CHAPTER 1

1.1 Introduction

In many developing countries, herbal medicines are of vital importance in primary health care (Kamboj, 2000). This is supported by literature in behavioural and pharmacological sciences with animals and people using a number of different plants for the control of disease symptoms and related illnesses in their environment (Hart, 2004; Cousins and Huffman, 2002). One of such disorder is diabetes mellitus, which is a chronic disease characterised by prolonged hyperglycaemia, especially post-prandial, in association with the consumption of diets that promote obesity.

The World Health Organisation (WHO) estimated that 171 million people were diagnosed with diabetes mellitus in 2000 and predicts that the prevalence of the disease will increase to 366 million in 2030 (a staggering increase of 144% over 30 years) if no action is taken (Wild et al., 2004). As a result, diabetes is one of the most rapidly growing non-communicable diseases and the fourth/fifth leading cause of death globally (Amos et al., 1997). While for numerous years, diabetes was considered a disease of the affluent in first world countries, the disease is also on the increase in Africa where the number of people with diabetes is predicted to increase from 13.3 million in 2000 to 33.3 million in 2030 (an increase of 148%) (Wild et al., 2004). In South Africa, the number of people suffering from diabetes is also high and estimated to have a prevalence of 3.4% in the 24 million South Africans between the ages of 20 to 79 (Rheeder, 2006). Type II diabetes is the most common form of diabetes which goes undiagnosed at the early stage because there are no physical clinical manifestation (Alberti and Zimmet, 1998). However, when hyperglycaemia is severe, it usually presents with symptoms of diabetes (e.g. excessive thirst, frequent urination and weight loss) or a complication related to diabetes (retinopathy, nephropathy) or as a chance finding in an asymptomatic individual (Alberti and Zimmet, 1998; Baynes, 2006). Although numerous hypoglycaemic therapies are available for the management of Type II diabetes they all have a number of limitations, such as adverse effects and high rates of secondary failure (Nathan et al., 2006; Virally et al., 2007), with the main reason for their failure being tolerance. From current evidence it appears that the latter results from a decreased sensitivity to insulin.

As an alternate to the use of drugs that mimic the insulin effect or enhances insulin release, it has been suggested that weight modulation may be a better treatment modality, as weight loss is known to improve insulin sensitivity, overall glycaemic control and decrease mortality rates (Norris et al., 2004). The latter is so well accepted that most doctors will unequivocally prescribe a change in diet in addition to pharmaceutical management in their diabetic patient (Anderson et al., 2003). Unfortunately diet changes only result in moderate weight loss as it is difficult to achieve long term weight loss control, as weight lost is slowly regained due to resumption of poor eating habits and/or the physiological abnormalities induced by the obesity (Norris et al., 2004).
It has been proposed that weight controlling medication may be a better or adjunct means of controlling diabetes than the current pharmaceutical agents (Lee and Aronne, 2007). The polyphenols present in plants may possibly be this alternate source. As a class they are naturally occurring phytochemicals of which some, such as catechins, anthocyanins, have been shown to regulate pathways that are involved in energy metabolism, adiposity and obesity (Meydani and Hasan, 2010), in addition to exerting an antidiabetic effect (Mai et al., 2007). Several species of the genus Ficus are used traditionally in a wide variety of ethnomedical remedies all over the world (Hansson et al., 2005; Koné et al., 2004). They have long been used in folk medicine in the treatment of different ailments including diabetes (Watt and Breyer-Brandwijk, 1962; Trivedi et al., 1969). From historical studies, these plants are known to have several phytochemical constituents including polyphenols which are among the naturally occurring antidiabetic agents (Ramadan et al., 2009; Andrade-Cetto et al., 2008). The antidiabetic potential of these species though has been confirmed in vivo but the mechanism of activity remains speculative.

1.2 Hypothesis
Plants within the Ficus species possess in vitro antidiabetic activity and in vivo weight reducing activity.

1.3 Aim
To evaluate the effectiveness of selected South African Ficus species in the management of Type II diabetes using either in vitro or in vivo models of efficacy.

1.4 Objectives
1. To evaluate the antioxidant activity of crude plant extracts of selected Ficus species in relation to their inherent total polyphenolic concentration.
2. To evaluate the in vitro α- amylase and α- glucosidase inhibitory activity of crude plant extracts of selected Ficus species.
3. To evaluate the efficacy of selected crude plant extracts of selected Ficus species for their ability to stimulate glucose uptake in established fat (3T3-L1), muscle (C2C12) and liver (H-4-II-E) cells.
4. To evaluate the efficacy of selected crude plant extracts of selected Ficus species in stimulating insulin release in an established insulin producing pancreatic cell line (RIN-m5F)
5. To evaluate the efficacy of selected crude plant extracts of selected Ficus species for their ability to stimulate glucose uptake in primary rat muscle and fat cell cultures.
6. To further evaluate the most active extracts from the above mentioned assays in the same assays through fractionation and the isolation of active compounds.
7. To evaluate the *in vitro* cytotoxicity of crude plant extracts and selected fractions using Vero monkey kidney and C3A human liver hepatoma cell lines.

8. To evaluate the most active and non-toxic fraction from the above mentioned assays for its ability to stimulate weight loss in a model of mouse obesity.
CHAPTER 2

2 Literature review

2.1 Introduction

Every living organism is made up of membrane bound cells which are the structural and functional unit of life. Mammals are multicellular organism containing different types of cells which vary in size, shape and specialised functions, to such an extent that cells that are similar in structure and functions aggregate to form tissues while different tissues with similar functions aggregate to form organs (Nelson and Cox, 2005). Nonetheless despite their specialised functions, these different cells are all characterised by a similar biological structure, which is classified as eukaryotes. The unique characteristic of the eukaryotic cells is the presence of an organised nucleus and specialised organelles which function synergistically:

![Figure 2-1: The features of eukaryotic animal cell structure (Alberts et al., 1998).](image)

- Cell membrane. – Each cell is surrounded by a phospholipid bilayer in which ion channels, receptors and other transporters are present. In addition to keeping the cytoplasm and organelles contained within the cell, the cell membrane is extremely important in controlling how substances enter via diffusion, facilitated diffusion, active transport or pinocytosis in addition to containing specific signalling proteins known as receptors.
• **Cytoplasm.** – The cytoplasm is enclosed by the plasma membrane and it is the content of the eukaryotic cell excluding the organelles. The cytoplasm is the site for many metabolic reactions that are fundamental to the cell’s existence e.g. protein biosynthesis, glycolysis and pentose phosphate pathway (Alberts et al., 1998).

• **Nucleus.** – The nucleus is the most prominent organelle in animal cell enclosed by double membrane called the nuclear envelope. The nucleus contains the cell’s genetic material (where they are stored and replicated), extremely long linear DNA (deoxyribonucleic acid) molecules which when complex with proteins such as histones form chromosomes. The nucleus is the control centre of the cell. The nucleus contains sub-nuclear bodies such the nucleoli which are involved in assembly of ribosomes exported to the cytoplasm where they translate mRNA (messenger ribonucleic acid) into proteins (Alberts et al., 1998).

• **Mitochondria.** – These are organelles containing their own DNA and RNA (ribonucleic acid) and a complete transcription and translation system including ribosomes enabling them to synthesize their own protein. The main function of the mitochondria is to synthesize energy in form of adenosine triphosphate (ATP) which is the main energy source of the cell (Hüttemann et al., 2007).

• **Endoplasmic reticulum.** – Endoplasmic reticulum (ER) is an irregular maze of space (cisternal space or lumen) enclosed by a membrane. The ER is involved in the synthesis of secretory and membrane proteins; biosynthesis of phospholipids, cholesterol and steroids; the degradation of glycogen; detoxification reaction; and the regulation of intracellular calcium (Baumann and Walz, 2001).

• **Golgi apparatus.** – This comprises stacks of flattened membrane cisternae surrounded by small vesicles (Nebenfuhr and Staehelin, 2001). The Golgi apparatus is the site for the processing, modification and attachment of carbohydrate molecules to proteins and lipids through the secretory pathway. It also serves as a station for sorting and transport of proteins which it receives from the ER and delivers to the plasma membrane or other intracellular sites (Altan-Bonnet at al., 2004).

• **Ribosomes.** – These are small particles in the cell which are composed of protein and RNA molecules called ribonucleoprotein complexes (Byrne, 2009). Ribosomes can exist as either free or membrane bound ribosomes with the free ribosomes synthesizing protein that are released in the cytoplasm where they are used while the membrane bound ribosomes are
attached to the ER (called rough ER) synthesize protein for export to other organelles or excretion (Alberts et al., 1998).

- Centrosome. –Centrosomes are small structures (1-2 μm³) on one side of the nucleus when the cell is not in mitosis. The centrosome plays an important role in organising both the interphase cytoskeleton and the bipolar mitotic spindle, ensuring correct segregation of chromosomes to prevent aneuploidy (Loffler et al., 2006).

- Cytoskeleton. – The cytoskeleton is responsible for the cell’s ability to organise its intracellular components including the organelles, to adopt a variety of shapes and to carry out coordinated movements such as transport of organelles from one place to another, the segregation of chromosomes into two daughter cells in mitosis, and the pinching apart of animal cells at cell division (Nelson and Cox, 2005).

- Lysosomes. – These are membranous sacs of hydrolytic enzymes that degrade proteins, phospholipids, nucleic acids and oligosaccharides (Alberts et al., 1998).

- Vacuoles. – These are enclosed compartments that are filled with water which contains inorganic molecules, organic molecules and enzymes. Sometimes they may contain solids which have been engulfed (Alberts et al., 1998).

While each organelle has a specific function within the cell, their functionality is dependent on the availability of adenosine triphosphate (ATP) as an energy source. At the subcellular level this ATP is formed by the mitochondria from glucose. The glucose involved in ATP generation is derived from dietary sources in a process known as glucose metabolism (Tiwari and Rao, 2002).

2.2 Glucose Metabolism

2.2.1 Digestion

Digestion is the mechanical (chewing) and chemical (enzymatic action) breakdown of food into smaller components that are more easily absorbed into the bloodstream (Reed and Wickham, 2009). While the digestion of all classes of food begins in the mouth with the mechanical process of mastication, for certain carbohydrate such as starches and dextrins, chemical digestion also begins in the mouth (Pedersen et al., 2002). Here the food mixes with saliva containing the α-amylase enzyme called ptyalin (Pedersen et al., 2002), which begins the conversion of starch and dextrins into short chain polysaccharide fragments, maltotriose (the di- and trisaccharides of α-D-(1→4) glucose), oligosaccharides called limit dextrins, fragments of amylopectin containing α-D-(1→6) branch
point sor disaccharide maltose, all through the hydrolysis of the internal glycosidic linkages of the carbohydrate molecule (Nelson and Cox, 2005) (Figure 2-2). However, because food remains in the mouth for only a short time, only about 5% of the starches eaten get hydrolysed by the time of swallowing and about 20% by the time of reaching the stomach (Guyton and Hall, 2000).  

![Image of carbohydrate structures](Image)

**Figure 2-2:** Illustration of the point of hydrolysis of the internal glycosidic linkages of starch.  
α-Amylase from saliva and the pancreas hydrolyse the α-D-(1→4) bond of amylose and; α-D-(1→6) and α-D-(1→4) bonds of amylpectin yielding short chain polysaccharides fragments and disaccharides (Kadokawa, 2012).

After deglutition carbohydrate metabolism is temporarily terminated by the acidic environment of the stomach, and only reinitiates once the chyme enters the duodenum, where it is once again exposed to α-amylase enzyme this time produced by the pancreas. In comparison, the pancreatic α-amylase is more active than the salivary α-amylase with more efficient hydrolyses of the carbohydrates into maltose, sucrose and lactose (Hanhineva et al., 2010).

Hereafter the formed maltose, sucrose and other disaccharides are exposed to a family of brush border enzymes collectively known as the glucosidases. These specific enzymes are specific to a disaccharide and are responsible for the splitting of disaccharides to their constituent monosaccharides e.g. lactose is metabolised by lactase into a molecule of glucose and a molecule of galactose, maltose by maltase into two molecules of glucose, sucrose by sucrase into a molecule of glucose and a molecule of fructose, and α-dextrin by α-dextrinase to several molecules of glucose (Figure 2-3). Of all the formed monosaccharides, glucose is the major product which is absorbed for further utilisation (Nelson and Cox 2005).
2.2.2 Absorption and transport of glucose

The newly formed glucose is rapidly absorbed across the wall of the small intestine with absorption being complete before reaching the terminal ileum. Due to the water soluble nature of glucose, its absorption is reliant on the presence of a specific co-transport protein that is driven by the facilitated absorption of sodium (symport dependent on sodium transport) i.e. in the absence of sodium no glucose absorption results (Nelson and Cox, 2005). The transporter has been named the Sodium-Glucose symporter (SGLUT). This driving force for the movement of sodium into the cell is created by the active sodium-potassium pump (Na⁺/K⁺ATPase) which depletes the cytoplasm of sodium by moving three sodium molecules out of the cytoplasm and two potassium molecules therein in order to maintain electrical neutrality (Figure 2-4). The glucose then leaves the cell via the basal cell membrane through the passive and specific glucose transporter GLUT2 (Nelson and Cox, 2005) and enters into the blood capillaries.
Figure 2-4: Illustration of the transport of glucose across the the intestinal epithelium.
Entry of glucose into epithelial cells is mediated by SGLUT located in the apical surface. Glucose is co-transported with sodium ion (Na⁺) across the apical plasma membrane into the epithelial cell. It moves through the cell to the basal surface, where it passes into the blood via GLUT2, a passive glucose transporter. The Na⁺/K⁺ ATPase continues to pump Na⁺ outward to maintain the Na⁺ gradient that drives glucose uptake (Nelson and Cox, 2005).

2.2.3 Entry of Glucose into cells

Glucose has to be transported from the blood across the cell membrane into the cellular cytoplasm before it can be used by the body’s tissue cells (Guyton and Hall, 2000). However, with the membrane of cells being impermeable to glucose, the cell is reliant on transmembrane proteins transporters for diffusion into the cell. The glucose transporters in non-epithelial mammalian tissues are part of the family of glucose transporters known as the GLUT receptors. These receptors may or may not be insulin dependent (requires insulin for function)(For more details please refer to section 2.3.3):

- GLUT1 transporters are present in the cell membrane of almost all cell types (highest expression is in the red blood and brain microvessels). Under basal physiological conditions the GLUT1 transporter continuously allows for glucose entry. These receptors are non-insulin dependent.

- GLUT2 transporters, which are also non-insulin dependent are found in the pancreatic β-cell, liver, kidney and small intestine (basolateral membrane). In these tissues, the uptake of glucose is dependent on plasma glucose concentrations i.e. facilitated diffusion (Scheepers et al., 2004).
• GLUT3 transporters are present in the neurons of the brain and foetal muscle. In these tissues, the uptake of glucose is dependent on plasma glucose concentrations i.e. facilitated diffusion (Scheepers et al., 2004).

• GLUT4 transporters are present in muscle and fat cells. GLUT4 transporters are normally sequestered in intracellular organelles where they remain inactive (Scheepers et al., 2004; Saltiel and Kahn, 2001). However, on binding of insulin to the insulin cell surface receptor, the GLUT4 transporter translocates from its intracellular location to the cell surface. Once in its active position the GLUT4 immediately and rapidly allows for the transport of glucose (for as long as insulin is bound to its receptor) intracellular at a rate of 10 times or more than in its absence (Scheepers et al., 2004).

Once within the cell, glucose gets phosphorylated by glucokinase (in the liver) and hexokinase (in other cells) into glucose-6-phosphate which can no longer leave the cell. The phosphorylation step is almost completely irreversible (except in certain cells e.g. hepatocytes) and serves to ensure that glucose is retained by cells for either energy generation or storage (Guyton and Hall, 2000).

2.2.3.1 Energy Utilisation

While glucose is the main fuel source of the body, cells do not have the capacity to make use of glucose directly but only in the form of ATP, which is generated under aerobic or anaerobic conditions.

2.2.3.1.1 Glycolysis (Energy generation under anaerobic conditions)

Glycolysis is a ten-steps, two stages, sequential enzymatic breakdown of a molecule of glucose into two molecules of pyruvate, and takes place within the cytoplasm (Figure 2-5). The first stage requires energy in the form of ATP to start the process. In this pathway, glucose is first phosphorylated to glucose-6-phosphate, then isomerised to fructose-6-phosphate and further phosphorylated to fructose-1, 6-bisphosphate. At the end of the first phase, two molecules of glyceraldehyde-3-phosphate are produced with two molecules of ATP being utilised.
Glucose is phosphorylated to glucose-6-phosphate, isomerased to fructose-6-phosphate, phosphorylated to fructose-1,6-bisphosphate and then split to glyceraldehyde-3-phosphate (GLAP) and dihydroxyacetone phosphate (DHAP). DHAP is isomerase to GLAP. After 5 steps enzyme reactions pyruvate is produced. For each glucose molecule that passes through the first phase, two glyceraldehyde-3-phosphate are formed. Both pass through the second phase and 2 pyruvate, 2 ATP and 2 NADH molecules are the end product (Nelson and Cox, 2005).

The second stage is the energy generation phase. The two molecules of glyceraldehyde-3-phosphate produced in the first stage undergo enzymatic oxidation and two molecules of pyruvate are produced.
with four molecules of ATP and two molecules of NADH (reduced nicotinamide adenine dinucleotide) generated as energy. With two molecules of ATP being utilised in the first phase, this process yields two molecules of ATP and two molecules of NADH as energy. The pyruvate molecules formed eventually get reduced to lactic acid under anaerobic condition by accepting electrons from NADH, while the NADH itself is oxidised to NAD⁺ (nicotinamide adenine dinucleotide), regenerating the NAD⁺ that is required to maintain the glycolytic process (Nelson and Cox, 2005).

2.2.3.1.2 ATP generation under aerobic conditions

Pyruvate is the principal mitochondrial substrate and is produced by glycolysis as mentioned above. The pyruvate molecules formed in the cytoplasm undergo carrier mediated transport across the inner mitochondrial membrane into the mitochondrial matrix where they undergo oxidative decarboxylation with a loss of the carboxyl group as a carbon dioxide (waste product) and generation of one molecule of NADH (Maechler and Wollheim, 2001). A total of two molecules of carbon dioxide and two molecules of NADH are reproduced from the two pyruvate molecules per one glucose molecule. The remaining two carbon atoms become the acetyl group of acetyl coenzyme A (acetyl CoA), a crucial intermediate metabolite for the citric acid cycle in the mitochondrial matrix (Figure 2-6).

Immediately acetyl coenzyme A enters the citric acid cycle, where it donates its acetyl group to a four carbon compound oxaloacetate, to commence the eight step enzymatic oxidation reaction and at the end of the cycle, oxaloacetate is regenerated which starts a new cycle. For each one acetyl group that enters the citric acid cycle, two molecules of carbon dioxide are released as waste products. One molecule of GTP (guanosine triphosphate) a close relative of ATP, three molecules of NADH and one molecule of FADH₂ (reduced flavin adenine dinucleotide) are generated as energy. At the end of glycolysis and the citric acid cycle, one molecule of glucose would have generates four molecules of ATP and twenty-four high energy electrons in the form of hydride ions which are carried by NADH and FADH₂ for entry into the electron transport chain (Nelson and Cox, 2005).
The electron transport chain is responsible for oxidative phosphorylation and is embedded within the inner mitochondrial membrane. Oxidative phosphorylation is a two stage enzymatic generation of ATP. The first stage is the oxidation of hydrogen by the splitting of hydrogen atoms (derived from NADH and FADH$_2$) into hydrogen ions (protons) and electrons, and the second stage is the utilisation of the energy generated during the oxidative reactions to phosphorylate ADP to ATP (Guyton and Hall, 2000). The electron transport chain consists of electron carriers, which are large enzyme complexes namely complex I, II, III, IV and V (Figure 2-7) arranged in order of increasing redox potential (Alberts et al., 1998). Oxidative phosphorylation begins with the entry of electrons into the respiratory chain at complex I (NADH) and II (FADH$_2$). Complex II, succinate dehydrogenase, is also an integral part of the citric acid cycle. The electrons from both complexes I and II move to complex III.
The flow of electrons along the respiratory chain drives the expulsion of protons from the mitochondrial matrix, which establishes a steep electrochemical gradient across the inner mitochondrial membrane i.e. the mitochondrial matrix becomes negative. More importantly since the inner membrane is impermeable to protons their re-entry into the matrix, to re-establish electrical neutrality, is dependent on entry through the dedicated transmembrane carrier ATP synthase, which is located in the inner mitochondrial membrane projecting into the matrix. The flow of the protons down the electrochemical gradient through a pore in the ATP synthase causes the ATP synthase to spin thereby providing energy to drive the synthesis of ATP from ADP and inorganic phosphate.

Figure 2-7: Illustration of the mechanism of oxidative phosphorylation (Nelson and Cox, 2005). As a high energy electron is passed along the electron transport chain starting with the electron donated by NADH, some energy is released to drive the respiratory enzyme complexes that pump H⁺ out of the matrix space. The resulting electrochemical proton gradient across the inner membrane drives H⁺ back through ATP synthetase, a transmembrane protein complex that uses the energy of the H⁺ to synthesise ATP from ADP and Pi in the matrix (Alberts et al., 1998).

The ATP produced is subsequently transferred into the cytoplasm in exchange for ADP by the enzyme adenine nucleotide translocator (Maechler and Wollheim, 2001). For every two electrons that pass through the electron transport chain (representing hydrogen atoms carried each by NADH and FADH₂), about three molecules of ATP are synthesized. The breakdown of glucose by glycolysis and citric acid cycle produce a total of twenty-four hydrogen atoms and twenty of these hydrogen atoms are undergo oxidative phosphorylation in the mitochondrial matrix. At the end of oxidative phosphorylation, additional 30 ATP molecules are metabolised accounting for almost 90% of the total energy generated (Guyton and Hall, 2000).
2.2.3.2 Storage of glucose

After entry into the cells, glucose can either be immediately converted to ATP for use by the cell (see section 2.2.3.1 above) or be stored in the form of glycogen (low osmotic active polymer of glucose) or fat. Glycogenesis is the process of glycogen formation from glucose. The glucose-6-phosphate formed (Figure 2-5) is converted to glucose-1-phosphate, then to uridine diphosphate glucose and finally converted into glycogen (Figure 2-8). All cells are capable of storing glycogen in large cytosolic granulesto various extents, with cells like the hepatocytes being capable of storing larger amount of glycogen (5% to 8% of their weight), and muscle cells to a lesser extent (1%-3% of their weight)(Guyton and Hall, 2000). The elementary particle of glycogen, the β-particle about 21 nm in diameter, consists of up to 55,000 glucose residues with about 2,000 non-reducing ends(Nelson and Cox, 2005).

The body does however only have limited capacity for the storage of glycogen(Guyton and Hall, 2000). Once the glycogen stores are saturated, the additional glucose is converted into triglycerides by the liver cells and transported as very low density lipoprotein for storage in the adipose tissue all over the body. The first step in the synthesis of triglycerides is the conversion of glucose to acetyl-CoA. Malonyl CoA and NADPH are the principal intermediates in the polymerisation process. The second step is the esterification of glycerol and the synthesised fatty acid with chains between 14 and 18 carbon atoms to form triglycerides. A major difference between glycogen and fat, is that glycogen is an immediately available store of glucose while fat is less readily available because in mammals fat cannot be metabolised anaerobically (Guyton and Hall, 2000).

![Cell membrane](image)

Glycogen

Uridine diphosphate

Glucose 1-phosphate

Bloodglucose

Figure 2-8: Illustration of the chemical reactions of glycogenesis and glycogenolysis.
The mechanism of inter-conversions between glycogen into glucose 6-phosphate (in all tissues) and liver glycogen into blood glucose. The latter is specific to the liver as the phosphatase enzyme required for release of glucose from within cell is present only in the hepatocytes (Guyton and Hall, 2000).
2.2.3.3 Regulation of glucose

Blood glucose in mammals is maintained within very tight limits (about 80 to 110 mg/dl), despite the large fluctuations that result following food intake (Tiwari and Rao, 2002, Guyton and Hall, 2000). The reasons being to supply the brain, retina and the germinal epithelium of the gonads with sufficient glucose to meet their energy requirements as entry of glucose into these tissues is non-insulin dependent and based purely on a passive concentration (see section 2.2.3). In addition, this specific concentration is strictly maintained to prevent intra- and extra-cellular dehydration, as glucose is highly osmotically active.

After the ingestion of a carbohydrate-rich diet, increases in the blood glucose concentration of about 140 mg/dl can be expected. This increase in blood glucose concentration stimulates the release of insulin, by the pancreatic β-cells, which subsequently decrease the concentration of blood glucose to the above-mentioned “normal limit”. Insulin reduces the blood glucose concentration by increasing the rate of glucose uptake in the muscle and fat cells and inhibiting glucose production (gluconeogenesis) by the hepatocytes. Conversely during the period of fasting, after overnight fast or in between meals, insulin levels are usually low and blood glucose concentrations are maintained by the supply of glucose from the liver through gluconeogenesis and glycogenolysis under the control of glucagon also produced by the β-cells (Nelson and Cox, 2005; Guyton and Hall, 2000).

• Glycogenolysis: This is the breakdown of the muscle and liver’s store of glycogen to produce glucose 6-phosphate in the cells which is subsequently used to provide energy. Glycogenolysis does not occur in reversal of the same pathway that formed glycogen (Figure 2-8); rather each succeeding glucose unit of the outer branches of the glycogen enters the glycolytic pathway through the action of three enzymes: glycogen phosphorylase, glycogen debranching enzyme and phosphoglucomutase (Nelson and Cox, 2005; Guyton and Hall, 2000).

When there is demand for glucose, two hormones; epinephrine (which acts on muscle and liver) and glucagon (which acts only on liver) are released into the blood and cause the formation of cyclic AMP within the muscle and liver cells. The formation of this substance initiates a cascade of reactions that activates glycogen phosphorylase which catalyzes the splitting (phosphorylation) of α-(1-4) glycolytic linkage between two glucose residues at a non-reducing end of glycogen removing the terminal glucose residue as α-D-glucose-1-phosphate. The glycogen phosphorylase continues to split the glucose unit until it reaches four...
glucose units away from an α-(1-6) branch point and its action stops. Further degradation by this enzyme can occur only after the debranching enzyme catalysed two successive reactions that transfers branches and hydrolysed the glucosyl residue at C-6. Glucose-1-phosphate the end product of glycogen phosphorylase reaction is converted to glucose-6-phosphate by phosphoglucomutase (Nelson and Cox, 2005; Guyton and Hall, 2000).

The glucose-6-phosphate formed from muscle glycogen can hereafter enter the glycolysis pathway. In the liver, however, glycogen breakdown serves to replenish glucose concentrations in the blood. A key enzyme glucose-6-phosphatase that is present as a transmembrane protein on the ER of the liver and kidney is responsible for the hydrolysis of glucose-6-phosphate. This glucose-6-phosphate is transported from the cytosol to the active site on the luminal side of the ER where the glucose-6-phosphate is hydrolysed to glucose and phosphate. The glucose formed is transported to the cytosol and out of the liver through GLUT2. Because muscle and the adipose tissues lack glucose-6-phosphatase, they cannot convert the glucose-6-phosphate formed by glycogen breakdown to glucose and therefore do not contribute glucose to the blood (Nelson and Cox, 2005).

- Gluconeogenesis: As mentioned above (see section 2.2.3.2 above) energy can also be stored in fat tissue (adipose tissue) as droplets of triacylglycerol (fat) within the adipocytes. Triacylglycerol molecules are formed from esterification of fatty acids and glycerol by enzymes found within the adipocytes. When fuel demand rises, especially during fasting, glucagon is released into the blood and activates hormone-sensitive lipases in the adipocytes which hydrolyse stored triacylglycerol with resultant release of large quantities of fatty acids and glycerol into the blood. The free fatty acids hereafter become the primary source of energy for the muscle and liver. However, because some cells like the brain cells mainly use glucose for energy, the liver degrades some non-essential amino acids by transamination and deamination and uses their carbon skeleton in addition to glycerol derived from the adipose tissue as substrates to produce glucose via gluconeogenesis.

After a few days of fasting, the acetyl CoA produced by fatty acid oxidation start to accumulate due to depletion of the citric acid cycle intermediates required for its conversion to glucose. As a result, the accumulating acetyl CoA molecules condensate to form acetoacetic acid, β-hydroxybutyrate or acetone, collectively known as the ketone bodies, which can be used as an energy source by the heart, skeletal muscle and brain during prolonged fasting and starvation. The ketone bodies like glucose can cross the blood brain
barrier and can be used by the brain cells with about two-third of the its energy being derived from $\beta$-hydroxybutyrate (Nelson and Cox, 2005; Guyton and Hall, 2000).

Insulin and glucagon therefore function as important feedback control systems for maintaining a normal blood glucose concentration. The liver also play an important role in glucose regulation by removing glucose from the blood when present in excess or returning it to the blood when low (Guyton and Hall, 2000). While glucagon and insulin are the main control hormones regulating glucose concentrations within the plasma, other hormones may also play a role in its control. The growth hormone (somatotropin), thyroxine and cortisol also increase plasma glucose concentration in conditions characterised by growth/production, increased activity or stress (Nelson and Cox, 2005; Burén and Eriksson, 2005).

When fat reserves are depleted, the degradation of essential proteins begins, protein synthesis is stopped and large quantities of proteins are released into the blood. The blood amino acids concentration rise considerable, and most of the excess amino acids can used either directly for energy (Nelson and Cox, 2005; Guyton and Hall, 2000) via gluconeogenesis pathway.

### 2.3 Insulin

#### 2.3.1 Structure and biochemistry

Insulin is a small protein with 51 amino acids composed of two chains (A and B) linked by disulphide bonds with molecular weight of 5802 (Wilcox, 2005). Insulin synthesis begins in the pancreatic $\beta$-cells in the form of an inactive single chain 110 amino acids precursor, preproinsulin (molecular weight of about 11,500) with an amino acid terminal called signal sequence (Figure 2-9). This terminal signal sequence with 24 amino acids directs the synthesis of preproinsulin into the lumen of the rough ER where it is converted to proinsulin by the cleavage of the terminal signal sequence and folded to acquire its characteristic 3-dimensional structure. Secretory vesicles transfer the proinsulin from the rough ER to the Golgi apparatus, where proinsulin is processed to form hexamers (Wilcox, 2009). The proinsulin hexamers are stored in secretory granule within the $\beta$-cell until they are released. While within the secretory granule, the C (connecting) chain (35 amino acids) that links the A and B chains together is cleaved to form the insulin in a process known as maturation. When insulin is released, C peptide and proinsulin are also secreted into circulation by exocytosis (Reiter and Gardner, 2003), although the proinsulin is virtually inactive while the C peptide is devoid of activity.
2.3.2 Mechanism of insulin secretion

As mentioned above, increases in blood glucose stimulates the release of insulin in concentration dependent manner from the pancreas. The absorbed glucose reaches the pancreas via the hepatic portal vein and enters the β-cells by facilitated transport, mediated by the GLUT2 transporter (see section 2.2.2 and 2.2.3 above). Once within the cell, the glucose is phosphorylated by the hexokinase IV enzyme initiating the process of glycolysis (Maechler and Wollheim, 2001). After the complete oxidation of glucose in the pancreatic β-cell mitochondria, the produced ATP is exported to the cytoplasm whereby the increasing concentration induce the closing of the ATP-gated efflux K⁺ channels, with subsequent depolarization of β-cells, and opening of voltage-sensitive Ca²⁺ channels in the cell membrane. This resultant influx of Ca²⁺ induces further mobilization of Ca²⁺ from the sarcoplasmic reticulum, microfilament contraction and the exocytosis of insulin from its storage granules (Figure 2-10) (Nelson and Cox, 2005; Saltiel and Kahn, 2001).
2.3.3 Mechanism of insulin action

Insulin released into the bloodstream gets carried to the liver, muscle and fat cells (Figure 2-10) (Saltiel and Kahn, 2001) where it binds reversibly to cell surface transmembrane receptors. This insulin receptor is a ligand-activated tyrosine kinase receptor. The binding of insulin to the receptor induces conformational changes in the insulin receptor molecule in the β-subunit with subsequent increase in its tyrosine kinase activity leading to the autophosphorylation of multiple tyrosine residues within its intracellular region (Saltiel and Kahn, 2001). This phosphorylation finally stimulates the translocation of the glucose transporter GLUT4 from the intracellular location up to the plasma membrane in muscle and adipocytes, allowing glucose to enter the cells across the concentration gradient (Bryant et al., 2002).

2.3.4 Physiology of normal insulin secretion

During periods of preprandial, blood glucose level is maintained at 80 to 90 mg/dl while basal insulin is secreted at 25 ng/min/kg of body weight (a level that has only a slight physiological activity (see
section 2.3.2 above). Immediately postprandial, blood glucose concentration is increased to two or three times the normal range. This stimulates the release of insulin in two stages (biphasic pattern) (Guyton and Hall, 2000);

1. Insulin concentrations increase rapidly initially to almost tenfold basal levels within 3 to 5 minutes of the elevation of blood glucose, and results from the immediate release of insulin from the matured secretory granules (Pratley and Weyer, 2001). However, this initial high secretion of insulin is not sustained, and the insulin concentration decreases back to the basal levels after a further 5 to 10 minutes (Pratley and Weyer, 2001).

2. Continued high blood glucose levels leads to a slower second phase of insulin secretion after approximately 15 minutes and subsequently a much higher concentration than the initial phase, with peaks being achieved within 2 to 3 hours or as long as the high concentration of glucose persist. This second phase insulin secretion is derived from stored secretory granules and from activation of the enzyme system that synthesizes (see section 2.3.2 above) and releases new insulin from the cells (Pratley and Weyer, 2001).

The early phase insulin secretion is pivotal in the transition from fasting state to fed state, as it serves to suppress hepatic glucose production, lipolysis in addition to preparing target cells for the action of insulin. The second phase is responsible for the overall decrease in postprandial hyperglycaemia (Virally et al., 2007).

2.4 Diseases of improper glucose metabolism

Diabetes mellitus is a metabolic disorder of multiple aetiologies characterised clinically as a disease of disturbances in carbohydrate, fat and protein metabolism brought about through defects in insulin secretion (i.e. β-cell dysfunction), insulin action (i.e. peripheral insulin resistance in muscle and fat cells), or both. Type I and Type II diabetes mellitus are the most predominant in the world (Sherwin, 2004).

2.4.1 Type I diabetes mellitus

2.4.1.1 Epidemiology

Type I diabetes, also known as insulin dependent diabetes mellitus (IDDM), is generally diagnosed in children or young adults with the most common acute symptoms being polyuria, polydipsia, tiredness, blurred vision and weight loss (Roche et al., 2005).
While data on sub-Saharan Africa are scarce (Mbanya et al., 2010), this form of the disease in Europe had an incidence of 94,000 in under age 15 years in 2005 and predicted to rise to 160,000 in 2030 (Patterson et al., 2009). In a study by Amos et al. (1997), the incidence of Type I diabetes in sub-Saharan Africa was estimated at 85,000 individuals for 1995. This study further speculated that the number of affected persons would increase to 219,000 by 2010. In contrast to the global population, an unusual finding amongst the African population is that the local population appears to develop the disease at an average age of 22 to 29 years (Motala, 2002).

### 2.4.1.2 Pathophysiology

Type I diabetes is a complicated disease and is usually a combination of a number of different factors, of which immunological factors are most important:

- **Immunological:** Type I diabetes is often the result of an autoimmune disease that causes the selective destruction of the insulin-producing β-cells of the pancreas (Morran et al., 2008). Numerous autoantibodies have been isolated from people with Type I diabetes, and include insulin autoantibodies (IAA) (which neutralises the produced insulin), islet cell antibodies (ICA) (which target and destroys the β-cells), and autoantibodies that target other pancreatic β-cell associated components such as glutamic acid decarboxylase (GAD) and tyrosine phosphatase-related IA-2 molecules (IA-2A) (all proteins found in pancreatic β-cells) (Morran et al., 2008; Knip and Siljander, 2008), and a pancreatic β-cell specific zinc transporter (ZnT8-Slc30A8) (Wenzlau et al., 2007).

- **Genetic:** Current evidence suggests the presence of a genetic predisposition to developing Type I diabetes. In Caucasians, the general incidence for the occurrence of Type I diabetes is only 0.4% for the entire population but substantially higher at 5 to 6% if one has an affected relative (Kelly et al., 2001). Genome-wide screens for susceptibility genes have identified more than ten chromosomal loci potentially associated with the disease (Cox et al., 2001; Van Belle et al., 2011), of which the two most important ones are the human leukocyte antigen (HLA) locus and the insulin gene locus, contributing approximately 42% and 10% respectively to familial inheritance (Morran et al., 2008). For the HLA locus, it has been speculated that mutations ona region located on chromosome 6may result in abnormalities which subsequently leads to the autoimmune response accounting for more than 50% of genetic susceptibility to Type I diabetes (Reimann et al., 2009). While the gene encoding insulin, located on chromosome 11 is another susceptible factor contributing 10% to genetic susceptibility (Reimann et al., 2009).
• **Environmental factors**: While speculative, a number of environmental factors have been tentatively linked to the induction of Type I diabetes (Reimann *et al.*, 2009). Factors blamed include viral exposure (such as enterovirus, adenovirus, Coxsackie B virus, cytomegalovirus, hepatitis C Virus, mumps virus, rotavirus and rubella virus) (Morran *et al.*, 2008), exposure to food contaminants bafilomycin A1 (bafA1, a macrolide antibiotic) or streptozotocin (Zimmet *et al.*, 2001) or other pathogens early in life that prime the immune system (Reimann *et al.*, 2009). Short term breastfeeding (~3 months) with early weaning onto cow milk (increased numbers of antibodies towards cow milk detected in children with newly diagnosed Type I diabetes) (Virtanen and Knip, 2003) and wheat-based diet with gluten (Van Belle *et al.*, 2011) may also induce immune intolerance.

Irrespective of the cause, the pathophysiology of Type I diabetes is linked to the absence of insulin production by the beta cells. With the absence of insulin, glucose is unable to enter into the cells, with resultant accumulation of glucose in the plasma leading to an initial hyperglycaemia, intracellular dehydration and osmotic diuresis (Guyton and Hall, 2000). As a result of inability to use an essential food source, the body shift energy utilisation to the fat stores. Unfortunately the use of fatty acids as an energy source is also eventually exhausted due to the depletion of the citric acid cycle intermediates, with subsequent accumulation and overproduction of the ketone bodies. In the absence of insulin, virtually all protein synthesis is also stopped while catabolism of protein increases. This degradation of amino acids leads to enhanced urea excretion in the urine, with resulting protein wasting and extreme weakness (Guyton and Hall, 2000). Finally the ketone bodies accumulate to such an extent that the body succumbs for keto-acidosis.

2.4.1.3 **Clinical presentation**

Patients with Type I diabetes, typically present with severe weight loss, polyuria and polydipsia while in severe cases patients may collapsed due to ketoacidosis (ketoacidosis is a potentially life-threatening condition caused by the build-up of ketone bodies with resultant drop of the physiological pH to 6.9) (Nelson and Cox, 2005; Guyton and Hall, 2000).

2.4.2 **Type II diabetes mellitus**

2.4.2.1 **Epidemiology**

Type II diabetes mellitus, also known as non-insulin dependent diabetes mellitus (NIDDM) or adult onset, is a slower form of the disease with milder symptoms (Nelson and Cox, 2005). Type II diabetes is associated with predominant insulin resistance with a relative insulin deficiency; or a predominant insulin secretory defect with or without insulin resistance (Zimmet *et al.*, 2001). Type IIis estimated to
account for approximately 90% of all diabetes mellitus cases globally (Goldstein, 2007). Most importantly this form of the disease is becoming more prevalent in children, teenagers and adolescents due to an increasing sedentary lifestyle and associated obesity (Pinhas-Haniel and Zeltler, 2005). In 2007, it was estimated that approximately 197 million people worldwide had impaired glucose tolerance (Hossain et al., 2007).

2.4.2.2 Pathophysiology of Type II diabetes

2.4.2.2.1 Factors Predisposing to Diabetes

- **Genetic predisposition:** Type II diabetes is foremost a disease that occurs within family groups which confers a 2.4 fold increased risk for the disease (Stumvoll et al., 2005). Children with one parent with Type II diabetes have a 38% risk of developing the disease while those with both parents having the disease have a 60% chance of developing the disease (Stumvoll et al., 2005).

- **Obesity:** About 90% of Type II diabetes is attributable to excess body weight, as a result of the interaction of fat and insulin function (Hossain et al., 2007). While excess fat in any region of the body increases the risk of developing Type II diabetes, abdominal fat (‘central’ obesity) appears to be a bigger risk factor (Montague and O’Rahily, 2000). From a medical standpoint, a person is considered obese when they have a body mass index (BMI) greater than 30 kg/m² as opposed to BMI not greater than 25kg/m² in healthy individuals (BMI is calculated as body weight in kilograms divided by the height in metres squared [kg/m²]) (Dixon et al., 2011).

  The levels of plasma free fatty acid are usually elevated in the obese and are most likely due to the free fatty acid release associated with excess visceral fat mass (Boden and Shulman, 2002). Studies have speculated that elevated circulating free fatty acid may contribute to the underlying pathophysiology of Type II diabetes, in particular, the development of insulin resistance both in muscle and the liver (Shulman, 2000; Boden and Shulman, 2002), due to pronounced lipolysis that directly blocks insulin signalling pathways (Milnar et al., 2007). The numerous hormones and inflammatory factors released by the fat tissue are also indicative of a link between obesity and diabetes (Reimann et al., 2009).

- **Urbanization:** Diabetes is a disease of urbanization with several studies associating the disease with higher prevalence in urban than in the rural areas within the same country (Jain and Saraf, 2010). While speculative, it has been suggested that urbanization is accompanied by a shift from labour intensive occupational activities to more sedentary service-based
occupations that require less energy due to computerization and mechanization and improved transport (Abubakari et al., 2009; Zimmet et al., 2001).

- **Low birth weight and thrifty phenotype**: Foetal under-nutrition and small birth weight for gestational age has been proposed as risk factors for Type II diabetes among adults and youth, as poor foetal growth appears to correlate with a reduced numbers of pancreatic β-cells and a concurrent diminished capacity to make insulin (Hussain et al., 2007). According to the thrifty phenotype hypothesis (Hussain et al., 2007; Hales and Barker, 2001), the combination of being undernourished in utero followed by a nutritionally overabundant environment later in life may unmask certain foetally programmed predispositions such as central adiposity, decreased pancreas β-cell growth, sub-normal insulin secretory response and insulin receptors functions (Hussain et al., 2007). These abnormalities in turn, may increase susceptibility to insulin resistance and Type II diabetes (Hussain et al., 2007; Eriksson et al., 2001). While the pathophysiology is not clear, it appears that the undernourished foetus may have less stimulus to produce insulin due to lower overall nutritional load within the body i.e. foetal β-cell development is stimulated by the level of foetal nutrition. As a result of the lower number β-cells, the foetus, when exposed to better nutrition post-natally, is more susceptible to obesity and subsequent diabetes later in life. It has also been speculated that people born thin at birth tend to be insulin resistant in adulthood as their metabolism is geared towards fuel conservation (Phillips, 1996).

- **Physical inactivity**: Physical inactivity independent of body size has been identified as a risk factor for Type II diabetes, with regular physical activity reducing the risk of Type II diabetes by 20-60% (Hussain et al., 2007). The WHO estimates that 41% of the global population are inactive, and up to 60% fail to achieve the recommended 30 minutes of moderate activity for most days of the week (Abubakari et al., 2009). Amongst those already afflicted by Type II diabetics, less fit individuals (people with less than 30 minutes activity per week) appear to have twice the risk of death in comparison to the fit (Wei et al., 2000). Physical activity of moderate and vigorously intensity and duration is associated with decreased risk of conversion of impaired glucose tolerance to diabetes, even in the absence of significant weight loss (Hussain et al., 2007). Exercise, or being physically active, enhances glucose uptake in skeletal muscle cells by increasing insulin dependent translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane, improves insulin mediated muscle blood flow, increases non-oxidative glycolysis and enhances glucose storage as muscle glycogen. More importantly, acute exercise increases glucose uptake into muscle by a mechanism that is distinct from the insulin stimulated phosphoinositol-3-kinase pathway (Hussain et al., 2007).
2.4.2.2 Factors resulting in TypeII diabetes

**Insulin resistance:** Insulin resistance is considered to be present when the biologically normal concentrations or supra-normal concentrations of insulin are inadequate to stimulate both glucose (≥140 mg/dl) uptake into the skeletal muscle and the suppression of endogenous gluconeogenesis within the liver, which results in high circulating blood glucose levels (Bryant et al., 2002). As for the decreased insulin production, decreased insulin response results in hyperglycaemia for the same reasons. The insulin resistance in muscle and liver does not result from a decrease in insulin receptor number but from a defect in the insulin signalling pathway (Virally et al., 2007). Insulin resistance in the muscle is characterised by defects at many levels including decreases in kinase activity, the concentration and phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), glucose transporter (GLUT4) translocation and the activity of intracellular enzymes (Virally et al., 2007). Insulin resistance in the liver is also characterised by defects at many levels including increases in glucagon concentration, glucose-6 phosphate and phosphoenolpyruvate carboxykinase (PEPCK) activity (enzymes of gluconeogenesis), concentration of plasma free fatty acids, hepatic fatty acid oxidation and acetyl-CoA (Virally et al., 2007).

**Pancreatic β-cell dysfunction (impaired insulin secretion):** In non-diabetic patients the β-cell adapts its secretory rate to the level determined by insulin sensitivity of the target tissues and blood glucose concentrations (Virally et al., 2007). Similarly in insulin-resistant states, the normal pancreatic β-cell responds to chronic blood glucose levels by increasing insulin secretion to maintain normoglycaemia in a process termed β-cell compensation. This compensation involves the expansion of β-cell mass, enhanced insulin biosynthesis, and enhanced β-cell function (Figure 2-11)(Prentki and Nolan, 2006). Type II diabetes results when the β-cell compensation can no longer be sustained at the level required to maintain normoglycaemia (Prentki and Nolan, 2006). While it is unknown if this dysfunction is genetic and/or acquired (Virally et al., 2007), β-cell dysfunction appears to be present early in the pathophysiology of the disease i.e. even before changes in blood glucose levels can be determined via clinical measurements (Prentki and Nolan, 2006).
Type II diabetes develops in response to excessive calorie intake and physical inactivity in people that have underlying genetic and/or acquired predisposition to both insulin resistance and/or hyperinsulinaemia and β-cell dysfunction. Over time, pancreatic β-cell compensation fails, resulting in a gradual decline in β-cell function. Consequently, there is a degeneration from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and finally to established Type II diabetes (T2D) (Prentki and Nolan, 2006).

2.4.2.2.3 Cellular mechanisms involved in β-cell dysfunction

- **The β-cell exhaustion and ER stress**: The compensating β-cell’s prolonged and chronic stimulation by the high blood glucose levels leads to the physical depletion of insulin stores so that further release of insulin is no longer possible (known as tachyphylaxis). The β-cell function does return to normal, once sufficient times is allowed for the re-synthesis and storage of insulin (Robertson et al., 2003). However, until such time that insulin stores are replenished, the circulation of glucose goes uncontrolled by the body. ER stress has been proposed as the potential cellular mechanism for β-cell exhaustion resulting from defect in proinsulin synthesis due to the high demand placed on the ER for the synthesis of proinsulin by the compensating islet of pancreatic β-cell (Prentki and Nolan, 2006).

- **Glucose toxicity**: The speculation that hyperglycaemia itself can desensitize β-cell and increase apoptosis led to the concept of glucose toxicity (Marchetti et al., 2008). It has been speculated that the high glucose concentration produces deleterious effects through several
mechanisms including increased oxidative phosphorylation, over activation of the hexosamine pathway and enhanced activity of protein kinase C (Marchetti et al., 2008). All these pathways lead to increased production of reactive oxygen species and consequently oxidative stress, changes in the expression of free radical scavenging enzymes and alteration of mitochondria (Marchetti et al., 2008). The major manifestation of glucose toxicity occurs in the β-cell evident as a defective insulin gene expression (defective pancreas duodenum homeobox-1 mRNA [PDX-1 mRNA]), diminished insulin content and/or defective insulin secretion (Robertson and Harmon, 2006).

- **Mitochondrial dysfunction with production of reactive oxygen species (ROS):** Prolonged hyperglycaemia in the obese promotes increased metabolism of glucose and free fatty acids through mitochondrial oxidation with resultant increased mitochondrial potential and superoxide production (Lowell and Shulman, 2005; Brownlee, 2003). Increased superoxide production causes increased exposure of the β-cell to ROS and activation of uncoupling protein-2 (UCP-2) (protein which uncouples oxidative phosphorylation) (Krauss et al., 2003) (Error! Reference source not found.). Chronically elevated levels of free fatty acids and glucose induce the UCP-2 genes in the β-cell. Increased UCP-2 helps to safely dissipate the elevated mitochondrial membrane potential, ROS and promote fuel detoxification (oxidation of these fuels becomes increasingly coupled to heat production rather than ATP production) at the expense of ATP synthesis and insulin secretion (Error! Reference source not found.)(Krauss et al., 2003; Prentki and Nolan, 2006).

![Figure 2-12: Superoxide production by the mitochondrial electron transport chain.](image)

Hyperglycaemia increases the levels of electron donors (NADH and FADH₂). This generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane and consequently inhibits electron transport at complex III, increasing the half-life of free radical intermediates of coenzyme Q which reduces O₂ to superoxide (Brownlee, 2001).
• **Islet amyloid:** Amyloid deposits are found in most but not all individuals with TypeII diabetes (Knowles *et al.*, 2002). This islets amyloid consists of deposits of amyloid polypeptide, also known as amylin that is normally co-secreted with insulin at a tenfold lower rate. While the physiological role of islet amyloid polypeptide is unclear, it is postulated that the initial demands of insulin secretion lead to increase in production and subsequent aggregates which appear to be cytotoxic.

### 2.4.3 Other forms of diabetes

Gestational diabetes is another form of diabetes, defined as a state of glucose intolerance during pregnancy that usually subsides after delivery but has a major implication for subsequent risk of Type II diabetes, as pregnancy serves as an ‘environmental’ stressor that reveals a genetic predisposition (Permutt *et al.*, 2005). Other less common forms of diabetes include the rare genetically determined disease maturity onset diabetes of the young (MODY), diabetes resulting from surgery, and other illnesses that constitute only 1-5% of cases (Permutt *et al.*, 2005).

### 2.4.4 Complications of diabetes

The following are some of the most common complications of diabetes

• **Retinopathy:** This is the most common complication of diabetes that damages the eyes. It is a disorder that can affect the peripheral retina, the macular, or both and is a leading cause of visual disablement and blindness in people with diabetes (Cade, 2008). Total or partial loss of vision is characterised vitreous haemorrhage or retinal detachment while central loss of vision is through retinal vessel leakage and macular edema (Cade, 2008).

• **Nephropathy:** This is a complication of diabetes that damages the kidneys. The kidney is made up of hundreds of thousands of small units called nephrons. These nephrons filter blood and remove waste from the body. However in people with diabetes, the nephrons are modified by the thickening of the basement membrane and result in reduced filtration, albuminuria and renal failure (Ahmed, 2005).

• **Neuropathy:** This is a complication of diabetes that damages the nerves. It is characterised by segmental demyelination and axonal degeneration of peripheral neurons thus resulting in reduced nerve conduction and blood flow. It may present clinically as pain or numbness or limbs or as impotence in men (Ahmed, 2005).
• Wound healing: Normal wound healing occurs in a complex co-ordinated sequence of events, cells migrate into the wound, inflammatory response, the proliferation of different cell types, formation of matrix components, remodelling and closure of the wound. Impaired wound healing is common in diabetic patients and it is the leading cause of amputations (Ahmed, 2005).

2.4.4.1 Oxidative stress and complication of diabetes

Oxidative stress is the excessive generation of highly reactive molecules (free radicals) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), or insufficient removal of them (Johansen et al., 2005). Studies have shown that prolonged chronic hyperglycaemia can lead to oxidative stress provoked by hyperglycaemia-induced generation of free radicals (Error! Reference source not found.) or by the impairment of endogenous antioxidant defense, and has been implicated in the development and progression of diabetic complication (Johansen et al., 2005).

Figure 2-13: Speculated pathways by which glucose metabolism can lead to the production of reactive oxygen species (ROS) (Robertson, 2004).

Under physiological conditions, glucose primarily undergoes glycolysis and oxidative phosphorylation (6). Under pathological conditions of hyperglycaemia, high glucose levels can overwhelm the glycolytic process and inhibit glyceraldehyde catabolism, which causes glucose, fructose 1,6-bis phosphate and glyceraldehyde 3-phosphate to be shunted to other pathways: (1) enolization and α-ketoaldehyde formation, (2) PKC activation, (3) dicarbonyl formation and glycation, (4) sorbitol metabolism, and (5) hexosamine metabolism.
2.4.4.2 The mechanisms involved in the complications of diabetes

The following are two of the speculated mechanisms through which hyperglycaemia-associated free radical can lead to diabetic complications in insulin independent tissues.

- Increased intracellular advanced glycation endproducts (AGEs) formation: With hyperglycaemia, intracellular glucose concentration rises and glucose reacts non-enzymatically with proteins to form advanced glycation endproducts (AGEs). AGEs bind to structural components of the connective tissue matrix or basement membrane, such as collagen, resulting in sclerosis of the renal glomeruli, thickening of the capillary basement and atherosclerosis (Lapolla et al., 2005). These AGEs can also bind to specific receptors (RAGEs) in smooth muscles, monocytes, macrophages and endothelial cells leading to the an increase in the production of NFκB, a transcription factor involved in vascular function, inflammation and pathology (Giacco and Brownlee, 2010).

- Increased polyol pathway flux: The polyol pathway is a sub-pathway for glycolysis (Mizuno et al., 1999) catalysed by aldose reductase that reduces glucose by NADPH to sorbitol (polyol). Aldose reductase is found in tissues such as nerves, retina, lens, glomerulus and vascular cells (Giacco and Brownlee, 2010). In these tissues that are insulin independent, hyperglycaemia activates the aldose reductase, increases polyol pathway flux and damages the cells through increase in redox stress (Giacco and Brownlee, 2010). In addition the polyol pathway also oxidises sorbitol to fructose by sorbitol dehydrogenase. The formation of both sorbitol and fructose deplete NADPH in cells causing redox imbalance (Edwards et al., 2008). The activation of the sorbitol pathway has been implicated in the lens cataract formation, peripheral neuropathy and nephropathy (Maritim et al., 2003).

2.5 Management and treatment of diabetes

The aim of therapy in Type II diabetes is to reach normal glycaemia levels. With insulin resistance being fundamental in the pathogenesis of Type II diabetes, intervention is initially aimed towards improvement in tissue sensitivity/responsiveness. This often involves lifestyle intervention, with moderate exercise and weight loss, and pharmacological management (Tuomilehto et al., 2001).

2.5.1 Therapy for weight loss

Lifestyle intervention through a low calorie diet and regular exercise is the best approach for patients with obesity and Type II diabetes. However, the physiological abnormalities of diabetes may make
weight loss more difficult and harder to maintain, therefore therapy (weight reducing drugs and surgery) promoting weight loss can be an effective adjunct when appropriate (Lee and Aronne, 2007).

2.5.1 Pharmacologic agents for weight loss

Numerous pharmacotherapies for weight loss in overweight or obese patients are available and have different mechanisms including:

2.5.1.1 Appetite suppressants

Most appetite suppressants work primarily by increasing the availability of anorexogenic neurotransmitters which are norepinephrine, serotonin, dopamine, or some combination of these neurotransmitters in the central nervous system (Yanovski and Yanovski, 2002). Noradrenergic agents include phendimetrazine and benzphetamine. Serotonergic agents act by increasing the release of serotonin, inhibiting its reuptake or both. The mixed noradrenergic-serotonergic agents e.g. sibutramine, is an inhibitor of both norepinephrine reuptake and serotonin reuptake and also weakly inhibits dopamine reuptake (Yanovski and Yanovski, 2002). Tachycardia, dry mouth, constipation, nervousness, insomnia and high blood pressure are some of the associated side effects (Moyers 2005). For safety reasons fenfluramine, dexfenfluramine and silbutramine have been withdrawn for sale in numerous countries (Moyers, 2005).

2.5.1.1.2 Decreased absorption of fats

Gastric and pancreatic lipases are required to hydrolyse triglycerides from dietary fat into absorbable free fatty acids and monoglycerides (DeWald et al., 2006). Through the inhibition of these lipases, the digestion and absorption of ingested dietary fat and fat soluble vitamins is reduced (DeWald et al., 2006). Tetrahydrolipstatin (Orlistat) inhibits gastric, pancreatic and pancreatic carboxylester lipase locally in the lumen of the stomach and small intestine with an estimated reduction of fat absorption by about 30% (Leung et al., 2003). The side effects associated with use of orlistat include reduction in absorption of fat soluble vitamins, flatus with discharge, increased defaecation and oily spotting (Moyers, 2005).

2.5.1.1.3 Increased metabolism/thermogenesis

Ephedrine and caffeine are thermogenic agents that promote weight loss by enhancing energy expenditure. Thermogenesis may be achieved through central activation of the sympathetic nervous system or peripherally by stimulation of atypical adrenoreceptors (DeWald et al., 2006). The side
effects from this combination include tremor, tachycardia, increased systolic blood pressure, insomnia and dizziness (DeWald et al., 2006).

2.5.1.2 Surgical therapy

There is evidence that significant weight loss achieved using lifestyle and pharmacological methods by obese, particularly severely obese people is modest and rarely sustained because of the constant upward pressure of counter-regulatory mechanisms of body weight (Lee and Aronne, 2007) making surgery an option (Dixon et al., 2011).

Bariatric surgery for weight loss can be classified into 2 groups; intestinal malabsorption and gastric restriction (DeWald et al., 2006).

2.5.1.2.1 Malabsorptive procedures

The aim of the malabsorptive procedures is to limit the surface area available for absorption in the small intestines. The procedures that have been used are:

- **Jejunoileal bypass**: In this procedure the proximal jejunum is anastomosed to the terminal ileum. The complications of this procedure include liver failure, cirrhosis, oxalate kidney stone, protein malnutrition, metabolic bone disease and renal dysfunction (DeWald et al., 2006).

- **Biliopancreatic diversion**: This involves diverting biliary and pancreatic secretions to distal 50 cm of ileum(DeWald et al., 2006). The complications associated with this procedure include protein-calorie malnutrition, metabolic bone disease, deficiencies in fat soluble vitamins, iron, calcium and vitamin B12.

- **Duodenal switch**: The duodenal-switch procedure connects the jejunum rather than the ileum to the proximal duodenum (DeWald et al., 2006).

2.5.1.2.2 Restrictive procedure

In contrast to limiting absorption, the restrictive procedures limit the total amount of food a person may consume:

- **Gastroplasty**: this restricts the storage capacity of the stomach to decrease the ability to consume solid foods. Because the procedure uses surgical stapling devices, it is also called a gastric stapling. This procedure consists of construction of a small pouch of the upper stomach
with a restricted outlet along the lesser curvature. The stomach is partitioned with staples and a window is created in the stomach to create the outlet (Ellison and Ellison, 2008). Complications include staple line disruption, pouch dilatation, anaemia, outlet obstruction, incisional hernia and gastroesophageal reflux disease (Ellison and Ellison, 2008).

- **Gastric banding**: this procedure involves an adjustable hollow silastic band placed around the proximal stomach, creating a restrictive pouch. A tube and port are extended from the band to the anterior rectus sheath. The band is secured with gastrogastriac sutures to prevent slippage. The port can be accessed through the skin to adjust the band size based on weight loss results (Ellison and Ellison, 2008). The absence of staple line and the associated risk of staple-line dehiscence are theoretical advantage of this technique (DeWald et al., 2006). Complications include gastric slippage, gastric stoma obstruction, band erosion, gastric injury and necrosis, esophageal and gastric pouch dilatation (Ellison and Ellison, 2008).

- **Gastric bypass**: this procedure consists of construction of a proximal gastric pouch with a Y-shaped outlet of jejunum of varying length. The stomach is partitioned by a staple line bypassing the lower stomach. This procedure is both a restrictive and malabsorptive procedure with the degree of malabsorption determined by the length of the jejunum attached at the stomach outlet (Ellison and Ellison, 2008). Complications include wound infections, leaks from the staple/suture anastomosis line, acute gastrointestinal obstruction, gastrointestinal hemorrhage, stomal/marginal ulcer, vitamins and mineral deficiencies and cholesterol stone (Ellison and Ellison, 2008).

Although bariatric surgical procedures have been shown to effectively achieve weight loss in many obese Type II diabetes patients as they decrease food consumption (Rubino, 2006), there are a number of associated complications.

### 2.5.2 Pharmacological therapy of insulin response or release

#### 2.5.2.1 Reduction of absorption of carbohydrate

Restriction of carbohydrate-containing foods is a method of reducing alimentary absorption of carbohydrate (Virally et al., 2007). Starting from the very beginning of carbohydrate metabolism, release of glucose and transport across the intestinal brush border membrane down to the blood stream, have been targets to reduce the post-prandial hyperglycaemic peak(s). This has been achieved by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase, or manipulating the glucose transporter (SGLUT)in the
digestive tract. Acarbose, miglitol and voglibose are competitive inhibitors of the α-glucosidases, the enzymes located in the brush border of enterocytes responsible for cleavage of oligosaccharides. This inhibitory effects delays glucose absorption and consequently decreases the postprandial glucose peak and insulin response to a meal (Virally et al., 2007). Flatulence, abdominal distension and diarrhoea resulting from the fermentation of unabsorbed carbohydrate in the bowel and occasionally increased liver enzymes are some of the adverse side effects resulting in treatment cessation or poor compliance (Virally et al., 2007).

2.5.2.2 Increased pancreatic insulin secretion

The sulfonylurea class of drugs (e.g. glibenclamide, tolbutamide, talazamide, glyburide, glipizide) increase in insulin secretion by binding to a specific sulfonylurea receptor (SUR-1) of the K⁺-channel of the pancreatic β-cell (Virally et al., 2007), thereby stimulating the closing of the ATP-sensitive potassium channel (K_ATP). The decrease in potassium results in depolarization of the β-cell membrane, calcium flux and exocytosis of insulin (Virally et al., 2007). The efficacy of sulfonylurea drugs is therefore limited to the number of functional β-cells, which due to progressive loss of β-cells over time renders them ineffective after a period of time. Hypoglycaemia, weight gain, hyperinsulinaemia and hepatic and renal impairment are some of the adverse effects of sulfonylureas (Stumvoll et al., 2005).

2.5.2.3 Nonsulfonylurea insulin secretagogue

Glitinides (e.g. repaglinide, netaglinitde) are non-sulfonylurea insulin secretagogues that require the presence of glucose for action and work by closing an ATP-sensitive potassium channel. This class of drug use at least two receptor binding sites on the pancreatic β-cell, and one of these sites is the sulfonylurea receptor (SUR-1) (Virally et al., 2007). These agents have a shorter action than the sulfonylurea drugs and are associated with lower risk of hypoglycaemia (Rendell, 2004). Hypoglycaemia, weight gain and hyperinsulinaemia are some of the associated adverse side effects (Virally et al., 2007).

2.5.2.4 Reduced hepatic glucose output

Thebiguanides (e.g. metformin) reduce of hepatic glucose production (gluconeogenesis and glycogenolysis) and to a lesser extent improves peripheral insulin sensitivity (Hundal and Inzucchi, 2003). The mechanism of action of metformin while speculative has been attributed to the stimulation of adenosine monophosphate activated protein kinase (AMPK) (Hundal and Inzucchi, 2003). Metallic taste, abdominal discomfort, anorexia and diarrhoea are adverse side effects and sometimes lead to drug discontinuation by patients (Virally et al., 2007).
2.5.2.5 Increased insulin sensitivity

The thiazolidinedione (e.g. pioglitazone, rosiglitazone) primarily activate PPARγ (peroxisome proliferators activated receptor γ) receptors in adipose tissue and subsequently alter adipose metabolism and distribution (Nolan et al., 2000). The thiazolidinediones also reduce circulating concentrations of pro-inflammatory cytokines that promote insulin resistance (e.g. TNF-α and interleukin 6) and at the same time increase concentrations of adiponectin, which has insulin-sensitising and anti-inflammatory properties. The multiple effects of thiazolidinediones on adipose tissue metabolism and cross-talk of these signals with liver and skeletal muscle, as well as pancreatic β-cells, may account for the enhancement of insulin action and improvement in insulin secretion (Stumvoll et al., 2007). Unlike metformin, thiazolidinediones act mainly at peripheral level and to a lesser degree on hepatic glucose production (Virally et al., 2007). Fluid retention, weight gain, congestive heart failure, oedema and anaemia are some of the associated adverse side effects.

2.5.2.6 Exogenous insulin

Exogenous insulin is the only available treatment for patients with the absolute insulin deficiency (Type I diabetes) while in Type II, because of the progressive nature of the disease diabetes, most patients will eventually require insulin (Fonseca and Kulkarni, 2008). However in contrast to physiological insulin which is under fine endogenous control, the administration of exogenous insulin is intermittent with resultant poorer responses. This can result in hypoglycaemia (sometimes fatal) from mismatch occurring between carbohydrate intake and circulating insulin concentrations. Insulin analogues with different pharmacokinetic profiles that are less likely to cause hypoglycaemia when used appropriately have been developed; however, cost currently restricts their use in developing countries (Bolli et al., 1999; Sachs and Bonner, 2000). Weight gain and hypoglycaemia are some of the risk factors of insulin therapy.

2.5.2.7 Limitation of conventional therapy

While these therapies can control many aspects of diabetes, none has so far convincingly demonstrated that they can significantly alter the progressive loss of pancreatic insulin secretion that would eventually require exogenous insulin supplementation. Furthermore despite the availability of therapeutic agents, the pathological complications associated with the disease and the mortality continues to increase. In addition the conventional treatments available for the management of Type II diabetes have their own inherent side effects. As a result, newer and more effective drugs/compounds need to be developed.
One of the suggested methods to overcome this problem would be the use of herbal remedies, and these herbal drugs have been prescribed widely because of their perceived effectiveness, fewer side effects and relatively low cost (Venkatesh et al., 2004). Proof for this comes from the extraction of metformin, from *Galega officinalis* (Goat’s Rue, French Lilac) which was used as a treatment for diabetes in Medieval Europe (Bailey and Day, 2004).

### 2.6 Medicinal plants and diabetes

Traditional medicine, derived mainly from plants, play a major role in the management of diabetes mellitus in developing countries where most people have limited resources and do not have access to modern treatment (Ali et al., 2006). Furthermore, about 80% of the world’s population makes use (wholly or partly) of plant-based drugs (Kamboj, 2000). There is also an increase in demand in industrially-developed countries to use alternative approaches to treat diabetes, such as plant-based medicines, due to the side effects associated with the use of insulin and oral hypoglycaemic agents (Ali et al., 2006).

In South Africa, like many African countries, medicinal plants and herbal formulations play an important role in the daily health care of the people (Anonymous, 2002). The country is rich in floral biodiversity which has provided health practitioners and traditional healers with an immense pool of “natural pharmacy” from which ingredients are selected for the preparation of herbal medicine (Mahomed and Ojewole, 2006). It is estimated that more than 4000 plant taxa are used in herbal formulations countrywide (Mulholland, 2005). Katerere and Eloff (2006) identified many African species that have been used traditionally to treat diabetes.

At present more than 200 pure compounds isolated from plant sources have been reported to show blood glucose lowering activity *in vivo* (Broadhurst et al., 2000). Amongst these compounds are polyphenols, alkaloids, glycosides, saponins, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions (Mukherjee et al., 2006). Of these, the polyphenols have appeared to be the most promising agents.

#### 2.6.1 Polyphenols

Polyphenols are a large and heterogeneous group of phytochemicals of plant based food such as tea, coffee, wine, cereal grains, vegetables, legumes and fruits (Hanhineva et al., 2010). Polyphenols are plant secondary metabolitessynthesized as protective agents from biological and environmental stresses (Pandey and Rizvi, 2009). The structural diversity of the polyphenols extends from simple...
one-phenol hydroxybenzoic and hydroxycinnamic acids to large polymeric molecules like proanthocyanidins and ellagitannins (Hanhineva et al., 2010). The two main types of polyphenols are flavonoids and phenolic acids with flavonoids distributed among various classes of flavones, flavonols, flavanols, flavanones, isoflavones, proanthocyanidins and anthocyanins (Scalbert et al., 2005). Some of the common flavonoids are quercetin, a flavanol in onion, tea and apple; catechin, a flavanol found in tea and several fruits; hesperetin, a flavanone in citrus fruits; cyanidin, an anthocyanin responsible for the colour of many red fruits; daidzein, isoflavone in soybean; and proanthocyanidin responsible for the astringency or bitterness of grape or cocoa (Scalbert et al., 2005). Phenolic acid such as caffeic acid is present in many fruits and vegetables, and ferulic acid is present in cereals (Scalbert et al., 2005).

2.6.2 Polyphenols and diabetes

Numerous mechanisms have been evaluated for the ability of the polyphenols to reduce blood glucose:

- **Precipitation of proteins:** One of the best-known properties of polyphenolic compounds, with the exception of the low molecular weight phenols, is their capacity to bind and precipitate protein (Bravo, 1998) with the highly polymerized tannins being the most effective. With the gastrointestinal tract being rich in digestive enzymes, polyphenols may be able to inhibit these enzymes with subsequent reduction in dietary carbohydrate hydrolysis and the postprandial glycaemic response (Bravo, 1998). A variety of polyphenols have been shown to inhibit α-amylase and α-glucosidase activities *in vitro* (Hanhineva et al., 2010). Matsui et al. (2001a) demonstrated that a polyphenolic rich extracts from the sweet potato (*Ipomoea batatas* L.) roots and morning glory flowers (* Pharbitis nil* cv. Scarlett O’Hara) enriched in anthocyanins were effective inhibitors of rat intestinal α-glucosidase and human α-amylase. Subsequently, they demonstrated that diacylated anthocyanins from these sources were most effective against α-glucosidase (Matsui et al., 2001b) and were capable of inducing an anti-hyperglycaemic effect in rats (Matsui et al., 2002). Specific isolated anthocyanins that inhibited α-glucosidase activity *in vitro* are Cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside (Cazarolli et al., 2008). Other polyphenols such as (+) catechin, (-) epicatechin, (-) epigallocatechin and epicatechin gallate from tea, isoflavones from soybeans, phenolic compounds, tannic acids and chlorogenic acid have also been shown to be potent inhibitors of α-glucosidase (Tiwari and Rao, 2002).

- **Competition with glucose:** Flavonoids are speculated to be absorbed in the intestine and in some cases compete with glucose in unknown absorption mechanisms (Cazarolli et al., 2008). A soybean extract containing genistein and daidzedin isoflavonoids decreased *in vitro* glucose
absorption into the brush border membrane vesicles of rabbits while naringenin reduced glucose uptake in the intestinal brush border vesicles of diabetic rats (Cazarolli et al., 2008).

- **Glucose transport**: The influence of polyphenols on glucose transporters had been studies *in vitro* and several flavonoids and phenolic acids have been associated with inhibition of glucose transport. (-) Epicatechin gallate (-) epigallocatechin gallate and (-) epigallocatechin markedly reduced glucose absorption through the competitive inhibition of sodium-dependent glucose transporter-1 (SGLUT1) (Cazarolli et al., 2008). The sodium-dependent SGLUT1 mediated glucose transport was also inhibited by chlorogenic, ferrulic, caffeic and tannic acids, quercetin monoglucosides, tea catechins and naringenin while glucose transporter GLUT2 was inhibited by quercetin, myricetin, apigenin and tea catechins (Hanhineva et al., 2010).

- **Insulin utilisation**: Polyphenolic fractions from plants may also alter glucose utilization in mammals through an insulin-like effect (Broadhurst et al., 2000). The supplementation of genistein and rutin in diabetic rats increased plasma insulin concentrations (Cazarolli et al., 2008). Within the anthocyanins and anthocyanidins group, cyanidin-3-glycoside and delphinidin-3-glycoside potent insulin secretagogues while pelargonidin-3-glycoside and its aglycon, pelargonidin, have also been able to significant increase in insulin secretion (Cazarolli et al., 2008).

- **Regeneration of pancreatic beta cells**: Polyphenols from some plants reportedly also have the ability to stimulate pancreatic β-cells regeneration. Intraperitoneal injection of (-) epicatechin in alloxan treated mice demonstrated β-cell regenerative capacity; similarly soybean isoflavoniods, genistein and daidzed in preserved the insulin production by β-cells (Hanhineva et al., 2010), while quercetin increased the number of pancreatic islets in both normoglycaemic and diabetic rats (Cazarolli et al., 2008).

### 2.6.3 Polyphenols and obesity

Some dietary polyphenols have been shown to modulate physiological and molecular pathways that are involved in energy metabolism, adiposity and obesity, which may facilitate weight loss and prevent weight gain (Meydani and Hassan, 2010). Several studies have demonstrated that consumption of green and white teas containing catechins, in particular epigallocatechin gallate (EGCG), fruits such as blueberries with anthocyanins, red grapes and wine with resveratrol and spices like turmeric which contain curcumins may stimulate weight loss (Meydani and Hassan, 2010). The mechanisms through which these polyphenols induce weight loss was speculated to include
suppression of fat absorption from the gut, uptake of glucose by skeletal muscles, suppression of anabolic pathways, stimulation of apoptosis of matured adipocytes, and reduction of chronic inflammation associated with adiposity (Meydani and Hassan, 2010).

2.6.4 Polyphenols and antioxidant defence

Polyphenols with catechol groups (aromatic rings with two hydroxyl groups in the ortho position) have greater antioxidant potency than those with simple phenol groups (aromatic rings with a single hydroxyl group). Quercetin and epigallocatechin, for example, have been shown to be excellent traps for ABTS+ and are almost five times more active than trolox, a water-soluble vitamin E analogue, at equivalent molar concentrations (Scalbert et al., 2005). It has been shown in several in vitro studies that the relatively polar polyphenols regenerate or spare lipophilic antioxidants, such as vitamin E while antioxidant effects of polyphenols may be more easily observed when the status in other antioxidants is low (Scalbert et al., 2005). The antioxidant efficiency of polyphenols may depend on the extent of absorption and metabolism of these compounds, as well as the activity of methoxylated and conjugated forms circulating in plasma (Bravo, 1998).

It has been demonstrated that for some plant extracts at least, a positive correlation exists between antidiabetic activity and flavonoid content (Sezik et al., 2005; Aslan et al., 2007). The phenolic groups in the polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components (Scalbert et al., 2005). As antioxidants, these compounds may prevent the progressive impairment of pancreatic beta-cell function due to oxidative stress (Song et al., 2005).

2.6.5 Potential risk associated with polyphenols

While several studies have focused mainly on the beneficial effects of polyphenols, there are likely to be side effects with increased intake of polyphenols, particularly beyond common levels of exposure associated with the diet. Many of the proposed side effects are speculative. Flavonoids may influence the thyroid function and have goitrogenic effects. A reduction in thyroid peroxidase activity was observed in rats fed a diet supplemented with genistein, and these effects of genistein and other related compounds (such as daidzein, kaempferol, quercetin or naringenin) on thyroid function are more pronounced when iodine is deficient (Scalbert et al., 2005). High intake of catechins or green tea extracts containing very high concentration of EGCG causes hepatotoxicity, inflammation of and necrotic damage to the liver (Meydani Hasan, 2010). The catechol group of polyphenols (coffee, tea, wine) directly chelate iron from the gut thus inhibiting non-heme iron absorption especially when polyphenols are consumed together with the source of iron, though the impact of dietary polyphenols
on iron status is not as firmly established (Scalbert et al., 2005). There are reports of animals consuming plants rich in tannins showing signs of acute toxicity while no such acute toxicity has been reported in human consumption of dietary polyphenols (Scalbert et al., 2005).

2.7 Selection of plant species

The use of *Ficus* species as food or pharmacological agents to improve human health has a history of about eleven thousand years in Ayurvedic and Traditional Chinese Medicine (TCM) (Lansky et al., 2008). The genus *Ficus*, commonly referred to as fig, is a genus of about 800 species of woody trees, shrubs and vines in the family Moraceae found in all tropical habitat types with about 100 species in Africa and the surrounding islands (Serrato et al., 2004). Many of these species possess auxiliary aerial root systems extending to the ground from their branches or trunks. All *Ficus* species possess latex-like material within their vasculature, affording protection and self-healing from physical assaults (Lansky et al., 2008).

2.7.1 Ethnomedical uses of *Ficus* species

In folk medicine, *Ficus* species are reported to have hypotensive, antidiabetic, mild laxative, antirheumatic, antibacterial and digestive activities. They are also used to treat cough, chest conditions and as an anthelmintic against intestinal parasites. They have been used as anti-inflammatory agents in urinary tract infections, sore throats, ulcerated noses, to reduce fever, and to cure tuberculosis and piles. Externally, they have been used to treat eczema, to cure tinea and leprosy, to treat cracks in the soles of the feet, and as a dressing for boils (Watt and Breyer-Brandwijk, 1962; Ramadan et al., 2009).

From what is known about the chemistry of the different species and from the genetic commonalities between different *Ficus* species, there seem to be some overlap, and similarities between different *Ficus* species do occur (Lansky and Paavilainen, 2010). Therefore, if a particular activity has been demonstrated to occur in one *Ficus* species, another *Ficus* species could be an alternative and possibly better source of the same activity and may be more easily obtainable (Lansky and Paavilainen, 2010). Phytochemical investigations of some *Ficus* species revealed that polyphenolic compounds constitute the major components of stembark (Li et al., 2006; Sandabe et al., 2006). Also present are triterpenes of different types, sterols, flavonoids, coumarins, alkaloids and other miscellaneous compounds (Ramadan et al., 2009). Some other studies reported the presence of antioxidant activity of some *Ficus* species which attributed the antioxidant activity to the polyphenolic content (Abdel-Hameed, 2009).

A survey of the literature indicates that about 12 *Ficus* species have *in vivo* antidiabetic activity from laboratory animal diabetic studies. These are *Ficus banghalensis* L. (Elder, 2004), *Ficus carica* L. (Perez et al., 1996), *Ficus racemosa* L (Rao et al., 2002), *Ficus hispida* L. f. (Ghosh et al., 2004),
Ficus deltoidea (Adam et al., 2012), Ficus microcarpa L.f. (Kumar et al., 2007), Ficus arnottiana Miq. (Mazunder et al., 2009), Ficus religiosa L. (Pandit et al., 2010), Ficus thonningii Blume (Musabayane et al., 2007), Ficus glumosa Del. (Madubunyi et al., 2012), Ficus glomerata Roxb. (Kar et al., 2003) and Ficus sycomorus L. (Adoun et al., 2011). However, of the 23 Ficus species growing in South Africa (Jordaan, 2000), very little research has been done on their antidiabetic activity.

From a search of the available literature, there was only one report by Musabayane et al. (2007) on the effect of Ficus thonningii stem bark ethanolic extract on blood glucose. Based on the scant ethnobotanical and ethnomedical information available for the South African species, ten South African Ficus species were selected for antidiabetic investigation. These plants are: Ficus capreifolia, Ficus cordata, Ficus craterostoma, Ficus glumosa, Ficus lutea, Ficus natalensis, Ficus poita, Ficus religiosa, Ficus sycomorus and Ficus thonningii.

2.8 Conclusion

Diabetes mellitus is a state of persistent and chronic hyperglycaemia which once thought to be an elite disease is now one of the most prevalent non-communicable diseases in recent times with severe clinical signs. Weight loss through increased physical activity in addition to low calorie food has been long suggested to improve glucose tolerance. However, since it is not easy for people to change habits formed over years, drugs and bariatric surgery are alternative weight loss therapy suggested for obese Type II diabetic. Unfortunately due to severe side effects associated with the use of these commercially available therapies, failure of the current agents to alter the natural progress of the disease newer therapies are needed. Herbal medicines, used traditionally for the treatment of many diseases including diabetes may serve as a source of these alternates. For this study 10 Ficus species will be explored as alternative therapies for diabetes. The description of the species used is provided in chapter 3.
Chapter 3

3 Materials and Methods

3.1 Materials

3.1.1 Reagents and chemicals

Folin-Ciocalteu reagent, gallic acid, sodium carbonate (Na₂CO₃), potassium persulphate, ABTS [2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)], potato starch, porcine pancreatic α-amylase enzyme (type VI), sodium chloride (NaCl), sodium phosphate, 3, 5-dinitrosalicylic acid (DNS), sodium potassium tartrate, sodium hydroxide (NaOH), acarbose, rat intestinal acetone powder, potassium phosphate, TRIS (hydroxymethyl) aminomethane, the glucose oxidase kit (GAGO 20) and trolox (a synthetic water soluble vitamin E analogue), bovine serum albumin (BSA), minimal essential medium (MEM), Dulbecco’s MEM (DMEM), Roswell Park Memorial Institute medium 1640 (RPM1640), foetal calf serum (FCS), glutamine, trypsin-EDTA, berberine chloride, phosphate buffered saline (PBS), sodium pyruvate, Hanks balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES), collagenase (type 11), penicillin/streptomycin, glucose oxidase kits and D-glucose were purchased from Sigma (South Africa). Gentamicin (Virbac), insulin (Sanof; Aventis), Trypan blue (Fluka), doxorubicin (Pfizer) and Insulin (Rat) ELISA kit (DRG Instruments GmbH, Germany Frauenbergst). Sodium hydrogen carbonate (NaHCO₃), potassium chloride (KCl), calcium chloride (CaCl₂), magnesium sulphate (MgSO₄), potassium hydrogen phosphate (KH₂PO₄), dimethyl sulphoxide (DMSO), acetone, methanol, hydrogen chloride (HCl), sulphuric acid (H₂SO₄) and Whatman No. 1 filter paper were purchased from Merck (South Africa). Corn oil was purchased from the University of Pretoria, Faculty of Veterinary Sciences pharmacy, condensed milk (clover) was bought from a local supermarket and the standard rodent chow was sourced from Epol foods. The absorbance measurements were read using a microtitre plate reader (VERSAmax, Molecular Devices Labotec).

3.1.2 Cell lines and primary cell cultures

C2C12 mouse muscle myoblast (CRL-1772), 3T3-L1 mouse pre-adipocytes fibroblast (CL-173), H411E rat hepatoma (CRL-1548), RIN-m5F rat insulinoma (CRL-11065), C3A human liver cells (CRL-10741) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and Vero African green monkey kidney cells were obtained from the Department of Veterinary Tropical Diseases (Faculty of Veterinary Sciences, University of Pretoria)(The cultures have been maintained for over ten years and are not deposited at the ATCC). The tissues/organs (abdominal muscle and epididymal fat pad) used in this study for isolation of cells were collected as waste by-products from
other animal studies. Ethics approval was received from the Animal Use and Care Committee (AUCC), University of Pretoria, for animal use with approval number V069-09.

3.1.3 Plant material

The leaves of ten *Ficus* species were collected at the Manie van der Schijff Botanical Garden (University of Pretoria), South Africa in February 2009, and voucher specimens were conserved in the HGWJ Schweikerdt Herbarium of the University of Pretoria. The names of plant species and voucher numbers are as follow: *Ficus capreifolia* Delile - PRU 33124; *Ficus cordata* Thunb - PRU 35501; *Ficus craterostoma* Mildbr. & Burret - PRU 48293; *Ficus glumosa* Delile - PRU 38554, *Ficus lutea* Vahl - PRU 074568; *Ficus natalensis* Hochst - PRU 078082; *Ficus polita* Vahl - PRU 35945; *Ficus religiosa* L. - PRU 115415; *Ficus sycomorus* L. - PRU 066173; and *Ficus thonningii* Blume - PRU 57036. The leaves were separated from the stems and dried at room temperature. The dried plant materials were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eierz® Bramley) and stored at room temperature in closed glass bottles in the dark until extracted (Eloff, 1998a).

3.1.4 The selected *Ficus* species

3.1.4.1 *Ficus capreifolia* Delile (sandpaper fig)

It is a shrub, but may become a small, slender tree up to 7 m; occurring in swamps, and frequently forming tangled thickets along river banks and on sandy islands in the larger rivers. The bark is greenish grey to whitish and smooth, and the branches are often long and whip-like. The leaves are alternate or opposite (unusual in *Ficus*); elliptic with the surface very rough and scabrid resembling sandpaper. Figs are borne singly on fairly long slender stalks in the axils of the leaves, and are pear-shaped, and green to yellow in colour (Coates-Palgrave, 2002).

Figure 3-1 *Ficus capreifolia* (van Noort and Rasplus, 2004)
Traditional use includes treatment of infections (Duke, 1975). In Tanzania the leaf sap and a decoction of the root are taken for the treatment of schistosomiasis, and the leaf sap or the powdered root is sprinkled on syphilitic ulcers (Brink, 2010) (Figure 3-1).

3.1.4.2 *Ficus cordata* Thunberg (Namaqua Rock fig)

It is usually a medium-sized tree, about 10 m in height and rather slender, but old species can be up to 20 m, with a wide spread, characteristically associated with rocks where its pale roots flattened against the rock-faces are distinctive; common in dry, stony deserts. The bark is pale grey and rather smooth. The leaves are ovate, or heart-shaped, dark green and hairless. Figs are paired in the axils of the leaves, without stalks, finely velvety and brown to yellowish in colour (Coates-Palgrave, 2002) (Figure 3-2).

The stem bark of *Ficus cordata* is used traditionally in Cameroon, in the treatment of diarrhoeal infections, tuberculosis and as an anthelmintic (Kuete *et al.*, 2008).

![Figure 3-2 *Ficus cordata* (van Noort and Rasplus, 2004)](image)

3.1.4.3 *Ficus craterostoma* Warb. Ex Mildbraed & Burret (Forest fig)

It is usually a small to medium-sized tree reaching 12 m in height, occurring in evergreen forest margins, swamp forest and in deep, and heavily wooded mountain ravines or on rocky outcrops in the open; it can be a strangler. The bark is grey and, relatively smooth. The leaves are spirally arranged to alternate and even sub-opposite, thinly leathery and hairless. Figs occur in the axils of the leaves, without stalks, and are not hairy, slightly warty and yellow to reddish in colour (Coates-Palgrave, 2002) (Figure 3-3).
In South Africa, the Xhosa people boil the fresh leaves of *Ficus craterostoma* and take the decoction orally for stomach-ache (Bhat and Jacobs, 1995).

![Figure 3-3 Ficus craterostoma](van Noort and Rasplus, 2004)

### 3.1.4.4 *Ficus glumosa* Delile (Hairy rock fig)

This is a small to medium-sized tree 5-10 m tall or sometimes a large tree reaching 13 m, occurring on rocky outcrops when it can become a rock-splitter, along dry watercourses, or in open country frequently in valleys where it reaches its greatest size. The bark is pale grey to yellowish grey, smooth to slightly rough, flaking off. Branchlets are finely hairy and may be marked with large leaf scars. Leaves are oval to oblong, roundish, green or greyish green, matured leaves are usually hairless above, and the under-surface is finely hairy, sometimes with scattered long hairs. Figs are in pairs in the leaf axils and often clustered at the end of the branches (Coates-Palgrave, 2002) (Figure 3-4).

![Figure 3-4 Ficus glumosa](van Noort and Rasplus, 2004)

The traditional medicinal uses are numerous. In Côte d’Ivoire the latex is applied to alleviate pain from sprains while the latex diluted in water in the Central African Republic is used to treat diarrhoea (Jansen and Cardon, 2005). In Zimbabwe the latex is dropped into the eyes for the treatment of sore
eyes (Jansen and Cardon, 2005). Powdered bark mixed with latex is used in Nigeria to plug carious teeth while in the Central African Republic a decoction of the bark is used as a mouthwash against toothache (Jansen and Cardon, 2005). A bark macerate is applied on the eyes of new born babies to prevent conjunctivitis while the pounded bark is applied topically against headache. In addition pounded bark, soaked in water, is drunk against stomach disorders while a decoction of pounded bark is used to wash sores daily until recovery (Jansen and Cardon, 2005). It is also used in the treatment of skin diseases (Hutchings et al., 1996) and diabetes (Madubunyi et al., 2010).

### 3.1.4.5 *Ficus lutea* Vahl (Giant leaved fig)

This species is a large, spreading tree reaching 25 m occurring in evergreen and riverine fringe forest. The bark is dark grey; the bole usually short and often buttressed, with the branches widely spreading. Leaves are very large, crowded at the end of the branches, ovate to elliptic or oblong and shiny green. Figs occur in ones or twos per leaf axil, and are tightly crowded at the tips of the branchlets or just below the leaves, and furry or smooth and yellow in colour (Coates-Palgrave, 2002) (Figure 3-5).

The leaves of *Ficus lutea* are used traditionally in the treatment of sores and boils (Marwah et al., 2006). In combination with other plants, macerations are taken orally for the treatment of rabies, madness and anaplasmosis. A decoction of the ground leaves is used to massage the abdomen to treat placenta retention (Chifundera, 1998).

![Figure 3-5 Ficus lutea (van Noort and Rasplus, 2004)](image_url)

### 3.1.4.6 *Ficus natalensis* Hochstetter (Coastal strangler fig)

This species is very variable in size and habit, from a small to medium-sized tree up to almost 20 m in height and densely leafy, often a strangler; occurring in a variety of habitats. The bark is grey and smooth, often with thin aerial roots hanging down the branches. Leaves are spirally arranged but
tending to be alternate or even sub-opposite, very variable, oblong to narrowly obovate, generally rather small, and dark green, thinly leathery and hairless. Figs are in pairs in the axils of or just below the leaves, hairless and reddish orange (Coates-Palgrave, 2002) (Figure 3-6).

The leaves are used as compresses and poultices for wounds, boils, warts and other growths (Hutchings et al., 1996). Roots are used for colic and when added to another plant, as a snake bite remedy while the bark is used for influenza (Watt and Breyer-Brandwijk, 1962). The fruits and leaves are also used for treatment of pain and venereal diseases (Cousins and Huffman, 2002).

![Ficus natalensis](van Noort and Rasplus, 2004)

### 3.1.4.7 **Ficus polita** Vahl (Heart-leaved fig)

This is a large tree, 10-16 m tall, occurs in evergreen forest, coastal forest and dune forest. The bark is light brown to grey-brown, flaking in small sections, the stem is inclined to the buttressed and the main branches with warty protuberances bear the figs. The leaves are broadly ovate, dark green and glossy above. Figs are in clusters, borne on dwarf knobby branchlets either on the main branches or on the trunk, without hairs, and are yellowish-green to purplish-green in colour when mature (Coates-Palgrave, 2002) (Figure 3-7).

The plant is used for treatment of dyspepsia, infectious diseases, abdominal pains and diarrhoea (Kuete et al., 2011), respiratory disorders and certain skin diseases (Hutchings et al., 1996).
3.1.4.8  *Ficus religiosa* L. (bo tree or sacred fig)

This is a large or medium sized, evergreen or deciduous tree, 20 m tall and 1.5-2 m diameter, irregularly-shaped, with wide-spreading branches and without aerial roots from the branches. The trunk is regularly shaped, often with low buttresses. The bark is grey with brownish specks, smooth, exfoliating in irregular rounded flakes. Leaves are alternate, spirally arranged and broadly ovate, glossy, coriaceous (leathery), dark green leaves. Figs occur in pairs, and are rounded, flat-topped green, smooth, and ripen to purple with red dots (Warrier *et al.*, 1995; Singh *et al.*, 2011; Makhija *et al.*, 2010) (Figure 3-8).

*Ficus religiosa* is reported to have numerous therapeutic uses in folk medicine. The leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, ear-ache and toothache, migraine, eye troubles, gastric problems and scabies (Sirisha *et al.*, 2010). The leaf decoction has been used as an analgesic for toothache; fruits for the treatment of asthma, other respiratory disorders and scabies; stem bark is used in gonorrhoea, bleeding, paralysis, diabetes, diarrhoea, bone fracture, and as an antiseptic and astringent (Sirisha *et al.*, 2010).
3.1.4.9 *Ficus sycomorus* L (sycamore fig)

This is a tree of about 5-25 m in height, fine and spreading; frequently occurs along river banks, forming a distinctive part of the riverine thicket, and also in mixed woodland often on termite mounds. The bark is distinctive yellow to greenish yellow, occasionally creamy brown, smooth, powdery or flaking; the stem may develop buttresses in the very large specimens. Leaves are spirally arranged, sometimes almost alternate, large, ovate to elliptic or almost circular, dark green, rough and harsh to the touch. Figs are quite large, up to 3 cm in diameter, and yellowish to reddish in colour (Coates-Palgrave, 2002) (Figure 3-9).

The powdered stembark of *Ficus sycomorus* is soaked in water and the aqueous solution is used in both human and animal medicine. In human it is used for the treatment of ailments such as diabetes (Etuk *et al.*, 2010), mental illness, wound dressing, diarrhoea (Sandabe and Kwari, 2000). It is used to stimulate milk production in cows (Venter and Venter, 1996). A decoction of the bark and latex is used for chest conditions, coughing and scrofula (Venter and Venter, 1996). Leaves are used against jaundice and as an antidote for snake bite while roots are used as an anthelmintic (Venter and Venter, 1996). Inflamed areas are treated with the milky latex and ringworm is treated with the bark and milky latex (Venter and Venter, 1996).

![Figure 3-9 Ficus sycomorus](van Noort and Rasplus, 2004)

3.1.4.10 *Ficus thonningii* Blume (bark-cloth fig)

This species is an evergreen tree about 6-21 m in height, with a rounded to spreading and dense crown. It is sometimes epiphytic, often a strangler; and the trunk is fluted or multi-stemmed. The bark on the young branches is hairy, but smooth and grey on older branches and stems, often with aerial roots hanging down from branches. Leaves are simple, glossy, dark green, thin and papery or slightly leathery. Figs are in leaf axils, sometimes below the leaves and are yellow or red in colour (Coates-Palgrave, 2002) (Figure 3-10).
The bark is important in local medicine, and an infusion of the bark is used in treating diabetes (Etuk et al., 2010), colds, sore throat, dysentery, wounds, constipation, nose-bleed and to stimulate lactation (Venter and Venter, 1996). The latex is used for wound fever, while an infusion of the root and fibre is taken orally to help prevent abortion (Venter and Venter, 1996). The powdered root is taken in porridge to stop nosebleed; and the milky latex is dropped into the eye to treat cataracts. The fruits and leaves are used to treat bronchitis and urinary tract infections (Venter and Venter, 1996).

Figure 3-10 *Ficus thonningii* (van Noort and Rasplus, 2004)

### 3.2 Methods

#### 3.2.1 Preparation of leaf extracts

The powdered plant material (2 g) was individually extracted with 20 ml of acetone, hexane or chloroform (technical grade - Merck) in polyester centrifuge tubes using a platform shaker (Labotec model 20.2 shaking machine) at room temperature for 30 min (Suffness and Douros, 1979; Eloff, 1998c). The extracts were centrifuged at 500 x g for 5 min in a Rotofix 32 A Hettich centrifuge and the supernatant filtered through Whatman No. 1 filter paper. This procedure was repeated three times on the same plant material with fresh solvent to ensure that all possible compounds were exhaustively extracted. The filtered extracts of each species were combined into pre-weighed glass vials and the solvent was left to evaporate at room temperature under a stream of cold air. The percentage yield was expressed in terms of air-dried mass of the leaves. The extracts obtained were used for the subsequent assays.
3.2.2 Phytochemical analysis of extracts

Ten microlitres of extracts, reconstituted to 10 mg/ml in acetone, were loaded onto the TLC plates (Merck, silica gel 60 F<sub>254</sub>) and eluted with one of the following solvents (Kotze and Eloff, 2002):

1. Polar/neutral = ethyl acetate: methanol: water (EMW) (10:1.35:1, v/v/v)
2. Intermediate polarity/acidic = chloroform: ethyl acetate: formic acid (CEF) (10:8:2, v/v/v)
3. Non-polar/basic = benzene: ethanol: ammonium hydroxide (BEA) (18:2:0.2, v/v/v)
4. Polar = ethyl acetate: water: formic acid: acetic acid (FAWE) (70:20:3:2 v/v/v) (adapted from Phytomedicine Programme protocol)

Following elution, the developed plates were spra yed with vanillin-sulphuric acid [0.1 g vanillin (Sigma) dissolved in 28 ml methanol and 1 ml sulphuric acid added] and heated for approximately 3 min at 110ºC for optimal colour development. A second set of plates were also sprayed with 0.2% 1,1-diphenyl-2-picryl-hydrazyl (Sigma) (DPPH) in methanol as an indicator of antioxidant activity (Deby and Margotteaux, 1970).

3.2.3 Total polyphenolic content

The amount of total polyphenolic compounds was determined using the method described by Djeridane <i>et al.</i> (2006). This method depends on the reduction of Folin-Ciocalteu reagent (a 2N phosphomolybdic-phosphotungstic reagent) by phenols to a mixture of blue oxides. The commercial reagent was diluted 10-fold with double-distilled water. A standard curve was developed using gallic acid as the standard. Different concentrations of gallic acid were prepared in 80% methanol in a 96-well microtitre plate, and their absorbance was immediately recorded at 760 nm. The crude acetone extracts were dissolved in 80% methanol to a final concentration of 1 mg/ml. The plant extract (100 µl of the 1 mg/ml) was dissolved in 500 µl (1/10 dilution) of the Folin-Ciocalteu reagent. After the addition of 1000 µl of distilled water the mixture was allowed to stand for 1 min at room temperature, where after 1500 µl of a 20% Na<sub>2</sub>CO<sub>3</sub> solution was added. The final mixture was shaken, followed by an incubation period of 1h in the dark at room temperature. The absorbance of all samples was measured at 760 nm. All analyses were performed in triplicate and repeated on three different occasions. The results are expressed in mg of gallic acid equivalent (GAE) per gram of dry weight of plant material.

3.2.4 Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant activity was estimated using the trolox equivalent antioxidant capacity (TEAC) test of Re <i>et al.</i> (1999). In this assay, the relative capacity of antioxidant to scavenge the ABTS<sup>•</sup> [2, 2–
azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical compared to the antioxidant potency of trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a synthetic water soluble vitamin E analogue, was measured. The ABTS⁺ radical was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulphate in the dark for 12 - 16 h at room temperature. Prior to assay, the pre-formed ABTS⁺ radical was diluted in methanol (about 1:89 v/v) and equilibrated to give an initial absorbance (Aᵢ) of 0.700 (±0.02) at 734 nm. The crude acetone extracts were made up in methanol to a concentration of 0.0625 mg/ml to 1 mg/ml and the trolox was made up to a concentration of 0.0625 mg/ml to 0.5 mg/ml in methanol. The crude acetone extract or trolox standard (20 μl) was added to the ABTS⁺ radical (180 μl) and the mixture allowed to react for 6 minutes, and the absorbance (A) read at 734 nm in a spectrophotometer at one-minute intervals. Appropriate solvent blanks were included in each assay. The percentage change in absorbance was calculated for each concentration of extracts and trolox. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance versus concentration plot for the extract under investigation is divided by the gradient of the plot for trolox (Re et al., 1999). As a result, the TEAC of a substance is a ratio value and has no unit. All assays were carried out in triplicate and repeated three times. The percentage change in absorbance (A) was measured as follows:

\[
\% \text{ change in } A = \left( \frac{Aᵢ \text{ABTS}⁺(734 \text{nm}) - \text{New mean } A \text{ABTS}⁺}{Aᵢ \text{ABTS}⁺(734 \text{nm})} \right) \times 100
\]

3.2.5 \(\alpha\)-Amylase inhibition assay

An adapted \(\alpha\)-amylase inhibition assay as described by Ali et al. (2006) was utilised. The dried crude acetone extracts were re-dissolved in undilutedDMSO to a concentration of 20 mg/ml and used for the \(\alpha\)-amylase inhibition assay. Ice cold porcine pancreatic \(\alpha\)-amylase solution (200 μl) at 4 unit/ml (type VI) was pre-incubated with 40 μl of crude acetone extracts and 160 μl of distilled water, and mixed in a screw-top plastic tube. The reaction was started by the addition of 400 μl of potato starch (0.5%, w/v) in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride, and thereafter incubated at 25°C for 3 min. Final concentrations in the incubation mixture were plant extract (1 mg/ml), 0.25% (w/v) starch and 1 unit/ml enzyme. An aliquot of the mixture (200 μl) was removed and placed into a separate tube containing 100 μl DNS colour reagent solution (96 mM 3, 5-dinitrosalicylic acid, 5.31M sodium potassium tartrate in 2M NaOH) and placed into an 85°C water bath. After 15 min, this mixture was removed from the water bath, cooled and diluted with 900 μl distilled water. Alpha-amylase activity was ascertained indirectly by measuring the concentration of the non-metabolised starch in the mixture at 540 nm. Control incubations, representing 100% enzyme activity were conducted by replacing the plant extract with undilutedDMSO (40 μl). For the blanks
(negative controls) the enzyme solution was replaced with distilled water and the same procedure was carried out as above. Acarbose was used as positive control (acarbose 1 mg/ml: α-amylase 1 unit/ml). The α-amylase inhibition activity was expressed as:

\[
\% \text{ Inhibition} = 100 \times \frac{(\Delta A_{\text{Control}} - \Delta A_{\text{Sample}})}{\Delta A_{\text{Control}}}
\]

\[
\Delta A_{\text{Control}} = A_{\text{Test}} - A_{\text{Blank}}
\]

\[
\Delta A_{\text{Sample}} = A_{\text{Test}} - A_{\text{Blank}}
\]

### 3.2.6 α- Glucosidase inhibition assay

A commercial rat intestine acetone powder was purchased and used to prepare enzyme solution according to the method of Oki et al. (1999) with slight modifications. In brief, rat intestinal acetone powder (25 mg/ml) was homogenized in ice-cold 50 mM phosphate buffer. After centrifugation at 6000 x g for 15 min, the clear supernatant obtained was used as the enzyme solution for further analysis. The rat intestinal sucrase inhibitory activity was determined using the method of Bhandari et al. (2008), with a slight modification. Sucrose (200 µl of a 56 mM solution) in 0.1 M potassium phosphate buffer (pH 7) was mixed with 100 µl of crude acetone extracts (2.5 mg/ml) in 50% aqueous dimethyl sulphoxide (DMSO) in a test tube. After pre-incubation at 37°C for 5 min, 200 µl of rat intestinal α-glucosidase solution prepared as described above was added. Whereas 100 µl DMSO (50% in water) was used in place of the plant extract to represent 100% enzyme activity, acarbose (0.1 mg/ml) was used as the positive control and for the blanks (negative controls) the enzyme solution was replaced with distilled water and the same procedure was carried out as above. Final concentrations in the incubation mixture for plant extracts were 0.5 mg/ml and 0.02 mg/ml for acarbose.

After thoroughly mixing, the test sample, solvent and positive control test tubes were incubated at 37°C for 20 min and the reaction stopped by adding 750 µl of 2 M Tris–HCl buffer (pH 6.9). The amount of liberated glucose was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) according to the manufacturer’s instructions. The absorbance of the wells was measured at 540 nm and the inhibitory activity was calculated using the following formula:

\[
\% \text{ Inhibition} = 100 \times \left( \frac{\Delta A_{\text{Control}} - \Delta A_{\text{Sample}}}{\Delta A_{\text{Control}}} \right)
\]

\[
\Delta A_{\text{Control}} = A_{\text{Test}} - A_{\text{Blank}}
\]

\[
\Delta A_{\text{Sample}} = A_{\text{Test}} - A_{\text{Blank}}
\]
3.2.7 Kinetics of inhibition against α- amylase and α- glucosidase activities

The kinetics of inhibition of the crude acetone extract of *F. lutea* against α- amylase and α-glucosidase activities were measured by increasing substrate concentrations of starch (0.016% - 0.5%) and sucrose (7 mM – 56 mM) respectively in the absence and presence of extract of *F. lutea* at concentrations of 25 - 100 µg/ml for α-amylase inhibitory assay and 62.5 – 250 µg/ml for α-glucosidase inhibitory assay. The type of inhibition was determined by Lineweaver-Burk double reciprocal plot analysis of the data, which was calculated according to Michaelis-Menten equation (Kim et al., 2005; Goutelle et al., 2008). Where \( v = \) initial velocity of reaction, \( V_{\text{max}} = \) the maximum rate of reaction, \([S]\) = substrate concentration and \( K_m = \) the substrate concentration at which the rate of the enzymatic reaction is half \( V_{\text{max}} \) (Goutelle et al., 2008).

3.2.8 Calculation of EC$_{50}$

The concentration of crude/fractions/compounds that inhibited 50% of the enzyme activity (EC$_{50}$) was determined for α- amylase and α-glucosidase inhibitory assay in the programme Kinetica 5 (Thermo). The concentration versus response relationship was plotted and fitted to a Hill’s model (Goutelle et al., 2008). The model was described by the following equation; 

\[
E = E_0 \frac{E_{\text{max}} C^\alpha}{EC_{50}^\alpha + C^\alpha}
\]

where: \( E = \) predicted effect of the drug, \( E_0 = \) baseline response (effect at a drug concentration of 0), \( E_{\text{max}} = \) maximum drug induced effect, \( C = \) drug concentration at time \( t \), \( EC_{50} = \) plasma drug concentration at which 50% maximal effect is obtained, and \( \alpha = \) sigmoidicity factor (Hill exponent). Concentration \( C \) and EC$_{50}$ are expressed either as µg/ml/mg/ml while \( \alpha \) has no unit (Goutelle et al., 2008).

3.2.9 Cytotoxicity assay

3.2.9.1 Vero monkey kidney cells

The acetone extracts of the ten *Ficus* species were screened for cytotoxic activity in human liver C3A and Vero African green monkey kidney cell lines using the MTT assay (Mosmann, 1983; McGaw et al., 2007). Cells were maintained in MEM supplemented with 0.1% gentamicin and 5% FCS. Previous established confluent monolayer were trypsinized for 20 min, and re-suspended in fresh MEM for final seeding at a density of 5.0×10$^4$ cells/ml into each well (200 µl) of 96-well microtitre plate for the toxicity assays. Wellsof columns 1 and 12 were only filled with growth media (200 µl) to minimise the ‘edge effect’ and maintain humidity. Following overnight incubation at 37ºC in a 5% CO$_2$ the cells in the microtitre plates were used for cytotoxicity assay. The media was removed and replaced with 200 µl of fresh media containing plant extracts. Prior to fresh medium with plant extracts, stock solutions of dried crude plant extracts were prepared by reconstitution with undiluted
DMSO to a concentration of 100 mg/ml. This was further diluted with growth media
to concentrations (1 to 1000 μg/ml) and used for the assays. Since DMSO was used as the
reconstitution solvent, it was used as solvent (negative) control at concurrent concentrations as for the
plant extracts with 1% DMSO in growth media as the highest concentration, while doxorubicin was
used as the positive control. The cells were thereafter incubated for 2 days. All extracts were tested in
quadruplicate and repeated at a later occasion.

After the above stated incubation period, the medium was removed by aspiration and the wells rinsed
with PBS (200 µl) to remove traces of the plant extract, prior to fresh media together with 30 µl of
MTT (5 mg/ml in PBS) being placed into each well. After a further incubation period of 4h, the
medium was carefully aspirated without disturbing the MTT crystals at the bottom of the wells and
replaced with 50 µl of undiluted DMSO. The concentration of MTT reduced was measured at 570 nm
after gentle shaking using a microtitre plate reader with a 1cm corrected pathlength (VERSAmax).
The wells containing only medium and MTT were used to blank the reader. The percentage cell
viability was calculated as the percentage change in MTT concentrations in comparison to the
untreated cells. The LC₅₀ values were calculated from a plot of log of concentration versus average
absorbance represents the concentration of plant extract that resulted in a 50% reduction of absorbance
in comparison to the untreated cells.

3.2.9.2 C3A liver cell line

The C3A liver cells are an established human liver cell lines that have retained some of the drug
metabolising enzymes and are used as relevant model for evaluating the cytotoxic effects of products
of metabolic alteration of chemicals (Flynn and Ferguson, 2008). The C3A cells were cultured in
MEM supplemented with 10% FCS in the absence of antibiotics. Previous established 70-80%
confluent monolayer were trypsinized for 20 min, and re-suspended in fresh MEM at a density of
5.0×10⁴ cells/ml into a 96-well microtitre plate (200 µl). Hereafter the same method as for the Vero
monkey kidney cells was used.

3.2.10 Glucose uptake in primary cell cultures

3.2.10.1 Preparation of rat abdominal skeletal muscle

Rat abdominal muscle was isolated and prepared by modification of the method described by Gray
and Flatt (1997). Briefly, 8 week old non-fasting male Sprague-Dawley rats (n=12) (170-250g)
supplied by the University of Pretoria Biomedical Research Centre (UPBRC) were euthanized by
anisoflurane overdose. Working under sterile conditions, abdominal muscle was excised and placed
into a sterile beaker containing PBS. Muscle tissue was washed twice in 10 ml PBS to remove blood, sectioned into pieces of approximately 10-20 mg and transferred into 20 ml KRB-BSA in 50 ml Schott bottles for gentle agitation at 37°C for 20 min in 5% carbon dioxide in oxygen (carbogen).

3.2.10.1.1 Glucose uptake assay

Each muscle square (20mg) was dispensed into a well of a 48-well microtitre plate containing 450 µl KRB-BSA supplemented with 2mM-sodium pyruvate at glucose concentration of 1 mM. Plant extracts (125 – 2000 µg/ml), insulin (1, 10, 100, 1000 µM; positive control), 10% DMSO in media (solvent control), or medium only (50 µl) was added into wells and incubated at 30°C for 45 min. The final concentrations in the incubation mixture were plant extracts (12.5- 200 µg/ml), insulin (0.1, 1, 10, 100 µM) and 1% DMSO. After the incubation period, the glucose concentration in the medium was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) according to the manual instructions. The absorbance of the wells was measured at 540 nm in a microplate reader (VERSAmax) and the glucose uptake was calculated using the following formula:

$$\text{% Glucose utilised} = 100 \times \left( \frac{\Delta A_{\text{Control untreated cells}} - \Delta A_{\text{Sample treated cells}}}{\Delta A_{\text{Control untreated cells}}} \right)$$

All experiments were carried out in triplicate on each of three different occasions.

3.2.10.2 Isolated rat (epididymal) adipose cells

Isolated adipocytes were obtained under sterile conditions using a method modified from Rodball (1964) and maintained in culture as described by Martz et al. (1986) with modification. Briefly, the epididymal fat pads removed under sterile conditions from Sprague-Dawley rats (170-250g) euthanized by anisoflurane overdose, were weighed and rinsed in phosphate buffered saline. Isolated cells were obtained by shaking finely minced tissue in sterile polypropylene containers in an incubator at 37°C for 1 h in DMEM containing 25mM HEPES, collagenase type 11 (1 mg/ml), and albumin (40 mg/ml). After the shaking, cells were filtered through nylon mesh (1000 µm) and centrifuged at 400 x g for 1 min for layered separation. The bottom layer was carefully aspirated from the container to leave only the cells in the top layer. The isolated cells were washed three times in HEPES buffered Kreb-Ringer (KRH) solution, pH 7.0, consisting of 20 mM HEPES, 120 mM NaCl, 1.2 mM MgSO₄, 2.0 mM CaCl₂, 2.5 mM KCl, 1 mM NaH₂PO₄, 1 mM sodium pyruvate and 1% bovine serum albumin by always removing the bottom layer by aspiration. Penicillin (20 units/ml) and streptomycin (20 mg/ml) were added to all buffers. The cells were finally re-suspended in polypropylene tubes containing the same buffer (4 ml of buffer per 1 g of the original weight) and allowed 40 min to
calibrate at 37°C without shaking. Adipocyte number and viability were determined by trypan blue exclusion.

3.2.10.2.1 Glucose uptake assay

To prepare the primary adipocytes for the glucose uptake assay, the cells were suspended in medium supplemented with 1 mM D-glucose in sterile polypropylene containers and placed in a shaking water bath and incubated at 37°C for 20 min. The cell suspension (200 µl) was plated at a density of $1.0 \times 10^3$ cells/well of a 96-well microtitre plate and 50 µl of plant extracts (62.5 – 1000 µg/ml), insulin (0.5, 5, 50, 500 µM; positive control), 5% DMSO in media (solvent control), or media only was added. Final concentrations in the incubation mixture were plant extracts (12.5 – 200 µg/ml), insulin (0.1, 1, 10, 100 µM) and 1% DMSO. The microtitre plates were incubated at 37°C in a 5% CO₂ for 1 h. After the incubation, the glucose concentration in the medium was determined by the glucose oxidase described above. Each experiment was repeated three times.

3.2.11 Glucose uptake in established cell lines

3.2.11.1 Complete growth medium

The C2C12 myocytes, 3T3-L1 pre-adipocytes and H-4-II-E hepatoma cell lines are used for glucose uptake because they have different glucose transporters. While undifferentiated C2C12 myocytes and 3T3-L1 pre-adipocytes predominantly express GLUT1 glucose transporter that is strictly involved in the regulation of basal glucose transport, the differentiated C2C12 myocytes and the adipocytes express GLUT4 insulin-responsive glucose transporter (Ariga et al., 2008). Thus, differentiation changes the number and composition of GLUT proteins, especially increasing the cellular GLUT4 content, contributing to the developing the insulin-responsive glucose transport system in myotubes and adipocytes (Ariga et al., 2008). The H-4-II-E hepatoma cells predominantly express GLUT2 glucose transporter (Scheepers et al., 2004).

The C2C12 myocytes were maintained in DMEM culture medium supplemented with 10% foetal bovine serum (FBS), 4 mM glutamine. The 3T3-L1 pre-adipocytes were maintained in DMEM culture medium supplemented 10% bovine calf serum, 4 mM glutamine. The H-4-II-E hepatoma cells were maintained in MEM culture medium supplemented with 10% FBS, 2 mM glutamine. The RIN-m5F insulinoma cell were maintained in RPMI-1640 with 2 mM glutamine supplemented with 10% FBS, 10 mM HEPES and 1 mM sodium pyruvate.
3.2.11.2 Culturing of cells

Frozen vials were revived according to ATCC guideline. Each cell line was revived on different days. The vial containing cells was rapidly thawed under gentle agitation in a 37°C water bath. The content of vial was transferred to a centrifuge tube containing 9 ml of complete growth medium for each cell type (pre-incubated for 15 min at 37°C under humidity at 5% CO₂ to avoid excessive alkalinity) and centrifuged at 125 x g for 5-7 min. The supernatant was discarded while the pellet was re-suspended in fresh growth medium (2 ml) of which 1 ml (3.0 x 10³ - 5.0 x 10³ cells/ml) was dispensed into T25 flasks containing 9 ml growth medium for incubation at 37°C in a humidified atmosphere at 5% CO₂. The following day the medium was replaced with fresh medium. Medium was changed every 2-3 days, until the monolayers required trypsinisation for sub-culturing or experiments at approximately 80% confluence. Trypsinisation made use of trypsin-EDTA (3 ml) for 5-10 min, centrifuged at 1500 for 2 min, prior to suspension in fresh growth medium (2ml) for counting.

3.2.11.3 Glucose uptake assay

The amount of glucose uptake was determined by the methods of Yin et al. (2008) and Deutschlander et al. (2009). The C2C12 muscle myocytes (25,000 cells/ml), the 3T3-L1 pre-adipocytes (30,000 cells/ml) and the H-4-11-E hepatoma cells (30,000 cells/ml) suspended in DMEM supplemented with 0.25% BSA were seeded (200 µl) into wells of 96-well microtitre plates. Only media (200 µl) was added into wells of column 1 and 12 to minimise the “edge effect”. After incubation at 37°C in a 5% CO₂ incubator for 4 days (C2C12 and 3T3-L1) and 2 days (H-4-11-E), the cells in the microtitre plates were used for glucose uptake assay.

Prior to glucose uptake assay, plant extracts were prepared by reconstitution in undiluted DMSO to a concentration of 100 mg/ml which was further diluted with appropriate growth medium before using them for assay. The medium in each of the test wells were removed by aspiration and immediately replaced with 100 µl of growth medium containing plant extracts at concentrations of 15, 31, 63, 125, 250 and 500 µg/ml. The cells were incubated at 37°C in a 5% CO₂ incubator for 1 h (C2C12), 1½ h (3T3-L1) and 3 h (H-4-11-E) with the various treatments. Insulin (0.1, 1, 10, 100 µM) served as the positive control for the C2C12 and 3T3-L1 cells and was incubated for 1 h and 1½ h respectively while metformin and insulin (0.1, 1, 10, 100 µM) were the positive control for H-4-11-E cells. Cells treated with metformin were incubated for 24 h. Since DMSO was the solvent of reconstitution, it served as the solvent control in all cases. The final highest DMSO content of plant extracts was 1% and this served as the highest concentration of DMSO used in solvent controls. After incubation, the glucose concentration in the medium was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) as mentioned above.
3.2.12 Insulin secretion assay

The amount of Insulin secretion by RIN-m5F was determined by the method of Persaud et al. (1999). The RIN-m5F insulinoma cells (100,000 cells/ml) were suspended in fresh growth medium (RPMI-1640 supplemented with 10% FBS, 10 mM HEPES and 1 mM sodium pyruvate) seeded (200 μl) into 96-well microtitre plate. Only media (200 μl) was added into wells of column 1 and 12 to minimise the “edge effect”. Cells were left in the incubator at 37°C in a 5% CO₂ to adhere for 48 h. After 48 h incubation, the medium was removed by aspiration and cells were pre-incubated in a glucose free Kreb-Ringer bicarbonate buffer (135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂ and 1.5 mM CaCl₂) pH 7.4 supplemented with 1mg/ml BSA and 10 mM HEPES for 2 h. The medium in test wells of column 3-11 were removed and replaced with 100 μl of glucose free Kreb-Ringer bicarbonate buffer containing plant extract at 4 concentrations (62.5, 125, 250 and 500 μg/ml). Glibenclamide (0.1, 1, 10 μM) was used as positive control and test wells of column 2 were replaced with plain medium (untreated cells control). The cells were incubated at 37°C in a 5% CO₂ 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 assays were run in duplicate and repeated three times.

\[
\% \text{ Insulin secreted} = \left[ \left( \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{control}}} \right) \times 100 \right] - 100
\]

The toxicity of the extract and pure compound to the RIN-m5F pancreatic β-cells after the 2h of exposure was concurrently determined using the MTT assay as described for the Vero monkey kidney cells. The percentage cell viability was calculated as the percentage change in MTT concentrations in comparison to the untreated cells.

3.2.13 Solvent-solvent fractionation and isolation of compounds

One and half kilograms of the dried powdered leaves of *F. lutea* were extracted with acetone and concentrated using a rotary evaporator (Büchi R-114). The partially dried extract was allowed to dry at room temperature under a stream of cold air. The weighed dried crude acetone extract was re-dissolved in 50% acetone in water, and successively and exhaustively partitioned (by liquid-liquid extraction) with hexane, chloroform/ dichloromethane, ethyl acetate and n-butanol (in order of increasing polarity). To get an indication of the chemical composition, the fractions were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates using the following elution system BEA, CEF, EMW and FAWE sprayed with vanillin-sulphuric acid. A second set of TLC were
sprayed with 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (Sigma) (DPPH) to evaluate for antioxidant activity (Deby and Margotteaux, 1970).

3.2.14 Isolation of compounds

Due to highest activity, the ethyl acetate fraction was subjected to a silica gel column chromatography and eluted with an increasing polarity of n-hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) to afford 115 fractions of 500 mL each. Fraction 18 eluted with n-hex : EtOAc (85:15) crystallized in the same solvent system to yield compound 1 (23 mg) while a combined fractions 16-19 [n-hex : EtOAc (90:10, 85:15)] and 20-25 [n-hex : EtOAc (80:20)] crystallized also in n-hex:EtOAc (85:15) to afford compound 1 (10 mg) and compound 2 (25 mg), respectively. Fractions 10-30 (1g) eluted with n-hex:EtOAc (95:5) was further subjected to a purification silica gel column chromatography using n-hexane and EtOAc (0 - 100%) to afford 139 fractions of 50 ml each and sub-fraction 76 (23 mg) yield compound 3 (17.4 mg) after preparative TLC (Merck, silica gel 60 F254). Fractions 46-52 eluted with n-hex:EtOAc (70:30) was also subjected to similar silica gel column chromatography as fractions 10-30 followed by preparative TLC to afford compound 4 (15 mg) and compound 5 (21 mg).

3.2.15 General experimental procedures

1H and 13C nuclear magnetic resonance (NMR) spectra were recorded with a Bruker spectrometer at 500 MHz and Variant spectrometer at 400 MHz. Chemical shifts (δ) are quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS). Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh. Preparative TLC was performed using high-purity grade powder silica gel (60 A, 2-25 µm) Sigma-Aldrich, Germany. Precolated plates of TLC silica gel 60 F254 (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with 30 % H2SO4 followed by heating up to 110 °C.

3.2.16 In vivo assay

3.2.16.1 Maintenance of animals

Ethics approval was received from the Animal Use and Care Committee (AUCC) of the University of Pretoria (V060) in compliance with the South African National Standard on the housing and care of laboratory animals. Healthy male CD1 male mice (n = 40) of age 5 weeks old were purchased from Onderstepoort Veterinary Institute (OVI), Pretoria and housed at the University of Pretoria Biomedical Research Centre (UPBRC) in conventional Eurostandard type II cages (Techniplast) at the
temperature of 22°C (± 2°C), controlled relative humidity (50%-60%) in a light/dark cycle of 12 hours. Environmental enrichment, e.g. suspended bells, cardboards and sticks, were provided to keep the mice busy. Animals were housed on cloth bedding for the duration of the study for easy collection of faecal samples. Animals were initially housed in pairs but later single housed due to fighting. The mice had free access to water and standard rodent chow (radiated) for one week during the one week acclimatisation period prior to the introduction of the experimental diets.

3.2.16.2 Experimental design

After acclimatisation, all mice were fed a high caloric diet until obesity was induced (on or before 13 weeks) prior to treatment. The high caloric diet was the standard rodent chow to which fat (42%) and sugar (36%) was added (Table 3-1). Obesity was defined as the animal being 5 g higher than the weight for the species at the specific age (Table 3-2). However, since the mice failed to attain an obese state at the same time, the animals were treated only when they were individually considered obese according to a growth chart (Table 3-2). Hereafter, the mice were randomly assigned (Table 3-3) into one of the four treatment groups below (as coded in Table 3-4) and kept on the diet for the particular treatment plan (Table 3-1) for the next 7 weeks. The treatment groups were based on two treatment plans and their associated controls, and are as follows:

- **Treatment Plan 1:** High caloric diet intake with ethyl acetate fraction. The following treatment aimed to show that weight loss was possible in obese animals maintained on a high-caloric diet in the presence (Group 2) of ethyl acetate fraction of *F. lutea* extract (1 g/kg) in comparison to the high-caloric diet alone (Group 1). This treatment option was to stimulate the practice of failing to reduce caloric intake in a weight loss programme.

- **Treatment Plan 2:** Normal caloric diet intake with ethyl acetate fraction. The following treatment aimed to show that weight loss was possible in obese animals maintained on a normal-caloric diet in the presence (Group 4) of the ethyl acetate fraction of *F. lutea* extract in comparison to the normal-caloric diet alone. This treatment option was essentially to stimulate the standard practice of decreasing caloric intake in a weight loss programme.
Table 3-1: Nutritional contents of food (g) to induce obesity and after inducement of obesity

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>High calorie diet (g)</th>
<th>High calorie diet with ethyl acetate fraction (g)</th>
<th>Normal diet (g)</th>
<th>Normal diet with ethyl acetate fraction (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard rodent chow</td>
<td>60</td>
<td>59.79</td>
<td>210</td>
<td>209.79</td>
</tr>
<tr>
<td>Condensed milk (clover)</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corn oil</td>
<td>55</td>
<td>55</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>-</td>
<td>0.21</td>
<td>-</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*The condensed milk contained 8.2g total fat and 57g sugar per 100g milk

3.2.16.3 Preparation of diets

The standard rodent chow was crushed with mortar and pestle, milled to powder in a Kenwood blender, packaged in plastic bag and stored in dry place at 4°C. The food was prepared for each treatment separately. The high calorie diet was prepared by adding the entire ingredient (Table 3-1 and Table 3-5) into a Kenwood mixer and mixing at the lowest speed. The resulting dough was cut into small pieces and moulded into pellets. The normal diet was prepared in the same manner in the absence of the added fat. For fortification, the treatment was prepared by mixing 1 g of ethyl acetate fraction of crude extract of *F. lutea* per kilogram of either high fat or normal diet in a Kenwood mixer prior to moulding. The prepared diets were oven dried at 70°C for 24 hour, allowed to cool, and packaged into labelled plastic bag. The diets were prepared every two days and animal food was changed at the interval of 3 days (every Mondays, Wednesdays and Fridays) to prevent oxidation of the fats and deterioration of the ethyl acetate fraction of *F. lutea* extract.

Table 3-2: Weight prediction chart for the CD1 mice

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Weight</th>
<th>Treatment weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>32.9</td>
<td>37.9</td>
</tr>
<tr>
<td>7</td>
<td>35.7</td>
<td>40.7</td>
</tr>
<tr>
<td>8</td>
<td>37.6</td>
<td>42.6</td>
</tr>
<tr>
<td>9</td>
<td>38.3</td>
<td>43.3</td>
</tr>
<tr>
<td>10</td>
<td>40.3</td>
<td>45.3</td>
</tr>
<tr>
<td>11</td>
<td>41.2</td>
<td>46.2</td>
</tr>
<tr>
<td>12</td>
<td>41.8</td>
<td>46.8</td>
</tr>
</tbody>
</table>

Source: Anonymous, 2011
Table 3-3 Chart for placement of CD1 mice into treatment group once obesity was induced

<table>
<thead>
<tr>
<th>Order</th>
<th>Code for treatment</th>
<th>Order</th>
<th>Code for treatment</th>
<th>Order</th>
<th>Code for treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>13</td>
<td>1</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>14</td>
<td>3</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>15</td>
<td>4</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>17</td>
<td>1</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>18</td>
<td>3</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
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<td>19</td>
<td>3</td>
<td>32</td>
<td>2</td>
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<td>8</td>
<td>3</td>
<td>20</td>
<td>3</td>
<td>33</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>21</td>
<td>4</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>22</td>
<td>2</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>23</td>
<td>3</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>25</td>
<td>1</td>
<td>37</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3-4 Codes assigned to the treatments group and the numbers of animals

<table>
<thead>
<tr>
<th>Code</th>
<th>Treatment group</th>
<th>Total number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High caloric diet</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>High caloric diet + ethyl acetate fraction</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Normal Diet</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Normal Diet + ethyl acetate fraction</td>
<td>10</td>
</tr>
</tbody>
</table>

3.2.16.4 Body weight, food intake and faecal weight

Body weight, food intake and faecal weight for each mouse were measured every Mondays, Wednesdays and Fridays using a rodentscale (Jadever Snug II precision balance). Body weight was reported as measured on the scale. Food eaten was determined by the difference between place and remaining food. For correctness, any food found in the bottom of the cage on the cloth bedding was collected and weight along with the remaining food. Faecal weight was reported as the amount of faeces on the cloth bedding as measured with the scale.
3.2.16.5 Baseline blood glucose and glucose tolerance test (GTT).

After the induction of obesity and prior to commencement of treatments, a glucose tolerance test was performed per mouse using a single drop of blood collected by needle prick from the tail. Mice were starved for 6 hours prior to the determination of baseline blood glucose levels. After the evaluation of the baseline sample, each mouse was treated with a sterile solution (SABAX 50% dextrose) of 2 g of glucose/kg body weight intraperitoneally (i.p). Blood glucose concentrations were sequentially sampled from each animal at 5, 10, 15, 30, 60 and 90 minutes. Blood glucose levels were measured by dropping blood on Contour TS glucose test strips (Bayer) and the blood glucose values displayed on the screen of the glucometer (Bayer, Contour TS) was immediately recorded. The entire process was repeated after 6 weeks of treatment. The area under the plasma glucose versus time graph was estimated using the linear trapezoidal rule and used for comparisons.

3.2.16.6 Clinical pathology

The animals were terminally bled under isoflurane anaesthesia after the 7 weeks of feeding. The haematological parameters evaluated were red blood cell count (RBC or RCC), red cell distribution (RCD), white blood cell count (WBC or WCC), haemoglobin (Hb), haematocrit (ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils (neut), eosinophils (eos), basophiles (baso), lymphocytes (lymph), monocytes (mono), platelets count (Plt C) and mean platelet volume (MPV) using which machine. Serum chemistry parameters evaluated were alkaline phosphatase activity (ALP), alanine aminotransferase (ALT), urea, plasma glucose, creatinine, total protein, cholesterol, globulin, albumin and triglycerides using which machines. All parameters were performed using standard laboratory procedures at the clinical pathology laboratory of the Department of Companion Animal Studies, Faculty of Veterinary Science of the University of Pretoria.

3.2.16.7 Pathology

All test mice were submitted for necropsy. All gross pathological changes were recorded for each animal and subsequently microscopy examination of the organs showing evidence of gross pathology in animals was conducted. In addition, the liver, kidneys, pancreas, heart and blood vessel to the legs were collected for histological pathological examination. The selected organs were collected in 10% buffered formalin. Tissue fixation was for 48 hours, thereafter, organs were cut and processed in an automated histological tissue processor according to standard operating procedure of the Section of Pathology, Faculty of Veterinary Science of the University of Pretoria. After tissue processing the wax blocks were sectioned at 6 μm and stained with Haematoxylin and Eosin in an automated histological
stainer and then mounted before histological evaluation was conducted. Histopathological evaluations were undertaken by Dr Botha of IDEXX VetPath.

3.2.17 Statistical analyses

All in vitro experiments were performed in triplicate and repeated on three different occasions to yield nine dose-response curves. Statistical analyses of antioxidant activity, total polyphenols content, α-amylase and α-glucosidase inhibitory activities, glucose uptake assays, insulin secretion, body weight, food intake, faecal output as well as haematological and serum chemistry parameters for high calorie diet, high calorie diet with treatment and normal diet and normal diet with treatment were evaluated by one-way analysis of variance (ANOVA) and considered to be significantly different at \( p < 0.05 \). Non normal data were log-transformed prior to statistical testing. When significance was found, location of significance was determined by Bonferroni and Tukey HSD multiple comparison post hoc tests. The correlation coefficients (R²) between antioxidant, polyphenols, α-amylase and α-glucosidase inhibitory activities, and between viability RIN-m5F pancreatic β-cells and insulin secretion were also determined. All analyses were undertaken in SPPS 20 (IBM). Data are presented as the mean ± standard error of mean (S.E.M.). For the glucose tolerance test differences before and after treatment were ascertained using a paired t-test.