EFFICACY OF SELECTED KENYAN MEDICINAL PLANTS

USED IN THE TREATMENT AND MANAGEMENT

OF TYPE II DIABETES

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Efficacy of Selected Kenyan Medicinal Plants

Used in the Treatment and Management of Type II Diabetes

By

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Problem Summary

In Kenya, the prevalence of diabetes is estimated at 3-10% of the population. These figures could be higher because most type 2 diabetics are diagnosed many years after onset. Out of this number, 15% are people below 30 years of age who need prompt education to avoid complications that are associated with diabetes (DMI centre, 2004).

Due to inadequate or lack of proper information, most patients especially those with type 2 diabetes are diagnosed through complications. Untreated or poorly managed diabetes is now the leading cause of eye disease and kidney failure in the world. Diabetes is the largest cause of kidney failure in the developed world, and is the fourth leading cause of global death by disease in the world (IDF, 2007). At the Kenyatta National Hospital in Nairobi, Kenya, it is the leading cause of all non-accident related amputations.

It is with such statistics in mind and the grim reality of poor and inadequate health services that this research is based. The wide use of selected medicinal plants for the treatment and management of diabetes warrants the further study of these plants for potential use and commercialization. The data obtained can also be invaluable for use and reference when using these plants for medicinal purposes.

The medicinal plant studied in the research is widely used in Kenya by many communities and was chosen based on ethno-pharmacological references using traditional medicinal practitioners as well as patient’s recommendations. Different in vitro and in vivo assays were studied to try and elucidate the mechanisms of action as well as the organs targeted during treatment using this plant.
LIST OF SYMBOLS AND ABBREVIATIONS

%  Percentage
µg  Microgram
µl  Microliters
AA  Aminoantipyrine
ACC  Acetyl CoA carboxylase
ADA  American diabetes association
AGE  Advanced glycation end products
ALT  Alanine transferase
AMPK  AMP activated kinase
AP  Amadori product
aPKC  Atypical protein kinase C
ATP  Adenosine triphosphate
BSA  Bovine serum albumin
CBBG  Coomassie brilliant blue G dye
DCCT  Diabetes control and complications trial
DEX  Dexamethasone
DHAP  Dihydroxyacetone phosphate
DMSO  Dimethylsulphoxide
DNA  Deoxyribonucleic Acid
DPPH  2, 2 - diphenylpicrylhydrazine
EDTA  Ethylene diamine tetra-acetic acid
ELISA  Enzyme linked immunosorbent assay
FFA  Free fatty acid(s)
FRAP  Ferrie Reducing Ability of Plasma
g  Gram
G6Pase  Glucose -6- phosphatase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl] piperazine- N-[ethanesulphonic acid]</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment index</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IC50</td>
<td>Concentration to produce 50% inhibition</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>IGTT</td>
<td>Intravenous Glucose Tolerance Test</td>
</tr>
<tr>
<td>IPR</td>
<td>International Property Rights</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Resistance</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP sensitive potassium channel</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo daltons</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LFD</td>
<td>Low fat diet</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver function transferases</td>
</tr>
<tr>
<td>m</td>
<td>Milli</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>M6P</td>
<td>Mannose-6-phosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated kinase</td>
</tr>
<tr>
<td>mM</td>
<td>Millimole(s)</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
</tbody>
</table>
NaCl   Sodium chloride  
NAD   Nicotinamide adenine dinucleotide 
NADP   Nicotinamide adenine dinucleotide phosphate 
NaHCO₃   Sodium bicarbonate  
NBT   Nitro blue tetrazolium  
NEFA   Non essential fatty acids  
NIDDM   Non insulin dependent diabetes mellitus  
OGTT   Oral glucose tolerance test  
P   Probability  
PBS   Complete phosphate buffered saline  
PBSA   Phosphate buffered saline  
PDK   3- Phosphoinosidate dependent protein kinase  
PI3K   Phosphoinosidate-3-kinase  
PIP2   Phosphatidylinositol-4-5-biphosphate  
PIP3   Phosphatidylinositol-3-4-5-triphosphate  
PKB   Protein kinase B  
PKC   Protein kinase C  
POD   Peroxidase  
PPARγ   Peroxisome proliferator-activated receptor γ  
QUICKI   Quantitative insulin sensitivity check index  
r²   Correlation coefficient  
RAGE   Receptor for advanced end product glycation proteins  
RIA   Radioimmunoassay  
ROS   Reactive oxygen species  
rpm   Revolutions per minute  
RPMI 1640   Roswell park memorial institute culture medium  
SD   Standard deviation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol response element-binding protein-1</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SUR</td>
<td>Sulphonylurea receptor</td>
</tr>
<tr>
<td>TBHB</td>
<td>2, 4, 6 tribromo-3-hydrobenzoic</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerol(s)</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2, 4, 6-Tri (2-pyridyl)-5-triazine</td>
</tr>
<tr>
<td>TRAP</td>
<td>Total reducing ability of plasma</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>UKPDS</td>
<td>U.K prospective diabetes study</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
</tbody>
</table>
CHAPTER 1
LITERATURE REVIEW

1.1. *Diabetes mellitus*
1.2. Pharmacological agents and traditional medicines
1.3. Screening methodology for antidiabetic properties in plants
1.4. The rat as a model for type 2 diabetes
CHAPTER 1

LITERATURE REVIEW

1.1. DIABETES MELLITUS

1.1.1. Introduction

Diabetes mellitus is a common and very prevalent disease affecting citizens of both developed and developing countries. The International Diabetes Foundation (IDF) estimates that diabetes affects about 246 million people worldwide and this is expected to rise to 380 million by 2025. Diabetes was expected to cause 3.8 million deaths worldwide in 2007, translating to about 6% of total global mortality, about the same as HIV/AIDS (IDF, 2007).

The World Health Organization (WHO) classification divides Diabetes mellitus into Insulin-dependent Diabetes mellitus (IDDM) or type 1 and Non insulin-dependent Diabetes mellitus (NIDDM) or type 2.

Insulin-dependent Diabetes mellitus is usually first diagnosed in children, teenagers and young adults. In these patients the β cells of the pancreas no longer make insulin because the body’s immune system has destroyed them. It has been suggested that many cases follow a viral infection which has destroyed the pancreatic beta cells or generated immunogenic epitopes similar to endogenous epitopes on pancreatic β cells. Treatment for IDDM usually involves taking insulin shots or use of an insulin pump, wise food choices, regular exercise and controlling blood pressure and cholesterol (Greenspan et al., 2001).

Non Insulin-dependent Diabetes Mellitus is the most common type of diabetes. It usually develops later in life though there is an increase in numbers of young patients. Insulin resistance defined as an inadequate response to circulating insulin by insulin target tissues like adipose, skeletal muscle and liver usually preceded the characteristic hyperglycemia in type 2 diabetes (Schenk et al., 2008).
Diabetes mellitus is caused by an abnormality of carbohydrate metabolism linked to low blood insulin level or insensitivity of target organs to insulin (Schenk et al., 2008). It occurs due to absolute or relative insulin deficiency. It has been defined by the WHO, on the basis of laboratory findings, as a fasting venous blood glucose concentration greater than 7.0 mmol/litre; or a concentration equal or greater than 11.1 mmol/litre two hours after a carbohydrate meal or two hours after oral ingestion of the equivalent of 75 g of glucose, even if the fasting concentration is normal. Chronic complications of Diabetes mellitus are varied, with changes involving the cardiovascular system for the most part, though they also affect the nerves, skin and eyes (Greenspan et al., 2001).

The physiologic effects of insulin in the body are far reaching. These also directly correlate to the effects seen in the body of either too much or too little insulin in circulation. The net effect of the hormone involves the storage and utilization of carbohydrates, proteins and fats. Figure 1.1 shows the role of insulin in a normal healthy condition and in both type 1 and type 2 diabetic states (Permutt et al., 2005).

In normal non diabetic individuals, 50% of an ingested glucose load is normally burned to carbon dioxide and water; 5% is converted to glycogen and 30-40% is converted to fat in the fat depots. In diabetes, less than 5% is converted to fat even though the amount burned to carbon dioxide and water is also decreased and that converted to glycogen not increased. The result is that glucose accumulates in the bloodstream, evidenced as hyperglycemia and some of the glucose spills over into the urine (Ganong, 1997).
Figure 1.1: Role of insulin in the normal healthy condition, in T1D and T2D (Permutt et al., 2005)
1.1.2. Major organs affected by Diabetes mellitus

Most of the effects of insulin are seen in the adipose tissue, skeletal, cardiac and smooth muscle, liver and the pancreas. The effect of insulin on various tissues is summarized below (Greenspan et al., 2001).

Table 1.1: Most important effects of insulin on insulin target tissues

<table>
<thead>
<tr>
<th>Adipose tissue</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased glucose entry</td>
<td>Decreased ketogenesis</td>
</tr>
<tr>
<td>Increased fatty acid synthesis</td>
<td>Increased protein synthesis</td>
</tr>
<tr>
<td>Increased triglyceride deposition</td>
<td>Increased lipid synthesis</td>
</tr>
<tr>
<td>Activation of lipoprotein lipase</td>
<td>Decreased glucose output</td>
</tr>
<tr>
<td>Inhibition of hormone sensitive lipase</td>
<td></td>
</tr>
</tbody>
</table>

**Pancreas**
- Decreased insulin synthesis
- Decreased insulin secretion
- Decreased beta cell growth proliferation
- Increased glucose entry

**Muscle**
- Increased protein synthesis
- Increased glycogen synthesis

1.1.2.1 Adipose tissue

Adipose issue is one of the major tissues in the body that is involved in the maintenance of normal glucose levels in the body. Its primary role is however the storage of energy in the form of triglycerides with glucose disposal being the primary role for skeletal muscle (Huang and Czech, 2007).

Adipose tissue has a number of glucose transporters such as GLUT 4, GLUT 8 and GLUT 12 that are responsible for shuttling glucose into the cells. GLUT 4 is the
main hexose transporter and is highly expressed in the adipose tissue (Huang et al., 2007)

Visceral fat depots found in adipose tissue have very high lipolytic rates resulting in the release of large amounts of fatty acids into the system. Insulin normally suppresses the lipase and adipocytes triglyceride lipase enzyme that hydrolyses intracellular triglyceride but in the insulin resistant state, the activity of this enzyme is enhanced resulting in a free fatty acid (FFA) flux (Duncan et al., 2007).

Adipose tissue releases large amounts of a protein known as tumour necrosis factor (TNFα) that plays a major role in the repression of many genes in the body which are responsible for the uptake and storage of glucose as well as fatty acids. TNFα also mediates the inflammatory process which is associated with obesity and type 2 diabetes (Ruan and Lodish, 2002).

1.1.2.2. Muscle

The skeletal muscle is the major tissue involved in glucose metabolism, accounting for about 75% of whole body insulin stimulated glucose uptake (Perriot et al., 2001).

Skeletal muscle stores glucose as glycogen which it oxidizes when needed to produce energy (Huang and Czech, 2007). About 500-600 gm of glycogen is stored in the muscle tissue of a 70 kg man, but because of the lack of glucose-6-phosphatase (G-6-Pase) in this tissue, it cannot be used as a source of blood glucose, except by indirectly supplying the liver with lactate for conversion to glucose (Greenspan et al., 2001). Muscle glycogen can only supply local needs.

Glucose is transported into the cells through a specialized transmembrane sugar transporter known as GLUT 4. GLUT 4 is one of many sugar transporters in the body that catalyses the transport of glucose through the plasma membrane. This transporter works in tandem with others like GLUT 1, 5 and 12 to enhance glucose transport via facilitative diffusion (Stuart et al., 2006).
Insulin has many effects on the muscle, with the most important ones being the increased entry of glucose. When insulin binds to its receptors, tyrosine phosphorylation of protein substrates occurs and this activates the PI3 kinase pathway. Subsequent signalling pathways are activated with GLUT 4 eventually moving from its intracellular stores to the plasma membrane (Perriot et al., 2001).

A characteristic feature of this transporter is that it has unique sequences in its N and COOH terminals. These sequences are important in directing both endocytotic and exocytotic processes in which this transporter is involved (Huang and Czech, 2007).

The important role of GLUT 4 in glucose transport was demonstrated using transgenic mice with high expression levels of GLUT 4. In these mice, sensitivity to insulin is markedly higher as is the response in taking up glucose in to the cells. When the levels of this transporter are decreased, the rats become insulin resistant (Abel et al., 2001). In type 2 diabetic patients there is as much as a 90% reduction in levels of GLUT 4 that are responsive to insulin. Translocation of transporters and subsequent signalling pathways are interfered with. This is one way that results in the characteristic insulin resistant (IR) state found in type 2 diabetics (Ryder et al., 2000).

During muscular activity glycogenolysis is stimulated by adrenaline. When the muscle is contracted, there is an increase in intracellular calcium concentration as well as an increase in AMP/ATP ratios. These changes enhance the translocation of glucose transporters from the intracellular stores to the plasma membrane. There is increased GLUT 4 expression in muscle in response to exercise (Huang and Czech, 2007).

With insulin resistance, hyperinsulinemia and the accumulation of fatty acids in the muscle affects glucose uptake and insulin sensitivity by interfering with insulin binding and signalling. This is discussed in section 1.1.4 (Black, 2006).
1.1.2.3 Liver

The liver is the first major organ reached by insulin via the bloodstream. Insulin exerts effects on the liver by either promoting anabolism or by inhibiting catabolism. The liver helps the body to maintain normal blood glucose concentrations in fasting and postprandial states. When insulin levels are low, then glycogenolysis and increased hepatic glucose production are the result (Lewis et al., 2002).

The liver has a maximum storage capacity of 100-110 gm of glycogen or approximately 440 kcal of energy. Insulin promotes glycogen synthesis and storage as well as inhibits glycogen breakdown to glucose. These effects are mediated by changes in the activity of enzymes in the glycogen synthesis pathway (Schenk et al., 2008). Insulin inhibits the expression of key gluconeogenic enzymes such as G-6-Pase leading to elevated levels of glucose production in the liver (Luca et al., 2007).

When insulin resistance develops, all these processes that are mediated by insulin are affected. The lack of inhibition of G-6-Pase enzyme in insulin resistance results in a higher glucose output in the liver and associated insulin sensitivity. Insulin increases both protein and triglyceride synthesis and very low density lipoproteins (VLDL) formation by the liver (Greenspan et al., 2001).

It also inhibits gluconeogenesis and promotes glycolysis through its effects on enzymes of the glycolytic pathway. In animal models, chronic hyperinsulinemia is found to predispose the liver to relative resistance to insulin (Lewis et al., 2002). High levels of free fatty acids (FFA) found in the insulin resistant state is known to be directly toxic to hepatocytes. Mechanisms involved in this toxicity include cell membrane disruption and mitochondrial dysfunction (Qatanani et al., 2007).

Individuals with type 2 diabetes have a higher incidence of liver function transferases (LFTs) abnormalities than individuals without diabetes. The most common abnormality is elevated alanine aminotransferase (ALT). Anti-diabetic agents have generally been shown to decrease ALT levels as tighter blood glucose levels are achieved (Harris, 2005).
1.1.2.4. Pancreas

The human pancreas is made up of two types of tissues, namely exocrine and endocrine. The exocrine tissue (acini) secretes digestive enzymes that help to breakdown proteins, carbohydrates, fats and acids in the duodenum while the endocrine pancreas (islets of Langerhans) has a hormonal function. It produces insulin, somatostatin, gastrin and glucagon. These hormones have important roles to play in maintaining glucose and salt homeostasis in the body (Ganong, 1998).

About 5% of the total pancreatic mass is comprised of endocrine cells, the pancreatic islets. These islets secrete the hormones into the bloodstream and not into the tubes or ducts like the digestive pancreas. Insulin is secreted by the β cells of the pancreas. The function of β cells has been found to be markedly decreased in type 2 diabetes (Butler et al., 2003). This is due to a decrease in cell mass, as well as defects in insulin gene transcription, biosynthesis and increased rate of β cell apoptosis (Leibowitz et al., 2001, Donath et al., 2003).

Since type 2 diabetes results from an inability to further increase insulin secretion during insulin resistant phases, these changes in the pancreatic islets further compound the problem (Cerasi et al., 1995, Kahn et al., 2003). These features are reproduced by a chronic exposure of β cells to fatty acids, suggesting that hyperlipidemia might be a contributing factor to this decreased cellular function (Kahn et al., 2003).

The various cellular changes in the pancreas during diabetic state facilitated by various conditions have been widely studied especially in rat models. It has been found that β cell apoptosis is largely responsible for the development of insulin dependent Diabetes mellitus in the STZ rat model (Morimoto et al., 2005). Beta cells are extremely susceptible to oxidative changes because they have very low anti oxidative abilities (Kajimoto, 2004).

This loss of β cell mass is well described in animal models where obesity is associated with islet hyperplasia and progression to diabetes followed by cell destruction. This phenomenon has also been recently confirmed in humans (Butler et al., 2003).
1.1.3. **Major contributing factors to Diabetes mellitus**

Increase in diabetes cases in the developing world can be largely attributed to urbanization, westernization and economic development. The major contributing risk factors related to this are population ageing, obesity, sedentary lifestyles, certain dietary components, smoking; psychological stress and low birth weight (Rituparna et al., 2007). The three major factors are discussed below.

1.1.3.1. **Obesity**

Obesity is defined as a body mass index (BMI - kg/m²) of more than or equal to 30. An increase in obesity status around the world is without a doubt a great contributor to the increase noted in insulin resistance and the metabolic syndrome as well as in type 2 diabetes (Mokdad et al., 2001).

Obesity is a condition that is greatly associated with other health risks such as high blood pressure, IR and type 2 diabetes. It is characterized by an increase in visceral body fat and an accumulation of adipose tissue. Obesity is associated by higher concentrations of fatty acids released from the increased fat mass. These fatty acids are driven towards a number of synthetic processes such as the synthesis of signalling molecules such as Akt, whose levels are much higher in obese individuals (Corcoran et al., 2007).

In obesity associated type 2 diabetes, there is an increased accumulation of visceral fat which contains proinflammatory molecules that mediate the inflammatory process like tumour necrosis factor (TNFα) when activated from adipocytes and macrophages (Black, 2006).

These adipokines have a role in the regulation of insulin sensitivity in the body. Adiponectin for example, whose levels are low in obesity, improves insulin sensitivity, reduces glucose output and fatty acid oxidation in the liver (Qatanani et al., 2007).
1.1.3.2. Diet

Poor dietary choice is one of the major contributing factors to the obesity and its associated disorders like type 2 diabetes. The inclusion of foods rich in trans fatty acids and high ratios of saturated to unsaturated fats results in weight gain and predisposition to obesity. Foods such as red meats, refined grains, sweets and high fat dairy products have been linked to risk of type 2 diabetes (Fung et al., 2004).

Collected epidemiological data also links saturated fatty acid intake with the increased risk of insulin resistance, diabetes and impaired glucose tolerance (Lichtenstein, 2000). Saturated fatty acids cause more potentiation than the unsaturated ones. This has led to speculation that dietary saturated fatty acids may lead to insulin resistance through chronic stimulation of insulin secretion (Frayn, 2003).

When a restriction in diet occurs, there is an improvement in insulin sensitivity and action. This improvement is seen even with only a slight decrease in weight. This could be due to changes in molecular nutrient sensors for insulin action such as fatty acids and amino acids as well as a reduction in reactive oxygen species (Gumbs et al., 2005).

Weight loss is characterised by a reduction in fat cell mass, especially visceral fat which contain inflammatory markers associated with insulin resistance and decreased insulin sensitivity. Reduced visceral fat due to weight loss is accompanied by decreased adipose TNFα release resulting in improved insulin sensitivity (Mlinar et al., 2007)

1.1.3.3. Exercise

After the diet of the diabetic patient has been modified, physical activity levels should also be addressed. Lack of exercise has been shown as one of the major causes of diabetes. Including both a change in diet as well as an appropriate exercise regimen is important because it helps to keep excess weight off as well as helping to prevent weight regain (Klein et al., 2004).
The American Diabetes Association (ADA) highly recommends that exercise should be part of the patient’s daily regimen although those patients with increased risk factors like coronary disease or neuropathy should seek medical advice before embarking on a strenuous physical exercise regimen (Zinman et al., 2004). Exercise decreases the risk of the development of diabetes in women (Hu et al., 1999), as well as improving the levels of haemoglobin A1C (HbA1C) in the body even though the body mass is reduced (Sigal et al., 2006).

When a patient does not have severe insulin deficiency, moderate exercise can decrease plasma glucose concentrations without complications. As long as the person has been deemed healthy, well hydrated and without ketones in the blood and/or urine, then one can exercise (Sigal et al., 2006). Care should, however, be taken with type I diabetes patients i.e. those taking insulin and/or its secretagogues. This is especially if the dose of the medication or the diet has not been altered accordingly. This may result in hypoglycaemia.

Exercise increases glucose uptake and lowers other parameters such as FFAs, TGs and VLDLs which are elevated in insulin resistance and diabetes (Black et al., 2006).

A marked upregulation of GLUT 4 expression in skeletal muscle in particular has been shown in response to exercise (Holmes et al., 2004). Since glucose transporters are important in the facilitated transport of glucose from the blood to the tissues, their upregulation in response to exercise will improve the glucose profiles in obese individuals.

1.1.4 Insulin Resistance

Insulin resistance is defined as a condition whereby the cells are unable to take up glucose in response to insulin in target tissues such as liver, skeletal muscle and adipose tissue. It is characterised by blood insulin concentrations higher than those required for normal response so as to maintain normoglycemic states in the body (Eckel et al., 2005; Schenk et al., 2008). IR is associated with body fat accumulation especially in the
muscle cells seen in obese states where there is a high rate of breakdown and uptake of fatty acids (Schenk et al., 2008).

There are a number of factors that contribute to the development of IR. These factors could be genetic or acquired. The most common acquired factors to the predisposition of IR are sedentary lifestyles, aging and obesity, especially visceral obesity (Schenk et al., 2008). Visceral fat is rich in blood and nerve supply, high numbers of adrenergic receptors and a high concentration of glucocorticoid receptors (Black, 2006).

The main characteristics of IR include impaired insulin stimulated glucose uptake by the muscle, impaired lipolysis in adipose tissue and impaired gluconeogenesis (Mlinar et al., 2006; Schenk et al., 2008). The availability, uptake and oxidation of fatty acids are major contributors to the IR state (Bruce et al., 2006).

FFAs interfere with insulin binding and its removal form the circulation. This contributes to hyperinsulinemia, a characteristic of insulin resistance. Dyslipidemias are also present in IR and these include increased plasma concentrations of TGs and decreased concentrations of HDL-C (Black, 2006).

Insulin resistance is associated with inflammation. Stress hormones, pro inflammatory cytokines and fatty acids are some of the mediators involved in the inflammatory process. Some of these pro inflammatory molecules include cytokines like tumour necrosis factor (TNFα), interleukin 6, resistin and leptin. They may mediate insulin resistance by disrupting insulin binding and signalling processes. TNFα for example, serine phosphorylates the insulin receptor and insulin receptor substrate associated proteins (Black, 2006).

There are various models that are used to evaluate IR including hyperinsulinaemic euglycaemic clamp, fasting plasma insulin concentration, homeostasis model assessment index (HOMA), the quantitative insulin sensitivity check index (QUICKI) and the McAuley index (Mlinar et al., 2006). The basic mechanism by which they all work is by measuring insulin, glucose and/or triglyceride levels. These parameters are then correlated using various computations depending on the index in use. These are presented in Figure 1.2.
HOMA index = insulin (mU/L) x [glucose (mmol/L)/22.5]

QUICKI = 1/(log insulin (mU/L) + log glucose (mg/Dl)).

McAuley index = $e^{[2.63 - 0.28 \ln (\text{insulin} \, \text{mU/L}) - 0.31 \ln (\text{triglycerides} \, \text{mmol/L})]}$

**Figure 1.2:** Common indices for IR evaluation (Adapted from Milner et al., 2006).

### 1.2. PHARMACOLOGICAL TREATMENTS AND TRADITIONAL MEDICINE

The aim of diabetes treatment according to the global partnership for effective diabetes management is the attainment of glycemic goals, early treatment with combination therapy and investigating underlying pathologies (McGill et al., 2006). Various drugs have been used effectively in the management of diabetes and are discussed herein.

#### 1.2.1. Pharmacological agents

There are many pharmacological agents available for the treatment and management of diabetes. These include insulin, sulphonylureas, biguanides and thiazolidinediones. There are many mechanisms through which these drugs act to elicit the desired glucose homeostasis in the body. The drugs may exert their effect via different mechanisms with a similar end result such as the improvement of the glycemic index as shown in Figure 1.3 below.
Figure 1.3: Some common drugs used in diabetes treatment and their main target tissues (Adapted from Evans and Rushakoff, 2002). The red arrows denote a decrease while green arrows denote in the physiological actions found at the specific sites. For example, after a carbohydrate load, digestion of polysaccharides takes place in the intestines; there are increased FFA levels in the adipose tissue, increased glucose production in the liver, impaired insulin secretion in the pancreas and decreased glucose uptake and utilization in the skeletal muscle.

1.2.1.1. Insulin

The overall effect of diabetes treatment with insulin (injectables and secretagogues – see 1.2.1.2) is increased insulin response to glycemic changes and hepatic glucose absorption (Childs et al., 2007).

Endogenous insulin is a key hormone involved in glucose homeostasis in the body. Its dysfunction or complete lack of function leads to an imbalance in the body resulting in many conditions, one of which is Diabetes mellitus. Insulin is a 51 amino acid polypeptide hormone with a molecular weight of 5700 produced by beta cells of the pancreas in a region called the islets of Langerhans (Nelson and Cox, 2000).
It is initially formed in the pancreas as pre-proinsulin with 110 amino acids. This passes through the endoplasmic reticulum where a 24 amino acid signal peptide is removed by enzyme action leaving a molecule called proinsulin. Proinsulin is packaged into secretory granules that bud off from the golgi apparatus where a further 33 amino acids known as the C peptide sequence are removed and disulphide linkages formed. The resultant chains designated as A and B chains are the basic structural unit of insulin (See Figure 1.4). Carboxypeptidase E enzyme removes another two amino acids from the sequence leaving the final amino acid sequence that forms insulin (Greenspan et al., 2001).

Insulin along with C peptide as well as some basic amino acids is released into the extracellular fluid when insulin is being secreted. The concentrations of endogenous insulin and those of the C peptide are directly related. This is important especially for diabetic patients since endogenous insulin levels in the body is a major determinant of the diabetic condition. It is the C peptide levels that are measured to determine the endogenous insulin levels (Greenspan et al., 2001).

Insulin is secreted in the body in response to high glucose concentrations in blood. Other factors that influence insulin secretion in the body include gene expression, hormonal regulation as well as cAMP levels. The primary function of insulin in the body is the reduction of glucose levels in blood. It is however also involved in protein, lipid, carbohydrate as well as glucagon metabolic processes (Nelson and Cox, 2000).

Insulin exerts its action via its receptor. Insulin receptor is defined as a ligand activated receptor from the tyrosine kinase family of transmembrane signalling proteins. It has two identical alpha chains and two transmembrane units as well as disulphide bonds. Insulin binds to the alpha sub units on the insulin receptor, stimulating tyrosine kinase enzyme. This enzyme initiates the phosphorylation mechanism that leads to the translocation on of glucose transporter 4 (GLUT 4), which in turn increases glucose uptake (Greenspan et al., 2001).

The insulin receptor is also responsible for mediating post receptor signalling pathways whose function is to further regulate glucose homeostasis in the body. This is done via the phosphatidyl inositol- 3- kinase (PI-3-K) activity as well as the mitogen activating protein kinase (MAPK) pathway (Schenk et al., 2008)
The close similarity between bovine, porcine and human insulin led to their use in replacement therapy for a long time. The differences were however enough to cause a number of patients’ immune systems to produce antibodies against them. The intended action of the insulin was therefore neutralised and inflammatory responses were seen at injection sites. Synthetic insulin made by recombinant DNA technology eliminates the inflammatory responses and is now widely used as treatment for diabetes (Pittman et al., 2004).

**Figure 1.4:** Primary structure of porcine pro-insulin (Adapted from Pittman et al., 2004). There are many similarities between human insulin and that from a number of different species. Human and porcine insulin differ by only one amino acid at the C terminal of the connecting peptide (C peptide). The C peptide however differs a lot more between various species.

The overall effect of diabetes treatment with insulin is increased insulin secretion from the pancreas, increased insulin sensitivity to glycemic changes and hepatic glucose absorption (Childs et al., 2007). Various forms of insulin (secretagogues and injectables) have been used over the years some with better efficacy than others. Table 1.2 shows some of these preparations.
Table 1.2: Examples of some insulin preparations available on the market (Ref: Visalli et al., 2002.)

<table>
<thead>
<tr>
<th>Product</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin</td>
<td>Recombinant human insulin</td>
</tr>
<tr>
<td>Insulin aspart</td>
<td>Recombinant human insulin analogue</td>
</tr>
<tr>
<td>Insulin lispro</td>
<td>Recombinant human insulin analogue</td>
</tr>
<tr>
<td>Pork insulin</td>
<td>Pork insulin</td>
</tr>
<tr>
<td>Glargine</td>
<td>Recombinant human insulin analogue</td>
</tr>
</tbody>
</table>

1.2.1.2. Sulphonylureas

This group of drugs, which are insulin secretagogues works by inhibiting $K_{ATP}$ channels in plasma membranes of pancreatic β cells (see Figure 1.5). This inhibition works to stimulate the secretion of insulin, which is similar to that produced by glucose in the body, but is of a distinct mechanism. This difference in mechanism is why these drugs are used successfully by type 2 diabetes patients who are not able to respond optimally to glucose stimuli (Foye et al., 1995).

Both glucose and sulphonylurea drugs work together to increase the release of insulin from the pancreas. The presence of glucose is paramount in the optimal release of insulin under these conditions. Insulin release is markedly reduced even in the presence of sulphonylurea drugs in the absence of glucose (Melander, 2004).

Sulphonylurea drugs all have a sulphonic acid-urea nucleus, hence their name. Addition of various chemical groups at different positions on this nucleus is what results in the different types of drugs in this group. The actions of these resultant drugs may have the same desired result in the patient but the potency and therefore the efficacy differs significantly (Melander, 2004).
Figure 1.5: Schematic of drug effects on insulin secretion in the pancreatic β cell (Adapted from McClenaghan (2007).

The mechanism of action of these drugs is thought to be through increasing the release of insulin from the β cells of the pancreas as well as improving its action on the target cells. There are receptors on the surface of pancreatic β cells on which sulphonylurea drugs can bind specifically, depending on their affinity. These receptors consist of two proteins, one which binds the sulphonylurea and is called the sulphonylurea receptor and the other is an ATP sensitive potassium channel called Kir 6.2. The general structure of this receptor is four molecules of the Kir 6.2 forming the pore while associated with four molecules of the sulphonylurea receptor (SUR) (Greenspan et al., 2001).

Once the beta cells have been activated by the drug, potassium channels close leading to the depolarization of the β cell membrane. Calcium ions can therefore now enter the cell and insulin release is promoted in this way. Sulphonylurea drugs are typically not indicated for type 1 diabetic patients since they require the functioning of the β cells to produce the desired effect on blood glucose. These drugs have been found to be most effective in non obese patients with mild maturity onset diabetes and whose high
glucose levels have not responded appropriately to diet alterations (Greenspan et al., 2001).

Figure 1.6: The chemical structures of some sulfonylurea drugs. All share one basic structure (Melander, 2004).

1.2.1.3. Biguanides

Biguanides are a group of drugs that are known to alter the action of insulin. One of the most widely used drugs in this group is metformin (1, 1- dimethylbiguanide hydrochloride). Metformin is not metabolized in humans (Greenspan et al., 2001).

The activity of metformin does not seem to be altered in the diabetic state although the use of other drug formulations does. This drug has a very high oral bioavailability of up to 60% as well as complete absorption in the gastrointestinal tract 6 hours after
ingestion. It does not bind to plasma proteins and is therefore distributed quite easily in the body. The lack of metabolism in the liver is a major differentiating point between metformin and other biguanides. This drug is excreted via the kidney 4 to 9 hours after administration (Scheen, 1996).

![Chemical Formula: C₄H₁₁N₅](image)

**Figure 1.7:** Chemical structure of Metformin (Adapted from: Arlt et al., 2001).

Metformin is widely used in the treatment of patients with insulin resistance because it can be safely used as an adjunct to diet therapy in obese patients to control their high glucose levels especially those who are not responsive to other therapies. Its use is not associated with the weight gain seen with the use of insulin and sulphonylurea drugs and this is an added advantage. Improvements are also seen in fasting as well as postprandial hyperglycemia as well as hypertriglyceridemia (Greenspan et al., 2001).

There are various ways through which metformin seems to effect its action. It has been shown to reduce both fasting blood glucose levels as well as improve postprandial hyperglycemia in patients with type 2 diabetes. This drug alleviates hyperglycemia through increasing the conversion of glucose to lactate in intestinal cells after oral administration (Greenspan et al., 2001).

The exact mode of action of this drug seems to be in dispute. A report from 2001 (Zhou et al., 2001) indicated that it acts by activating AMP-activated protein kinase (AMPK) in liver cells, leading to increased fatty acid oxidation and glucose uptake by cells. An overall reduction in lipogenesis and hepatic glucose production would then be observed.
Figure 1.8: Schematic of the mechanisms through which metformin affects lipid and glucose metabolism in the body (Adapted from: Zhou et al., 2001). SREBP-1c is an important insulin-stimulated transcription factor that is implicated in the pathogenesis of insulin resistance, dyslipidemia, and type 2 diabetes. Target genes that are induced by SREBP-1 include those that encode lipogenic enzymes (Shimomura, 2000).

Metformin has antioxidant properties which are useful in its use as treatment for diabetes and cardiovascular disease. Some of its antioxidant mechanisms of action include the inhibition of xanthine oxidase and phosphodiesterase, inhibition of advanced glycation end product formation and decreased production of tumour necrosis factor (Rahimi et al., 2005).

There are a few side effects associated with the use of metformin, the most frequent of which are gastrointestinal symptoms. These include nausea, vomiting, diarrhoea and abdominal discomfort. These symptoms usually improve after a short period of metformin use. Overall, metformin is an effective and safe drug evidenced by its wide use as an antihyperglycemic drug. It should however not be prescribed to patients with conditions that might lead to lactic acidosis such as heart failure or kidney disorders (Greenspan et al., 2001).
1.2.1.4 Thiazolidinediones

These drugs act by increasing the sensitivity of peripheral tissues to insulin by affecting the expression of specific genes. They achieve this by binding and activating peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor (Qatanani et al., 2007). Some of the effects of this gene expression include the increase in the expression of the glucose transporters, decreased hepatic glucose output as well as the increased differentiation of pre-adipocytes into adipocytes (Greenspan et al., 2001). This is why a thiazolidinedione drug is chosen as a positive control in adipocyte differentiation.

The high affinity of these drugs to PPAR-γ is important in insulin resistance management since large adipocytes that differentiate from smaller ones produce substances like TNFα which are resistant to insulin (Suzuki et al., 2002). Thiazolidinediones suppress the expression of these adipokines that contribute to insulin resistance (Sharma et al., 2007).

1.2.2. Alpha glucosidase

Alpha glucosidase inhibitors act by inhibiting alpha (α) glucosidase enzyme in the brush border of the small intestine. This delays the absorption of glucose by decreasing the breakdown of complex carbohydrates by enteric digestive enzymes. Only monosaccharides are absorbed from the intestinal lumen for transport into blood (Shibao et al., 2007).

Control of postprandial hyperglycemia is essential in the management of early diabetes. Associated parameters such as hypoglycaemia, obesity and postprandial fluctuations make glycemic control more challenging for the diabetes patient. Postprandial hyperglycaemia which in diabetes is characterised by a large rapid increase in blood glucose levels is determined by a number of factors such as timing, quality and composition of meals as well as the resulting insulin secretion. Proper control of postprandial plasma glucose levels is optimal when medication, dietary regulation and exercise are used in combination (Ceriello, 2006).
Research has shown that the inhibition of some or all intestinal disaccharides as well as could regulate carbohydrate absorption in the body. These inhibitors for example acarbose and miglitol have found use in the control of postprandial hyperglycemia in type 2 diabetes (Van de Laar et al., 2005). Acarbose is an oligosaccharide analogue that has a much higher (1000 x) ability to bind to intestinal disaccharides than to products of carbohydrate digestion (Greenspan et al., 2001).

Some of the more commonly used α glucosidase inhibitors like acarbose have severe gastrointestinal side effects such as diarrhoea flatulence and abdominal pains. This raises the need for other sources of theses inhibitors that have fewer side effects. The most obvious choice for these alternatives would be plants, especially known medicinal plants (Fujisawa et al., 2005).

1.2.3. Antioxidants

1.2.3.1. Oxidative stress a complication of diabetes

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the body’s capacity for defence against antioxidants in vivo resulting in a shift of the body’s systems to stress. It plays a major role in multiple diseases and is associated with many conditions such as aging as well as diseases such as cancer, cardiovascular diseases, diabetes (both type 1 and type 2) and complications associated with diabetes (Rahimi et al., 2005).

There are a number of ways through which oxidative stress in involved in the diabetic condition. Glycation of proteins such as superoxide dismutase (SOD) in hyperglycaemia can result in oxidative stress even though the levels of ROS are within range. High insulin levels associated with type 2 diabetes can also stimulate oxidative stress by inducing hydrogen peroxide (H₂O₂) during the activation of insulin receptors. Erythrocyte catalase levels are reduced markedly in diabetes and so the removal of the harmful peroxide is hampered (Wiernsperger et al., 2003). It is involved in beta cell death due to the reduced antioxidant potential in these cells. Insulin uptake is also affected by oxidative stress affecting its delivery to target tissues. Oxidative stress has a
role in impairment of GLUT 4 mediated glucose transport in the body (Bertelsen et al., 2001).

1.2.3.2. Role of antioxidants to combat insulin resistance complications

Antioxidants are one of the primary defence systems in the body against damaging free radicals such as reactive oxygen species (ROS). Antioxidants that function to neutralise ROS in the body can be broadly divided into two groups, enzymatic and non enzymatic. Examples of enzymes that act as antioxidants in the body include catalase, SOD, glutathione reductase and glutathione peroxidase while vitamins are examples of non enzymatic antioxidants (Wiernsperger et al., 2003).

Plants are known to possess antioxidant properties that they use for protection in their own systems against internal stresses such as free radicals as well as external stresses such as insects and pests. The antioxidant properties of traditional medicines may help to explain the said benefits of these plants since reactive oxygen and/or nitrogen and chloride species are involved in disease (McCune et al., 2007).

There are many sources of ROS in the body. Some of the cytosolic sources of ROS include glycolysis, glucose-6-phosphate dehydrogenase, advanced glycation and xanthine oxidase (Forbes et al., 2008). Once reactive species are produced in the body and allowed to accumulate they attack biological molecules such as DNA in the body. Cellular material and debris accumulates in the cell and some of the visible evidence of this is in the processes of ageing, inflammation as well as some degenerative disorders (Kohen et al., 2002).

Studies done using gene analysis suggest that levels of reactive oxygen species are increased in diabetes states; both type 1 and type 2. This is because in the diabetic state there is a reduction in endogenous antioxidants and increased levels of oxygen stress (McCune et al., 2007). This has been confirmed by measuring various parameters of the cellular redox state. The methods used include the treatment of cell cultures with treatments designed to alter ROS levels (Houstis et al., 2006).
Cellular damage in hyperglycaemic conditions has been attributed to four main mechanisms: increased polyol pathway, increased advanced glycation end product (AGE) formation, PKC isoform activation and increased hexosamine pathway flux. In hyperglycaemia, there is increased conversion to sorbitol by aldolase reductase and sorbitol dehydrogenase enzymes. This conversion uses up NADPH that is needed to make reduced glutathione (GSH) in the body. It is these decreased levels of glutathione in the body that contribute to oxidative stress (Rituparna et al., 2007). Oxidative stress is generated during AGE formation which is accompanied by ROS generating reactions as well as the interaction between AGEs and their respective receptors (Forbes et al., 2008).

Some of the drugs used in the treatment of diabetes have antioxidant properties. Allopurinol for example, inhibits xanthine oxidase enzyme while glibenclamide scavenges free radicals. Metformin, antioxidant properties have found potential in treating cardiovascular complications associated with diabetes. Refer to section 1.1.2.2 (Rahimi et al., 2005).

1.2.4. Traditional medicines

1.2.4.1 Significance

An ethno-botanical study aims to add value on the information that already exists about a variety of local plants known to have specific medicinal properties. Plants produce a wide range of secondary metabolites that are bioactive which provide them with protection against external aggression from bacteria, fungi, viruses and insect pests. This then suggests that plants contain biologically active substances that can be used to protect humans, livestock, and crop plants against microbial and other disease causing agents. The possibility of relying on plant extracts for use in medicine has been and is continually being investigated (Evans et al., 1997).

Many plants are however still unexplored, with many unique and potentially useful medicinal properties. Although scientific data is still lacking for the bulk of these plants, there are records of medicinal plant uses in various herbariums as well as institutions of
learning. Most of the knowledge is however held by local herbalists who pass it on from generation to generation. In this regard there have been concerted efforts by governments as well as communities to put together a database of the traditional plants in use in many countries.

Although traditional herbalists may not be familiar with the active biological compounds in the medicinal plants they use, knowledge of such bioactive compounds would enhance the application and effectiveness of traditional herbal medicines against a wide range of human and animal ailments. Not all the chemical compounds that are produced by or constitute the plants are of interest medicinally.

There are many plants and plants extracts which possess marked hypoglycaemic activity. Anecdotal as well as ethno-botanical data appears to support the use of traditional medicines by traditional health practitioners for diabetes syndrome. Some have shown remarkable results in decreasing blood sugar levels as well as mediating other cellular metabolic functions that generally enhance the diabetic patients overall health status. It is therefore prudent to do extensive research on the various plants in use and test their efficacy and document their phytochemical compositions and mode of action.

The mode of action of the extracts from these plants is uncertain. However, many anti-diabetic plants act, at least in part, through their fibre, vitamin or mineral contents and some secondary metabolites. Mineral deficiencies are common in diabetic patients which aggravates insulin deficiency. Several minerals found in some medicinal plants have been reported to be cofactors that signal intermediaries of insulin action and key enzymes of glucose metabolism (Day, 1998).

There are a number of valid concerns that have been put forward on the use of complementary medicine for the treatment of diabetes. Some remedies have reported side effects and may induce drug interactions when taken in combination with other drugs. These interactions result in either exaggerated or therapeutic effects below what is expected. Product quality and control is also a major concern since the active components of most of these plants have not been fully determined. Quality control in terms of the plant parts used, storage and processing techniques is not always adhered to by the practitioners (Shane-McWhorter, 2001).
Due to inherent International Property Rights (IPR) protocols followed during the course of this research, the plant extracts used will be referred to herein in code.

1.2.4.2. Statistics and prevalence of use

The plants studied are used by Kenyan communities and were chosen based on ethno-botanical documentation of their use and anecdotal reference to their effectiveness by traditional medicine practitioners and diabetic patients who have used them in various forms during the course of their therapy.

Medicinal plants were ranked fourth in relation to their uses by about 87.5% of respondents in a study on their status, uses and management of indigenous plants in the upper imenti forest reserve, a prime ecological region in Kenya (Mworia, 2000). The plant extracts used by the communities come from various part of the plant either the leaves, roots or barks.

1.2.4.3 Plant bioactive compounds

Basic metabolic pathways in plants provide both primary and secondary metabolites that are used by the plant to make useful compounds such as proteins and carbohydrates. Most plant compounds with medicinal properties are primary and secondary metabolites. The bioactive principles found in plants are usually from the main groups of compounds namely: alkaloids, saponins, flavonoids, anthaquinones, terpenes, carotenoids and glycosides. Other compounds include resins, rubbers, lignans, gums, waxes, dyes, flavours, fragrances, proteins, amino acids, bioactive peptides, phyto hormones and sugars. There are some classes of compounds that appear to be richer in secondary metabolites such as alkaloids, phenolics and terpenoids (Evans, 1997).

The wide range of structures of the various plant constituents which appear to be the active hypoglycaemic principles suggests different sites of action within the body. Plant constituents that have been found to have this activity include polysaccharides, flavonoids, steroids, saponins, diterpenoids, alkaloids (Lamba et al., 2000).
Figure 1.9: Shikimic acid pathway (adapted from Craig et al., 1999). This pathway shows how some of the bioactive compounds in plants are synthesized.

1.2.4.4. Plants extracts used in this study.

Dr M Mbaabu, my co-supervisor and senior lecturer at the University of Nairobi, identified 3 plants that are commonly used for diabetes in Kenya. The plants were chosen based on a survey showing their widespread use and alleged efficacy. He made these plants extracts available to the researcher to test their efficacy.
1.3. SCREENING METHODOLOGY FOR ANTI DIABETIC PROPERTIES IN PLANTS

1.3.1. Antioxidant assays of plant extracts

1.3.1.1. End product glycation

Advanced glycation end products (AGEs) are defined as the products of glycation and oxidation formed via a process known as the Maillard reaction. The formation of AGEs is initiated when glucose interacts with specific amino acids on proteins (Rahimi et al., 2005). The resultant schiff base is readily reversible to an early glycosylation compound called an amadori product (AP). The AP can be further rearranged to irreversible protein compounds called AGEs.

Major AGEs found \textit{in vivo} are formed from highly reactive intermediate carbonyl groups called oxoaldehydes such as glyoxal and methyloxal (Brownlee, 2001). AGEs from exogenous sources also contribute to AGE levels in the body. Tobacco smoke and diet are some of the major sources of exogenous AGEs (Vlassara et al., 2002).

Accumulation of AGEs in the body is one of the causes of diabetic complications. Some of the sites of this accumulation include the kidney, retina as well as atherosclerotic plaques (Bucala \textit{et al.}, 1995). The modification of proteins during the formation of AGEs is one of the consequences of hyperglycemia seen in \textit{Diabetes mellitus}. AGEs may cause damage through the formation of cross links in collagen that has been linked with vascular stiffness associated with hypertension found in diabetic patients (Cooper \textit{et al.}, 2001). Examples of AGEs are N-(carboxymethyl) lysine and pentosidine (Matsuura \textit{et al.}, 2002).

In hyperglycaemic conditions, glycoxidation products whose formation is enhanced by oxidising conditions and reactive oxygen species accumulate in tissues in diabetes. The increased levels of AGEs in the body modify intracellular proteins altering their function and they also modify extracellular matrix components. Modifications of
cellular processes such as cell signalling occur when AGEs interact with their specific receptors (Rahimi et al., 2005).

Once the AGEs have been formed in the body, their accumulation bound to proteins depends on the half life of the specific protein. This accumulation results in the cross linking of AGEs with amino acids of other proteins. Pentosidine, an AGE, is a marker for this cross linkage and is found in high levels in patients with heart disease (Lapolla et al., 2007). Pentosidine has an intrinsic fluorescence that is used in the measurement of its levels in plasma and tissues. Other AGEs without this intrinsic fluorescence can be measured by other means such as enzyme linked immunosorbent assay (ELISA) (Goh et al., 2008).

Research in animal and in vitro models have shown that AGEs activate cytokine production and transcription factors though binding to their specific receptors (RAGE). RAGE is a member of the immunoglobulin super family of receptors. It is through these receptors that AGEs trigger the activation of secondary messenger pathways such as protein kinase C (Goldin et al., 2006). Some of the effects of AGEs are shown in Figure 1.10 below (Meerwaldt et al., 2008; Haartog et al., 2007).

![Image of Figure 1.10: Pathogenetic effects of advanced glycation end products (AGEs) accumulation in the body (Adapted from Meerwaldt et al., 2008).](image)

Patients with type 2 diabetes and heart disease have been found to have elevated levels of serum pentosidine, a marker of glyoxidation and malondialdehyde (MDA) an indicator of lipid peroxidation and oxidative stress in vivo (Kilholvd et al., 1999).
Accumulation of AGEs can be controlled in the body by intensive monitoring of glucose levels as well as the inhibition of glycation reactions by using compounds such as pyridoxamine. Some of the medicines commonly used for diabetes such as metformin have been shown to cause a decrease in AGE accumulation by reducing methylglyoxal levels and scavenging for ROS (Meerwaldt et al., 2008; Bonnefont- Rousselot et al., 2003).

AGE inhibitors are finding use in research and medicine in the control of AGE accumulation and related complications. Aminoguanidine has been shown to prevent diabetic vascular complications in animal studies. Its mode of action is thought to be targeting of carbonyl intermediates as a nucleophilic trap (Goh et al., 2008). Pyridoxamine, another AGE inhibitor prevents the degradation of APs to protein AGE products. It has been shown to reduce hyperlipidemia and prevent AGE formation in rat models (Stitt et al., 2002).

1.3.1.2 Xanthine oxidase assay

Xanthine oxidase is a cytosolic enzyme of the molybdenum iron- sulphur flavin hydroxylase group. This enzyme is involved in the catalysis of purine hydroxylation and is widely distributed in many organs including liver, kidneys and heart. It has two identical sub units of 145 KDa each (Pacher et al., 2006).

Xanthine oxidase (XO) catalyses the reduction of oxygen to water with the concomitant release of superoxide anions and hydrogen peroxide. It is therefore a source of free radicals in the body and contributes to oxidative stress (Kadam et al., 2007). The conversion of hypoxanthine to xanthine and then to uric acid is facilitated by this enzyme as shown in figure below.
Figure 1.11: Purine degradation pathway. Xanthine oxidase enzyme is one of the enzymes responsible for converting xanthine to uric acid (Adapted from Pachar et al., 2006).

Allopurinol is a xanthine oxidase inhibitor used in the clinical management of hyperuricaemia and related conditions such as gout. It is rapidly oxidised by XO in vivo into its active metabolite oxyipurinol. Allopurinol is readily absorbed and has a short half life in plasma of about 2-3 hours.

In type 1 diabetes patients, elevated levels xanthine oxidase in plasma and liver have been observed. Allopurinol has been shown to decrease the levels of oxidative markers such as haemoglobin glycation and glutathione oxidation in type 1 diabetic patients (Desco et al., 2002).

High uric acid levels associated with decreased xanthine oxidase activity have a damaging effect on vascular function as well as contribute to the antioxidant capacity of blood (Pachar et al., 2006)
1.3.1.3 Superoxide anion scavenging effect

Superoxide anions are potent oxygen derived free radicals that contribute to oxidative stress in the body and may affect cell proliferation and adhesion (Cathcart, 2004). They can also be defined as highly reactive compounds produced when oxygen is reduced by a single electron. This may occur in normal catalytic functions of many enzymes in the body, even in the normal state. Superoxide dismutase, is an enzyme found in the body that helps to protect against the harmful effects of these free radicals (http://nih.gov/glossary)

1.3.1.4 DPPH assay

DPPH stands for Diphenylpicrylhydrazyl; a molecule with a stable free radical. This assay is used to measure the free radical scavenging ability of the plant extract. This assay is used to provide information on the mechanism of action of possible antioxidants, as well as the ability of a compound to donate hydrogen atom. The number of electrons a given molecule can donate can also be established using this assay (Mothana et al., 2008). This method is rapid, simple and works well with both hydrophilic and lipophilic samples (Buratti et al., 2007).

The characteristic purple colour of free DPPH is altered when an antioxidant with the capacity to donate electrons to DPPH is added to the reaction mixture. This change in colour from purple to yellow can be followed spectrophotometrically at 517 nm. The resulting decolourisation is stoichiometric with respect to the number of electrons captured by DPPH (Mothana et al., 2008).

This assay is used to provide information on the mechanism of action of possible antioxidants, as well as the ability of a compound to donate hydrogen atom. The number of electrons a given molecule can donate can also be established using this assay (Mothana et al., 2008).
1. Diphenylpicrylhydrazyl (free radical)  
2: Diphenylpicrylhydrazine (non radical)

**Figure 1.12:** Structures of free radical and non radical DPPH (Adapted from Molyneux, 2004).

1.3.1.5. FRAP assay

Ferric reducing ability of plasma (FRAP) is an assay that uses antioxidants as reductants in a calorimetric redox reaction. This reaction takes place at a low pH with the conversion of ferric form of tripyridyltriazine (TPTZ) to its ferrous form. With the ferrous ions in the reaction mixture being of known concentration, the absorbance changes seen in antioxidant mixtures are linear. Examples of standards that can be used in this assay include trolox and ascorbic acid. The stoichiometric value of these standards in this assay has been determined to be 2 (Benzie and Strain, 1999).

FRAP assay is sensitive, fast, easy to use and inexpensive with highly reproducible results. The three main reagents in the assay are sodium acetate acetic acid, TPTZ, and HCl. The scavenging effect of a plant extract and its reducing capacity are strongly related, hence measurable using this method (Oyawoye et al., 2003). Some studies have shown that this assay is more accurate when measuring antioxidant capacities of water soluble antioxidants (Bub et al., 2000). This was an added advantage since the plant extract tested were highly water soluble.

Total reducing antioxidant potential (TRAP), is similar to FRAP in mechanism of action and is an expression of the defence mechanism against glycolipid oxidation. Low TRAP levels have been found in both types 1 and 2 diabetes (Ghiselli et al., 1995).
There are various ways in which FRAP values can be expressed. An example is shown in the equation below:

\[
\text{FRAP value of sample (\(\mu\text{M}\)) = \frac{\text{(Change in absorbance of sample for } 0 - y \text{ minutes})}{\text{Change in absorbance of standard for } 0 - y \text{ minutes)}} \times \text{FRAP value of standard}
\]

1.3.2. Cell lines

Tissue culture methods were developed with the aim of studying the behaviour of animal cells, without the influence of other factors normally found in the animals. The cells in culture can be manipulated to test mechanisms and processes found in vivo. Cell culture also offers an easier way to replicate sampling as well as cell quantitation (Freshney et al., 2000).

Many different cell types can be grown in culture. Some of these include hepatocytes, skeletal tissue, fibroblasts, endocrine cells, cardiac and smooth muscle. Cells differentiate and propagate differently depending on cellular characteristics as well as the conditions of growth.

One of the main advantages of using cell cultures in cell biology studies is that one is able to control the physiochemical environment such as pH, CO\(_2\) and temperature as well as the physiological conditions. Another advantage is that after one or two passages, cultured cell lines have a homogenous constitution since the cells are randomly mixed during transfer (Freshney et al., 2000).

Continuous cell lines are ‘transformants’ of a primary culture after several sub culturing procedures. The formation of a cell line from a primary culture means that there is an overall increase in the cell numbers over several generations. The cells are much smaller and rounded, with a much higher growth rate. Other advantages of continuous cell lines are that due to higher growth rates, they have a higher cell density, resulting in higher yields. They need relatively little maintenance and can grow well in suspensions (Freshney et al., 2000)
Various cellular metabolic processes can be followed in the cells obtained from a continuous cell line for example, the glucose uptake test. Continuous cell lines are widely used in research; those used in this study represent some of the major organs and tissues in the body affected by diabetes: namely the liver and skeletal muscle.

1.3.2.1. Cell lines used

Chang liver cells are one of the first cell lines to be derived from non-malignant tissue. They are adherent epithelial cells obtained from human liver. These cells are widely used in virology, biochemistry and transplantation related to malignancy studies. These cells tend to pile up in cultures with high population density. However, this cell line was found to have a HeLa profile by DNA fingerprinting, suggesting that it may be a HeLa derivative and no longer the original cell line (Gartler et al., 1968).

C3A cells are derived from a patented human liver cell line from a hepatoblastoma, a tumour of early childhood. These cells have many characteristics similar to those of human liver and are widely used in the study of liver functions. Some of these characteristics include serum protein synthesis, aromatic amino acid metabolism and P450 based drug metabolism (Kelly, 1994). These cells are highly aerobic and have a strong contact inhibition. Alpha-fetoproteins are produce in large quantities by these cells indicative of their hepatoblastoma origins (Kelly et al., 1997).

The C2C12 continuous cell line was derived from murine skeletal muscle. It is a sub clone from a myoblast line established from normal adult C3H mouse leg muscle. These cells differentiate rapidly, produce characteristic muscle proteins and grow as a monolayer. As with Chang cells, C2C12 cultures are not allowed to become confluent since this depletes the myoblastic population. These cells provide good models for the study of vitro myogenesis and cell differentiation. The recommended split ratios are 1:3 and 1:6. This cell line was a good choice to study cell line maintenance as well as glucose uptake protocols (Ref: ATCC).
1.4. THE RAT AS A MODEL FOR TYPE 2 DIABETES

1.4.1. Rat model

Rats are widely used in scientific research due to their ease of handling as well as the degree of homology they share as mammals with humans. Selective in breeding has led to many strains and models of type 1 and 2 diabetes, obesity and insulin resistance. Investigations into treatments also use rat models to test efficacy and effect of the drugs and procedures. For example, STZ and alloxan are some of the drugs that have been widely used in research to induce hyperglycemia in rats. New animal models continue to emerge with genetic manipulation, such as knock in, generalised knock out as well as tissue specific knock out mice (Rees et al., 2005).

Rats fed on a high fat diet were used to model insulin resistance and the pre diabetic state. Feeding normal rats with high dietary levels of saturated fat leads to pathological conditions for example obesity, hypertriglyceridemia, hypercholesterolemia and hyperglycemia (Ghatta et al., 2004). The use of high fat diet fed rat model is ideal because it is a useful model of the effects of excessive fat intake in humans, and it represents NIDDM. The high fat diet (HFD) rat fed model for insulin resistance has been extensively used to study the mechanisms involved in the prevention and/or reversal of insulin resistance (Ghatta et al., 2004).

With high fat diet, the energy intake is shifted from carbohydrate to fat. There have been numerous reports showing that mice and rats fed on a HFD have increased visceral fat accumulation, insulin resistance and hyperinsulinemia within four weeks. Obesity is usually the first visible result of this HFD. Many studies have shown a correlation between insulin resistance and central or visceral obesity. This correlation is also seen with the increased fat accumulation in intra-abdominal adipocytes (Milagro et al., 2006).

Glucose transport is the initial step in glucose utilization and is rate determining for glucose utilization in muscle. The use of deoxyglucose allows measurement of coupled transport and phosphorylation since it is phosphorylated but not further metabolised by the cells (Perrot et al., 2001).
Figure 1.13: Photograph of the male wistar rat. Male wistar rats are an out bred strain of albino rats with characteristic wide heads, long ears and tails longer than their bodies (Source: www.google.com/images and www.iar.or.jp).

1.5. PROBLEM CONCLUSION

Diabetes is a multi organ, multi system disease that affects millions of people worldwide each year. The complications that are associated with the disease are varied. The medications used for the treatment and management of diabetes have different mechanisms of action.

Traditional medicine is still widely used in Kenya as the primary or secondary line of defence in treatment and management of diseases. There is therefore a need to validate the efficacy of these plant extracts for their antidiabetic properties

1.6. AIM OF THE STUDY

To identify and investigate one Kenyan medicinal plant for its efficacy as a hypoglycaemic agent. The results obtained aim to further validate its use in the treatment of diabetes mellitus.
1.7. EXPERIMENTAL APPROACH

*In vitro* and *in vivo* approaches to the study of the effect of one Kenyan traditional medicine were done. Glucose utilization assays in different cell lines representing some of the major organs affected by diabetes were studied.

Antioxidant assays to study the effect of these plants on oxidative systems. Oxidative stress has been found to be an important consequence of over nutrition which may contribute to the complications and or development of *Diabetes mellitus*. A plant extract with strong antioxidant properties would probably be effective as an anti diabetic treatment. The promotion of glucose utilization in hepatocytes and/or myoblasts as well as glucose uptake in tissues by this plant would give it further credibility for use in diabetes treatment.

High fat diet fed rats were used as models for insulin resistance, a condition that is prevalently associated with diabetes. Medication of these rats with the plant and subsequent analysis of blood and tissues would be followed to evaluate any anti diabetic properties.
CHAPTER 2

METHODS: PRINCIPLES AND METHODOLOGY

2.1. Plant analysis
2.2. Cell lines
2.3. The rat model
2.4. Rat tissue analysis
CHAPTER 2

METHODS: PRINCIPLES AND METHODOLOGY

2.1. PLANT ANALYSIS

2.1.1. End product glycation assay

A reaction mixture was prepared containing 400 µg BSA, 200 mM glucose and 10 µl of plant extract solubilised in water. The reaction mixture was heated at 60 °C on a heating block for 24 hours. A blank was also prepared containing BSA and glucose but without the plant extract and kept at 4 °C. After the samples were heated for the allocated time, they were cooled and 100 µl aliquots transferred to 1.5 ml plastic tubes. 10 µl of 100 % (w/v) TCA was then added to each tube. The reaction mixture was then mixed well and centrifuged at 15000 rpm, 4 °C for 4 minutes. The supernatant obtained after centrifugation contained glucose, inhibitor and other interfering substances. The precipitate obtained (AGEs- BSA) was dissolved in 400 µl of alkaline PBS and the fluorescence measured at an extinction wavelength of 350 nm and an emission wavelength of 450 nm using a microplate reader. Quercetin, a flavonoid was used as the positive control (Adapted from Matsuura et al., 2002).

2.1.2. Xanthine oxidase assay

Principle

The hypoxanthine-xanthine oxidase system generates superoxide anions which can be detected using NBT (Nitro blue tetrazolium) reduction. The superoxide anion reduces NBT changing its colour from a pale yellow to purple. This change is measured over time and read at 570 nm in a microplate reader.

The xanthine oxidase assay was used in this study to analyse the antioxidant potential of plant A extract. Since this plant is extensively used in diabetes treatment and the role of antioxidants in oxidative stress and diabetes has been well established, the XO assay sought to add more information on the plant’s antioxidant potential.
The plant extracts at varying concentrations were mixed with Na₂PO₄ – KH₂PO₄ buffer (66.67 mM, pH 7.5, 1.3 mls) and xanthine solution (0.15 mM in ddH₂O, 1.5 ml). This mixture was then incubated at 30°C for 10 minutes. Xanthine oxidase solution (0.28 U/ml in 66.67 mM phosphate buffer) was added to the mixture to initiate the reaction. The absorbance at 295 nm was measured each minute for 10 minutes using a microtiter plate reader. The % inhibition of this enzyme with plant extracts was calculated using the equation below. Allopurinol served as the positive control.

\[
\% \text{ Inhibition} = \left\{ \frac{(OD_{control} - OD_{sample})}{OD_{control}} \right\} \times 100
\]

2.1.3. Superoxide anion scavenging assay

The plant extracts at various concentrations were pre-incubated at room temperature with the reaction mixture of 20 µl Na₂EDTA (15mM) in KH₂PO₄/ KOH buffer (pH), 50 µl NBT 0.6 mM, 30 µl of 3 mM hypoxanthine in KH₂PO₄/ KOH buffer. 50 µl of 1 U in 10 ml buffer xanthine oxidase solution was then added to the mixture. The change in absorbance of NBT due to its reduction by superoxide anion was then read at 560 nm. The concentration of plant extracts that decreased NBT reduction by 50% (IC₅₀) was then determined (Adapted from Ljubuncic et al., 2005).

2.1.4. DPPH assay

Dilutions of plant extracts were prepared before analysis. 40 µl diluted sample or control was placed in the appropriate wells of the microtiter plate and 80 µl of Tris- HCl buffer (50 mM, pH 7.4) was added. 120 µl of DPPH solution (0.2 mM in absolute ethanol) was then added and the solution mixed well by repetitive pipetting. The mixture was then incubated at ambient temperature (25 ° C) in the dark for 30 minutes. Absorbance was measured at 518 nm.

A reading was done with water with Tris- HCl buffer (40 µl water + 80 µl buffer) to exclude any interfering components. As a blank, 120 µl DPPH solution was replaced
with 120 µl ethanol. Blank was included for each sample dilution. The % inhibition value was then calculated as follows:

\[
\text{% Inhibition} = \left[ \frac{A_{\text{control}} - A_{\text{Sample - Blank}}}{A_{\text{control}}} \right] \times 100
\]

Where A= Absorbance at 518 nm.

2.1.5. FRAP assay

On the day of the assay, the FRAP reagent was prepared fresh. FRAP reagent contains: 300 mM sodium acetate buffer, 40 mM HCl, 10 mM of the ferric form of tripyridyltriazine (TPTZ), 20 mM ferric chloride. The FRAP reagent was kept in a water bath at 37 °C. Prior to use, 10 µl of sample, control (Ascorbic acid), or FeCl₃ was transferred to a 96 well plate. 250 µl of the FRAP reagent was then added to each of the wells. Absorbance was read at 593 nm after five minutes of incubation. Measurements were taken at room temperature with samples protected from direct light. Water was used as a reference and 1 mM FeSO₄ was used to prepare the standard curve (Adapted from Benzie and Strain, 1996).

2.1.6. Alpha glucosidase inhibition

Alpha glucosidase enzyme (EC 3.2.1.20) is also known as maltase and catalyses the hydrolysis of terminal non reducing (1 to 4) – linked α- D- glucose residues with release of α- D- glucose (Ref: ATCC).

The first step is the preparation of the enzyme solution. 0.6 gm of rat intestinal acetone powder (Sigma Aldrich 1630- 10G) was weighed and 20 mls of 0.1 M maleate buffer pH 6.9 added. This mixture was then homogenized and the enzyme mixture sonicated and centrifuged for 20 min at 4° C at 3000 rpm. The supernatant was removed and solution stored in ice. The supernatant was used as the crude enzyme solution.
125 µl of 40% DMSO was added to all appropriate wells in a 96 well microtiter plate. Serial dilutions of the plant extracts tested were prepared in the plate. 20 µl of dilution was then taken from each well and added to a new 96 well plate. 40 µl of substrate (either maltose or sucrose) was then added to all wells except to those in which water was added to act as the blank. 20 µl of the enzyme preparation was then added into each well. (A 1:4 dilution of the enzyme for maltose and 1:2 for sucrose substrates was made).

The different dilutions were done due to the constituent nature of maltose with two glucose units compared to sucrose with one glucose and one fructose unit, yielding two identical units of glucose and equimolar mixture of glucose and fructose respectively. The mixtures were then incubated for 30 minutes for maltose and 60 minutes for sucrose. 70 µl of 0.5 Maleate/Tris pH 7.4 was added to stop the reaction. 10 µl (for maltose) and 20 µl (for sucrose) was taken from each well and added to a new plate. 150 µl of the glucose oxidase reagent was added and the mixture incubated for 15 minutes. Absorbance was read at 492 nm. The % inhibition was calculated as:

\[
\% \text{ Inhibition} = \left(\frac{A_c - A_s}{A_c}\right) \times 100
\]

Where \( A_c \) is absorbance of the control and \( A_s \) is the absorbance of the tested sample (Adapted from Shim et al., 2002 and Oki et al., 1999)

2.1 Cell lines

2.2.1. Cell maintenance

All cultures used in this study were incubated at 37°C in a humidified atmosphere with 5% CO₂. The increased humidity in the incubator acts to reduce evaporation from the petri dishes that would result in a hypertonic culture medium as well as stressed cells. The 5-10% CO₂ condition recommended for most cell lines helps to maintain pH at about 7.4 depending on the bicarbonate system in use. Sterile conditions were maintained in a class II laminar flow cabinet. The cultures were generally fed fresh medium every 2 to 3 days, with the growth medium used being dependent on the cell
type. The cell maintenance protocols were obtained from Freshney et al., 1994; 2000, as well as in-house modifications at the Department of Biochemistry, NMMU.

2.2.2. Sub culturing

Most cell lines and primary cultures grow as single thickness cell layers. Once the cells have reached sub-confluence, i.e. their growth has slowed though still in the log phase, sub culturing is done at regular intervals to ensure that the cells remain healthy and are growing actively to ensure transformation efficiency. This sub culturing process involves the breaking of bonds that attach cells to the substrate as well as to each other.

Proteolytic enzymes such as trypsin are usually used to break the intercellular bonds. This causes cells to detach from the growth surface. The disadvantage to this method of detachment is that exposed cell surface proteins might initiate cell surface digestion. The cells are incubated until they are rounded and loosened from the growth surface. Once the cells have been loosened, they are removed, viewed under the microscope, counted and then diluted appropriately with medium before being subdivided into new plates. Addition of growth medium stops the proteolytic activity of trypsin. If the cells are healthy and all the growth requirements are provided, then the cells are able to reattach and grow. Subsequent sub culturing is then possible on these plates (Freshney et al., 1994; 2000).

During the sub culturing process, the cells were washed twice with 5 ml PBSA. The PBSA was aspirated off after each washing. Trypsin was then added to the monolayer 10 cm (Sarstedt) culture plate to dislodge cells from the bottom of the plate as well as to break bonds between the cells. This was left on the cells for 30 seconds after which most of it was removed. The plate was then incubated at 37 °C to allow the cells to round up and loosen. 37 °C is the optimum temperature to increase activity of the enzyme solution. Progress of the trypsinization was checked every few minutes with an inverted phase contrast microscope. Once most cells had rounded up, the plate was tapped gently to detach cells from the surface. 5 ml of media was then added to the plate, followed by repeated pipetting over the culture surface to disperse the cells. This was especially important with those cells that have characteristic clumping growth patterns. An
appropriate split ratio of the cells was always done and plates were then incubated at 37 °C and an atmosphere of 5% CO₂.

2.2.3. Seeding

Before every seeding procedure, the culture plate was washed twice with 5 ml of PBSA solution. 1 ml of trypsin was added, left for 15 seconds and aspirated. 5 ml of media was then added to the plate to resuspend the cells. About 20 µl of cells from the parent plate was taken out to be used for cell count in combination with 20µl of trypan blue dye. A hemocytometer was used to do a total cell count as well as cell viability.

2.2.4. Preservation

Cells that were not immediately needed were preserved for later use at -80°C. The media in the plate was removed and the plate washed twice with PBSA. Trypsin was then added and aspirated followed by a five minute incubation period. Media was added to resuspend cells followed by cell counting before preservation. 900 µl of cell culture was put into a cryogenic vial and 100 µl of DMSO added. The vial was then insulated with cotton wool in a falcon tube, frozen at -80°C in a liquid nitrogen storage container.

2.2.5. Cytotoxicity assays

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is based on the ability of a mitochondrial dehydrogenase enzymes found in viable cells to cleave the tetrazolium rings of MTT, changing it from a pale yellow to purple colour after the formation of formazan crystals. Reducing equivalents like NADH and NADPH are produced during this reaction. Adding DMSO solubilises the cells leading to the release of the crystals. Cell viability was defined as the ratio of treated cells to untreated cells that served as control (Freshney, 2000).

Medium was aspirated from the wells in the 24 well microtiter plates (Nunclon) designated for the cytotoxicity assay. 0.5% MTT solution was prepared by adding 25 mg of MTT in 5 mls of incubation medium. 100 µl of MTT was then added to each
well, and then incubated at 37 °C for 3 hours. The MTT solution was subsequently aspirated from the wells and the purple crystals dissolved using 100 µl of Dimethylsulphoxide (DMSO). The plate was shaken prior to reading the absorbance at 540 nm to ensure that the formazan crystals were dissolved. (Ref: ATCC). Similar methods were used for both Chang liver and C3A cells.

2.2.6. Glucose utilization Chang liver cells

Prior to the glucose utilization experiment, Chang liver cells were seeded into 96 well plates at 200 µl of cell suspension per well at a density of 6000 cells per well. The cells were incubated at 37 °C for 5 days without changing the medium. However, on the third day, plant extracts dissolved in DMSO as well as the positive control were added to selected wells. The positive control, metformin was used at a concentration of 20 µM in RPMI 1640 with 10% fetal bovine serum. The plant extracts for initial screening were added at an initial concentration of 250 µg/ml then adjusted to fit a dose response method. The extracts and positive control were added to each well at a volume of 10 µl giving a final concentration of 2.5 µg per well and 1 µM for metformin.

For the glucose utilization experiment, all media was aspirated from the wells and 50 µl of incubation medium (8mM glucose, 0.1% BSA containing metformin or plant extract) was added per well according to the plate layout used. The plates were then incubated at 37° C for 3 hours. After the incubation, 10 µl of medium was transferred from each well to another 96 well plate into which 200 µl of Glucinet (Bayer) reagent was added. A further incubation for 15 minutes was done followed by reading the absorbance at 492 nm using a microplate reader (Adapted from Wilson, 2006).

2.2.7. Glucose utilization in C3A cells

For C3A cells seeding was done at a density of 30,000 cells per well. This is due to their growth characteristics at the specified conditions, which differs from that of the Chang liver cells. Glucose utilization was assayed as described in section 2.2.6.
2.2.8. Glucose utilization in C2C12 cells

Seeding of C2C12 cells into 96 well plates was done using a growth medium of RPMI 1640 with 11 mM glucose and 0.1% BSA. The cells were allowed to grow in the plate for two days before addition of plant extracts and/or the positive control. The cells were then further incubated for 24 hours before the glucose uptake experiment was carried out. After removing the spent medium, 50 microliters of incubation medium, insulin or extract was then added to selected wells. Then plates were incubated for an hour at 37 °C. 10 microliters of media was then transferred from each well to a clean 96 well plate. 200 microliters of Glucinet reagent was added to each well then plates incubated at 37 °C for 15 minutes. After this incubation period, absorbance was read at 492 nm using a microplate (Multiskan® MR microplate reader, Labsystems) reader (Adapted from Wilson, 2006).

2.3. The Rat Model

2.3.1. Rat maintenance

This project was approved by the Animal Ethics Committee of NMMU (A068BM-004). The care and maintenance of the rats was in accordance with the guidelines provided by NMMU Animal Ethics Committee as well as international rules relating to the care and use of laboratory animals.

Rats were bred at the Animal Unit of the Medical Research Council, Cape Town. After they had been weaned, they were transported to Port Elizabeth. A 1-week adjustment period was allowed after which the animals were fed either normal rat chow or the HFD for twelve weeks. A previous experiment done at NMMU showed that rats became insulin resistant on the HFD within twelve weeks (Chadwick et al., 2006). The rats had free access to food and water all the time during the duration of this study. The rats were weighed on a weekly basis.
The rats were housed in cages (not more than 4 rats per cage) according to their groups as set out in Table 3.2 below. They were maintained under standard conditions of temperature (22 ± 1°C), humidity and light with a 12-hour light and 12-hour dark cycle.

### 2.3.2. Rat diet

Rats were fed either on a low fat diet (LFD) or a high fat diet (HFD). The LFD consisted of commercial mice pellets (EPOL). The high fat diet (HFD) consisted of ground mice pellets (2 kgs); margarine (128 gms of wooden spoon® and 190 gms of Holsum®) and condensed milk (220 gms). The final constituents of the HFD are shown in Table 2.1 below. The LFD was lower in saturated fat and cholesterol and higher in dietary fibre than the HFD. Water and food was given to the rats *ad libitum*.

<table>
<thead>
<tr>
<th>Dietary constituent</th>
<th>% of the diet (LFD)</th>
<th>% of the diet (HFD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>21.3</td>
<td>15.8</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>72.4</td>
<td>45.3</td>
</tr>
<tr>
<td>Fats</td>
<td>6.3</td>
<td>38.9</td>
</tr>
</tbody>
</table>

Rats were fed either a LFD or a HFD for 3 months. To determine whether the rats were insulin resistant after 3 months, 7 of the rats fed a LFD diet (COL) and 7 rats on the HFD (COH) were sacrificed. Since the rats were found to be insulin resistant (rats were continued on the LFD to be the lean controls (CCL). The remaining rats were given a HFD (CCH). After 12 weeks on the HFD, treatment was began with plant A and metformin on the now IR rats.

### 2.3.3. Preparation of medication

The dosage of the traditional medicine (plant A) was calculated using an estimate obtained from regular users of this medication. A handful of the ground bark is usually
brewed in 250 ml of hot water or liquid and taken by the individual during the course of a day. This recipe was followed to prepare the medication and was then freeze dried. A yield of 9.375 g extract was obtained. By correlating this estimate with an assumed weight for the treatment of an average normal man of about 75 kg, we were able to estimate that rats should get 0.125 mg extract per gm of body weight per day as an appropriate dosage.

This same principle was applied for the determination of dosage for the metformin group, from known dosages administered to an average normal sized male of about 75 kg. The South African medicines formulary (Gibbon, 2003), recommends a dosage of 850 mg metformin daily. Using this recommendation the following calculations were done to determine the concentration of metformin to administer to the rats: Assuming that 850 mg of metformin is taken by a man 75 kg body weight/day, then 11.33 mg of metformin is taken per kg body weight/day. This is equivalent to 0.01133 mg of metformin/gm body weight/day. If each 500 mg tablet metformin had a mass of 550 mg, then 0.012 mg of metformin tablet should be taken per gm of body weight per day.

Both plant A and metformin are readily soluble in water and so this was used as the preferred solvent for the duration of the study. The medication was gavaged to the animals at a volume of 1 ml at 50 mg/ml concentration. Gavaging had the advantage of ensuring that all the rats received equal amount of the medication. This would have been harder to verify if the medication had been dissolved in the drinking water. This is because the rats did not consume the water and food with the same vigour.

2.3.4. Experimental setup

This experiment consisted of 70 rats. After they had been weaned the rats were put on a normal rat chow diet for 1 week. The rats were then divided in 2 main groups: 21 rats got the normal diet (LFD) and 49 got a high fat diet (HFD) as described in section 3.2.2. The average body weights of the two groups were the same.

The experimental setup is explained in table 2.2. Four rats became ill or died and had to be removed from the experiment. After 12 weeks 7 rats from each group were
sacrificed to confirm that the rats on HFD were insulin resistant: COL and COH of the normal diet and high fat diet respectively.

Having confirmed insulin resistance, the rats were divided in 4 groups between 12 and 14 rats per group: 14 rats on normal mouse chow (CCCL) which were the lean control rats, 14 rats on high fat diet (CCH) which were the insulin resistant control rats, 12 rats receiving the high fat diet and treated with the plant extract (C4), 13 rats receiving the high fat diet and treated with metformin (C5). The experiment continued for another 4 weeks. Medication was given by gavaging the rats. Control rats were gavaged with equal volume water.

2.3.5. Rat sacrifice

To sacrifice the rats two procedures were followed, one to obtain tissues from the rats while the other was to determine glucose uptake in the different tissues.

i) Sacrifice for tissue sampling

After an overnight fast, the rats were anaesthetized with ketamine administered intramuscularly. The volumes of ketamine used to put the rats down varied according to the body mass of the rat. One ml ketamine was administered to the rat, and if the rat was not asleep after 20 min, more ketamine was given. On average the rats received 1.4 ml of ketamine. A drop of blood was obtained from the tip of the tail and used to measure glucose content using a glucometer. Between 7 and 10 ml of blood was drawn directly from the heart as quickly as possible (see section 2.3.6). This procedure put the rat into a hypovolemic coma.

ii) Sacrifice for determining glucose uptake in different tissues

The rats were anaesthetized with an intramuscular injection of ketamine at 3 µl/g body weight after 12 hour starvation period. The femoral vein was exposed, after which the basal glucose levels were measured with the aid of an Optimum MediSense glucometer. A one millimetre solution containing 0.9% saline, 0.4 g/kg glucose and 1 µCi/500g 2-deoxy-D-[2, 6-3H] glucose was injected over one minute into the femoral vein. Blood glucose levels were subsequently measured with the glucometer every 5 minutes.

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Muscle, epididymal adipose tissue and liver tissue were removed from the rat an hour later and snap frozen in liquid nitrogen for subsequent analysis (Adapted from Chadwick et al., 2006).

**Table 2.2**: Summary of data on diet and treatment for the rats as at the time of sacrifice, day 0 and day 28 (where H\(^3\)DG: \([H^3]\) deoxyglucose)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Wks on diet</th>
<th>Treatment</th>
<th>Wks on treatment</th>
<th>Treatment on day of sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL (n=7)</td>
<td>LFD</td>
<td>12</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>COH (n=7)</td>
<td>HFD</td>
<td>12</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>CCL (n=7)</td>
<td>LFD</td>
<td>16</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>CCH (n=7)</td>
<td>HFD</td>
<td>16</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>C4 (n=6)</td>
<td>HFD</td>
<td>16</td>
<td>Plant A</td>
<td>4</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>C5 (n=7)</td>
<td>HFD</td>
<td>16</td>
<td>Metformin</td>
<td>4</td>
<td>Starved for 8 hours</td>
</tr>
<tr>
<td>CCL (n=7)</td>
<td>LFD</td>
<td>16</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours + H(^3)DG for 1 hour</td>
</tr>
<tr>
<td>CCH (n=6)</td>
<td>HFD</td>
<td>16</td>
<td>None</td>
<td>-</td>
<td>Starved for 8 hours + H(^3)DG for 1 hour</td>
</tr>
<tr>
<td>C4 (n=6)</td>
<td>HFD</td>
<td>16</td>
<td>Plant A</td>
<td>4</td>
<td>Starved for 8 hours + H(^3)DG for 1 hour</td>
</tr>
<tr>
<td>C5 (n=6)</td>
<td>HFD</td>
<td>16</td>
<td>Metformin</td>
<td>4</td>
<td>Starved for 8 hours + H(^3)DG for 1 hour</td>
</tr>
</tbody>
</table>

**2.3.6. Blood collection**

Blood was obtained from the rats via heart puncture. This was the fastest and least invasive method since the heart was still beating. It was important not to put the rats under any unnecessary stress that could affect other parameters in the body that needed to be measured such as the plasma glucose and insulin levels.

EDTA (0.45 g/ml) was added into the syringe before the heart puncture in order to prevent clotting of the blood in the syringe before transfer to EDTA containing vacutainers (purple top). The first 2 ml of obtained from the rat was aliquoted and used for the plasma glucose and insulin determination. This initial blood collected was expected to have insulin levels as at time of collection as well as lower stress hormones than blood drawn out later.

The blood samples were centrifuged at 5000 rpm for 10 minutes. The plasma samples obtained were aliquoted into cryo vials and stored at −80°C until for analysis at a later date.
2.4 Rat tissue analysis

2.4.1 Blood glucose measurement

Principle

A number of tests are routinely carried out to measure glucose and its metabolites in the monitoring and diagnosis of diabetes. Some of these tests include measuring fasting blood glucose, the random glucose test and oral glucose tolerance test (OGTT). There are relatively simple automated techniques of measuring glucose levels. The use of glucometers has been widely accepted since they are easy to use and clean and are relatively inexpensive.

The principle of the glucometer is based on measurement of electrical potential caused by the reaction of glucose with the reagents on the test strip. For example, glucose in the blood sample reacts with glucose oxidase and potassium ferricyanide on the test strip. Electrons are generated which produce a current that is proportional to the glucose in the sample. After a reaction time of 60 seconds or less, the glucose concentration is displayed on the screen.

With the MediSense glucometer, the normal blood glucose level in humans should fall within the range of around 5 to 8 mmol/litre. If one had fasted for approximately eight hours before the sample was obtained, the level of glucose in the blood would be about 3.5 mmol/litre. This glucometer is capable of registering a range of blood glucose levels from as low as 1.1 mmol/litre to 27.8 mmol/litre.

Method

The glucose level in the blood was measured as soon as the blood was obtained from the heart via heart puncture. A 5 ml syringe with a 22 gauge needle was used. The blood glucose levels were determined by an enzymatic glucose oxidase method on which the commercial glucometer (MediSense ® Optimum plus, Abbot Laboratories, Maidenhead, UK) used is based on (Adapted from Chadwick et al., 2007)
2.4.2. Glucose clearance

**Principle**

Glucose in the body is quickly utilized for many metabolic processes. It clearance in the body is an important pointer to the physiologic status of the body, more so in a diabetic person. Glucose clearance experiments are therefore important in determining how quickly glucose is cleared or taken up in the body. Since insulin regulates glucose clearance in the body, this is another useful method for measuring insulin sensitivity.

In this experiment $[^3]$H-deoxy-glucose and glucose were used since they are both taken up well by tissues. Deoxyglucose is phosphorylated to deoxyglucose-6-phosphate but cannot be further metabolised and as a result it accumulates in the cells lacking G-6-Pase such as adipocytes and myocytes. Accumulation in the liver is lower due to active Dephosphorylation using G-6-Pase (Nelson and Cox, 2000).

**Method**

The rats were anaesthetized with an intramuscular injection of ketamine at 3 µl/g body weight after 12 hour starvation period. The procedure followed is as explained in section 2.2.5.

2.4.3. Glucose uptake in muscle, liver and fat

**Principle**

Glucose uptake test measures the rate of glucose uptake into the cell using a radio labelled glucose analogue known as $[^3]$H deoxyglucose. This molecule is taken up by the cells through the same transporters as normal glucose with the same affinity. In fat and muscle tissues deoxyglucose can be phosphorylated but cannot be metabolized further (Nelson and Cox, 2000). It accumulates in the cell, and the measured amounts of this compound give a direct representation of the amount of glucose taken up.

Scintillation counting is the preferred method for measuring beta emissions. It has a higher counting efficiency, is easy to use, and separate isotopes can be counted in the
same sample. Its main disadvantage is a phenomenon known as quenching whereby the energy transfer process suffers interference such as loss of energy by absorption by chemicals in the solvent before the photon can reach the detector. Correcting for this phenomenon leads to loss of scintillation counting efficiency (Wilson and Walker, 2000).

Method

The samples to be analyzed were finely chopped and 1 gm was degraded using 1 ml of 30% w/v potassium hydroxide (KOH). The samples were placed in a boiling water bath to speed up the breakdown of tissue for 30 minutes. 3 ml of Packard Ultima Gold scintillation cocktail was added to appropriately labelled polyethylene vials. Blank with only the scintillation cocktail and another with the cocktail plus 200 µl KOH were also prepared. 200 µl of samples to be tested was then added into the appropriate vials, which were then mixed well by vortexing. The vials were then put into a Packard tricarb 2300TR liquid scintillation analyzer and readings taken. Once initial readings were complete, a 20 µl of 10000 dpm deoxyglucose was added to each vial. The solution turned milky upon addition of the tritium solution. The mixture was vortexed and read again.

2.4.4. Plasma insulin determination

Principle

Insulin is the principal hormone responsible for the control of glucose metabolism. Its secretion is mainly controlled by plasma glucose concentration. Insulin measurements are mainly done to investigate hypoglycemia. Other applications for its measurement include assessment of residual cell function, diagnosis of insulinoma and investigation of the pathophysiology of Diabetes mellitus such as insulin resistance.

The particular immunoassay technique used in determining plasma insulin levels relies on the use of a fixed concentration of a labeled antigen, $^{125}$ I. This labeled antigen is reacted with an antiserum of constant known concentration over an incubation period.
This means that the binding sites that are available on the antibody are limited. The introduction of an unlabelled antigen means that there will be competition for the binding sites on the antibody. The higher the amount of unlabelled antigen in the reaction system, the less the binding of labelled antigen to antibody binding sites.

It is then possible to quantify the decrease in labeled antigen antibody interaction. This is done by freeing the tracer from the antigen and then measuring its levels using a scintillation counter. A standard curve derived from serially increasing concentrations of unlabelled antigen can be used to calculate amounts of antigen in unknown samples.

**Method**

Blood obtained from the rats after sacrifice was centrifuged at 2000 g at 4 °C for 20 minutes. Plasma obtained was used for insulin determination using an immunoassay technique with the aid of a commercially available kit from Linco Research, St. Louis, MO (Linco, R1-13K). On day 1 of experiment, the experiment was carried out as follows as per the manufacturer’s instructions.

**Table 2.3:** Protocol followed for plasma insulin determination (Source: LINCO kit).

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Buffer</th>
<th>Standards (and samples)</th>
<th>[^{125}_I] tracer</th>
<th>Rat Insulin Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>-</td>
<td>-</td>
<td>100 μl</td>
<td>-</td>
</tr>
<tr>
<td>3,4 (NSB)</td>
<td>200 μl</td>
<td>-</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>5,6 (Bo)</td>
<td>100 μl</td>
<td>100 μl 0.1 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>7,8</td>
<td>-</td>
<td>100 μl 0.2 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>9,10</td>
<td>-</td>
<td>100 μl 0.5 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>11,12</td>
<td>-</td>
<td>100 μl 1.0 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>13,14</td>
<td>-</td>
<td>100 μl 2.0 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>15,16</td>
<td>-</td>
<td>100 μl 5.0 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>17,18</td>
<td>-</td>
<td>100 μl 10 ng/ ml</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>19-∞</td>
<td>-</td>
<td>Samples</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

On day two, after the addition of the rat insulin antibody, the reaction mixture is vortexed and incubated at 4 °C, for 24 hours. After the incubation period, 1.0 ml of precipitating buffer is added to all tubes. The tubes are vortexed and incubated for 20
minutes at 4 °C. The pellet obtained was resuspended in scintillation cocktail and read in the counter. The average values of the non specific binding tubes were subtracted from averages obtained for every tube. The equations used are shown below:

\[
\% B = \left\{ \frac{\text{Total binding counts (tubes 5–6)}}{\text{Total counts (tubes 1–2)}} \right\} \times 100
\]

To calculate the percentage of bound tracer

\[
\% \frac{B}{Bo} = \left( \frac{\text{Sample or standard}}{\text{Total binding}} \right) \times 100
\]

To calculate percentage total binding for both standards

![Standard curve for Plasma Insulin determination](image)

**Figure 2.1:** A log - log standard curve of \% \(\frac{B}{Bo}\) for each standard against the known concentrations of unlabelled antigen on the \(x\)-axis. Insulin concentrations for the samples were read from this standard curve.

2.4.5. Triglyceride content determination

**Principle**

The triglycerides are determined after enzymatic hydrolysis by lipoprotein lipases. The principle of the method can be summarized by the equations below:

- Triglycerides \(\text{Li}^\text{pase}\) Glycerol + Fatty Acids (FFA)
- Glycerol + ATP \(\text{Glycerokinase}\) Glycerol–3–phosphate + ADP
- Glycerol-3-phosphate \textit{Glycerol-3-phosphate oxidase} Dihydroxyacetone phosphate + H₂O₂
- 2 H₂O₂ + 4-Chlorophenol + 4-Aminoantipyrine \textit{Peroxidase} Quinoneimine + 4H₂O + HCL

The hydrogen peroxide is monitored in the presence of horse radish peroxidase with 4 aminophenazone as the chromogen system. The high absorbance of this chromogen at 510 nm means that results can be obtained with low sample to reagent volumes (Gallardo \textit{et al.}, 2008).

\textbf{Method}

KAT™ Triglycerides kit was used for this assay. The triglycerides are determined after enzymatic hydrolysis by lipoprotein lipase. Plasma samples from the rats were used for this assay. A range of standards (supplied as part of the kit) were also prepared for the assay ranging from 0.143 mmol/ l to 2.29 mmol/ l.

96-well microtiter plates were used for this assay. 3 µl of standard or samples were mixed with 250 µl of the working reagent and the mixture incubated for 5 minutes at 37 °C. The absorbance of the samples and of the standard was then measured at 510 nm against the reagent blank. The concentration of the triglyceride in the sample was calculated as follows:

\[
\text{Sample concentration} = \left( \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \right) \times \text{concentration of TG standard}.
\]

Absorbance was read at 510 nm. The average absorbance values obtained were used to calculate the TG content in mmol by using the equation derived from the standard curve. The protocol followed was as per manufacturer’s instructions.
2.4.6. Total cholesterol level determination

**Principle**

Cholesterol is a fatty substance found in blood, bile and brain tissue. It is a precursor to bile acids, steroids and vitamin D. There are various metabolic, infectious and coronary heart diseases that are associated with total cholesterol concentrations in serum (ADA, 2006).

During incubation of serum samples with cholesterol reagent, cholesterol esters are converted to cholesterol by the action of cholesterol esterase. The cholesterol so formed, plus the free cholesterol originally present, is oxidized in the presence of cholesterol oxidase with the simultaneous production of hydrogen peroxide. This reaction is coupled with the peroxidase/phenol/4-aminoantipyrine system, resulting in the formation of a colour, which is measured spectrophotometrically. The enzymatic hydrolytic process is explained by the equations below:

- \[ \text{Cholesterol – ester} + \text{H}_2\text{O} \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{fatty acids} \]
- \[ \text{Cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholestene-3–one} + \text{H}_2\text{O}_2 \]
- \[ 2\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4\text{H}_2\text{O} \]

**Method**

KAT™ Cholesterol kit was used for this assay. The total cholesterol is determined after enzymatic hydrolysis followed by an oxidation step. Serum obtained from the rats was used for the assay. The reagents in the kit were supplied ready to use though a range of dilutions was prepared for the standard.10 µl of the standard and sample was mixed with 250 µl of the working reagent, and the mixture incubated for 5 minutes at 37°C. The absorbance of the samples and standard was then read at 500 nm against the reagent blank.

The concentration of cholesterol in the sample was then calculated as follows:

Sample concentration = (Absorbance of sample/ Absorbance of standard) \times\ concentration of Cholesterol standard.
The protocol followed was as per manufacturer’s instructions.

2.4.7. HDL cholesterol level determination

Principle

In the plasma, cholesterol is transported by three lipoproteins: high-density lipoprotein (HDL-Cholesterol), low density lipoprotein (LDL-Cholesterol), and very low density lipoprotein (VLDL-Cholesterol) (Greenspan et al., 2001)

The role of HDL particles in lipid metabolism is primarily the uptake and transport of cholesterol from peripheral tissue to the liver. Low HDL-C levels have repeatedly been associated with an increased risk of coronary heart disease and coronary artery disease. Thus the determination of serum HDL cholesterol has been recognized as a useful tool in identifying high-risk patients (Luca et al., 2007).

The method described below is in a two-reagent format. The first reagent stabilizes LDL, VLDL, and chylomicrons. The second reagent contains modified enzymes that selectively react with the cholesterol present in the HDL particles. Consequently, only the HDL cholesterol is subject to cholesterol measurement

The principle of the method is that the polymer and polyanion in reagent 1 react with the serum producing a stable protection layer around the lipoprotein under the action of the surfactant. On addition of reagent 2, the HDL cholesterol is released rapidly and an enzymatic reaction follows. A colour reaction is seen with the intensity of the colour being directly proportional to the HDL Cholesterol concentration in the serum. Reagent 1 contains: Good’s buffer, phenol, polyanion, surfactant etc. Reagent 2 contains: Cholesterol esterase, cholesterol oxidase, peroxidase, 4-amino-antipyrine and surfactant.

Method

Rongsheng HDL cholesterol diagnostic kit was used for this assay. This kit is used for the in vitro detection of HDL-cholesterol in human plasma.
Reagents were provided ready to use though a range of dilutions was prepared for the standard from 0.065625 mmol/l to 1.05 mmol/ l. Plasma obtained from the rats was used for this assay.

3 µl of distilled water, standard and plasma respectively was mixed with 240 µl of reagent 1 in a 96-well plate. These were the blank, standard and sample respectively. The mixtures were then incubated for 5 minutes at 37 °C and read at 546 nm. 80 µl of reagent 2 was then added to each well and incubated for a further 5 minutes at 37 °C. Absorbance was again measured at 546 nm.

To calculate the HDL value:

\[
\text{HDL (mmol/l)} = \frac{(\text{Abs. 2 of plasma} - \text{Abs. 2 of blank})}{(\text{Abs. 1 of plasma} – \text{Abs. 1 of blank})}.
\]

This HDL value was calculated from the standard curve. The reference value was 0.78-2.00 mmol/l and was representative of the expected range using this particular method. The protocol followed was as per manufacturer’s instructions.

2.4.8. Free Fatty Acid level determination

Free fatty acid half micro test kit from Roche® was used for this assay. It is an optimized enzymatic colorimetric assay for the determination of free fatty acids in research samples from serum or plasma.

**Principle**

The principle of the method is that in the presence of the enzyme acyl synthase and ATP, FFA are converted to acyl- coenzyme A, adenosine-5’-monophosphate (AMP) and pyrophosphate. The acyl-coenzyme A reacts with oxygen in the presence of acyl-CoA oxidase enzyme to form 2, 3-enoyl-coenzyme A (enoyl-CoA). Hydrogen peroxide is produced by the reaction and this converts 2, 4, 6 – tribromo-3-hydroxy-benzoic (TBHB) acid and 4- aminoantipyrine (4-AA) to red dye in the presence of peroxidase (POD). The dye was measured in the visible wavelength range at 546 nm.
Method

The working solution for this assay involved reaction mixture A and B. For reaction mixture A, one tablet of bottle 2 was dissolved in bottle 1 and this was sufficient for 10 assays. For reaction B, one tablet of bottle 5 was dissolved in bottle 4 and this was sufficient for 10 assays.

100 µl of reaction mix A was added to the blank and sample wells and then 5 µl of double distilled water or sample was added to the appropriate wells. The reagents were then mixed well at 25 °C. This temperature was maintained for approximately 10 minutes in a water bath. To each cuvette 5 µl of ready to use N-ethyl-maleinimida-solution was added, mixed well and absorbance (A₁) measured at 546 nm. The next reaction step was then initiated by adding 5 µl of reaction mix B to the wells. The reaction was allowed to stand for 15 minutes and the absorbance (A₂) read at 546 nm.

The absorbance differences (A₂ - A₁) for both the blank and the samples were calculated and subtracted from the absorbance difference of the sample.

The standard solution required preparation of solutions 1 and 2. Solution 1 was prepared by dissolving 6.0 gm of Triton- X100 in 80 ml of double distilled water at 30° - 40° C. This was allowed to cool to 15 – 25° C and made up to 100 ml in a measuring cylinder. Solution 2 was prepared by weighing 36 gms of palmitic acid into a 100 ml beaker which was then dissolved in 24 ml of warm ethanol at about 35 – 40° C. The beaker was immediately sealed with parafilm and allowed to cool to 15 – 25° C.

For the standard solution, 80 ml of solution 1 was added to solution 2 while stirring slowly to avoid the formation of micro crystals at the point of entry. The solution was stirred using a magnetic stirrer for a further 30 minutes and transferred to a 100 ml volumetric flask. This was made up to the mark with solution 1. This standard solution was stable at 4-8° C for 3 days. The standard had a concentration of 0.35 mM. A standard curve was prepared from this standard solution and absorbance values obtained were read against this curve to provide free fatty acid concentration values in the samples tested.
Possible interferences include haemoglobin, bilirubin and ascorbic acid if they are above the normal range. Protocol followed was as per manufacturer’s instructions.

2.4.9. Glycogen determination in muscle and liver

Principle

Glycogen is a polymer of glucose residues linked mainly by an α (1-4) glycosidic linkage. There are α (1-6) linkages at branch points of the glucose residues. Glycogen is the storage form of glucose in the body especially in the muscle and liver tissues and cells. During low glucose periods in the body, glycogen is broken down to release glucose via the glycogenolysis process (Greenspan et al., 2001).

Method

Liver and muscle tissue obtained from control rats as well as high fat diet rats fed on Plant A were analyzed. Approximately 100 mg of muscle tissue was placed in screw-cap eppendorf tubes. 200 µl of 30% KOH was then added to each tube. The tubes were incubated at 100°C for 60 min, with vortexing every 15 minutes. The tubes were then cooled on ice and 67 µl 1 M Na₂SO₄ followed by 535 µl absolute ethanol. Samples were centrifuged at 14000 x g for 30 minutes (4°C) in eppendorf tubes. The resulting supernatant was discarded and the pellet dried in a 37 °C oven. The dry pellet was then stored at -20 °C until further analysis (Adapted and modified from Suzuki et al., 2002 and Plummer, 1987).

When doing the assay, the dry pellet was dissolved in 200 µl distilled water at 37 °C. 10 µl of sample or standard was added in duplicate into wells of a 96 well plate. 60 µl of the reaction mixture (containing 0.05 gm of amyloglucosidase in 6 ml distilled water containing 1 ml sodium acetate buffer pH 5.0) was added to each well and incubated at 37 °C for 2 hours. 150 µl Glucinet (Bayer) reagent was then added to each well and absorbance read at 492 nm after 15min.

The readings obtained were then extrapolated off a glycogen standard curve. For the glycogen standard curve, 50 µl stock glycogen solution was diluted in 9950 µl of
distilled water. The derived standard curve was used to calculate the glycogen content in the tissue samples. For the determination of liver glycogen content the procedure described above was followed except that 400 mg of liver tissue was used compared to 100 mg of muscle.
CHAPTER 3

RESULTS AND DISCUSSION

3.1. Plant analysis
3.2. Cell lines
3.3. Rat tissue analysis
CHAPTER 3

RESULTS AND DISCUSSION

3.1. PLANT ANALYSIS

Preliminary screening of three plant extracts A, B and C was done to pick one plant to be used for in vivo studies with HFD induced IR rats. Several antioxidant assays were performed and the preliminary results are described herein.

3.1.1. End product glycation assay

Table 3.1: % inhibition of formation of the glycated BSA

<table>
<thead>
<tr>
<th>[Plant A] µg/ml</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>-83.089</td>
</tr>
<tr>
<td>5</td>
<td>-42.509</td>
</tr>
<tr>
<td>10</td>
<td>4.951</td>
</tr>
<tr>
<td>20</td>
<td>10.231</td>
</tr>
<tr>
<td>30</td>
<td>26.577</td>
</tr>
<tr>
<td>40</td>
<td>33.902</td>
</tr>
<tr>
<td>50</td>
<td>38.257</td>
</tr>
<tr>
<td>60</td>
<td>39.370</td>
</tr>
<tr>
<td>80</td>
<td>49.844</td>
</tr>
<tr>
<td>100</td>
<td>51.243</td>
</tr>
</tbody>
</table>

Figure 3.1: % Inhibition of formation of the glycated BSA

There was a concentration dependent increase in % inhibition of formation of glycated BSA using plant A. The fluorescence measured is of the covalent bonds formed after
glucose and fructose have formed links with BSA molecules. These results indicate the potential ability of plant A to inhibit the formation of end glycation products that have been found to be increased in the diabetic condition (see section 2.1)

3.1.2. Xanthine oxidase assay

**Table 3.2:** Absorbance and % Inhibition values for the xanthine oxidase assay after treatment with various concentrations of plant A

<table>
<thead>
<tr>
<th>[Plant A] µg/ml</th>
<th>Average absorbance</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.1835</td>
<td>15.24249</td>
</tr>
<tr>
<td>10</td>
<td>0.1665</td>
<td>18.78049</td>
</tr>
<tr>
<td>25</td>
<td>0.166</td>
<td>-15.67944251</td>
</tr>
<tr>
<td>50</td>
<td>0.1705</td>
<td>-8.253968254</td>
</tr>
<tr>
<td>75</td>
<td>0.162</td>
<td>-7.641196013</td>
</tr>
<tr>
<td>100</td>
<td>0.189</td>
<td>-18.49529781</td>
</tr>
</tbody>
</table>

**Figure 3.2:** % Inhibition of xanthine oxidase after treatment with plants A, B and C at varying concentrations in µg/ml

Decreasing % inhibition of xanthine oxidase was observed with increasing concentrations of plants A, B and C. Positive inhibition was observed at the lower concentrations of these plant extracts. Due to the negative results obtained, this assay was not successful as an indicator of the plants’ antioxidant potential. Alternative assays are discussed herein.
3.1.3. Superoxide anion scavenging effect assay

Plants A and B both inhibited the rate of NBT reduction in a concentration dependent manner from 5 µg/ml to 50 µg/ml. There was a reduction in this inhibition from 50 µg/ml to 150 µg/ml. This decrease in inhibition was more in plant A compared to plant C. Inhibition of NBT by plant B was erratic over the concentrations used and was much lower than that found with plant B and C.

Table 3.3: % Inhibition of NBT values by plants A, B and C at various concentrations

<table>
<thead>
<tr>
<th>Plant</th>
<th>µg/ml</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant A</td>
<td>% Inhibition</td>
<td>5.109</td>
<td>13.869</td>
<td>20.438</td>
<td>22.628</td>
<td>15.328</td>
<td>-8.759</td>
</tr>
<tr>
<td>Plant B</td>
<td>% Inhibition</td>
<td>-0.730</td>
<td>-18.978</td>
<td>7.299</td>
<td>2.920</td>
<td>-4.380</td>
<td>-10.949</td>
</tr>
<tr>
<td>Plant C</td>
<td>% Inhibition</td>
<td>10.949</td>
<td>22.628</td>
<td>24.818</td>
<td>27.737</td>
<td>21.898</td>
<td>16.788</td>
</tr>
</tbody>
</table>

Figure 3.3: Plot of % Inhibition of NBT against varying concentrations of plants A, B and C

Plants A and C therefore, through this preliminary assay seem to have an inhibitory activity on NBT and can be said to have some superoxide anion scavenging ability. The higher concentrations yielded more inhibition of NBT than the lower ones. % inhibition results for plant B were erratic and not concentration dependent.
3.1.4. DPPH assay

Plant A showed much higher capacity to scavenge free radicals than plants B and C based on % inhibition values obtained. Inhibition by plant A seems to proceed in a concentration dependent manner unlike that of plants B and C. The concentration at which 50% inhibition occurred with plant A extract was 78 µg/ml.

Table 3.4: Average absorbance and % inhibition values obtained after analysis with different concentrations of plants A, B and C

<table>
<thead>
<tr>
<th>Plant</th>
<th>Plant A</th>
<th>Plant B</th>
<th>Plant C</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>% Inhibition</td>
<td>% Inhibition</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>5</td>
<td>-19.003</td>
<td>-20.445</td>
<td>-29.5868</td>
</tr>
<tr>
<td>10</td>
<td>-10.755</td>
<td>-16.7345</td>
<td>-36.1847</td>
</tr>
<tr>
<td>25</td>
<td>7.0455</td>
<td>-14.3978</td>
<td>-19.0026</td>
</tr>
<tr>
<td>30</td>
<td>9.588</td>
<td>-9.79296</td>
<td>-16.5971</td>
</tr>
<tr>
<td>40</td>
<td>18.798</td>
<td>-12.2672</td>
<td>-6.21907</td>
</tr>
<tr>
<td>50</td>
<td>23.197</td>
<td>-11.8548</td>
<td>-6.08162</td>
</tr>
<tr>
<td>75</td>
<td>48.558</td>
<td>-5.05069</td>
<td>4.640034</td>
</tr>
<tr>
<td>100</td>
<td>53.781</td>
<td>-9.6555</td>
<td>-2.57646</td>
</tr>
<tr>
<td>150</td>
<td>56.118</td>
<td>1.478522</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>55.293</td>
<td>8.901203</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3.4: % Inhibition of DPPH with a range of concentrations of plants A, B and C

This assay which measures the free radical scavenging capacity of the plant extract shows that plant A has a better antioxidant capacity than both plants A and B. This assumption is further validated by the results obtained after superoxide anion scavenging assay in section 3.1.3.
3.1.5. FRAP assay

Plants A, B and C all showed a concentration dependent decrease in % inhibition with increasing absorbance.

Table 3.5: Average absorbance and % inhibition values obtained from the FRAP assay using various concentrations of plants A, B and C.

<table>
<thead>
<tr>
<th>[Plant]</th>
<th>Plant A</th>
<th>Plant B</th>
<th>Plant C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Av. Abs</td>
<td>% Inhibition</td>
<td>Av. Ab</td>
</tr>
<tr>
<td>5</td>
<td>0.137</td>
<td>33.212</td>
<td>0.134</td>
</tr>
<tr>
<td>10</td>
<td>0.142</td>
<td>29.562</td>
<td>0.135</td>
</tr>
<tr>
<td>25</td>
<td>0.15</td>
<td>23.358</td>
<td>0.142</td>
</tr>
<tr>
<td>50</td>
<td>0.172</td>
<td>7.664</td>
<td>0.147</td>
</tr>
<tr>
<td>100</td>
<td>0.256</td>
<td>-53.650</td>
<td>0.162</td>
</tr>
<tr>
<td>150</td>
<td>0.437</td>
<td>-185.766</td>
<td>0.166</td>
</tr>
</tbody>
</table>

Figure 3.5: % Inhibition values obtained after FRAP analysis using plants A, B and C.

Table 3.6: FRAP values for plants A, B and C obtained using change in absorbance of samples and ascorbic acid standard

<table>
<thead>
<tr>
<th>[Plant] µg/ml</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>17</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>50</td>
<td>43</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>100</td>
<td>168</td>
<td>30</td>
<td>97</td>
</tr>
<tr>
<td>150</td>
<td>362</td>
<td>8</td>
<td>116</td>
</tr>
</tbody>
</table>
**Figure 3.6:** FRAP values against concentrations of plants A, B and C

All 3 plants showed a concentration dependent increase in FRAP values over the concentrations of plants A, B and C used. Plant A had the highest FRAP value indicating that it has higher electron donating antioxidants and therefore a higher reducing power than plants B and C. In the FRAP assay the reactions are linearly related to the molar concentration of the antioxidants present (Benzie and Strain, 1999) and therefore the inference that plant A has a higher antioxidant potential than both plants A and B is valid.

3.1.6 Alpha glucosidase assay

One of the mechanisms of action of traditional medicines used for the treatment of diabetes is hypothesised to be regulation of glucose uptake from the intestinal lumen by inhibiting carbohydrate digestion and absorption leading to normal glucose homeostasis in diabetic subjects. Alpha glucosidase is an important factor in glucose uptake in the intestinal mucosa since it is one of the major enzymes the degrade polysaccharides to monosaccharides.

It has been previously reported that postprandial hyperglycemias could contribute to the increase of haemoglobin glycosylation (HBA1C) by up to 255 in inadequately controlled patients with type 2 diabetes (Lebovitz, 1998). It has also been suggested that a good
control of postprandial glucose levels would be beneficial in early treatment of diabetes as well as prevention of complications associated with it (Baron, 1998).

Table 3.7: The inhibition of alpha glucosidase found in rat intestinal powder with plants A, B and C. Data is shown as percentage inhibitory concentrations using maltose as substrate respectively.

<table>
<thead>
<tr>
<th>[Plant] μg/ml</th>
<th>% Inhibition</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>0.15625</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.3125</td>
<td>15.23682</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>0.625</td>
<td>29.62388</td>
<td>11.34784</td>
<td>0</td>
</tr>
<tr>
<td>1.25</td>
<td>55.56312</td>
<td>20.8659</td>
<td>16.4032</td>
</tr>
<tr>
<td>2.5</td>
<td>74.91123</td>
<td>39.47492</td>
<td>77.2585</td>
</tr>
<tr>
<td>5</td>
<td>86.74689</td>
<td>50.82337</td>
<td>90.09517</td>
</tr>
</tbody>
</table>

**Figure 3.7:** Inhibitory activity of plants A, B and C extracts against alpha glucosidase found in rat intestinal powder using maltose as substrate.

Plants A, B and C all inhibited alpha glucosidase from rat intestinal powder when maltose was used as substrate. The results show that plant C caused a higher inhibition than plants A and C with maltose as substrate. The increase in % inhibition seems to be concentration dependent. Plant A showed a steady increase in % inhibition values compared to both plant B and C. The IC 50 values for alpha glucosidase inhibition with plants A, B and C are 1.9, 3.75 and 0.94 μg/ml respectively.
Table 3.8: % Inhibition of alpha glucosidase found in rat intestinal powder with sucrose as substrate over various concentrations of plants A, B and C

<table>
<thead>
<tr>
<th>[Plant] µg/ml</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0.0390625</td>
<td>0</td>
</tr>
<tr>
<td>0.078125</td>
<td>2.543</td>
</tr>
<tr>
<td>0.15625</td>
<td>6.705</td>
</tr>
<tr>
<td>0.3125</td>
<td>-</td>
</tr>
<tr>
<td>0.625</td>
<td>48.671</td>
</tr>
<tr>
<td>1.25</td>
<td>57.148</td>
</tr>
<tr>
<td>2.5</td>
<td>57.118</td>
</tr>
<tr>
<td>5</td>
<td>61.503</td>
</tr>
</tbody>
</table>

Figure 3.8: % Inhibition of alpha glucosidase found in rat intestinal powder with sucrose as substrate for various concentrations of plants A, B and C

There was a concentration dependent increase in % inhibition of alpha glucosidase using sucrose as substrate with plants A, B and C however the highest inhibition was shown by plant C. The IC 50 values of this inhibition were 0.16, 0.58 and 0.157 µg/ml for plants A, B and C respectively.

The results obtained indicate that these plants especially plant A can be used as medication to regulate postprandial hyperglycemia of the patients with diabetes. It could also be used for prevention and control of obesity.
3.2. CELL LINES

3.2.1. Glucose utilization in Chang liver cells

There was an increase in glucose utilization by Chang liver cells within the range of plant A concentrations used. This positive result is also reflected by metformin treated cells. This result is as expected since increasing glucose uptake into hepatocytes is one of the known mechanisms of action of this drug (Mithieux et al., 2006).

Table 3.9: Dose dependent response results of glucose utilization in Chang liver cells after treatment with plant A and metformin.

<table>
<thead>
<tr>
<th>Extract</th>
<th>% of Control</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Metformin</td>
<td>151.8</td>
<td>2.1</td>
</tr>
<tr>
<td>12.5 µg/ml</td>
<td>122.2</td>
<td>3.8</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>126.7</td>
<td>2.8</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>166.6</td>
<td>2.1</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>126.1</td>
<td>4.2</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>131.6</td>
<td>1.3</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>149.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Figure 3.9: Dose dependent response analysis of glucose utilization of Chang liver cells after treatment with plant A and metformin.
Although the increase shown due to plant A treatment was not dose dependent as would be expected, the overall improvement in the glucose utilization in these cells is an indicator of one of the possible mechanisms of action of this plant.

In hepatocytes, metformin acts by activating AMP-activated protein kinase (AMPK) in liver cells, leading to increased fatty acid oxidation and uptake by cells. An increase in liponeogenic and gluconeogenic processes then follow to take up the excess FFAs and glucose respectively (Zhou et al., 2005). The different mechanisms of action of metformin are discussed in section 1.2.1.2.

The varied results seen with the different plant concentrations are observed probably due to a synergistic or antagonistic interaction between the different plant constituents. This interaction may pass a certain threshold and may be the cause of the varied results in plant analysis. This could be due to the fact that we were not using a pure compound per se but a fraction of an extract in solution that did not have homogeneity in terms of composition or concentration of constituent compounds. The optimum concentration during this analysis was found to be 50 µg/ml.

The main glucose transporter in the liver is GLUT 2 which has a very low affinity for glucose (Greenspan et al., 2001). This could explain the relatively slight increase in glucose absorption in the liver after treatment with both plant A and metformin.

The principle of the MTT assay is discussed in section 2.1.5. The performance of mitochondrial Succinate dehydrogenase is tested by its conversion of yellow MTT to purple formazan product. The number of surviving cells is therefore directly proportional to the level of the formazan product and quantification on the basis of colour is now possible using a simple colorimetric assay (Mosmann, 1983).
Table 3.8: MTT data for Chang liver cells after treatment with metformin and plant A.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ave. Abs</th>
<th>SD</th>
<th>% Error</th>
<th>% of control</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.964</td>
<td>0.071</td>
<td>7.4</td>
<td>100.0</td>
<td>7.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Metformin</td>
<td>0.897</td>
<td>0.090</td>
<td>10.0</td>
<td>93.1</td>
<td>9.3</td>
<td>3.8</td>
</tr>
<tr>
<td>12.5</td>
<td>0.984</td>
<td>0.109</td>
<td>11.1</td>
<td>102.1</td>
<td>11.3</td>
<td>4.6</td>
</tr>
<tr>
<td>25</td>
<td>1.012</td>
<td>0.077</td>
<td>7.6</td>
<td>92.8</td>
<td>7.0</td>
<td>2.9</td>
</tr>
<tr>
<td>50</td>
<td>0.878</td>
<td>0.127</td>
<td>14.5</td>
<td>80.6</td>
<td>11.7</td>
<td>4.8</td>
</tr>
<tr>
<td>100</td>
<td>1.113</td>
<td>0.106</td>
<td>9.6</td>
<td>115.4</td>
<td>11.0</td>
<td>4.5</td>
</tr>
<tr>
<td>200</td>
<td>1.088</td>
<td>0.131</td>
<td>12.0</td>
<td>99.8</td>
<td>12.0</td>
<td>4.9</td>
</tr>
<tr>
<td>250</td>
<td>1.090</td>
<td>0.248</td>
<td>22.8</td>
<td>100.0</td>
<td>22.8</td>
<td>11.4</td>
</tr>
</tbody>
</table>

Figure 3.10: MTT analysis of metformin and Plant A treatment on Chang liver cells.

Cell viability was defined as the ratio (expressed as percentage) of absorbance of treated cells to untreated cells that were used as the control. From the data, the concentrations of treatments used were not toxic to the cells.

3.2.2. Glucose utilization in C3A cells

Glucose utilization in C3A cells was improved by treatment with metformin and plant A at certain concentrations. From the results, there was marked increase in uptake at the lowest and highest concentrations of plant A used. Albeit this discrepancy, there is some improvement in the glucose uptake profiles of C3A cells with plant A treatment.

Plant A at a concentration of 250 µg/ml showed higher glucose utilization in C3A compared to Chang liver cells as shown in section 3.1.1. Plant A has been shown to
improve glucose uptake profiles of in peripheral tissues of in vivo models, more so in muscle compared to liver tissues.

Table 3.11: Dose dependent response results of glucose uptake in C3A cells after treatment with plant A and metformin.

<table>
<thead>
<tr>
<th>Extract</th>
<th>% of control</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>7.4</td>
</tr>
<tr>
<td>Metformin</td>
<td>116.3</td>
<td>8.2</td>
</tr>
<tr>
<td>12.5 µg/ml</td>
<td>132.8</td>
<td>5.5</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>96.2</td>
<td>6</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>87.1</td>
<td>3.6</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>127.2</td>
<td>10.7</td>
</tr>
<tr>
<td>200 µg/ml</td>
<td>103.8</td>
<td>9</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>180.6</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Figure 3.11: Dose dependent response analysis of glucose utilization of C3A cells after treatment with plant A and metformin.

Plant A might be causing this improved uptake in these muscle cells by improving the translocation and internalization of GLUT 4 transporters (Huang et al., 2007). The facilitated transport of glucose is now enhanced resulting in an increase in glucose levels intracellularly. It is these intracellular glucose levels that are measured quantitatively using the glucose oxidase reagent and measurement at 540 nm.
3.2.3. Glucose utilization in C2C12 cells

Glucose utilization in C2C12 cells was improved by treatment with both insulin and plant A. The least increase of glucose utilization using Plant A was at 25 μg/ ml while the highest was at 150 μg/ ml. The increased uptake with insulin is as expected since this is one of the ways insulin improves glycemic states in the body.

**Table 3.12:** Dose dependent response absorbance values of glucose utilization in C2C12 cells after treatment with plant A and insulin. Results are shown in the table as percent of control values.

<table>
<thead>
<tr>
<th>Treatment (μg/ml)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Insulin 225.925</td>
<td>115.912</td>
</tr>
<tr>
<td>12.5</td>
<td>96.110</td>
</tr>
<tr>
<td>25</td>
<td>147.539</td>
</tr>
<tr>
<td>50</td>
<td>211.758</td>
</tr>
<tr>
<td>100</td>
<td>273.199</td>
</tr>
<tr>
<td>150</td>
<td>134.241</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

**Figure 3.12:** Dose dependent response analysis of glucose utilization of C212 cells after treatment with plant A and metformin.

Exogenous insulin has been shown to increase insulin secretion from the pancreas, increase insulin sensitivity to changes in glucose and improve hepatic glucose absorption (Childs *et al.*, 2007). The addition of insulin to the cells triggers the translocation of GLUT 4 transporters from the intracellular space to the plasma membrane. Glucose is then taken up from the medium by the cells hence the increase in
glucose uptake after treatment with both insulin and plant A. Plant A could therefore also be acting as a trigger for the signalling, translocation and binding of GLUT 4.

3.3. THE RAT MODEL

3.3.1. Comparison of rat weights

The primary purpose of feeding the rats with a HFD was to induce insulin resistance. It has been shown that overfeeding in rodents or feeding on high calories rapidly induces skeletal muscle and liver insulin resistance (Wang et al., 2001). The rats in this study were fed on a HFD for a period of 16 weeks showed signs of insulin resistance as evidenced by the glucose uptake results (see section 3.3.6).

Along with insulin resistance induction, there was an observable increase in body mass over 16 weeks of feeding on a HFD. This was attributed to the high caloric diet. Body weights were recorded weekly and mean weight increases were calculated before sacrifice. The effects of the normal rat chow and high-fat diets on body weight of rats are shown in table 3.13 below.

**Table 3.13:** Average weights of the rats, standard deviation and probability values in the different rat groups on the days 0 and 28 of sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Treatment</th>
<th>Ave. weight (g)</th>
<th>S DEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>LFD</td>
<td>None</td>
<td>504.6</td>
<td>25.679</td>
</tr>
<tr>
<td>COH</td>
<td>HFD</td>
<td>None</td>
<td>490.8</td>
<td>7.430</td>
</tr>
<tr>
<td>CCL</td>
<td>LFD</td>
<td>None</td>
<td>511</td>
<td>23.927</td>
</tr>
<tr>
<td>CCH</td>
<td>HFD</td>
<td>None</td>
<td>581</td>
<td>88.564</td>
</tr>
<tr>
<td>C4</td>
<td>HFD</td>
<td>Plant A</td>
<td>523.2</td>
<td>24.631</td>
</tr>
<tr>
<td>C5</td>
<td>HFD</td>
<td>Metformin</td>
<td>553.2</td>
<td>40.660</td>
</tr>
</tbody>
</table>
The change in body weights over 4 weeks of feeding was minimal. The rats fed on the HFD showed a significant (p = 0.009) increase in body weight after feeding on the HFD for 16 weeks, however this was not evident at 12 weeks (COH vs COL). There was also an increase in body and visceral fat as observed during sacrifice, compared to the control rats.

Table 3.14: Significance (P) values for rat weights in the control and treatment groups

<table>
<thead>
<tr>
<th></th>
<th>COL</th>
<th>COH</th>
<th>CCL</th>
<th>CCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>0.120</td>
<td>0.008</td>
<td>0.153</td>
<td>0.138</td>
</tr>
<tr>
<td>C5</td>
<td>0.006</td>
<td>0.001</td>
<td>0.01</td>
<td>0.878</td>
</tr>
</tbody>
</table>

There was a significant increase in body weight in the Plant A treated group (C4) compared to the LFD control on day 28 (CCH). The increase in body weight in the C4 group was expected due to feeding on a HFD. There was a decrease in body weight in the HFD fed rats after treatment with plant A.

Although there was a general increase in weight in all the rats on the HFD, that on plant A therapy weighed slightly less, however, this was not statistically significant. Metformin treated rats showed a slight decrease in body weight though not as much as was observed after treatment with plant A. This decrease in weight after metformin.
treatment is seen in diabetic patients as well and was expected though it was not significant.

**Figure 3.14:** Average rat body weights in the control and treatment groups (where n=7). Significance was calculated using Student’s t test where * p < 0.05

As shown in Table 3.12, there were a number of significant changes within the experimental groups. Significance values shown in the graph only reflect changes in groups with at least one similar parameter. For example both C4 and C5 groups were on a HFD so comparing their weights with the HFD control is warranted.

**Figure 3.15:** Rat body weights over a 16 week time period for the LFD and HFD controls at different time points i.e. COL, COH and CCL, CCH. Points plotted are mean values (where n= 8).
The results were according to what was expected. The HFD did elicit an increase in body weight in the HFD fed group compared to the LFD fed and control groups (See figure 3.15). The constituent nature of the HFD, with its high saturated fat content can explain this related increase in body weights. High fat diets rich in trans fatty acids and saturated fats result in fat accumulation and its storage in the body in adipose tissue. The weight gain resulting from HFD feeding is due to the increase in adipocytes mass in the body.

Although no significant difference in body weight between CCH and C4 treated rats over 4 weeks was observed, it seems that plant A extract may prevent weight gain when taking the extract with a high fat diet: There was also no significant difference between the body weights of CCL and C4 either, whereas the rats on the HFD gained an average of 90 g and differed significantly from CCL (p < 0.05). Similarly, rats that were on HFD plus metformin treatment gained an average of 63 g over 4 weeks of treatment and differed significantly from CCL (p < 0.05). As with plant A, there was a decrease in weight between C5 and CCH groups.

3.3.2. Blood glucose measurement

The fasting glucose levels seen in the HFD controls (COH and CCH) were higher than the LFD (COL and COH) controls group as shown in Table 3.15. This increase was significant between the COL and COH groups. Rats on the HFD and plant A (C4) therapy showed a decrease in fasting blood glucose levels compared to those on HFD alone, although this decrease was not significant. The HFD and metformin treated group (C5) showed an increase in blood glucose although it was not significant.

The only significant increase in blood glucose levels observed was between COL and COH confirms the induction of insulin resistance using the HFD diet. Although this IR seems to have been achieved early on in the experiment, there was a subsequent increase in glucose levels over time although this increase was not significant.
Table 3.15: Average blood glucose values of the various groups used in the study were COL (control rats on low fat diet); COH (control rats on high fat diet); CCL (control rats on low fat diet after 2 weeks); CCH (control rats on high fat diet after two weeks); C4 (rats on plant A therapy); C5 (metformin positive control rats).

<table>
<thead>
<tr>
<th></th>
<th>COL</th>
<th>COH</th>
<th>CCL</th>
<th>CCH</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Glucose] mM</td>
<td>3.8</td>
<td>4.4</td>
<td>5.3</td>
<td>6.7</td>
<td>5.4</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>5.8</td>
<td>5.8</td>
<td>5.4</td>
<td>6.1</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>6.1</td>
<td>5.8</td>
<td>5.4</td>
<td>4.4</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>5.3</td>
<td>6.1</td>
<td>5.7</td>
<td>5.6</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>5.2</td>
<td>4.9</td>
<td>6</td>
<td>5.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Average</td>
<td>3.96</td>
<td>5.36</td>
<td>5.58</td>
<td>5.76</td>
<td>5.36</td>
<td>6.16</td>
</tr>
<tr>
<td>SD</td>
<td>0.472</td>
<td>0.650</td>
<td>0.476</td>
<td>0.643</td>
<td>0.619</td>
<td>0.838</td>
</tr>
</tbody>
</table>

Figure 3.16: Glucose levels in control and treated groups on the day of sacrifice. Data shown as mean values (where n=5). Significance was calculated using students t test where * p < 0.05

Plant A therapy seems to cause a reduction in blood glucose levels after treatment for four weeks. This is an important result as it implies the ability of this plant to bring about glycemic change within a relatively short time. Although the change was not significant, it would be interesting to note further changes or fluctuations after treatment over a longer period of time. Since this decrease in glucose levels was not significant, it appears therefore that this effect in vivo is mainly due to stimulation of insulin secretion and the IR state of the rats.

Metformin did not seem to have an effect on the blood glucose levels in this experiment. This was not the expected results since treatment with metformin does bring about a change in the glycemic index of diabetic patients (Shimomura, 2000).
The Diabetes Control and Complications trial (DCCT) as well as the U.K. Prospective Diabetes Study (UKPDS) have both shown through extensive studies, that the control of blood glucose levels is critical in reducing morbidity as well as associated complications, especially with type II diabetes (The DCCT research group, 1993). This reduction in blood glucose levels is the basic mechanism of action of many drugs used in the treatment and management of diabetes, including traditional medicines.

The insulin resistance brought about by the HFD induced hyperglycemia. This increased glucose levels in the blood would in turn have affected the secretion of insulin from pancreatic β-cells prompting glucose uptake up to the point where cells and tissues are no longer sensitive to insulin. This is the case in both COH and CCH (see figure 3.19). This means the rats were only insulin resistant and therefore no significant increases in blood glucose levels are expected.

3.3.3. Glucose clearance

The results obtained show a significant increase in glucose clearance for the plant A treated group (p< 0.05) compared to the HFD fed rats as well as the lean controls. The HFD fed rats were IR at the time of sacrifice.

The slope was calculated from graphs of log [glucose] against time. The values obtained which represent glucose clearance were quite different between lean controls (COL and CCL) and HFD controls (COH and CCH) on the different sacrifice dates. This difference in values could be due to the age and body size difference as at time of sacrifice. The percentage in which the slope value of the HFD rats changed from the lean controls was calculated for each sacrifice. The percentage change observed between the LFD and the HFD was almost the same for the two sacrifice dates (37.54% and 36.75%). Table 3.16 shows that both the plant extract and metformin improved glucose clearance, and therefore insulin resistance.
Table 3.16: Mean glucose clearance rate values and standard deviations for control and treatment groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose clearance rate (mM/min)</th>
<th>Standard Deviation</th>
<th>% change from relative lean control</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.01998</td>
<td>0.005755</td>
<td>-</td>
</tr>
<tr>
<td>COH</td>
<td>0.01248</td>
<td>0.004292</td>
<td>37.54</td>
</tr>
<tr>
<td>CCL</td>
<td>0.024875</td>
<td>0.004723</td>
<td>-</td>
</tr>
<tr>
<td>CCH</td>
<td>0.015733</td>
<td>0.002861</td>
<td>36.75</td>
</tr>
<tr>
<td>C4</td>
<td>0.02225</td>
<td>0.00764</td>
<td>10.55</td>
</tr>
<tr>
<td>C5</td>
<td>0.0192</td>
<td>0.003081</td>
<td>23.62</td>
</tr>
</tbody>
</table>

Figure 3.17: Mean glucose clearance rates of LFD and HFD control groups.

There was a clear trend in decrease of glucose clearance rates with induction of a HFD. A significant decrease (P=0.048) was however only observed between CCL and CCH groups as shown in figure 3.9 above. The decrease in glucose clearance in the HFD fed rats can be attributed to the acquired insulin resistance after feeding on a high fat diet. The body’s response to insulin action peripherally was reduced and therefore the uptake of glucose by tissues was also impaired.
Glucose clearance rates improved with plant A and metformin treatments compared to the lean control (CCL). This improved clearance rate with plant A may be due to the improved insulin sensitivity and glucose uptake as is evident from the deoxy-glucose uptake measurement into the selected tissues (Refer to sections 3.3.4 and 3.2.5). Similarly, metformin is known to increase insulin sensitivity peripherally consequently improving glucose uptake into the tissues which would explain the increased glucose clearance rate and decreased AUC (0-60 minutes) obtained (Refer to section 1.2.1.2).

### 3.3.4. Plasma insulin determination

Fasting insulin levels were significantly elevated in the HFD fed rats compared to the control groups as shown in Figure 3.19. There have been many studies done that show an increase in the fasting insulin levels in HFD fed rats compared to the controls. An increase in fasting insulin levels was observed in the HFD fed rats compared to the controls as expected in insulin resistance. The link between increased saturated fatty acid levels in the diet and increased insulin has been postulated to be due to an increase in insulin released from the rat’s islets facilitated by increased intracellular calcium levels (Warnotte et al., 1994).
Table 3.17: Average insulin concentrations in ng/ml and their standard deviation values on days 0 and 28 of medicinal treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average [Insulin] ng/ml</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.622</td>
<td>0.133</td>
</tr>
<tr>
<td>COH</td>
<td>1.8</td>
<td>0.460</td>
</tr>
<tr>
<td>CCL</td>
<td>0.575</td>
<td>0.217</td>
</tr>
<tr>
<td>CCH</td>
<td>1.593</td>
<td>0.857</td>
</tr>
<tr>
<td>C4</td>
<td>4.363</td>
<td>1.286</td>
</tr>
<tr>
<td>C5</td>
<td>1.022</td>
<td>0.462</td>
</tr>
</tbody>
</table>

There was an increase in insulin levels in the HFD controls (both time points) compared to the LFD controls. This increase is due to the insulin resistance that is associated with HFD feeding. Since the peripheral tissues have reduced sensitivity to insulin in the IR state, insulin levels increase without the expected concomitant decrease in glucose levels. The increase in insulin levels in COL compared to COH and COL compared to CCH was significant (P=0.001 and P= 0.007 respectively).

Figure 3.19: Plasma insulin concentrations of control groups (COL, COH, CCL and CCH). Points plotted are mean values (where n=8). Significance was calculated using Student’s t test where * p < 0.05 and ** p < 0.001

Plant A appears to drastically increase insulin levels compared to the LFD and HFD controls. A significant increase (P= 0.0002) in fasting blood insulin levels was observed after treatment with plant A. This could be due to an increase in insulin synthesis and release from the cells of the pancreas that improves and maintains blood glucose
homeostasis. It needs to be noted that in spite of a higher insulin secretion the rats did not become hypoglycaemic.

![Graph showing average insulin concentrations (ng/ml) for CCL, CCH, plant A and metformin groups.](image)

**Figure 3.20**: Average insulin concentrations (ng/ml) for CCL, CCH, plant A and metformin groups. Points plotted are mean values (where n=8). Plasma samples were obtained after 16 weeks on the diets and 4 weeks on treatment. Significance was calculated using Student’s t test where * p < 0.05 and ** p < 0.001

Although use of plant A elevates blood insulin levels and would be beneficial in alleviating symptoms of diabetes, its prolonged use may damage the pancreas due to enhanced insulin production. This is one of the pathophysiologies of type 2 diabetes. It is therefore imperative that further studies be done on the effect of this plant on the pancreas as well as possible side effects with prolonged use.

3.3.5. Insulin resistance

QUICKI values were calculated from the plasma insulin and glucose levels as it is one of the reference point indicators for insulin resistance (Katz *et al.*, 2000). QUICKI (Quantitative Insulin sensitivity Check Index) is an index based on the logarithm and the reciprocal of the insulin-glucose product (Katz *et al.*, 2000). From the results of glucose clearance (section 3.3.3) and glucose uptake in the muscle, liver and adipose (section 3.3.6), there was a clear improvement in insulin sensitivity though QUICKI values show a weakening following treatment with plant A and metformin.
This reciprocal result is due to the equation used to calculate QUICKI values (Section 1.1.5). Calculation of QUICKI values with insulin and glucose levels is based on the principal insulin will increase and blood glucose values remain constant in the insulin resistant state or the blood glucose values will increase when the blood insulin values drop in the diabetic state. Both condition lead to a decrease in QUICKI values. However this formula can be compromised when a medication is used which elevates insulin levels.

**Table 3.18:** QUICKI values obtained from plasma glucose and plasma insulin analysis. Points plotted are mean values (where n=8). QUICKI values were calculated using insulin and glucose values.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.3071</td>
<td>0.0153</td>
</tr>
<tr>
<td>COH</td>
<td>0.2722</td>
<td>0.0159</td>
</tr>
<tr>
<td>CCL</td>
<td>0.3063</td>
<td>0.0215</td>
</tr>
<tr>
<td>CCH</td>
<td>0.2834</td>
<td>0.0242</td>
</tr>
<tr>
<td>C4</td>
<td>0.2520</td>
<td>0.0127</td>
</tr>
<tr>
<td>C5</td>
<td>0.2978</td>
<td>0.0262</td>
</tr>
</tbody>
</table>

**Figure 3.21:** Average QUICKI values for control groups (COL, COH, CCL and CCH). Points plotted are mean values (n=8). Significance was calculated using students t test where ** p < 0.01.
Figure 3.22: QUICKI values for control and treated groups. Points plotted are mean values. Significance was calculated using Student’s t test where * p < 0.05.

For the positive control used, metformin the results were not as good as expected based on the insulin and QUICKI analysis. The QUICKI values obtained were higher than those of the HFD fed control. This was so because there was a decrease in insulin and blood glucose levels after metformin treatment. In diabetes treatment, metformin works to reduce glucose output by the liver (Morioka et al., 2005).

The lower QUICKI values for the plant A treated group are as expected. This is because of the dramatic increase in insulin levels after treatment with plant A even though there was only a slight reduction in glucose levels following this treatment. Measurement of insulin sensitivity is based on the quantity of insulin needed to maintain glucose homeostasis. However, when the levels of insulin are increased for reasons other than glucose homeostasis, such as medication or b-cell dysfunction, it becomes difficult to quantify the actual degree of insulin resistance since the changes in insulin levels are no longer due to glucose homeostasis alone.

3.3.6. Glucose uptake in muscle, liver and fat tissues

A significant increase in glucose uptake as measured by the accumulation of $^3$H deoxy-glucose, was observed between the HFD fed rats and plant A treated rats on all the tissues measured. A higher count was obtained for the LFD fed rats. The results in table
below also show decreased glucose uptake in HFD fed rats compared to the controls confirming the insulin resistant nature of these rats.

**Table 3.19**: Glucose uptake (dpm/g tissue) results for diet/control and treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>CCL</th>
<th>CCH</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1305.216</td>
<td>955.012</td>
<td>1438.81</td>
<td>1176.35</td>
</tr>
<tr>
<td>Muscle</td>
<td>1754.845</td>
<td>965.659</td>
<td>1994.702</td>
<td>1094.558</td>
</tr>
<tr>
<td>Fat</td>
<td>2800.739</td>
<td>1088.731</td>
<td>8276.945</td>
<td>327.570</td>
</tr>
</tbody>
</table>

The increase in $^3$H deoxy-glucose count in plant A and metformin treated groups implies an improvement in glucose uptake and suggests a recovery of insulin sensitivity in these target tissues. It is also possible that the elevated levels of insulin measured in the plant A treated group may have contributed to the enhanced glucose uptake. In this regard it is surprising that while there was only a modest increase in the glucose uptake in muscle tissue, there was a dramatic increase in adipose tissue. Given that both tissues are responsive to insulin, one may have expected a similar increase in muscle glucose uptake if it were simply due to higher blood insulin levels. This tissue specific effect suggests that plant A treatment may also influence insulin independent glucose uptake. The plant may induce greater insulin sensitivity in fat tissue compared to muscle and liver probably due to inhibition of hormone sensitive lipases.

In both muscle and liver tissues there was a significant increase in glucose uptake in the treatment groups compared to the HFD fed group. The values obtained seem to indicate a movement towards insulin levels found in the LFD state. Perhaps normalization to these levels would have been achieved over a longer period of treatment.

Both starvation and diabetes are characterized by low concentrations of insulin and high concentrations of fatty acids and ketone bodies in serum. The inhibition of glucose oxidation and decrease of pyruvate dehydrogenase activity observed in starvation and diabetes may be related to changes in the acetyl coenzyme A and NADH/NAD+ ratios in muscle mitochondria. Increased NADH/NAD+ and acetyl CoA /CoA ratios might explain decreased glucose uptake in the body (Lewis *et al.*, 2002).
Figure 3.23: $^1$H counts from liver, muscle and fat tissues obtained from rats sacrificed on day 28 after an overnight fast. Data points are mean values. Significance was calculated using Student’s t test where * $p < 0.05$.

In hyperinsulinemia a common feature in IR, glucose production is driven further forward resulting in hyperglycemia and decreased glucose uptake into the cells (Luca et al., 2007). Since plant A and metformin have been shown to increase insulin sensitivity in the muscle and liver tissues (section 3.3.4) then it is possible that the improved glucose uptake in these tissues is due to the improved response to insulin by the cells and associated signalling and transporter molecules. Glucose uptake in tissues from the metformin treated rats (C5) did not show a significant deviation form the HFD fed rats with the exception of fat tissue.

An improvement in the signalling, translocation and internalization processes for GLUT 4 transporters by improved insulin sensitivity might also explain the improved glucose uptake in peripheral tissues. GLUT 4 is the main glucose transporter in the body and is highly expressed more so in adipose tissue muscle while GLUT 2 is predominantly in the liver (Huang et al., 2007).

More GLUT 4 transporters are sequestered intracellularly in their unstimulated state compared to other glucose transporters. This implies that once activation occurs in response to insulin and other stimuli, they are moved to the plasma membrane much faster than other transporters (Bryant et al., 2002). This might explain the improved glucose uptake in fat and muscle compared to the liver.
The expression of GLUT 4 in the body is tissue specific. Adipose tissue of obese and type 2 diabetes shows significantly low levels of GLUT 4 mRNA and proteins. The resulting down regulation of GLUT 4 expression especially in obese and type 2 diabetics is more significant in adipocytes compared to myocytes (Huang et al., 2007). This explains the much higher glucose uptake values seen in fat tissue.

Plant A seems to enhance the expression of GLUT 4 processes in fat more than the other tissues. An increased presence of GLUT 4 transporters in the plasma membrane leads to increased transport of glucose into the tissues. Plant A might also upregulate the expression of other glucose transporters present in adipose tissue such as GLUT 8 and GLUT 12 thereby increasing the glucose uptake capacity in the fat tissue tested.

The main glucose transporter in the liver is GLUT 2 which has a very low affinity for glucose and exerts most of its action postprandially (Greenspan et al., 2001). This could explain the relatively slight increase in glucose absorption in the liver after treatment with both plant A and metformin.

3.3.7. Triglyceride content determination

There was a significant increase in TG content in the rats after feeding on a HFD. After treatment with plant A, there was a significant reduction in the TG levels as compared to the HFD control. Plant A had a role to play in this decrease and might therefore be helpful in decreasing TGs levels as treatment for type 2 diabetes.

Table 3.20: TG content in mmol and derived standard deviations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average [TG] mmol/l</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.662</td>
<td>0.175</td>
</tr>
<tr>
<td>COH</td>
<td>0.712</td>
<td>0.313</td>
</tr>
<tr>
<td>CCL</td>
<td>0.585</td>
<td>0.073</td>
</tr>
<tr>
<td>CCH</td>
<td>0.860</td>
<td>0.196</td>
</tr>
<tr>
<td>C4</td>
<td>0.592</td>
<td>0.146</td>
</tr>
<tr>
<td>C5</td>
<td>0.733</td>
<td>0.267</td>
</tr>
</tbody>
</table>
Figure 3.24: Average TG concentrations in control groups and (C4) plant A treated group. Points plotted are mean values (where n=6). Significance was calculated using Student’s t test where * p < 0.05.

Hypertriglyceridemia is a common condition found in the insulin resistance syndrome and is also highly correlated to type 2 diabetes and obesity, especially central obesity (Bruzell, 2007). Due to the constituent nature of the HFD, the TG levels are expected to be higher than those of rats fed on normal chow.

High levels of triglycerides in muscle cells have been associated with decreased insulin sensitivity in the skeletal muscle. The increase in TG levels is probably due to an increase in the uptake of fatty acids as well as the decrease in mitochondrial lipid oxidation. The excess fatty acids that result are esterified or metabolized to molecules that may interfere with specific cellular proteins such as insulin mediated signal transduction. This interference results in the disruption of glucose metabolism (Corcoran et al., 2007).

3.3.7. Total cholesterol level determination

There was a significant increase in the cholesterol content in HFD fed control samples compared to the LFD controls. Plant A was shown to significantly reduce cholesterol levels in rats fed on the HFD then treated.
Table 3.21: Total cholesterol values for control and treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>[Cholesterol] mmol/l</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>1.271</td>
<td>0.131</td>
</tr>
<tr>
<td>COH</td>
<td>1.53997</td>
<td>0.20972</td>
</tr>
<tr>
<td>CCL</td>
<td>1.2120</td>
<td>0.2481</td>
</tr>
<tr>
<td>CCH</td>
<td>1.4960</td>
<td>0.1493</td>
</tr>
<tr>
<td>C4</td>
<td>1.1751</td>
<td>0.0741</td>
</tr>
<tr>
<td>C5</td>
<td>1.323</td>
<td>0.132</td>
</tr>
</tbody>
</table>

Figure 3.25: Average cholesterol concentrations in CCL, CCH, C4 and C5 groups. Points plotted are mean values (where n=6). Significance was calculated using Student’s t test where * p < 0.05 and ** p < 0.005

Insulin resistance is widely acknowledged to be associated with dyslipidemia (Garvey et al., 2003). Saturated and Trans fatty acids and saturated fats are major dietary determinants of plasma LDL cholesterol. Since the HFD diet fed to the rats was high in saturated fats, this explains the increased cholesterol levels.

Saturated and Trans fatty acids and saturated fats are major dietary determinants of plasma LDL cholesterol. The link between LDL cholesterol levels, total lipid profiles and metabolic disorders such as diabetes is so high that major nutrition recommendations and interventions for diabetes are discussed with these parameters in mind (ADA, 2006). Since the HFD diet fed to the rats was high in saturated fats, this explains the increased cholesterol levels.
In humans, plant sterols decrease intestinal absorption of both dietary and hepatobiliary cholesterol (Fowler, 2007). Plant sterols are plant esters and might be in abundance in plant A extract. This could also explain the decreased cholesterol levels in plasma after treatment with plant A.

3.3.9. HDL cholesterol level determination

The role of HDL particles in lipid metabolism is primarily the uptake and transport of cholesterol from peripheral tissue to the liver. HDL is known as the good cholesterol and an improvement or maintenance of its profile is an indicator of one’s health status. Very low HDL-C levels have repeatedly been associated with an increased risk of coronary heart disease and coronary artery disease. Thus the determination of serum HDL cholesterol has been recognized as a useful tool in identifying high-risk patients (Luca et al., 2007).

There was a significant improvement in HDL levels in the blood following plant A treatment with both the lean control, CCL (0.002) and the HFD control (0.002). This analysis shows that plant A could be helpful in decreasing elevated HDL levels associated with type 2 diabetes, obesity and insulin resistance. Decreasing HDL concentration as a consequence of treatment is advantageous only if it is back to homeostatic levels.

HDL and insulin concentrations have an inverse relationship. The low HDL concentrations are most likely due to the inability of HDL to be formed from VLDL (Barter et al., 2004).

Table 3.22: HDL cholesterol concentrations in the control (CCL and CCH) groups and plant A treated group (C4). Values calculated from the standard curve shown in the appendix

<table>
<thead>
<tr>
<th>Groups</th>
<th>[HDL] mmol/l</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.223</td>
<td>0.032</td>
</tr>
<tr>
<td>COH</td>
<td>0.236</td>
<td>0.053</td>
</tr>
<tr>
<td>CCL</td>
<td>0.303</td>
<td>0.050</td>
</tr>
<tr>
<td>CCH</td>
<td>0.298</td>
<td>0.032</td>
</tr>
<tr>
<td>C4</td>
<td>0.174</td>
<td>0.049</td>
</tr>
<tr>
<td>C5</td>
<td>0.408</td>
<td>0.092</td>
</tr>
</tbody>
</table>
Figure 3.26: Average HDL cholesterol levels in CCL, CCH, C4 and C5 groups (where n=6).

Since plant A decreases HDL levels in both lean and HFD induced IR states, its use should be regulated and the HDL levels monitored so that they are not decreased below the homeostatic levels in the body. That it decreases grossly elevated HDL levels found in obesity is an advantage and further study into its role in this should be carried out.

3.3.10. Free Fatty Acid level determination

The plasma FFA content in the HFD fed control rats was significantly higher than that of control rats on normal chow (see figure 3.27). This is an expected change due to the high fat content of the diet. Although treatment with plant A reduced free fatty acid levels in the HFD rats, the change was not significant.

It is possible that the fatty acid profiles can change with longer treatment periods with plant A coupled with an improved diet. The results seem to correlate with research and known mechanisms of fatty acids in insulin resistant states as discussed below. There is significance in the measurement of plasma FFA levels since they are elevated in obesity and insulin resistant states due to increased fatty acid release (Qatanani et al., 2008).
Table 3.23: FFA concentrations of CCL, CCH control groups and plant A treated group obtained from the standard curve

<table>
<thead>
<tr>
<th>Groups</th>
<th>[FFA] mM</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.070</td>
<td>0.020</td>
</tr>
<tr>
<td>COH</td>
<td>0.076</td>
<td>0.006</td>
</tr>
<tr>
<td>CCL</td>
<td>0.0691</td>
<td>0.0109</td>
</tr>
<tr>
<td>CCH</td>
<td>0.0853</td>
<td>0.0117</td>
</tr>
<tr>
<td>C4</td>
<td>0.0748</td>
<td>0.0086</td>
</tr>
<tr>
<td>C5</td>
<td>0.051</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Figure 3.27: Average FFA concentrations (mM) for CCL, CCH and C4 groups. Points plotted are mean values (where n=6). Significance was calculated using Student’s t test where * p < 0.05.

*In vivo* experiments previously conducted in healthy persons showing that increasing plasma FFA concentrations can induce IR similar to that seen in type 2 diabetes (Roden et al., 1996). Elevated levels of FFA in healthy individuals have been shown to inhibit insulin stimulated glucose oxidation, glucose uptake inhibition as well as glycogen synthesis through the inhibition of glucose transport and/or phosphorylation and a decrease in the activity of glycogen synthase activity in the muscle (Berge et al., 2004).

The uptake of glucose more so than its metabolism is now regarded as the rate limiting step for FA induced insulin resistance (Shulman et al., 2000). From the glucose uptake results in section 4.6.4, it is evident that the uptake of glucose in tissues obtained from HFD fed rats was significantly lower than that seen in LFD fed rats as well as plants A
treated rats. This decreased glucose uptake could be rightly attributed to the increased FFA levels.

Large depots of adipose tissue characteristically found in obesity, release cytokines such as TNFα which in turn trigger the secretion of FFA from adipose tissue into the circulation (Arkan et al., 2005). This could explain the increased FFA levels found in plasma since there was an increase in adipose tissue mass in the HFD fed rats as observed during sacrifice.

Lipase, a hormone sensitive enzyme is responsible for the facilitated transport of fatty acids from the adipose tissue to the blood stream. Its enhanced activity in lipolytic processes that follow the fasting stage may also be responsible for the increased fatty acid profile seen (Berge et al., 2005). Clinical studies have shown that the inhibition of lipolysis using pharmacological agents decreases the levels of fatty acids and improves insulin sensitivity in a range of test subjects (Assali et al., 2001).

The increased FFAs in the body are usually diverted to fuel production via the beta oxidative process and thus compete with glucose in the body, more especially in the muscles. Human in vitro studies have been conducted that suggest the fatty acid profiles of peripheral tissues influence insulin sensitivity in those tissues (Lichtenstein, 2000).

The increased glucagon levels in the diabetic state also contribute to the mobilization of free fatty acids. These free fatty acid levels are then almost as high as the plasma glucose level in diabetes and in some ways are a better indicator of the severity of the diabetic state (Ganong, 1997).

3.3.11. Glycogen determination in muscle and liver

An improvement was observed in glycogen content in the rats fed on a high fat diet compared to the control rats. The COL and COH control groups were used to show that the animal models used were representative of the insulin resistant state. Due to the fact that they were about 4 weeks younger than the other control groups, CCL and CCH group averages of glycogen concentrations are lower. The calculated glycogen contents are shown in tables 3.24 and 3.25.
Table 3.24: Muscle glycogen concentrations in control and plant A treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>[Glycogen] mg/g</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.3297</td>
<td>0.1171</td>
</tr>
<tr>
<td>COH</td>
<td>0.1627</td>
<td>0.0849</td>
</tr>
<tr>
<td>CCL</td>
<td>0.3247</td>
<td>0.0414</td>
</tr>
<tr>
<td>CCH</td>
<td>0.1851</td>
<td>0.0715</td>
</tr>
<tr>
<td>C4</td>
<td>0.5556</td>
<td>0.2412</td>
</tr>
</tbody>
</table>

Table 3.25: Liver glycogen concentrations in control and plant A treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>[Glycogen] mg/g</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL</td>
<td>0.0987</td>
<td>0.1039</td>
</tr>
<tr>
<td>COH</td>
<td>0.2564</td>
<td>0.1326</td>
</tr>
<tr>
<td>CCL</td>
<td>0.1646</td>
<td>0.1279</td>
</tr>
<tr>
<td>CCH</td>
<td>0.3674</td>
<td>0.2394</td>
</tr>
<tr>
<td>C4</td>
<td>0.2828</td>
<td>0.1659</td>
</tr>
</tbody>
</table>

Figure 3.28: Average muscle glycogen concentrations in control groups compared to C4, the plant A treated group. Points plotted are mean values (where n= 5). Significance was calculated using students t test where * p < 0.05

There was a significant increase (p= 0.045) in liver glycogen levels in the early HFD controls compared to the lean control. This increase early on the in experiment is due to the increased caloric and nutrient intake. The excess energy derived from the HFD is stored in the liver in the form of glycogen.
A significant decrease (p=0.023) in muscle glycogen levels was observed in COH compared to COL control groups after induction of the HFD. This decrease

![Graph showing liver glycogen concentrations](image)

**Figure 3.39:** Liver glycogen concentrations in control groups compared to C4, the plant A treated group. Points plotted are mean values (where n=6). Significance was calculated using Student’s t test where * p < 0.05

The overnight fast to which the sacrificed rats were subjected to prior to sacrifice might explain the higher levels of glycogen in the muscle compared to the liver hepatic glycogen levels were lower in plant A treated group compared to the HFD fed group.

The enzyme G-6-Pase found in the liver might be inhibited therefore glycogen is not broken down efficiently. The glycolytic process is affected by the decreased flux of glycogen 6 phosphate. This could explain the increased glycogen values in plant A treated rats compared to the controls. Some of the commonly used medications for type II diabetes e.g. metformin, also works by decreasing the production of glucose by inhibiting this enzyme (Adisakwattana et al., 2005). In HFD fed rats, metformin has been shown to inhibit G-6-Pase activity resulting in glycogen accumulation (Mithieux et al., 2002).

The increase observed in muscle glycogen content seen in the HFD fed rats after Plant A therapy can be explained by the elevated insulin levels (Refer to section 3.3.4). The uptake of glucose into tissues is increased by this elevated insulin level and this would lead to increased glycogenesis and subsequent storage of the excess.
When FFAs are in excess due to high fat feeding, they accumulate in the muscle where insulin stimulated glucose uptake and glycogen synthetic processes are affected. The HFD fed rats had a significant reduction in glucose uptake as shown in section 3.3.6. This inhibition of glycogen synthesis by high FA levels might explain the decreased glycogen levels observed in muscle and liver samples from HFD fed rats.

Glycogen synthesis is expected to be reduced as a consequence of low glucose availability. The decreased glycogen levels obtained from HFD diet fed rats are as expected. Plant A had a positive effect on glycogen levels probably as a result of improved glucose uptake and insulin sensitivity.
CHAPTER 4

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY
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Diabetes is one of the major causes of death by disease in the world. Changes in lifestyles, poor eating habits involving fatty and refined foods, sedentary lifestyles, population ageing and obesity are some of the factors contributing to this pandemic.

The diabetes problem is very much a medical, social and economic concern. Due to the absence of proper documentation, it is difficult to state definitively the effect felt by the people and the country, now and in the future. What is clear though is that there is an increase in incidence of diabetes diagnosis as well as the various complications associated with this condition.

It is clear that intervention from various corners is needed if we are to tame this condition. Government and other stake holders have to look at all avenues that provide a medical solution as well as those that show promise in palliative care. Traditional herbal medicine is one such avenue that continues to show a lot of promise and has been used by the vast majority of people in the country as their first line of defence.

Diabetes impacts various facets of the lives of those who have it as well as their families. Interference with work, time away from school as well as paid work and leisure are some of the few social- economic areas directly affected by diabetes. In developing economies where disability reforms are inadequate, it is the families of diabetic patients who bear the brunt of medical care.

The disparities in diabetic care between the developing and developed world is extremely large. More than 80% of expenditure for diabetes medical care is made in the richest countries in the world, while only 20% in the middle to low income countries, where an estimated 80% of people with diabetes are expected to live (Diabetes atlas, IDF, 2006). Developing countries therefore need to harness the resources available to them to bridge this gap.

The importance of extensive studies on the herbal remedies used by our local communities cannot be overstated. With these studies it follows that scientific data
should be collected and used to support the use of these remedies. There in lies another challenge, in documentation of the plants, their use as well as their perceived and corroborated effects. The modes of action and active components of these plants can also be elucidated giving further credit to the use of these remedies.

Over and above the obvious far reaching effects of traditional herbal remedies on health care, there are several financial aspects that can benefit either directly or indirectly from scientific research on these plants. The patients, who can ill afford the conventional medicines for treatable ailments such as diabetes, will have a viable and proven alternative. Potential for job creation with large scale cultivation of these plants as well as in further research will also improve the financial status of communities.

The enhancement of medical research in the African continent on natural plant resources should be a priority by governments as well as scientists. This would ensure that the vast ethno botanical resources we have are not lost or overused. We have a rich and vast history of successful use of traditional medicine in the continent. However, a lot of this information is not documented, with a large part of it held by specialist groups of healers who pass it orally to selected recipients. Therefore, this information could be lost in the transfer mechanism.

The global pharmaceutical industry is a multi billion dollar industry, of which Africa and its scientists play a very small role. We can change this by using the information and resources available to prove that further research into traditional medicine is a viable and profit making route for the pharmaceutical companies. The impact of multinational companies getting involved locally in research and development would be felt directly and indirectly by the community. Involvement by large pharmaceutical companies will also directly increase the use and marketability of the plants and their products. It can create jobs locally and therefore aid in alleviating poverty.

The World Health Organization (WHO) has also provided guidelines that would act as incentives by government and scientists to further research in the continent (WHO Country Cooperation Strategy for the Republic of Kenya, 2002-2005). The traditional medicine strategy launched in 2002 is designed to assist countries to:
• Develop national policies on evaluation and regulation of traditional medicines and practices.
• Create a stronger evidence base on the safety, efficacy and quality of the traditional medicines and practices.
• Ensure availability and affordability of these traditional medicines including essential herbal medicines.
• Promote therapeutically sound use of these medicines by providers and consumers.
• Document traditional medicines and remedies.

Initial screening of selected plant extracts was done to determine which one to use for in vivo studies. The screening process involved antioxidant assays and glucose utilization analyses. The plant extracts A, B and C, all showed antioxidant potentials with the antioxidant assays. There was inhibition of xanthine oxidase, NBT as well as alpha glucosidase enzyme.

The next step was to do glucose utilization analyses using the three plant extracts. All 3 showed improved glucose utilization in Chang, C3A and C2C12 cells albeit in varying degrees. Different concentrations of the plant extracts were used. The dose response analyses were not always concentration dependent. This is a common feature in plant studies especially when dealing with a crude extract. Further analysis can be made on glucose utilization in these and other cell lines using processed extracts and the results compared to those obtained with the crude extracts.

The constituent active principles of the plant extracts should also be elucidated and characterised. This can be done through mass spectroscopy (MS) or high performance liquid spectroscopy (HPLC) studies. The separated and/or characterised compounds can then be used on cell lines and their effects on parameters such as glucose utilization or insulin secretion observed.

From results of antioxidant and cell culture studies, plant A was picked as the extract of choice for use in in vivo experiments involving HFD induced IR rats. Once IR had been established in the rats, plant A was administered over 4 weeks. Experiments on glucose, insulin and glycogen profiles were done after the 4 week treatment period.
There was a slight reduction in weight after treatment of HFD fed IR rats with plant A. This decrease in weight may be beneficial to diabetic patients since some of the commonly used drugs such as metformin have been associated with weight gain over long periods of treatment. Further study can be done on the effect of plant A treatment on weights of HFD induced IR rats over a longer period of time.

The reduction in blood glucose levels after plant A treatment though not significant showed the potential antidiabetic effect of this extract. That this reduction occurred over the relatively short period of time of 4 weeks is also important since the plant seems to be able to improve the glycemic index quite quickly. It would be interesting to observe the effect of this plant on the glucose profiles of IR rats over a longer period of treatment.

The improved glucose clearance shown by plant A treatment means an overall improvement in the IR state. In IR the body’s response to insulin action peripherally was reduced and treatment with plant A improved this response. There was a concomitant increase in insulin levels after treatment of HFD fed IR rats probably due to increased insulin synthesis and release from beta cells of the pancreas. Further analysis on this apparent increase in insulin secretion can be done in vitro using INS pancreatic cells. This enhanced insulin production over a long period of time may cause damage to the structural and functional integrity of the pancreas. Varying concentrations and treatment periods need to be tested on INS cells to study the effect of plant A under these and other conditions.

The improvement in glucose uptake peripherally by muscle, liver and fat tissues can be explained by an improvement in insulin sensitivity and response after treatment with plant A. It is possible that there is an improvement in the response of glucose transporters in the tissues. The translocation of glucose transporters in the cells can be analysed to validate this hypothesis. Further tests need to be done on fat tissues and cells such as 3T3 cells to explain the marked increase in glucose uptake in fat tissue compared to muscle and liver tissues tested after treatment with plant A.

An improvement in the overall lipid profiles of rats treated with plant A was observed. There was a decrease in TG levels compared to the HFD controls, a decrease in total cholesterol levels, a decrease in HDL as well as FFA levels. IR is associated with
dyslipidemia and therefore the reduction of the stated lipid parameters would prove beneficial to the diabetic patient. The HDL levels decreased in both the lean and HFD groups. It is important therefore that the use of this plant is regulated and monitored to avoid the decrease of these parameters below homeostatic levels in the body. The effect of plant A on lipid profiles of HFD IR rats should be monitored over a longer period of treatment in conjunction with diet variations aimed at improving IR brought about by a high fat diet.

In conclusion, it has been shown that plant A has potential as an antioxidant and antidiabetic drug from the experiments carried out over the course of this study. More research needs to be undertaken and documented on this plant as it is widely used in many Kenyan communities for the treatment and management of diabetes. This would validate its use as well as open avenues for product research and commercialization.


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