D. After week 4 the food intakes of the groups were 15.26 ± 1.74g for group A; 17.91 ± 3.36g for group B; 17.05 ± 2.50g for group C and 19.13 ± 3.76g for group D. There was no significant difference between ($P>0.05$) the treatments (groups B, C or D) and group A after 4 weeks of treatments. There was a general trend for decrease in faecal output for all the groups (Figure 4).



SP+W: Standard pellet diet and water; SP+W+C: Standard pellet diet, water and compound; SP+W+PE: Standard pellet diet, water and plant extract ; SP+W+PC: Standard pellet diet, water and positive control

Figure 3: Relationship between the basal blood glucose concentrations (mmol/l) and the weights (g) of spontaneous diabetic mice after 1 week (a), 2 weeks (b) and 4 weeks (c) of treatments.

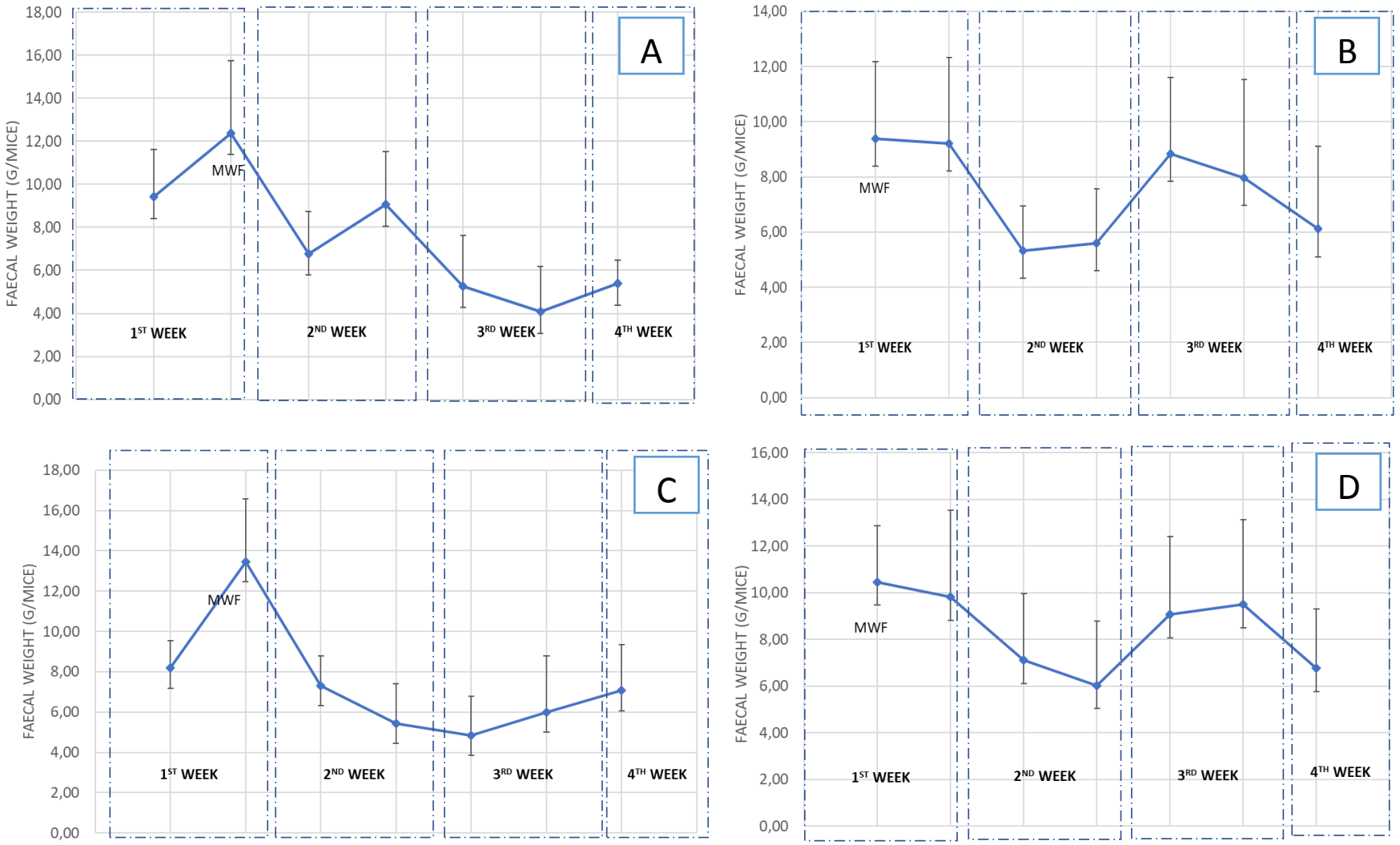


Figure 4: Effect of food (treatment and control) administration on the faecal weights of mice for 4 weeks. Data are presented as mean ± SD (n = 11). Group A: Standard pellet diet; Group B: Compound; Group C: Plant extract and Group D: Positive control. Key: MWF = Faeces mostly mixed with food.

5.4.4 Clinical pathology

The haematological parameters obtained for the study are presented in Table 2 and clinical chemistry in Table 3. There were no changes in the haematology evaluations attributable to administration of treatments. Clinical chemistry evaluations indicated slight treatment-related changes (Groups B and D) in triglycerides and total cholesterol, relative to the control (Group A). In general, triglycerides and total cholesterol were low for all groups. When using citrate buffer in blood tubes, overall glucose levels were normal and they were dropped back into the reference range for groups B, C and D, however, slightly higher for group A.

5.4.5 Pathological changes

Necropsy findings are presented in Table 4. There was a clear line of demarcation between the morphological changes in mice and diet consumed especially on various amounts of internal body fat. Group A, B and D showed moderate amounts of internal body fat but group C showed large amounts of internal fat. Histopathological descriptions of each mice are presented in Table 5. In general, there were pathological signs in some of the mice particularly in myocardial fibres, renal tubular, glomerular and hepatocyte granularity. However, there was no significance trend between the groups. However, the compound treated animals had less pancreatic tissue inflammation than the untreated group.

Groups A and C had some islets of Langerhans which were small and had fewer cells as shown in Figure 5A and C. Qualitative evaluation of individual cells within pancreatic islets showed a moderate decrease in size for animal group C (5.80 µm ±0.62) when

compared with group A (6.28 µm ±0.35), group B (6.14 µm ±0.64) and group D (6.04 µm±0.70) (Figure 9C).

Histopathologic changes of islets for groups A (Figure 6) and D (Figure 8), showed hyperplasia and atrophy. In some cases, groups B and C showed hyperplasia (Figure 7). A qualitative evaluation of animals islets of Langerhans in group A (38.30 µm; 838.62 µm and 170.25 µm) and group D (36.22 µm; 584.99 µm and 147.65 µm), displayed a combination of small, enlarged and also normal mean diameter of islets of Langerhans (Figure 9B). Group B (675.20 µm and 144.57 µm) and group C (540.00 µm and 138.03 µm) displayed only enlarged and normal mean diameter of islets of Langerhans (Figure 9B). Morphometric analysis revealed an increase in the total islet number of 84 per 6 µm tissue thickness sections cut in animals of group B, compared to groups A, C and D with 66, 50, 73 total islet number (Figure 9A).

Table 2: Haematological parameters of mice.

	Group A	Group B	Group C	Group D	Reference range (Ref**)
Full blood count Parameters					
Haemoglobin (g/L)	134,9±10.38	136,125±5,10	131,60±5,10	134,91±8,57	135-163
Red Cell Count (x10Λ12/L)	9,46±0,78	9,19±0,44	9,33±0,44	9,41±0,56	9.82-10.65
Haematocrit (L/L)	0,44±0,04	0,44±0,02	0,42±0,02	0,44±0,03	0,40-0,46
Mean corpuscular volume (fL)	46,67±0,50	46,2±1,04	45,16±1,04	46,29±0,51	43,7-45,6
Mean corpuscular haemoglobin (pg)	14,25±0,14	14,425±0,17	14,10±0,17	14,35±0,18	14,8-16.0
Mean corpuscular haemoglobin concentration (g/dL)	30,56±0,54	31,19±0,73	31,21±0,73	31,00±0,40	33,3-34,9
Red cell distribution width (%)	13,03±0,70	12,2±0,33	12,86±0,33	12,24±0,35	11.5-14.5
White cell count (x10Λ9/L)	8,207±1,78	8,25±1,30	6,50±1,30	7,89±1,07	7,80-15,61
Segmented neutrophil (x10Λ9/L)	1,706±0,71	1,99±0,67	1,39±0,67	1,64±0,65	1,50-4,02
Band neutrophil (x10Λ9/L)	0±0,00	0±0,00	0,00±0,00	0,05±0,08	0-1
Lymphocyte (x10Λ9/L)	5,36±1,77	5,57±0,90	4,26±0,90	5,48±1,37	6,63-12,46
Monocyte (x10Λ9/L)	0,51±0,27	0,475±0,16	0,32±0,16	0,32±0,13	0-0,45
Eosinophil (x10Λ9/L)	0,58±0,22	0,215±0,17	0,52±0,17	0,40±0,27	0-0,45
Basophil (x10Λ9/L)	0,06±0,10	0±0,04	0,01±0,04	0,01±0,02	0-0,09
Platelet count	1067,8±HSD	993,125±HSD	1195,90±HSD	957,55±HSD	862-1611
HSD = Very high standard deviation difference >100; Ref** Serfilippi et al., 2003.					

Table 3: Serum chemistry parameters of mice.

	Group A	Group B	Group C	Group D	Reference Range (Ref**)
Full blood count Parameters					
Total serum protein (g/l)	45,83±1,78	44,91±1,85	44,02±2,24	45,93±1,88	44-58
Albumin (g/l)	22,06±1,89	19,42±2,80	17,15±4,61	18,52±2,77	26-38
Globulin (g/l)	23,75±1,73	25,49±2,40	26,87±2,78	27,40±1,98	17-22
Alanine aminotransferase(U/l)	26,78±5,03	28,76±8,78	26,09±7,42	26,55±3,73	31-57
Alkaline phosphatase (U/l)	73,27±17,36	66,80±13,75	68,64±14,16	69,91±12,76	55-100
Urea nitrogen (mmol/l)	7±0,53	5,94±0,62	5,94±0,85	5,80±0,90	1,3-7
Creatinine (mmol/l)	<18±0,00	<20,00±1,41	<18±0,00	<18±0.00	0,016-0,02
Cholesterol (mmol/l)	2,19±0,16	3,10±1,98	2,19±0,20	2,55±0,19	4,05-5,49
Triglyceride (mmol/l)	1,31±0,21	1,11±0,44	1,16±0,21	1,08±0,29	2,05-5,4
Glucose (mmol/l)	8,63±0,21	7,91±2,81	7,35±1,18	7,20±0,62	4-7
Ref**Serfilippi et al., 2003.					

Table 4: Necropsy morphological findings.

POST MORTEM	Unique reference number for individual mouse											
Normal appearance of the lungs and heart	**11**	12	15	17	24	25	26	28	30	31	39	Highlighted white numbers: the mice with a slit like left-ventricular lumen of the heart
	2	9	13	20	22	23	**29**	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	**3**	8	**10**	14	16	**18**	32	35	41	45	
The thymus is small	11	12	15	**17**	24	25	26	28	30	31	39	Highlighted white numbers: minimal haemopericardium and haemothorax
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	Highlighted red number: mice prominent thymus
	1	3	8	10	14	16	18	32	35	41	45	
The spleen appears normal and active with prominent white pulp follicles	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
Moderate amounts of internal fat present	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	**5**	**6**	**19**	**21**	**27**	**36**	**38**	**40**	**42**	**43**	Highlighted red numbers: Mice with large amounts of internal fat present
	1	3	8	10	14	16	18	32	35	41	45	
The liver is pale tan in colour and of normal size and The spleen appears normal and active with prominent white pulp follicles	11	**12**	15	17	24	25	26	28	30	31	39	Highlighted white numbers: liver with poorly visible darker central pinpoint areas are disseminated on the parenchyma and highlighted in red: liver with accentuated lobulation
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	**8**	**10**	14	16	18	32	**35**	**41**	45	
The kidneys are normal in appearance and pale tan in colour	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
The adrenals appear small	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
The urinary bladder is empty	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
The stomach contains a moderate amount of normal appearing ingesta	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
Normal appearance of the oral cavity, small and large intestine (full of granular content) as well as genital tract and brain	11	12	15	17	24	25	26	28	30	31	39	
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	
Mild haemopericardium	11	12	15	**17**	24	25	26	28	30	31	39	Highlighted white numbers: severe haemothorax
	2	9	13	20	22	23	29	33	34	44	dead	
	4	5	6	19	21	27	36	38	40	42	43	
	1	3	8	10	14	16	18	32	35	41	45	

KEY:

Group A: Mice provided with Standard pellet diet and water

Group B: Mice provided with Standard pellet diet, water and compound

Group C: Mice provided with Standard pellet diet, water and plant extract

Group D: Mice provided with Standard pellet diet, water and positive control

Table 5: Histological findings.

HISTOPATHOLOGICAL DESCRIPTIONS	Unique reference number for individual mouse												
Heart: diffuse smudged cytoplasmic appearance, slightly coagulated, with mild increased eosinophilia and loss of cross striations in many fibres.	**11**	12	15	**17**	24	25	26	28	**30**	31	39	SD	Highlighted white numbers Multifocal fibres show mild hyalinisation with eosinophilia of the cytoplasm. In many fibres cross striations are not prominent or absent and single fibres show vacuolisation of the cytoplasm.
	2	9	13	20	22	23	29	33	34	44	dead	SD	
	4	5	6	19	21	27	36	38	40	42	43	SD	
	1	3	8	10	14	**16**	18	32	35	41	45	SD	
Liver: active granular appearance of the cytoplasm of hepatocytes.	11	12	15	17	24	**25**	26	28	30	31	39	NSD	Highlighted white numbers: With minimal vacuolisation in some cells
	2	9	13	20	22	23	29	33	34	44	dead	NSD	
	4	5	6	19	21	27	36	38	40	42	43	NSD	
	1	3	8	10	14	16	18	32	35	41	45	NSD	
Kidney: deep cortical tubules show mild increased eosinophilia of the cytoplasm and its slightly swollen.	**11**	**12**	15	17	**24**	**25**	**26**	28	**30**	**31**	**39**	SD	Highlighted white numbers: Some glomeruli show mildly increased eosinophilic appearance of the mesangium.
	2	9	**13**	20	**22**	**23**	29	**33**	**34**	**44**	dead	SD	
	4	5	6	19	21	27	36	38	40	42	43	SD	
	1	3	8	10	14	16	18	32	35	41	45	SD	
Adrenal gland: normal histological appearance.	11	12	15	17	24	25	26	28	30	31	39	NSD	Highlighted white numbers: the zona fasciculata appears slightly thin
	2	9	13	20	22	23	29	33	34	44	dead	NSD	
	4	5	6	19	21	27	36	38	40	42	43	NSD	
	1	3	8	10	14	16	18	32	35	41	45	NSD	
Pancreas: Mostly show normal appearance of exocrine pancreatic tissues.	11	12	15	17	24	25	26	28	30	31	39	SD	Highlighted white numbers: endocrine islets appear few Underlined numbers: inflammation
	2	9	13	20	22	23	29	33	34	44	dead	SD	
	4	5	6	**19**	**21**	**27**	36	38	**40**	42	43	SD	
	1	3	8	**10**	14	16	18	32	35	41	45	SD	
Peripheral nerve: normal histological appearance.	11	12	15	17	24	25	26	28	30	31	39	NSD	Highlighted white numbers: scattered small inconspicuous vacuoles are present in the neuropil
	2	9	13	20	22	**23**	29	33	34	**44**	dead	NSD	
	4	5	6	19	21	27	36	38	40	42	43	NSD	
	1	3	8	**10**	14	16	18	32	35	41	45	NSD	

KEY:

- Group A: Mice provided with Standard pellet diet, water
- Group B: Mice provided with Standard pellet diet, water and compound
- Group C: Mice provided with Standard pellet diet, water and plant extract
- Group D: Mice provided with Standard pellet diet, water and positive control

Chi square X^2; SD $p<0.05$ NSD $P > 0.05$

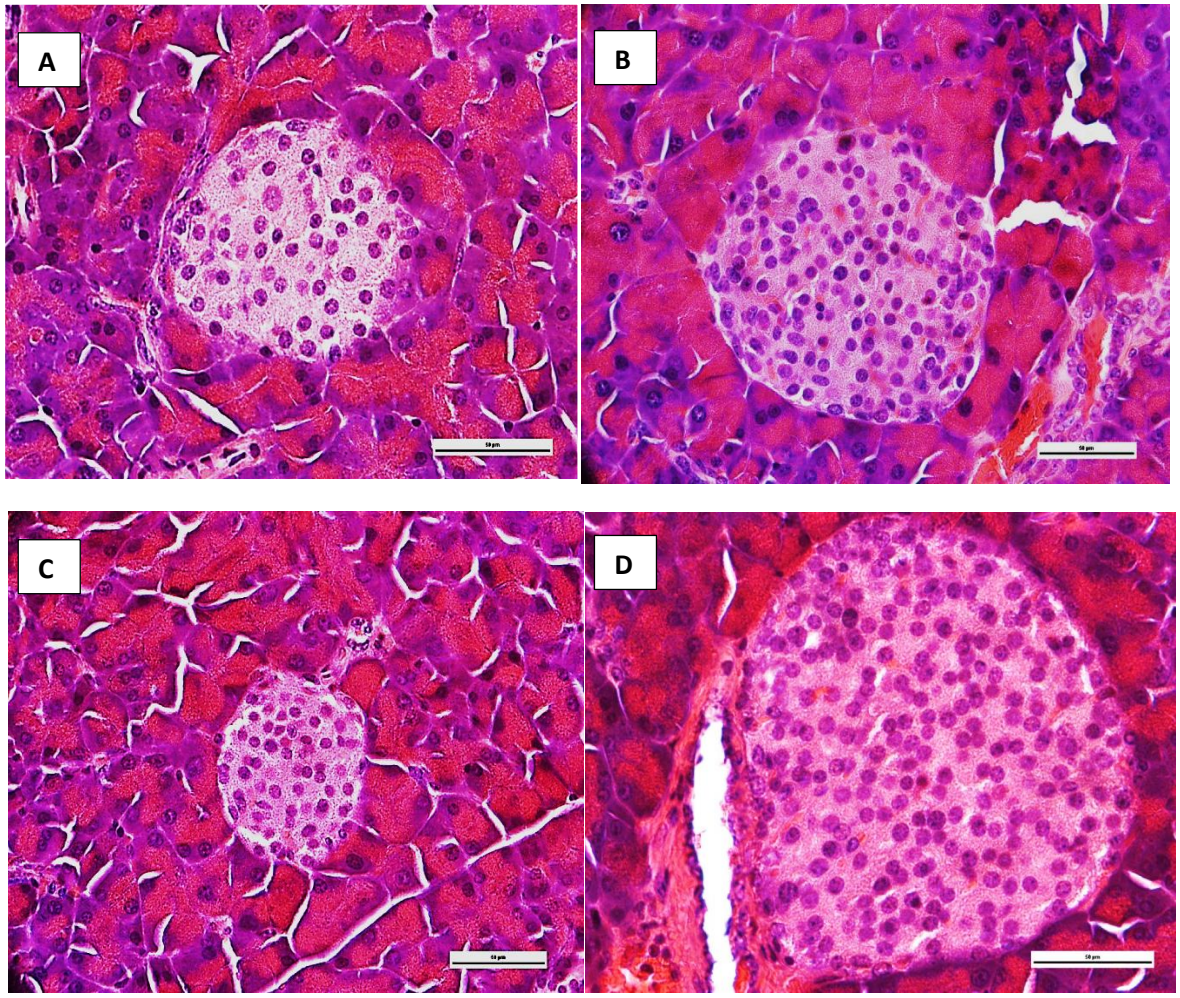


Figure 5: Example of the effect of food (treatment and control) administration in the islets of Langerhans in pancreas after treatment of mice for 4 weeks showing cells number. Group A: Standard pellet diet; Group B: Compound; Group C: Plant extract and Group D: Positive control. (Haematoxylin-eosin staining, 400× and 100x). Group A and C with fewer cells compared to Group B and D. Scale bars, 50 µm.

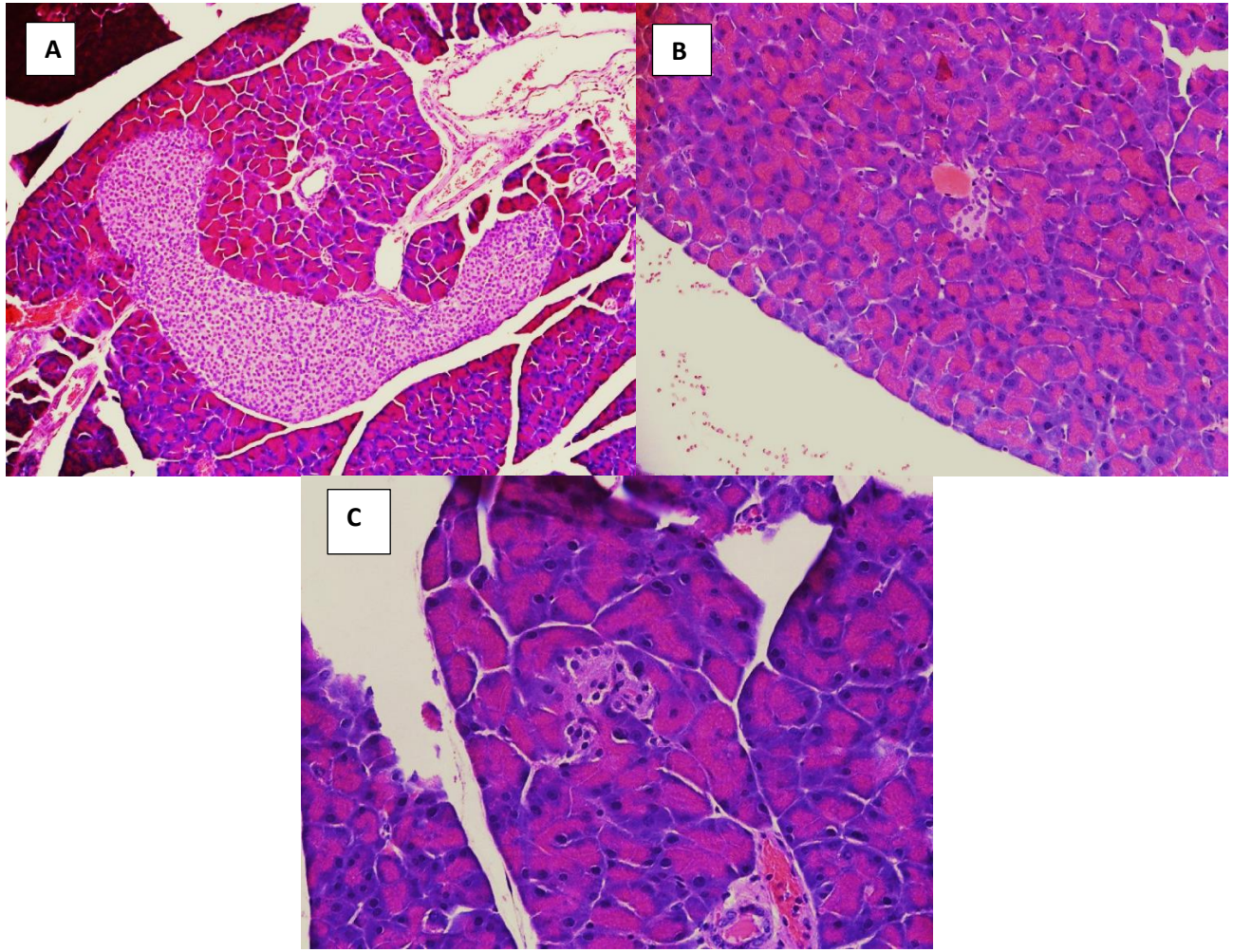


Figure 6: Example of the effect of food administration of **only standard pellet diet** in the islets of Langerhans in pancreas after treatment of mice for 4 weeks. (Slide A; diameter 1168.38 µm: Hyperplasia) and (Slides B and C; diameter <44.59 µm: Atrophy). (Haematoxylin-eosin staining, 400× and 100x). Scale bars, 50 µm.

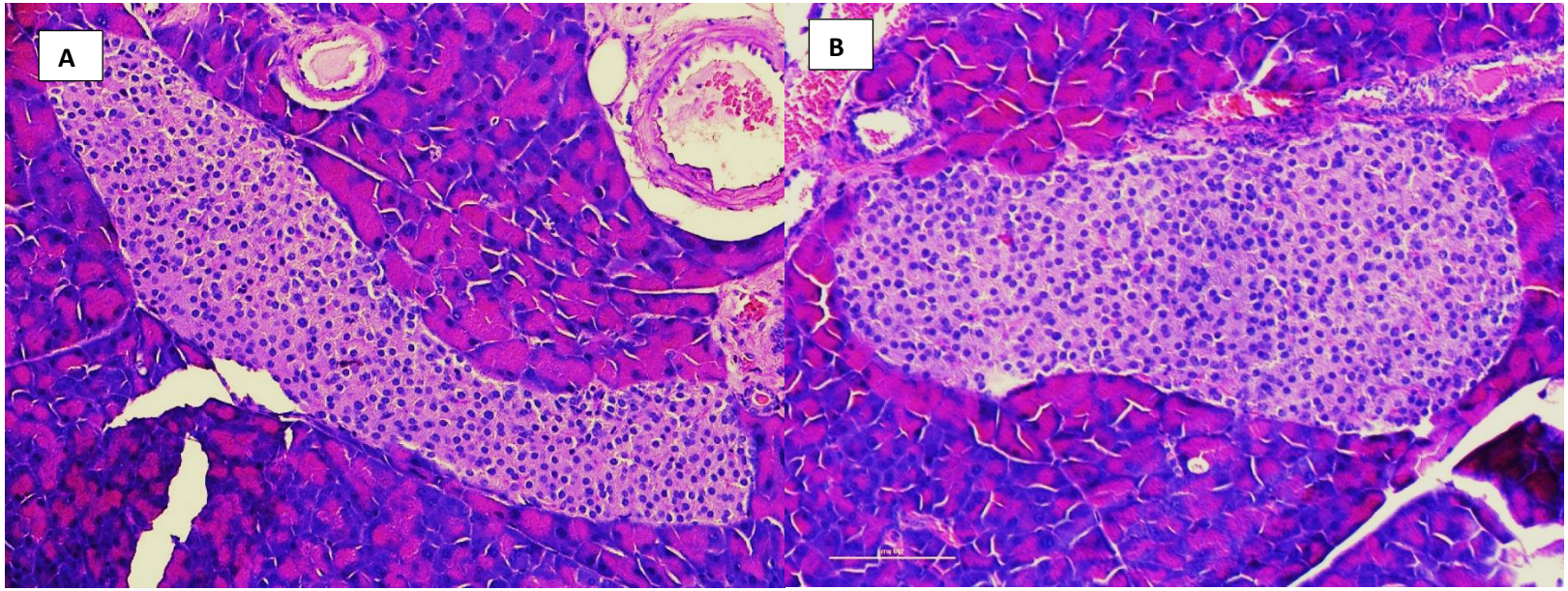


Figure 7: Example of the effect of food administration of **Compound** (Slide A; Group B) or **Plant extract** (Slide B; Group C) in the islets of Langerhans in pancreas after treatment of mice for 4 weeks. (Slide A; diameter 675.20 µm) and (Slide B; diameter 540.00 µm) Hyperplasia. (Haematoxylin-eosin staining, 400× and 100x). Scale bars, 50 µm.

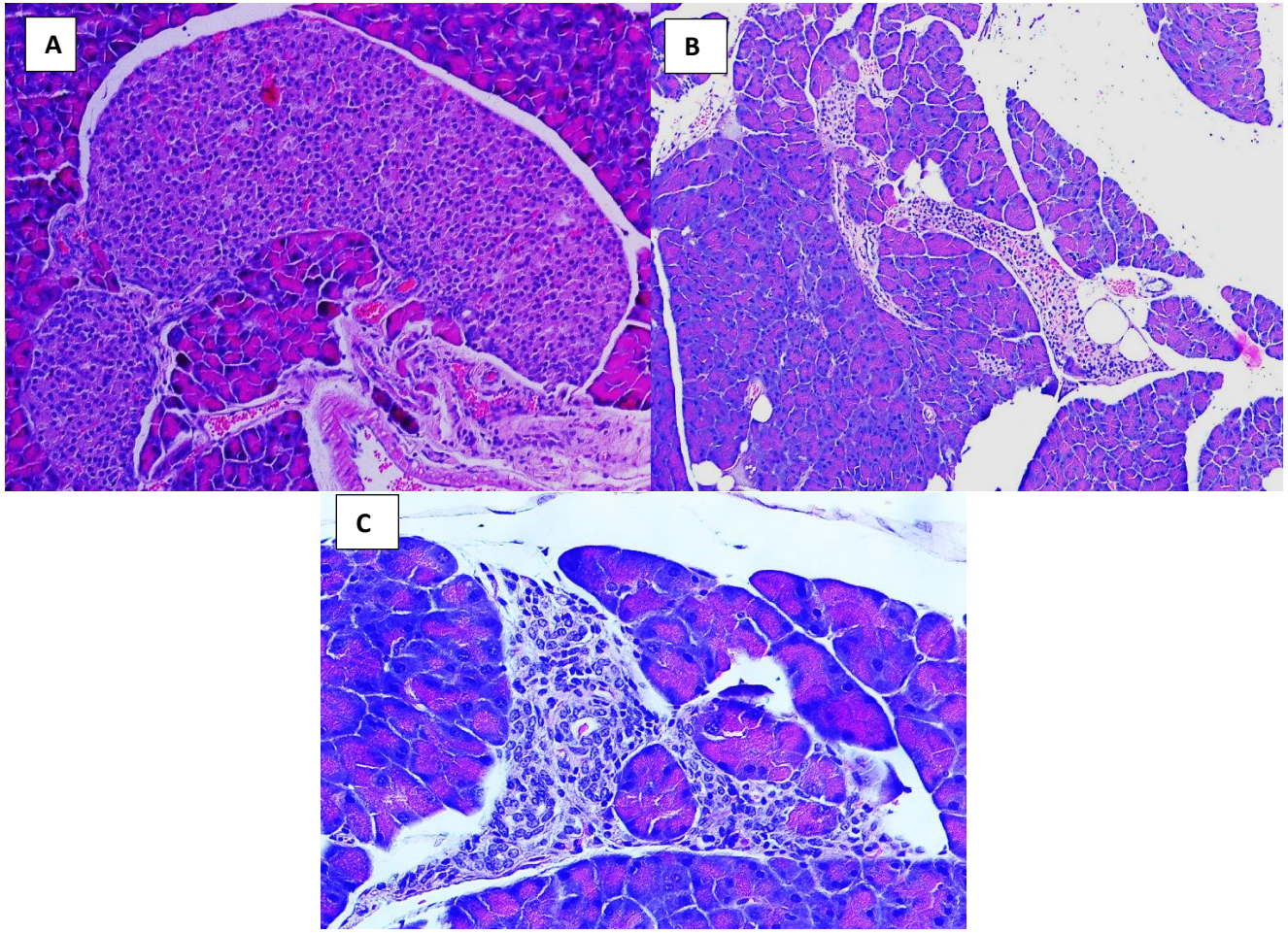


Figure 8: Example of the effect of food administration of **only positive control drugs** in the islets of Langerhans in pancreas after treatment of mice for 4 weeks. (Slide A; large islet diameter 709.03 µm, connected to small islet diameter 220.78 µm: Hyperplasia), (Slides B: Atrophy) (Slides C: with a few exocrine acini trapped near the periphery). (Haematoxylin-eosin staining, 400× and 100x). Scale bars, 50 µm.

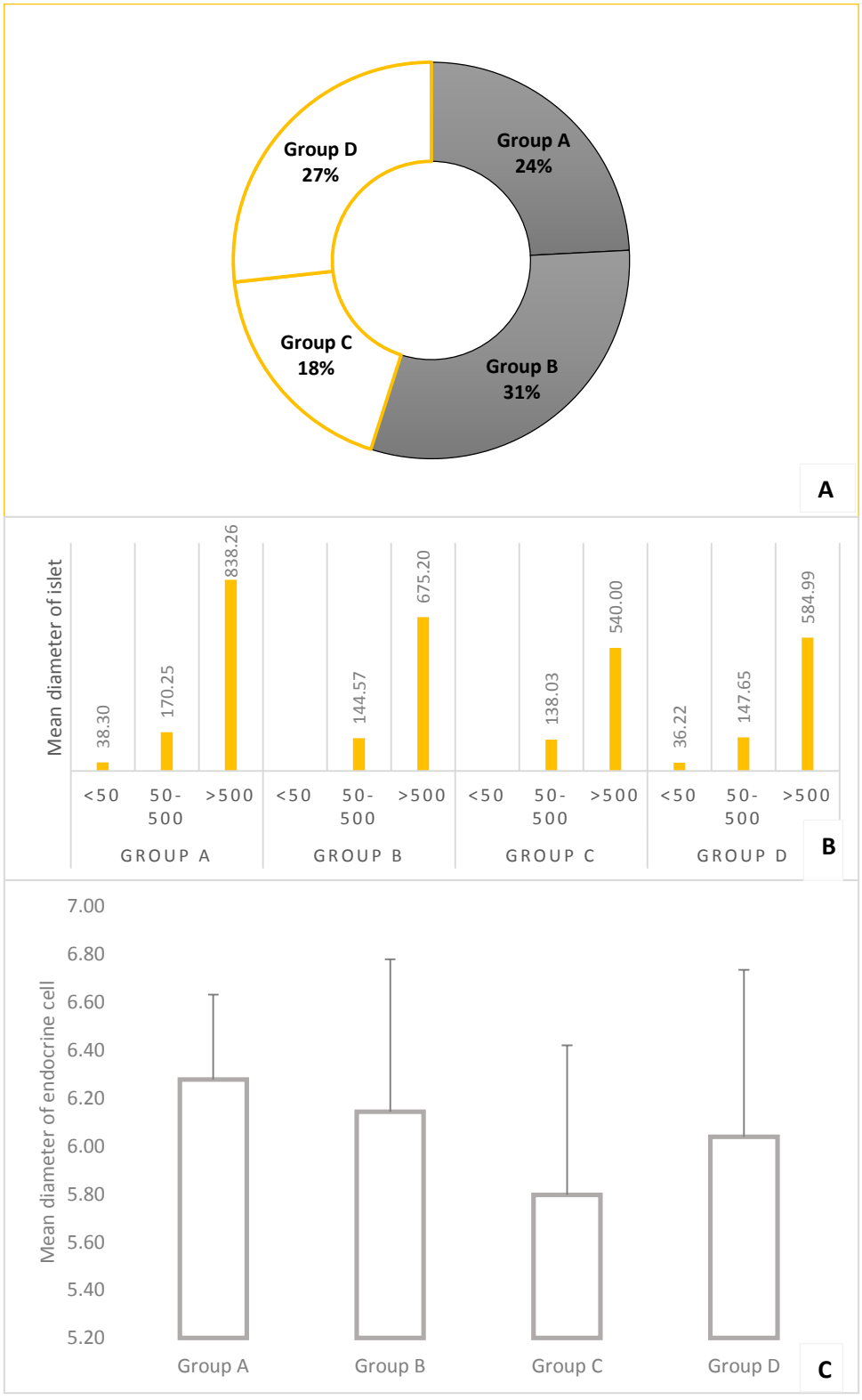


Figure 9: Endocrine cells in pancreatic islets and pancreatic islet of Langerhans: (A) Total islet percentage per 6 µm tissue thickness section cut (B) Mean diameter (µm) of islet of langerhans (C) Mean diameter (µm) of endocrine cells pancreatic islets.

5.5 Discussion

The use of animals in scientific research has contributed significantly to the development of science, promoting various advances in understanding the metabolic machinery and the discovery of treatments and preventive measures applied to human and veterinary medicine. Phenotypically, the diabetic mouse (C57BLKS db/db) is undistinguishable from the obese mouse (C57BL/6J ob/ob), however, the syndrome in C57BLKS db/db is far more severe than in the C57BL/6J ob/ob mouse. The clinical picture is reliant on the background strain in which the gene is expressed. If the ob gene is transferred to C57BLKS, Ks ob/ob this strain advances a more severe form of diabetes than that observed in the C57BL/6J strain, indicating that these two strains vary in their capacity to adapt to diabetogenic stimuli (Cohn and Cerami 1979). This designates that the clinical expression of the mutant gene is a result of the interaction of the gene (ob or db) and the genetic background (Coleman, 1982). Male mice used in the present study were DBM heterozygous (mice heterozygous for the diabetes allele (db) were crossed with mice homozygous for the misty (m) gene). With this, they become heterozygous for the db gene, which makes results in impaired glucose-stimulated insulin secretion and hyperglycemia, while not to give rise to diabetes. Nonetheless, they can show abnormalities from 3 months of age and above, which can also be primary restricted to males. This allows the model to be a good indicator of response to treatment in the earlier stage of type II diabetes.

For this study the plant extract and control led to an increased in blood glucose level despite treatment continuing for four weeks. The results from this study thus do not support previous studies that have indicated that *H. hemerocallidea* corm extract has clear antidiabetic properties (Mahomed and Ojewole, 2003). This may be as a result of the model used. For our study, the animals had true pathology and not induced

through chemical means, where the other study used STZ-induced diabetes in rats. With plant known to have antioxidant activity, the findings from previous studies may thus only show the ability to mitigate in diabetes developing in the presence of oxidative stress as opposed to treating disease once present.

Both the *H. hemerocallidea* corm extract and Epol diet also failed to reverse the weight gain of mice, also with no decrease in food intake and faecal output, throughout the experiment. In contrast, the other two groups showed some weight loss together with a reduction in the 120 min post-exposure blood glucose concentrations. This finding is likely explained by a late 2017 studies which have suggested that type 2 diabetes can be reversed, not through original pharmaceutical treatments, but through firm adherence to certain dietary interventions of low carbohydrate diet with 850 total daily calories for humans (Watson, 2018).

Our results also showed no significant change in basal glucose concentrations over time (4 weeks period) for β-sitosterol, while the control showed continuous increase. This would support efficacy, as β-sitosterol mitigated to an extent the increase in blood glucose concentrations which is likely an indication of disease progression in the control and extract treated group. This trend for stabilised blood glucose was also present for the positive control. In the study of Sanjay et al. (2012) β-sitosterol has antidiabetic activity in treated mice, in addition to reversing the weight loss. The reason for the lack of response in the extract group may be because the concentration of β-sitosterol was too low therein. This would support Drewes et al., 1984 who speculated that β-sitosterol is unlikely to account solely for antidiabetic activities of the corm's aqueous extract used for *in vivo* studies, since the compound is usually more abundant in alcoholic extracts, rather than in aqueous extracts of the corm. Moreover, this can

be due to the fact that the absolute bioavailability of β- sitosterol compound upon oral administration is about 9% (Ritschel et al., 1990).

Both the β-sitosterol and chlorpropamide groups also led to a slight decrease in animal weights. The mechanism behind this slight weight reduction is unknown and in theory could be due to changes in food consumption, inhibition of intestinal lipid absorption (Ikeda et al., 2005; Takami et al., 2008; Yang and Koo, 2000; Yokozawa, Nakagawa and Kitani 2002), an increase in the expenditure of energy (Dulloo et al., 1999) or stimulation of lipid oxidation (Dulloo et al., 1999; Murase et al., 2002). Our animals showed no change in consumption and faecal weight. Unfortunately, the activity of animals were not evaluated for this study.

There were no changes in the haematological parameters attributable to administration of tested treatments. The animals in general did not demonstrate severe pathology associated with diabetes and thus can be considered a good model for early stage diabetes. Moreover, there was a variation in the distribution pattern of endocrine islets of Langerhans. Some pancreatic tissues appeared normal although few did show mild non-specific inflammation with a decreased or increased endocrine cell numbers. The compound treated animals had less inflammation than the untreated group. This is important as one of the arguments for the use of anti-oxidants in treating diabetes is that it mitigates inflammatory damage to the cells. While, considering that islet inflammation is a significant factor in the pathogenesis of type 2 diabetes this increases a concern about the use of drugs potentially dangerous to the remaining β-cells. The histopathological observations in the present work showed some improvement in the animals treated with the compound when compared with the untreated group animals (Epol diet) and treated group (plant extract and positive control).

From qualitative evaluation there was no clear indication of regeneration of the endocrine cells in pancreatic islets by the compound. Prentki and Nolan (2006) demonstrated degeneration in the pancreatic tissue of diabetic mice, vacuolization of Langerhan's islet cells, decrease in the islets size, decrease in the β-cell numbers and also in the architecture of the islets. Loss of pancreatic β-cell mass and β-cell dysfunction are vital in the progress of type 2 diabetes and, in combination with peripheral insulin resistance which lead to hyperglycaemia. While, β-cells fail to accurately secrete insulin at a provided glucose level, there is also a progressive decline in the number of β-cells (Bonner-Weir and O'Brien, 2008). In the present study the histopathologic changes of islets have been observed in the animals treated with Epol diet only and with the positive control drug showing hyperplasia and atrophy, while those treated with plant extract and compound showed hyperplasia. Since the pancreas responds to peripheral insulin resistance by increasing insulin production (Wagner et al., 1996), the hyperplasia and absence of atrophy may be indicative of the anti-oxidant protective mentioned above.

An unexpected finding was the total increase in islet counts in the compound group. This can be due to the fact that new islets do grow under certain experimental conditions, such as neogenesis, β-cell replication and differentiation (Cockburn and Ferris, 2015; Bonner-Weir et al., 1993). Collombat et al. (2010) indicated that some plants induce regeneration of pancreatic islets in STZ-induced diabetic rats. However, contradictory opinions and data surrounding these regeneration theories are plentiful. The result in this study does tend to support Collombat assertions.

5.6 Conclusion

It can be concluded from this study that *H. hemerocallidae* is unlikely to be a suitable sole treatment agent in the management of diabetes. Since, none of the treatments could be considered highly effective for the management of type II diabetes as sole therapeutic intervention. Moreover, the diabetic models of natural progress are crucial for investigating and developing novel drugs for diabetes and its complications.

5.7 References

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Chapter 6:

General Conclusion

The aim of this study was to isolate, chemically and biologically characterize antidiabetic compounds present in corm of *H. hemerocallidea* in a bioassay guided process, with the following objectives:

Objective 1: To determine if the antioxidant activity of *H. hemerocallidea* extracts are influenced by plant stressors like, soil heavy metals concentrations, organic matter content and pH.

Objective 2: To investigate the potential antioxidant activity of *H*. *hemerocallidea* extracts in comparison to the intrinsic total phenolic content.

Objective 3: To isolate the constituents of interest in active extracts, and characterize the isolated compounds.

Objective 4: To evaluate the glucose uptake effect of the *H. hemerocallidea*, selected plant corm crude extract, fractions and compounds on pre-adipocytes (Fat 3T3-L1), myocytes (C_2C_{12}) and on its insulin sensitizing effect (RIN-m5F rat insulinoma).

Objective 5: To determine the cytotoxicity of *H. hemerocallidea* plant corm crude extract, fractions and isolated compounds on diabetic cell lines.

Objective 6: To determine the *in vivo* antidiabetic activity of the *H. hemerocallidea*, selected plant corm crude extract and compound/s on spontaneous diabetic mice.

The degree to which the above aim and objectives have been met is briefly outlined in the following sections:

To meet objective 1 and 2; the *in vitro* anti-oxidative activity of plant extracts were primarily systematically investigated, due to the vital role of oxidative stress to the two main pathogenic features of diabetes i.e. insulin resistance and pancreatic β-cell dysfunction, as well as other aspects of diabetes complications. Because certain minerals, are known to increase antioxidant capacity, the possible presence of heavy metals were investigated. This could impact on the safety of this plant from wild source. This as antioxidant activity is linked to plant stressors like soil heavy metals concentrations, organic matter content and pH. There was no link between the corm's antioxidant activity and environmental conditions, it would appear that wild harvesting may not pose a direct safety risk to the user. The study however demonstrated that free radical scavenging activity and the high phenolic content was associated with plants growing in soils with the lowest concentration of organic matter and lowest pH. The best anti-oxidative crude corm extract collected from Ga-rankuwa was selected for further studies.

To achieve objective 3, 4 and 5; we attempted to isolate and characterize the antidiabetic compounds from the corm collected from Ga-rankuwa. Using solvent-solvent fractionation and column chromatography, five different fractions were collected from the methanol extract of *H. hemerocallidea* corm and three compounds were isolated. The three compounds, corm crude extract, and fractions were all able to improve the glucose uptake and enhance the insulin secretion, with β- sitosterol having the best glucose uptake in 3T3-L1 pre-adipocytes and C_2C_{12} myocytes and insulin secretion by rat insulinoma RiNm5F cells. The crude extract, fractions and all the compounds had no clear evidence of cellular toxicity in any of the cell lines tested.

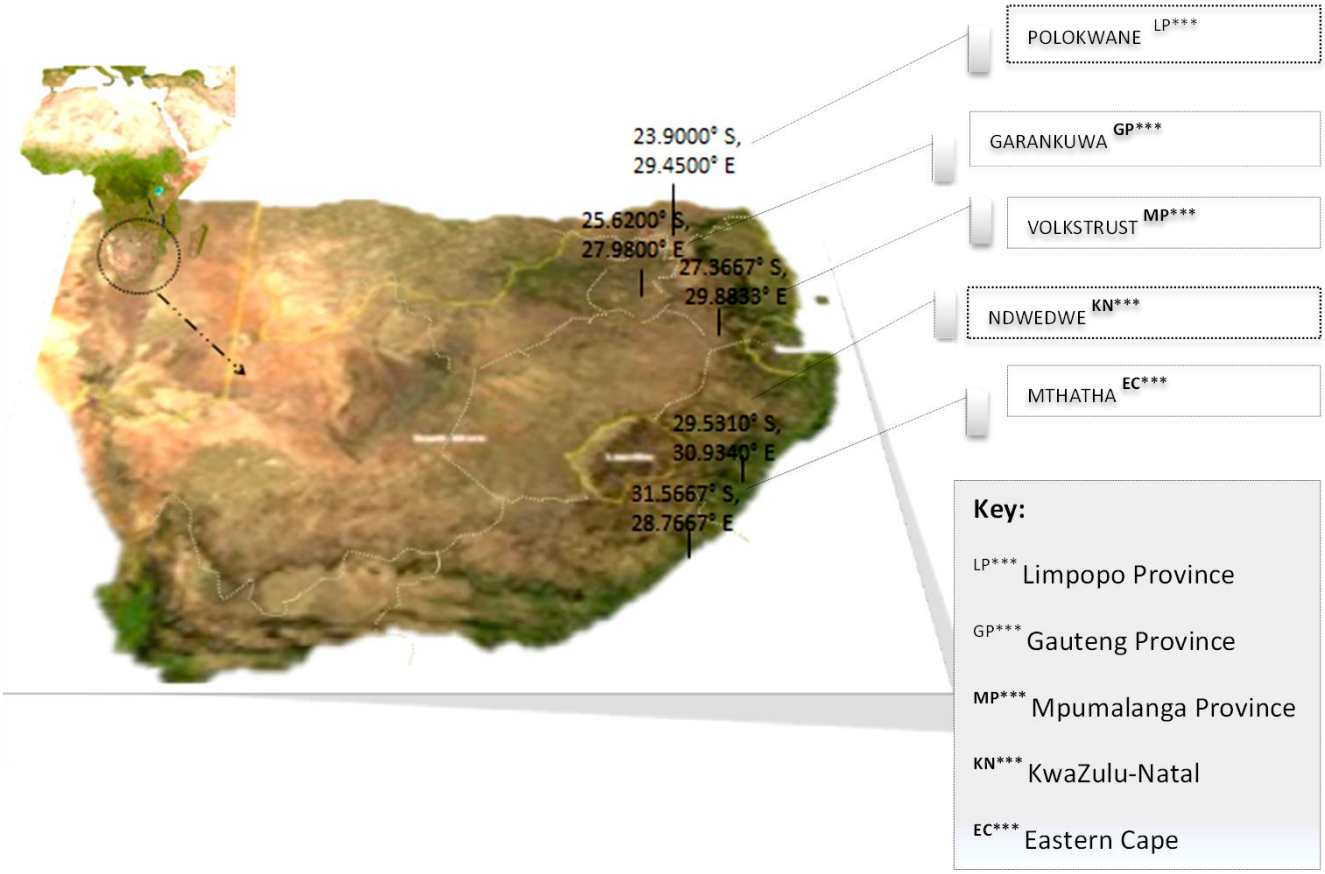
In investigating objective 6; the animal study led to no significant change in their plasma concentrations over the 4 weeks period following β-sitosterol treatment, while the animals on the crude corm extract and negative control (Epol diet) had a continuous increase in blood glucose level. No changes in the haematology were evident while clinical chemistry evaluations showed slight treatment-related changes in triglycerides and total cholesterol. Despite the effects seen, the biological response achieved was not sufficient to support the use of *H. hemerocallidae* or β-sitosterol as a suitable sole treatment agent in the management of diabetes. This study would also support current thinking that the chemical management of Type II diabetes must be undertaken in conjunction with lifestyle modification.

Our hypothesis postulated that *H. hemerocallidea* and its constituent can be a promising candidate for developing effective antidiabetic treatment. Our data did not provide conclusive support for the postulated hypothesis, since *H. hemerocallidea* and its constituent did not mitigate the hyperglycaemia associated within spontaneously diabetic mice. This finding was unexpected since our *In vitro* study revealed that *H. hemerocallidea* and its isolated β- sitosterol compound was effective with glucose uptake and insulin secretion. This can be due to the fact that the absolute bioavailability of β- sitosterol upon oral administration was previously reported at 9%, which could explain the poor response when orally administrated to spontaneous diabetic mice used in this study.

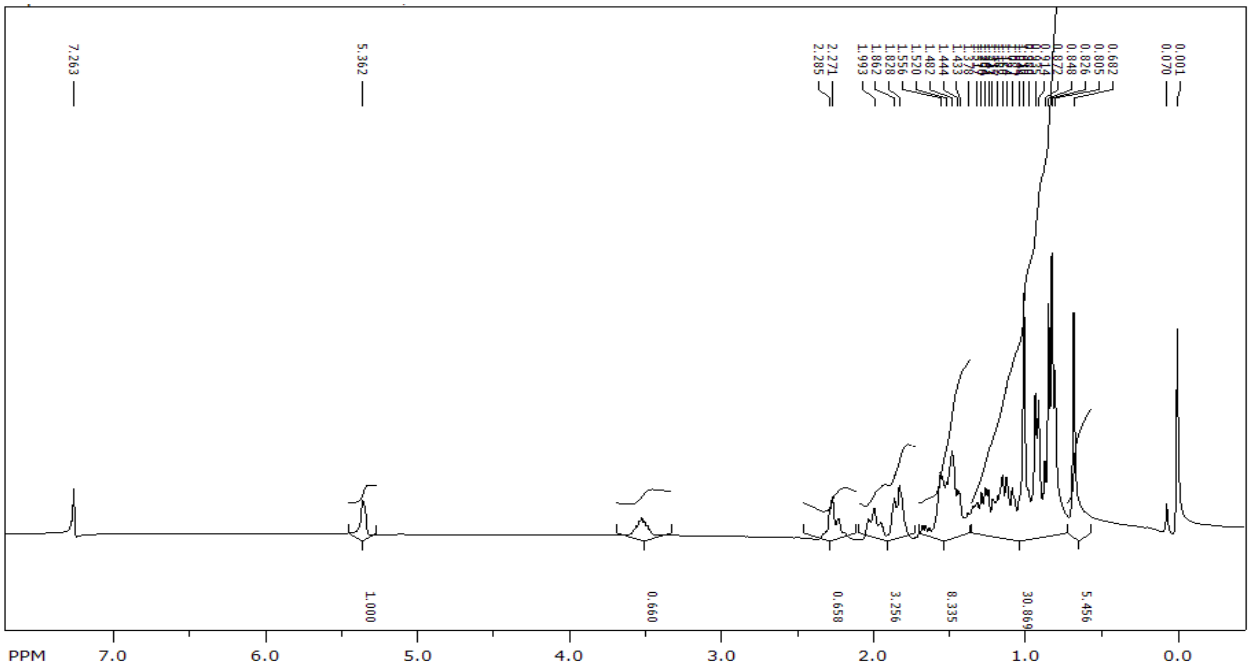
Further work:

1. Unfortunately, due to the low yield of some of the isolated compounds they were not characterized. Thus, further studies using better methods to isolate and elucidate the constituent(s) of plant are required in order to approve and expand these findings.
2. This study did not provide pharmacological support to the reported folkloric and anecdotal uses of the plant's corm in the treatment and/or management of diabetes mellitus, since the plant was evaluated as a sole therapeutic agent. Thus, further studies are needed to determine if the used plant have antidiabetic efficacy in the presence of lifestyle modification e.g. low fat diet and perhaps exercise.
3. The hypoglycaemic action could be due to release of insulin, insulin-sensitizing action or a combination of both. Hence further studies need to be undertaken to determine the mechanism of action by measurement of either insulin or 'C' peptide level in relation to a different doses. Further studies can also be undertaken at the molecular level, which may further elucidate its mechanism in detail.
4. Moreover, further work can also be done to evaluate pharmacokinetics and bioavailability of plant and beta-sitosterol in the used spontaneous diabetic mice.

Appendix A: Different areas in South Africa where plant (*Hypoxis hemerocallidea* corm) and soil samples were collected



Appendix B1: β-sitosterol compound (coded PRCD): 13C.



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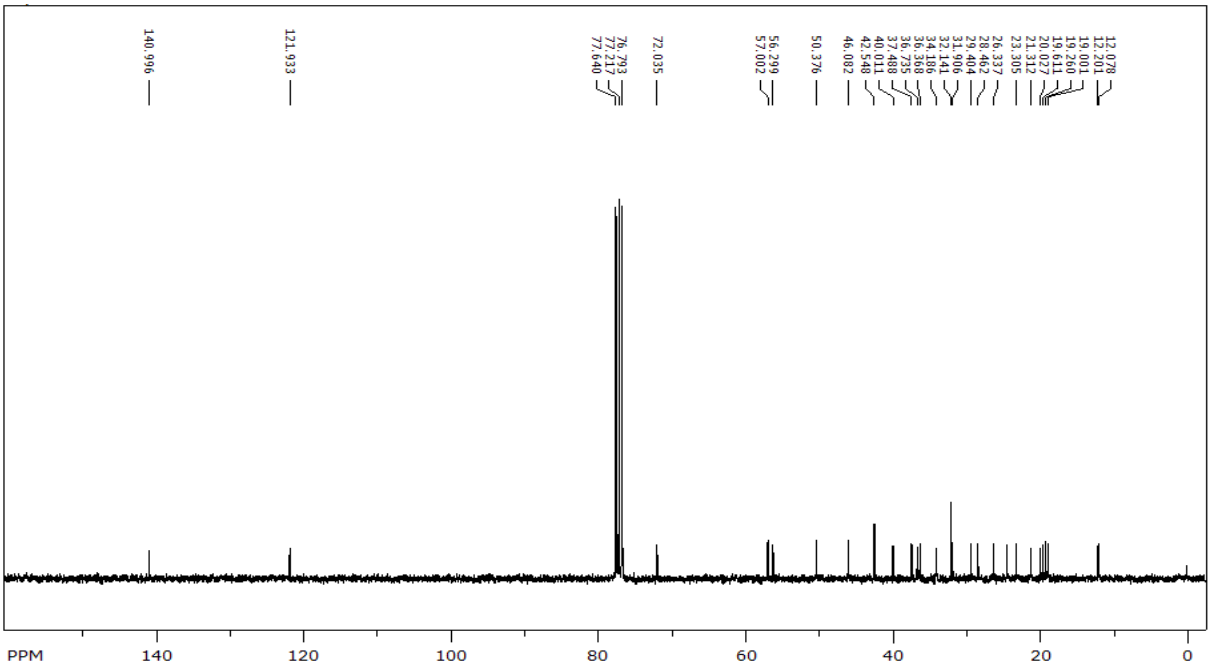
time domain size: 19194 points
width: 4803.07 Hz = 16.0067 ppm = 0.250238 Hz/pt
number of scans: 32

freq. of 0 ppm: 300.064108 MHz
processed size: 32768 complex points
LB: 1.500 GF: 0.0000

Appendix B2: *β-sitosterol compound (coded PRCD): 1H-NMR spectrum.*



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number of scans: 1000

freq. of 0 ppm: 75.451162 MHz
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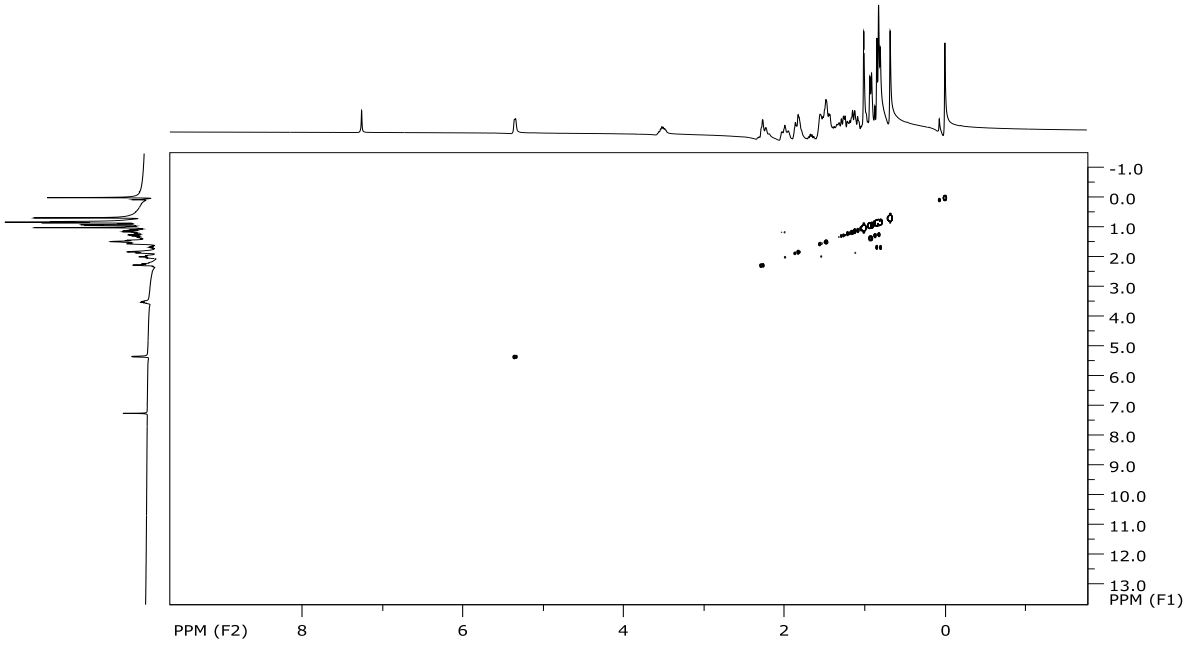
LB: 1.000 GF: 0.0000

Appendix B3: *1H-1H COSY spectrum of β-sitosterol compound.*



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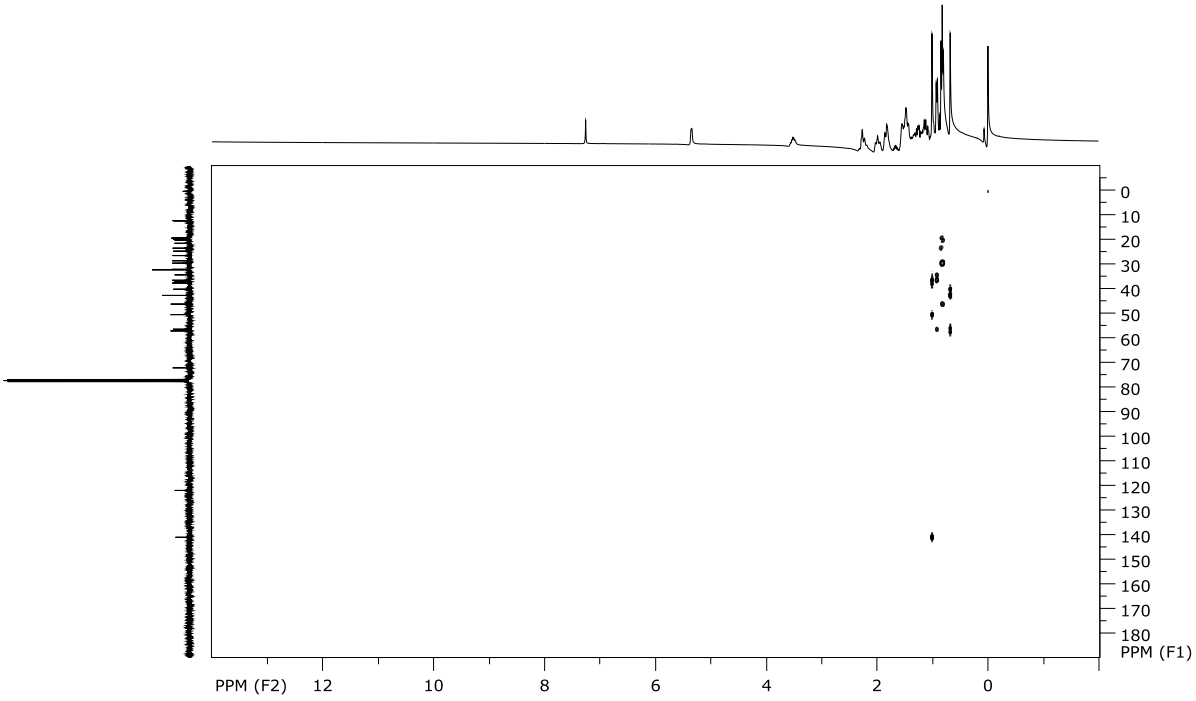
number of scans: 8

F2: freq. of 0 ppm: 300.0641084 MHz
processed size: 2048 complex points
window function: Sine
shift: 0.0 degrees

F1: freq. of 0 ppm: 300.0641061 MHz
processed size: 2048 complex points
window function: Sine
shift: 0.0 degrees

Appendix B4: *HMBC spectrum of β-sitosterol compound.*



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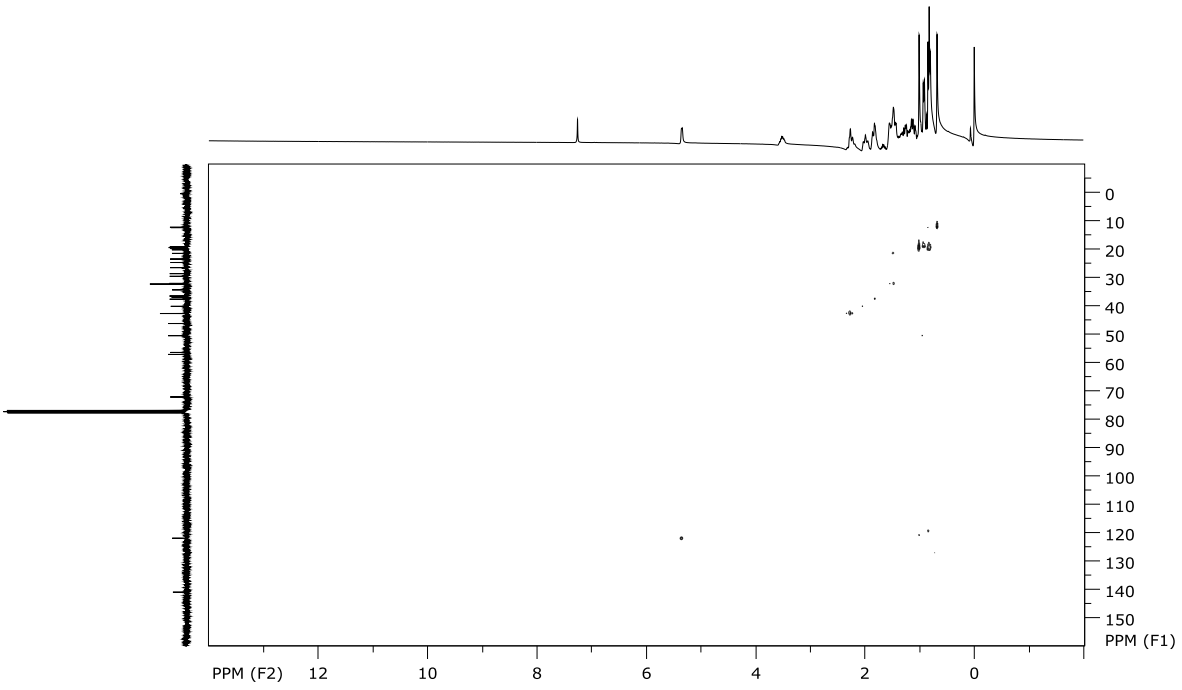
transmitter freq: 300.065910 MHz
time domain size: 2048 by 200 points
width (F2): 4807.69 Hz = 16.0221 ppm = 2.3475 Hz/pt
number of scans: 16

F2: freq. of 0 ppm: 300.0641084 MHz
processed size: 2048 complex points
window function: Sine
shift: 45.0 degrees

F1: freq. of 0 ppm: 75.4511625 MHz
processed size: 2048 complex points
window function: Sine
shift: 45.0 degrees

Appendix B5: HMQC spectrum of β-sitosterol compound.



file: ...nmrdata\HMQC-freddy-Monate.fid\fid block# 1
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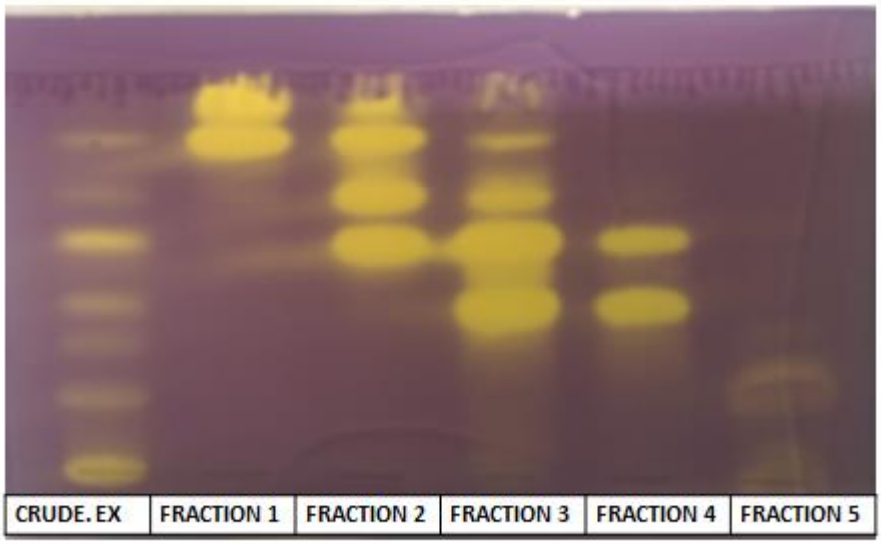
transmitter freq: 300.065910 MHz
time domain size: 2048 by 512 points
width (F2): 4807.69 Hz = 16.0221 ppm = 2.3475 Hz/pt
number of scans: 32

F2: freq. of 0 ppm: 300.0641084 MHz
processed size: 2048 complex points
window function: Sine Squared
shift: 90.0 degrees

F1: freq. of 0 ppm: 75.4511625 MHz
processed size: 2048 complex points
window function: Sine Squared
shift: 90.0 degrees

Appendix C: *Thin layer chromatography and anti-oxidant qualitative analysis from n-butanol fraction collected from column chromatography using BUTANONE solvent system*



Appendix D: *Vet import permit from Denmark and Animal ethics committee certificate*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF AGRICULTURE
DIRECTORATE: ANIMAL HEALTH
PRIVATE BAG X 138
2018 -08- 01
PRETORIA 0001
FORESTRY & FISHERIES

Directorate of Animal Health
Import-Export Policy Unit
Private Bag X138
Pretoria, 0001
Republic of South Africa

Tel: (27)-012-3197514
Fax: (27)-012-3298292

PERMIT NO: 13/1/1/30/0-201808000109
Valid from: **2018-08-01**
Expiry date: **2018-11-01**

IMPORTER:
SEFAKO MAKGATO HEALTH SCIENCES UNIVERSITY
P O BOX 139
MEDUNSA CAMPUS
0204

VETERINARY IMPORT PERMIT FOR LABORATORY MICE/RATS
[Issued in terms of the Animal Diseases Act, 1984 (Act 35 of 1984)]

Authority is hereby granted for you to import ALIVE MICE-DIABETIC 20 X LOT:20171211-EBUO402016-H6 / 25 X LOT: 20171225-EBUO402016-H6 into Republic of South Africa.
from:**TACONIC BIOSCIENCES , DENMARK**

subject to the following conditions :

1. Each consignment must be accompanied by:
   1.1 This original permit;
   1.2 a certificate issued by the Veterinary Authority of the country of export, certifying:
      1.2.1 the origin of the ALIVE MIICE-DIABETIC;
      1.2.2 that the ALIVE MICE-DIABETIC do not constitute any danger of introducing infectious or contagious diseases into South Africa;
      1.2.3 that the ALIVE MICE DIABETIC were securely packed in leakproof containers, sealed under official Veterinary supervision.
2. the consignment is to be airfreighted through port of entry **O.R. TAMBO INTERNATIONAL AIRPORT.**
3. **The mice/rats may only be imported as manifest cargo under an airwaybill number and may not be imported as personal luggage.**
4. the ALIVE MICE-DIABETIC must be kept and used for purposes of testing/research at the laboratories
5. On completion of tests/research the mice/rats and their progeny are to be euthanized and destroyed by incineration, including all contaminated/infectious things or animal products (as defined by the Animal Diseases Act, 1984 [Act No. 35 of 1984]) derived/produced from or that came into contact with the above-mentioned mice/rats. Records of the incinerations must be maintained for a period of 5 years, and made available for auditing to the Veterinary Authority upon request.
6. The State Veterinarian: **KEMPTON PARK** Tel: **(011)973 2827** must be advised timeously of the arrival of the consignment.
7. This permit is subject to amendment or cancellation by the Director Animal Health at any time and without prior notice being given.
8. This permit is valid for three (3) months from date of issue and FOR ONE CONSIGNMENT ONLY.

DIRECTOR: ANIMAL HEALTH

NOTE:
- All imports for research purposes require Section 20 permission in compliance with the Animal Diseases Act.
- Any consignment imported into South Africa packed with either wood packaging material or dunnage, will require treatment to remove any pests present (by heat or methyl bromide fumigation). Treatment must be indicated as per IPPC prescript on wood packaging material. [Directorate: Inspection Services Tel: 012 309 8754 or Fax 086 732 4768 or www.daff.gov.za]

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Isolation and identification of a novel anti-diabetic compound from *Hypoxis hemerocallidae* (Fisch and Mey) *(Hypoxidaceae)***
PROJECT NUMBER	**V113-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**NM Mkolo**

STUDENT NUMBER (where applicable)	**U_13172621**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Mice**	
NUMBER OF ANIMALS	**40**	
Approval period to use animals for research/testing purposes		**November 2017- November 2018**
SUPERVISOR	**Prof. V Naidoo**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 27 November 2017
CHAIRMAN: UP Animal Ethics Committee	Signature Nigel Bennett