INVESTIGATION OF ANTI-DIABETIC PROPERTIES OF
*PSIDIUM GUAJAVA* LEAF IN STREPTOZOTOCIN INDUCED
DIABETIC RATS

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INVESTIGATION OF ANTI-DIABETIC PROPERTIES OF PSIDIUM GUAIJAVA LEAF IN STREPTOZOTOCIN INDUCED DIABETIC RATS

BY

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SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY IN THE DISCIPLINE OF BIOCHEMISTRY, SCHOOL OF LIFE SCIENCES, COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE.

2015

As the candidate’s supervisor I have approved this thesis/dissertation for submission.

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Student: Mr Toluwani Tella  
Supervisor: Dr. B. Masola  
Co-supervisor: Prof. S. Mukaratirwa

Signature

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DEDICATION

This work is dedicated to my source of wisdom, inspiration and strength – The Almighty God. Your grace has sustained me throughout this project work. I love you Lord.

And to my wonderful parents, Rev. Dr and Mrs A.O. Tella.
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ABSTRACT

Diabetes mellitus results in chronic hyperglycaemia, leading to defects in carbohydrate, fat and protein metabolism. Diabetes mellitus is also linked with elevated plasma cholesterol and triglyceride levels, which may promote the development of cardiovascular disease. *Psidium guajava* (PG) leaf is known to have a blood-glucose lowering effect in diabetic rats. The aim of this study was to carry out a phytochemical study of PG leaf extract; investigate its protective effect on pancreas and also its effect on muscle and liver glycogen synthase and phosphorylase activities in streptozotocin induced diabetic male Sprague-Dawley rats; Serum biomarkers of liver and muscle dysfunction such as alanine amino transferase (ALT), aspartate amino transferase (AST) and lactate dehydrogenase (LDH) were also analyzed. The effect of PG on markers of lipid metabolism and on hormone sensitive lipase (HSL) enzyme was also investigated. A single dose of 40 mg/kg body weight of streptozotocin was administered to fasted male Sprague-Dawley rats intraperitoneally for diabetes induction. The aqueous extract of PG leaves was used to treat both normal and diabetic animals (400 mg/kg body weight) for 2 weeks while control animals were treated with the vehicle. After 2 weeks of treatment, PG was shown to enhance lowering of blood glucose in diabetic rats following a glucose load and protected pancreatic tissue from diabetic damage. GC-MS analysis of the aqueous extract of PG indicated the presence of phenolic compounds and triterpenes. In acute study, PG activated Protein kinase B (PKB/Akt) in skeletal muscle of streptozotocin induced diabetic rats. In the sub-chronic study, the treatment of rats with PG extract restored glycogen synthase activity depressed by diabetes and decreased glycogen phosphorylase activity in skeletal muscle. These changes in enzyme activity mirrored those in enzyme expression. It also restored glycogen synthase activity depressed by diabetes which was accompanied by reduced glycogen phosphorylase activity and increased glycogen levels in liver. PG decreased HSL activity in adipose tissue and liver and this was accompanied by reduced levels of serum triglycerides, total cholesterol, LDL-cholesterol, cardiac risk factor, atherogenesis and increased HDL-cholesterol. We conclude that PG has significant antidiabetic and hypolipidemic effects, and that these effects may be associated with the presence of triterpenes and phenolic compounds. PG increased GS activity, glycogen storage and reduced GP activity. It also reduced HSL activity and improved serum lipid profile.
CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1.0 DIABETES MELLITUS

Diabetes mellitus (DM) is a metabolic disorder, characterized by inadequate or inefficient insulin secretory response and high blood glucose level (Prabhakar and Doble, 2008). It is also characterized by impaired uptake and storage of glucose as well as reduced glucose utilization for energy purposes (Jacobsen et al., 2009; Sangeetha et al., 2010). Glucose homeostasis helps to maintain blood glucose levels constant, and this is achieved by a balance in endogenous glucose production and utilization (Roden and Bernroider, 2003). Diabetes-induced hyperlipidaemia is one of the major factors responsible for cerebrovascular and cardiovascular disorders. Two important lipids such as cholesterol and triacylglycerols have been implicated in these disorders (Sriplang et al., 2007). Recent studies reported a link between diabetes and gall bladder disease (Liu et al., 2012; Chen et al., 2014).

1.1 TYPES OF DIABETES MELLITUS

1.1.1 Type I and Type II diabetes mellitus

Type I and Type II diabetes mellitus are the two main types. A distinctive feature of type I diabetes is an absolute lack of insulin production, leaving the patient relying on exogenous insulin for survival (Varshosaz, 2007). Approximately, 10% of patients with diabetes mellitus suffer from Type I diabetes (Gilespie, 2006). Type II diabetes (non-insulin-dependent diabetes mellitus), is characterized by relative lack of insulin and/or insulin resistance (Kumar et al., 2005b). Type II diabetes also accounts for over 90% of all cases of diabetes mellitus (World Health Organization, 2014). Type II diabetes can be properly managed by regulating the patient’s diet and also treating with synthetic antidiabetic drugs such as biguanides (e.g metformin), sulphonylureas (e.g glibenclamide), alpha glucosidase inhibitor (e.g acarbose), dipeptidyl peptidase 4 inhibitors (e.g vildagliptin) and thiazolidinediones (e.g rosiglitazone). Hyperglycaemia affects the insulin signalling pathway through various mechanisms such as activation of e-Jun-N terminal kinase and oxidative stress driven pathways (Bensellam et al., 2012). The generation of reactive oxygen species causes damage to cellular lipids, proteins and nucleic acids, resulting in the activation of the
unfolded protein response (UPR) and endoplasmic reticulum stress (Hotamisligil, 2003; Ozcan et al., 2004).

1.1.2 Gestational diabetes mellitus (GDM)

GDM can be defined as any degree of glucose intolerance which is first recognized during pregnancy (Metzger and Coustan 1998). GDM usually occurs in women who are at risk of type II diabetes (Cheung, 2009). During pregnancy, GDM is linked with several complications for the child. The inability of insulin to cross the placenta barrier exposes the foetus to the maternal hyperglycaemia, but the foetal pancreas is capable of responding appropriately to this hyperglycaemia (Scollan-Kolippoulos et al., 2006). Hyperinsulinaemia is observed in the foetus, which promotes growth and subsequent macrosomia (Perkins et al., 2007). Foetus born to mother suffering from GDM has higher risk of developing hyperbilirubinaemia, clavicle fracture, macrosomia, shoulder dystonia with its attendant risk of brachial injury and neonatal hypoglycaemia (Hapo Study Cooperative Research Group, 2008; Ramos et al., 2012; Alberico et al., 2014).

1.1.3 Maturity onset diabetes of young (MODY)

MODY is also known as monogenic diabetes which is caused by mutations in an autosomal dominant gene (sex independent) altering insulin production (Barry and Dirk, 2008). Eleven genetic forms of MODY have been identified. About 10% of patients with diabetes cases suffer from MODY (Johnson, 2007). People suffering from MODY do not possess traits that can be found in type 2 diabetic patients such as high blood pressure and obesity (Fajans and Bell, 2011). MODY can be treated with oral anti-diabetic medications (America Diabetes Association, 2012). Next generation sequencing (NGS) technology is used to identify patients with MODY gene mutations (Colclough et al., 2014).

1.1.4 Neonatal diabetes mellitus (NDM)

This condition occurs in the first 6 months of life because infants with NDM do not produce enough insulin, there is increase in blood glucose level and therefore exogenous insulin is required for management. (Shield, 2000). There are two types of NDM, Transient Neonatal Diabetes Mellitus (TNDM) and Permanent Neonatal Diabetes Mellitus (PNDM) (Neve et al., 2005). The etiology of NDM is genetically heterogeneous characterized by absence or abnormal development of pancreas or islets, and β-cell dysfunction that reduces insulin
secretion (Aguilar-Bryan and Bryan, 2008). A report by Garin et al., (2012) revealed that mutation (p.L30Q) in the insulin gene (INS) caused PNDM in a four-month old female who was showing symptoms such as hyperglycaemia with glucosuria, polyphagia, irritability and ketonuria without acidosis.

1.2 COMPLICATIONS ASSOCIATED WITH DIABETES MELLITUS

1.2.1 Diabetic nephropathy

The factors that promote the development of diabetic nephropathy are dyslipidaemia (Chaturvedi et al., 2001, Appel et al., 2003), high blood pressure (Nelson et al., 1995; Ravid et al., 1998; Adler et al., 2000), hyperglycaemia (Gall et al., 1997; The Microalbuminuria Collaborative Study Group, 1999), epithelial-to-mesenchymal transition (Tang et al., 2013), smoking (Sawicki et al., 1994; Hovind et al., 2003), reactive oxygen species (ROS) (Singh and Winocour, 2011), and increased formation of advanced glycation end products (AGEs) (Yamagishi and Imaizumi 2005). Glomerular hyperfiltration is one of the problems that have been proposed to promote the development of diabetic nephropathy. Type I and II diabetic patients experience this problem in the early years of the disease (Vora et al., 1992; Nelson et al., 1996; Levine, 2008). Various hypotheses have been proposed promoting the development of glomerular hyperfiltration. In a study using animals, it was observed that afferent glomerular arterioles dilate more than efferent arterioles, thereby increasing filtration fraction, intraglomerular pressure and glomerular filtration rate (Hostetter et al., 1981). Such changes have been suggested to promote diabetic glomerulosclerosis in STZ-induced diabetic Munich-Wistar rats (Zatz et al., 1985). AGE products also initiate the development and progression of diabetic nephropathy; they are formed by non-enzymatic reaction of reducing sugars with the amino groups of proteins to form reversible Schiff bases and then Amadori products (Yamagishi and Imaizumi, 2005). These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to form AGEs (Rahbar and Figarola, 2003). There is increased formation and accumulation of AGEs during diabetes. Various studies reported that serum and tissue AGEs levels were significantly increased in type I and type II diabetic patients when compared with non-diabetic control subjects (Galler et al., 2003). One of the several mechanisms by which accumulation of AGEs in the kidney may contribute to the alteration in renal architecture and loss of renal functions in rodents and patients is by activating downstream signalings and cross-linking (β-sheets or cross-β structure) properties of matrix proteins (Bouma et al., 2003; Bohlender et
In addition, AGE formation on different types of matrix proteins impairs their degradation by matrix metalloproteinases, contributing to mesangial expansion and basement membrane thickening which are the hallmarks of diabetic nephropathy (Mott et al., 1997). Yamagishi et al., (2002) reported that AGEs stimulate monocyte chemoattractant protein-1 (MCP-1) expression in mesangial cells. Monocyte infiltration in association with increased MCP-1 expression in mesangium has been observed in the early phase of diabetic nephropathy (Banba et al., 2000). Ye et al., (2009) reported that urinary MCP-1/creatinine ratios in type II diabetic patients with microalbuminuria were much higher when compared to those in normal controls, but with intensive insulin treatment, there was a significant reduction in urinary MCP-1/creatinine ratios. Therefore, diabetic nephropathy is initiated by AGE accumulation in glomerulus and subsequent secretion of MCP-1 in mesangial cells.

1.2.2 Diabetic neuropathy

Diabetic neuropathy affects motor neurons, pain fibres and the autonomic nervous system (Said, 2007). During hyperglycaemia, when there is a decline in insulin secretion, the polyol pathway becomes activated: glucose is converted to sorbitol in a process catalysed by aldose reductase, because of the inability of sorbitol to cross cell membrane, it accumulates in the nervous tissue, leading to a condition known as osmotic stress. This condition increases Schwann cell damage, water influx, intracellular fluid molarity (The amount of solute per unit volume of solution within the cell) and nerve fibre degeneration (Oates, 2002). Current studies suggest that osmotic stress in cells and tissues may contribute to various human diseases (Brocker et al., 2012). Another way in which hyperglycaemia contributes to the development of diabetic neuropathy is the production of advanced glycation endproducts (AGEs) (Sugimoto et al., 2008), via attachment of reactive carbohydrate groups to nucleic acids, proteins or lipids. Extracellular AGEs bind to the receptor for AGE (RAGE), which activates NADPH oxidases, leading to a condition known as oxidative stress (Vincent et al., 2007). Dyslipideamia is also known to contribute to the pathogenesis of diabetic neuropathy (Wiggin et al., 2009). Plasma lipoproteins, especially low-density lipoproteins (LDLs), are modified by glycation or oxidation (oxLDL). These modified LDLs can bind to extracellular receptors such as Toll-like receptor 4 (Nowicki et al., 2010), oxLDL receptor LOX 1 (Vincent et al., 2009) and RAGE (Vincent et al., 2007). These receptors trigger signalling cascades that activate NADPH oxidase and oxidative stress (Vincent et al., 2009). Impaired insulin signalling is also a factor underlying diabetic neuropathy. Insulin has been shown to
promote neuronal growth and survival (Toth et al., 2006). Reduction of this neurotrophic signalling as a result of insulin deficiency or insulin resistance contributes to the development of diabetic neuropathy (Kim and Feldman, 2012).

### 1.2.3 Diabetic cardiomyopathy

This is a disease which affects the myocardium in diabetic patients causing several structural disorders such as left ventricular hypertrophy (LVH). Hyperglycaemia leads to the production of excess reactive oxygen species (ROS) in the mitochondria (Singh, 2001), this affects transcription of a segment of DNA, which leads to contractile dysfunction (Rosen et al., 1998). Elevated ROS levels leads to decreased nitric oxide (NO) levels, which promotes the development of endothelial dysfunction and myocardial inflammation via poly ADP-ribose polymerase (PARP). Inhibition of PARP has been reported to reverse diabetic endothelial dysfunction (Soriano et al., 2001). In a state of chronic hyperglycaemia, there is increased oxidative stress, which may lead to DNA breakage and render the DNA unstable, thereby activating PARP as a reparative response to the damage done to DNA (Chiu et al., 2008). Activation of PARP depletes NAD\(^+\), reducing the rate of glycolysis and mitochondrial function and eventually leading to apoptosis. The activity of glyceraldehyde-3-phosphate dehydrogenase is inhibited by PARP, which causes hyperglycaemia-induced activation of hexosamine pathway and protein kinase C (PKC) (Zheng and Kern, 2009). Previous studies reported the beneficial effects of PARP inhibition as illustrated by PARP-1 knockout mice; the inhibition of this enzyme protected the animals against streptozotocin-induced diabetes and myocardial ischemia/reperfusion injury (Yang et al., 2000). Recent studies implicate xanthine oxidase and NADPH oxidase in the pathogenesis of diabetic cardiomyopathy (Rajesh et al., 2009; Li et al., 2010; Ma et al., 2013).

### 1.3 GLUCOSE HOMEOSTASIS

#### 1.3.1 Insulin

Insulin is produced by beta cells in the pancreas (Aronoff et al., 2004; Wright et al., 2014). Insulin regulates blood glucose levels, by increasing glucose uptake, into adipose tissue, liver and muscle. It also inhibits glucagon secretion (Gerich, 1993). Insulin is first synthesized as preproinsulin in β-cells after which it is translocated to the rough endoplasmic reticulum to form proinsulin (Steiner and Oyer, 1967; Kahn et al., 2005). After about 5-10 min in the endoplasmic reticulum, proinsulin is transported to the trans-Golgi network (TGN) where it
develops into active insulin through the action of enzymes such as prohormone convertases and carboxypeptidase E (Steiner and Oyer, 1967). The insulin is packaged inside mature granules waiting for vagal nerve stimulation and metabolic signals such as mannose, arginine, leucine and glucose before it is released into circulation (Najjar, 2001).

Figure 1.1: Amino acid sequence of insulin (Adapted from Berg et al., 2002)

Fig 1.2: Insulin action in tissues. Adapted from Ganong, 2005). Review of Medical Physiology, 22nd edition
1.3.2 Glucagon

Glucagon is produced by the alpha cells of the pancreatic islets of Langerhans and is secreted during periods of hypoglycaemia (Cryer, 2002) resulting in stimulation of hepatic glucose production mainly through processes such as gluconeogenesis and glycogenolysis, thereby increasing blood glucose levels (Szablewski, 2011). Glucagon is also activated by epinephrine (through α1, α2 and β2 adrenergic receptors) (Skoglund et al., 1987; Layden et al., 2010) and acetylcholine (Honey and Weir, 1980). It is inhibited by Peroxisome proliferator-activated receptor gamma (PPARγ)/retinoid X receptor heterodimer (Kratzner et al., 2008) and insulin (Xu et al., 2006).

1.3.3 Incretin

The incretins are peptide hormones secreted from the gut and elicit an array of biological effects such as downregulating the activity of glucagon and stimulating a decrease in blood glucose levels by increasing the amount of insulin released from pancreatic beta cells (Baggio and Drucker, 2007). Various incretin hormones have been characterized, but G-I-P (Glucose dependent insulino-tropic polypeptide) and G-L-P 1 (Glucagon like peptide-1) are the two main types (Drucker, 2007). GIP and GLP-1 are both inactivated by dipeptidyl peptidase-4 (DPP-4) (Drucker and Nauck, 2006; Russell-Jones and Gough, 2012).

1.3.4 Glycogen metabolism in tissues

1.3.4.1 Glycogen metabolism in muscle

There is also an inverse relationship between muscle glycogen and both basal and insulin-stimulated glucose uptake (Jensen et al., 1997). Increased glycogen synthase activity has a stimulating influence on glycogen synthesis in the muscle. Skeletal muscle glycogen synthase activity is lowered in diabetic subjects studied under euglycemic conditions (Kelley and Mandarino, 1990). During low muscle glycogen conditions, there is increase in AMP-activated protein kinase activity and translocation of glucose transporter type 4 (GLUT-4), these mechanisms increase glucose uptake (Kawanaka et al., 2000; Wojtaszewski et al., 2003; Friedrichsen et al., 2013).
1.3.4.2 Glycogen metabolism in liver

Glycogen is mainly stored as glucose in the liver. The mechanism by which glycogen is synthesized and degraded is the same in all tissues (Smythe and Cohen, 1991; Alonso et al., 1995; Bollen and Stalmans, 1995). Furthermore, livers from diabetic animals show a decreased capacity for glycogen synthesis, (Friedman et al., 1967; Hornbrook, 1970; Postle and Bloxham 1980; Van de Werve et al., 1984). Luo and colleagues (2011) reported that during fasting, the activity of protein phosphatase 1 regulatory subunit 3G (PPP1R3G) in the liver is upregulated and downregulated after feeding.

1.4 PATHWAYS INVOLVED IN THE PATHOGENESIS OF DIABETES MELLITUS

1.4.1 Insulin signalling pathway

Insulin binds to insulin receptor (IR) which triggers the activation of intrinsic tyrosine kinase activity of IR. (Insulin receptor substrate-1 (IRS-1) phosphorylated by IR activates phosphoinositol-3-kinase (PI3K), which converts phosphatidyl inositol-4,5-bisphosphate
(PIP$_2$) to phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$) and subsequently activates protein kinase B (PKB/Akt). PKB phosphorylates GSK-3 on a Ser 9 residue, inactivating it. GSK-3, inactivated by phosphorylation, cannot phosphorylate glycogen synthase (GS), so GS remains active. This action of PKB, promotes increase in the rate of glycogen synthesis from glucose (Nelson and Cox, 2005).

1.4.1.1 PI3-K/AKT signalling cascade

1.4.1.1.1 Insulin receptor (IR)

The insulin receptor plays vital regulatory roles in development, cell division, and metabolism (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992; Fantl et al., 1993). The insulin receptor plays an important role in glucose homeostasis; under degenerate conditions, a range of diseases such as diabetes and cancer may occur (Malaguarnera and Belfiore, 2012). The enzymes involved in the control of glucose metabolism by insulin appear to be regulated by phosphorylation and dephosphorylation on serine and/or threonine residues (Hubbard and Cohen, 1993; Lawrence, 1992). The insulin receptor has two subunits; the α-subunit consists of 723 amino acids with a molecular mass of 130 kDa (Lawrence et al., 2007; De Meyts, 2008). It shares this feature with the insulin-like growth factor 1 (IGF-1) receptor and members of the epidermal growth factor (EGF) receptor family (Schlessinger and Ullrich, 1992). The β subunit contains 620 amino acids and has a molecular mass of 95 kDa (Lawrence et al., 2007; De Meyts, 2008). Each α subunit is joined to one β subunit by so called class II disulphide bonds (Massague and Czech 1982). The insulin receptor is derived from a single gene and two isoforms are produced by alternative splicing of exon 11 (Seino and Bell, 1989). The insulin receptor with exon 11 (HIR-B or Exll+) has 12 more amino acids at the carboxy-terminus of the α-subunit after Arg-723, compared with the other isoform (HIR-A or Exll-). The tissue distribution established by Northern blotting suggests that Exll- is the predominant species in liver and is very rare in leukocytes and muscle. Placenta and adipocytes express both isoforms of the insulin receptor at nearly equal levels (Benecke et al., 1992).

1.4.1.1.2 Insulin receptor substrates (IRSs)

The IRS family is made up of six family members, IRS-1, 2, 3, 4, 5 and 6 (Chakraborty et al., 2011), but the two main types present in most cells are IRS-1 and IRS-2 (Sun et al., 1991; Sun et al., 1995). It has been reported that mice lacking IRS-1 or IRS-2 proteins develop
insulin resistance (Waterfield & Greenfield 1991, Araki et al., 1994, Withers et al., 1998). In animal models and patients with type 2 diabetes and insulin resistance, insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 is impaired in adipose tissue, liver and skeletal muscle (Bjornholm et al., 1997; Kerouz et al., 1997; Cusi et al., 2000). Dearth et al., (2006) reported that the overexpression of IRS-1 in transgenic mice leads to the development of breast cancer. IRS-1 is essential for cell cycle progression and cell maturation (Fasshauer et al., 2001; Machado-Neto et al., 2011), while IRS-2 plays an important role in neural homeostasis (Martin et al., 2012; Murillo-Cuesta et al., 2012).

1.4.1.1.3 Phosphoinositide 3-kinase (PI3-K)

PI3Ks are divided into three classes (I–III). The class I family consists of four distinct protein species with a molecular weight of 110 kDa (p110α, p110β, p110δ and p110γ). Most structural features and substrate specificity of class I enzymes are similar (Rameh and Cantley, 1999; Fry, 2001; Katso et al., 2001). Based on their mechanisms of activation, the class I family members are further subdivided into two groups, Class 1A and class 1B. Active PI3-K triggers PKB activation (Reif et al., 1997, Astoul et al., 1999) which can accelerate the rate of glycogen formation. Previous studies reported that peripheral insulin resistance occurs at the proximal end of insulin signalling, where the activation of PI3-K is attenuated (Saltiel and Kahn 2001; Shulman, 2004). The EGF receptor that functions upstream of PI3-K, has been linked with a number of cancers, when it is overexpressed as a result of mutation (Walker et al., 2009; Bleeker et al., 2012; Bleeker et al., 2014).

1.4.1.1.4 Phosphoinositide-dependent protein kinase-1 (PDK1)

The 556-residue enzyme isolated from human tissues (hPDK1) contains a catalytic domain (residues 84–341) and a COOH-terminal pleckstrin homology or pleckstrin homology (PH) domain (residues 450–550). hPDK1 phosphorylates Akt at Thr308 and activates the enzyme in a PI3-kinase-dependent manner. In addition to Akt, hPDK1 phosphorylates and activates another PI3-kinase downstream target, p70S6K (Pullen et al., 1998). PDK1 activates and phosphorylates protein kinase A, B, C, G and p70 S6 kinase (Cheng et al., 1998; Chou et al., 1998; Le Good et al., 1998; Dutil et al., 1998; Dong et al., 1999; Kobayashi and Cohen, 1999; Park et al., 1999). Lack of PDK-1 in mice causes mild glucose intolerance, approximately 40 % decrease in body mass (Frodin et al., 2002; Mora et al., 2004) and it is also known to promote the development and growth of melanomas (Scortegagna et al., 2014).
The overexpression of phosphorylated PDK-1 has been implicated in human breast cancer (Lin et al., 2005; Maurer et al., 2009).

### 1.4.1.1.5 Protein kinase B (PKB)

Protein kinase B (also termed Akt) is involved in mediating the metabolic actions of insulin (Vanhaesebroeck and Alessi, 2000). It has three functional domains: an N-terminal PH domain, which comprises the lipid-binding region that facilitates the translocation of the protein to the membrane. The C-terminal hydrophobic motif, contains both the phosphorylation site Ser473 and a docking site for PDK-1, which phosphorylates PKB on Thr308 (Scheid and Woodgett, 2003). Akt1 and Akt2 play significant roles in insulin signalling in skeletal muscle and adipose tissue, whereas Akt3 is additionally activated by insulin in liver (Cho et al., 2001a; Cho et al., 2001b). Akt indirectly activates glycolytic enzymes through HIF transcription factors and phosphorylation of phosphofructokinase-2 (PFK 2) which activates phosphofructokinase-1 (PFK 1) (Simons et al., 2012).

### 1.4.1.1.6 Glycogen synthase kinase 3 (GSK-3)

This enzyme plays a key role in the regulation of glycogen metabolism. Glycogen synthase kinase 3 (GSK-3) is also necessary for proper axis formation during embryonic development (Frame and Cohen, 2001). Two forms of GSK-3 are found in mammals, GSK3α and GSK3β. GSK3β is found in the central nervous system (Leroy and Brion, 1999). GSK-3 is constitutively active in cells, and its activity can be suppressed by several extracellular stimuli, such as insulin, fibroblast growth factor (FGF), epidermal growth factor (EGF) and Wnt ligands (Frame and Cohen, 2001; Grimes and Jope, 2001; Woodgett, 2001). GSK-3 is inhibited by phosphorylation on a serine residue (Ser9 in GSK-3β and Ser21 in GSK-3α) located in the N-terminal domain. In certain cell types, such as adipocytes, insulin activates glycogen synthase, whereas EGF does not (Robinson et al., 1993). One of the main characteristics of diabetic muscle is the inhibition of glycogen synthase (GS) and the loss of glycogen synthesis (Shulman, et al., 1995). The role of GSK-3 in diabetes was further demonstrated in two model systems: in fat tissue of obese diabetic mice, where GSK-3 activity was found to be twofold higher than in control mice (Eldar-Finkelman, 1999); and in skeletal muscle of type II diabetics, where GSK-3 activity and expression levels were significantly higher than in healthy individuals (Nikoulina et al., 2000). GSK-3 inhibitors induce apoptosis in pancreatic and glioma cancer cells (Marchand et al., 2012). It is also used
in the treatment of type II diabetes (Rayasam et al., 2009). Studies have shown that competitive inhibitors of GSK-3 improve glucose tolerance in diabetic mice (Jope et al., 2007).

1.4.1.1.7 Glycogen synthase (GS)

Glycogen synthase is a rate-limiting enzyme and is key to the regulation of the rate of glycogen synthesis (Villar-Palasi and Larner, 1961; Leloir and Cardini 1962). GS requires glycogenin to initiate the synthesis of glycogen (Palm et al., 2013). The highest concentration of GS is found in the bloodstream 30 – 60 minutes following vigorous exercise (Jentjens and Jeukendrup, 2003). GS occurs in two forms: synthase I and synthase D (Rosell-Perez et al., 1962; Traut and Lipmann, 1963; Rosell-Perez and Larner 1964 b) and is activated mainly through allosteric regulation instead of covalent regulation (Bouskila et al., 2008; Bouskila et al., 2010). In humans with type II diabetes, hyperglycaemia has been shown to reduce both GS activity (Thorburn et al., 1990) and glycogen synthesis (Shulman et al., 1990; Rothman et al., 1992). Insulin activates GS by activating protein phosphatase-1 (PP-1) and inhibiting glycogen synthase kinases (Saltiel, 2001).

1.4.1.1.8 Glycogen phosphorylase (GP)

Glycogen phosphorylase is an enzyme that occurs in two forms, phosphorylase GP\textsubscript{a} and GP\textsubscript{b} (Kristiansen et al., 2004; Johnson, 2009). The isozymes of GP are found in brain, liver and muscle of mammals. The brain type can be found in embryonic tissues and adult brain, whereas, the muscle and liver types are located in skeletal muscle and adult liver respectively (David and Crerar, 1986). GP is present in resting skeletal muscle almost entirely in the \textit{b} form (Krebs and Fischer 1955). The inhibition of GP activity has been proposed as one method for treating type II diabetes (Somsak et al., 2003; Henke, 2012). Since glucose production in the liver has been shown to increase in type II diabetic patients (Moller, 2001), downregulating GP activity will stop the release of glucose from the liver’s glycogen supplies. Insulin terminates the glycogenolytic process in order to prevent further breakdown of glycogen. Docsa and colleagues (2011) reported that glucopyranosylidene-spirothiohydantoin (TH) was effective in lowering glycogen phosphorylase \textit{a} levels and also in activating glycogen synthase after intravenous administration of TH to Zucker diabetic fatty rats.
1.4.2 Glycogenolysis

This process involves the removal of glucose monomers to produce glucose-1-phosphate and glycogen with one less glucose molecule this reaction being catalyzed by the phosphorylated form of glycogen phosphorylase (Krebs, 1997). Adrenaline and glucagon are known to stimulate glycogenolysis by binding to a G-protein coupled receptor (Ring et al., 2013, Siu et al., 2013) which activates adenyly cyclase (Williamson et al., 1981, Krebs, 1997). Adenyly cyclase in turn converts ATP to cyclic AMP which activates protein kinase A (PKA), PKA then activates glycogen phosphorylase (Newgard et al., 1989). Lodish and colleagues (2007) also reported that glycogen degradation in myocytes may be stimulated by neural signals.

1.4.3 Gluconeogenesis

Gluconeogenesis describes the production of glucose from non-carbohydrate sources such as glucogenic amino acids, glycerol, lactate, glutamine and alanine. It takes place in the liver, intestine and kidney (Mithieux et al., 2004, Widmaier, 2006). When blood sugar levels are low, glucagon activates the gluconeogenic process and inhibits glycolysis (Mutel et al., 2011).

Fig 1.4: Insulin signalling pathway (Source: Nelson and Cox, 2005)
1.5 LIPID HOMEOSTASIS

1.5.1 Lipoproteins

There are four types of lipoproteins

1.5.1.1 Chylomicrons (CMs)

Chylomicrons (CMs) are composed of triglyceride (85-92 %), phospholipids (6-12 %), cholesterol (1-3 %) and proteins (1-2 %) (Mahmood, 2000). They are produced in the small intestines and promote the delivery of dietary fats to muscles or fat cells. Chylomicrons also enhance the delivery of dietary cholesterol from the intestines to the liver (Chung and Wasan, 2004). They have been implicated in the progression of atherosclerosis (Gower et al., 2011).

1.5.1.2 Very low density lipoproteins (VLDLs)

The liver is responsible for the production of very low density lipoproteins (VLDLs) (Gibbons et al., 2004). It facilitates the transportation of triglycerides, cholesterol and phospholipids from the liver to fat cells. VLDL transports about 10-15 % of the total cholesterol present in the blood (Antonopoulos, 2002). The main structural and regulatory proteins that can be found in VLDLs are apolipoprotein B100, apolipoprotein E and microsomal triglyceride transfer protein (Bobe et al., 2004). Accumulation of lipid in the liver occurs when triglyceride synthesis exceeds the rate of triglyceride clearance either through hydrolysis or secretion of VLDLs (van Dorland et al., 2011).

1.5.1.3 Low density lipoproteins (LDLs)

The main particle responsible for the movement of cholesterol from the liver to other cells of the body is low density lipoproteins (LDLs). LDLs carry about 60-70 % of serum cholesterol (Antonopoulos, 2002). They are strongly linked with atherosclerosis but ingestion of antioxidants may reduce LDL’s contribution to atherosclerosis (Esterbauer et al., 1991; Stocker and Keaney 2004). Green tea has been reported to protect human LDL against oxidative damage (Ohmori et al., 2005), as a result of radical scavenging ability of catechins (Zhu et al., 1999). Oral ingestion of catechins by healthy Japanese men protected them against the deleterious effect of LDL oxidation and improved their total antioxidant capacity (Suzuki-Sugihara et al., 2016).
1.5.1.4 High density lipoproteins (HDLs)

HDL particles are small in size, about 5-17 nm (Assmann and Gotto, 2004). HDL subclasses differ in their composition of lipids, apolipoprotein (apo A-I and apo A-II) and lipids transfer proteins such as cholesteryl ester transfer protein (CETP), lecithin cholesterol acetyl transferase (LCAT), PAF-AH, PON1, and phospholipid transfer protein (Link et al., 2007). The removal of excess cholesterol from the arterial wall is done by HDL. The anti-inflammatory effect of HDL has been reported in vivo. Patel et al., (2010) reported that infusion of apo-A1 inhibits acute vascular inflammation in normo-cholesteromic New Zealand white rabbits.

1.5.2 Lipoprotein lipase (LPL)

Lipoprotein lipase is a hydrophilic enzyme that plays an important role in lipid metabolism. LPL includes hepatic lipase, pancreatic lipase and endothelial lipase. LPL is synthesized mainly in muscle and adipose tissues (Goldberg, 1996; Fielding and Frayn, 1998). Insulin regulates the synthesis and activity of LPL in adipose tissue. In the fed state, the activity of LPL is upregulated in adipose tissue, while it is downregulated in muscle (Goldberg, 1996; Fielding and Frayn, 1998). During fasting, insulin levels are low; this leads to HSL activation and adipose tissue lipolysis stimulated by catecholamine begins. Fasting also increases LPL activity in muscle, while in adipose tissue, LPL activity is downregulated. LPL deficiency causes hypertriglyceridemia (Okubo et al., 2007), while overexpression of LPL in mice has been reported to promote obesity (Delezie et al., 2012) and affect insulin response (Ferreira et al., 2001; Kim et al., 2001).

1.5.3 Hormone sensitive lipase (HSL)

Hormone-sensitive lipase (HSL) is an intracellular lipase capable of hydrolyzing stored fat in the form of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG) and cholesteryl esters, to release free fatty acids (FFA) (Kraemer and Shen, 2002). HSL can be sub-divided into four regions, amino terminal domain, lipid binding domain, carboxyl terminal domain and the regulatory domain of the enzyme, which contains the phosphorylation sites of HSL (Antonis et al., 2011). Furthermore, previous studies reported that HSL may play a role in the polygenic background of obesity and type II diabetes (Klannemark et al., 1998; Magre et al., 1998). The activity of HSL is enhanced by adrenocorticotropic hormone, (ACTH) and catecholamines, whereas insulin suppresses its
activity (Holm et al., 2000). Evidence also suggests that hydrogen peroxide (H$_2$O$_2$) generated by insulin inhibited lipolysis (Zentella de Pina et al., 2008).

1.5.4 Insulin

Insulin activates lipogenic enzymes like acetyl CoA carboxylase and fatty acid synthase and also enhances the activity of lipoprotein lipase (Kersten, 2001). It also inhibits the activity of hormone sensitive lipase in adipose tissue (Anthonsen et al., 1998). During lipodystrophy, plasma fatty acid levels are high; this leads to accumulation of triglycerides in islet cells, muscle, plasma and liver, resulting in hypertriglyceridemia and insulin resistance (Garg and Misra, 2004).

1.6 Cholesterol

Cholesterol is the main component of cellular membranes and serves two functions. Firstly, it modulates the fluidity of membranes, enabling them to maintain their function over a wide range of temperatures. Secondly, it acts as a cellular insulator by preventing leakage of ions (Haines, 2001). About 20-25 % of the daily cholesterol synthesis occurs in the liver, other sites of synthesis include the reproductive organs, intestines and adrenal glands. The synthesis of cholesterol is regulated by SREBP (sterol regulatory element-binding protein) (Espenshade and Hughes, 2007). Cholesterol is susceptible to oxidation leading to the formation of oxysterols that participate in various aspects of lipid metabolism such as regulation of gene transcription and biosynthesis of bile acid (Russell, 2000). Hypercholesterolemia is associated with an increased risk of developing atherosclerosis and coronary heart disease (Lewis, 2011).

1.7 Triglycerides

Triglycerides are the major components of human skin oils, vegetable oil and animal fats (Lampe et al., 1983; Nelson and Cox, 2000). Triglycerides are the main constituents of chylomicrons and very low density lipoprotein (VLDL) and play a vital role in metabolism as energy sources and also facilitate the movement of dietary fats. They contain more calories per gram than carbohydrates (Drummond et al., 2014). Yki-Jarvinen (2010) reported that excessive accumulation of triglycerides in the liver is involved in the pathogenesis of insulin resistance.
1.8 TREATMENT OF DIABETES MELLITUS

1.8.1 Synthetic hypoglycaemic agents

Management of diabetes mellitus involves the use of insulin and synthetic hypoglycaemic agents such as biguanides (metformin), sulphonylureas (glibenclamide), alpha glucosidase inhibitor (acarbose), dipeptidyl peptidase 4 inhibitors (DPP-4) (vildagliptin) and thiazolidinediones (TZDs) (rosiglitazone). Several side effects are associated with these synthetic anti-diabetic drugs (Moller, 2001). Compared with synthetic compounds, natural products have significant antidiabetic potential with little side effects (Verspohl, 2002; Neelesh et al., 2010).

1.8.1.1 Insulin

Insulin is produced by the beta cells of the pancreas. It facilitates the uptake of glucose from the blood into adipose tissue and skeletal muscle. It also suppresses the production of glucose by the liver (Sonksen and Sonksen, 2000). Type I diabetic patients depend solely on insulin for survival (Varshosaz, 2007). Side effects associated with insulin include blurry vision, water retention, hypoglycaemia, sweating, hunger and weakness. Examples of biosynthetic insulin include rapid acting insulin analogues (aspart and lyspro) and long acting insulin analogues (detemir and glargine) (Hartman, 2008; Valla, 2010).
1.8.1.2 Sulphonylureas

Sulphonylureas are antidiabetic drugs used in the management of type II diabetes mellitus and trigger the release of insulin from the beta cells of the islets of Langerhans (Amod et al., 2012, South African Medicines Formulary, 2012). Evidence suggests that when sulphonylureas are used with another anti-diabetic drug, diabetic patients experience a better blood sugar lowering effect (Hanefeld, 2007). Some side effects associated with sulphonylureas include weight gain and hypoglycaemia (Fowler, 2010, Amod et al., 2012, South African Medicines Formulary, 2012). Examples of sulphonylureas include gliclazide, glipizide, glimepiride, glibenclamide and chlorpropamide.

1.8.1.3 Biguanides

Biguanides (e.g metformin) are oral hypoglycaemic drugs used for treating type II diabetes mellitus (Rang et al., 2003). They enhance insulin sensitivity, resulting in decreased plasma glucose levels, decreased gluconeogenesis and increased glucose uptake. Previous studies reported that biguanides reduce triglyceride and LDL cholesterol levels (El Messaoudi et al., 2011; Joint Formulary Commitee, 2013). One of the proposed mechanisms of action of
metformin is that it activates AMP-activated protein kinase (AMPK), which in turn increases small heterodimer partner (SHP) expression, thereby inhibiting the expression of gluconeogenic genes in the liver such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (Kim et al., 2008). This series of enzyme activities suppresses the production of glucose by the liver (Kirpichnikov et al., 2002). Side effects include diarrhoea, nausea and vomiting (Sumari, 2012).

1.8.1.4 Thiazolidinediones

Thiazolidinediones are synthetic oral hypoglycaemic agents used for the treatment of type II diabetes mellitus. Examples of thiazolidinediones include rosiglitazone, pioglitazone, lobeglitazone and troglitazone. The mechanism of action of thiazolidinediones entails the activation of peroxisome proliferator activated receptors (PPARs), specifically PPAR gamma. PPAR alpha is found in the liver (Lee et al., 2003), PPAR delta is found in the spleen, colon, heart, small intestine, brain, skeletal muscle, keratinocytes, thymus and lung (Girroir et al., 2008) while PPAR gamma is located in the heart, colon, liver, macrophages, adipocytes and skeletal muscle. Thiazolidinedione is a ligand which activates PPARγ. PPARγ complexes with retinoid X receptor (RXR) to form a heterodimer, which binds to DNA and then upregulates the transcriptional and translational activities of a number of proteins involved in lipid and glucose metabolism as well as cellular differentiation. Thiazolidinediones act by increasing insulin sensitivity, thereby enhancing glucose uptake into liver, muscle and adipose tissue, with concomitant decrease in glucose production (Nolan et al., 1994; Iwamoto et al., 1996; Yki-Jarvinen, 2004). Increased risk of fractures and oedema is associated with the use of thiazolidinediones, especially in women (Sumari, 2012).

1.8.1.5 Alpha glucosidase inhibitors

Alpha glucosidases are membrane bound intestinal enzymes that hydrolyze disaccharides, trisaccharides and oligosaccharides into glucose and some other monosaccharides for absorption by the body. They are hypoglycaemic drugs used for treating type II diabetes mellitus, and act by preventing carbohydrate absorption such as sugar from the gut (Campbell et al., 1996). Side effects include diarrhoea and flatulence (Sumari, 2012). Examples of alpha glucosidase inhibitors are voglibose, acarbose and miglitol.
1.8.1.6 Dipeptidyl peptidase 4 (DPP-4) inhibitors

These inhibitors are oral hypoglycaemic agents used for treating type II diabetes mellitus. Examples include sitagliptin, alogliptin, saxagliptin, anagliptin, vildagliptin and gemigliptin. They inhibit dipeptidyl peptidase 4. The mechanism of action of DPP-4 inhibitors is to enhance levels of incretin (GLP-1 and GIP) (Dupre et al., 1995; Behme et al., 2003; McIntosh et al., 2005), which in turn inhibit the release of glucagon, this leads to increased insulin secretion and decreased blood sugar levels. Side effects include nasopharyngitis, upper respiratory tract infection and headache (John, 2008). Oxidative stress leads to the production of reactive oxygen species such as superoxide radical and hydroxyl radical. Various DPP-4 inhibitors have been reported to reduce oxidative stress (Kroller-Schon et al., 2012; Maeda et al., 2012). Recently, a novel DPP-4 inhibitor (teneligliptin) was reported to possess hydroxyl radical (.OH) scavenging activity (Shinichiro et al., 2016).

1.9 REVIEW ON MEDICINAL PLANTS WITH ANTIDIABETIC POTENTIAL

1.9.1 Introduction

The ethnobotanical survey route has been identified as a major route in the search for plants that can provide new drugs for the treatment of diabetes. Several plants have been shown to have antidiabetic properties and some are discussed below.

1.9.2 Achyranthes aspera

This plant can be found growing in Australia, Asia, America, Baluchistan and Ceylon. It is a medicinal herb and can grow up to 2 m, the plant is crushed and boiled, and then taken for the treatment of pneumonia. It can also be used for treating night blindness and cutaneous diseases (Nadkarni, 2009). Previous studies reported the hypoglycaemic activity of aqueous and methanolic extracts of the whole plant (Akhtar and Iqbal, 1991). Treatment of normal and alloxan induced diabetic animals with this plant reduced blood glucose levels (Akhtar and Iqbal, 1991). A dose of 100 mg/kg of this plant reduced serum triglycerides, cholesterol, total lipids and phospholipids in hyperlipidemic rats (Khanna et al., 1992).

1.9.3 Artemisia afra

Artemisia afra (AN) grows in Ethiopia, Kenya, Tanzania, Uganda, Zimbabwe, Namibia, Lesotho, Swaziland and South Africa (Hilliard, 1977; van Wyk et al., 1997). It is also called
Umhlonyane. Leaves are dark green, producing butter-coloured yellow flowers (Hilliard, 1977). The leaves and roots are boiled and then taken for the treatment of diabetes. This plant has also demonstrated antioxidant, antimalarial and antimicrobial effects (Burits et al., 2001; Gathirwa et al., 2007; Vagionas et al., 2007).

1.9.4 Brachylaena discolor

Brachylaena discolor (BD) is also known as Umphahla. Leaves from this plant have been reported to possess antihyperglycaemic activity (Erasto et al., 2005). It is an evergreen tree and can grow up to 10 metres (van Wyk and van Wyk, 2007). It can be found growing in South Africa and Mozambique. Mellem et al. (2013) reported that the methanolic extract of this plant reduced blood sugar levels in STZ-induced diabetic rats.

1.9.5 Catharanthus roseus

Catharanthus roseus (CR) is also known as Isisushlungu. This plant originated from Madagascar, but can also be found growing in South Africa (van Wyk et al., 1997). It grows as high as 1m (van Wyk et al., 2002). Studies carried out on the ethanolic extracts of the leaves and flowers of C. roseus showed that the plant extracts possess anti-hyperglycaemic activity (Ghosh and Gupta, 1980; Chattopadhyay et al., 1991a; 1991b). It has also been reported to possess antibacterial activity (Chinnavenkataraman and Rajendran, 2012).

1.9.6 Helichrysum nudifolium

Helichrysum nudifolium (HN) is a highly nutritious plant. It is also known as Ichocholo, and its boiled leaves and roots have been reported to elicit an anti-hyperglycaemic effect (Erasto et al., 2005). It is found in tropical Africa and Lesotho.

1.9.7 Helichrysum odoratissimum

Helichrysum odoratissimum (HO) is also known as Imphepho in Zulu and is considered native to Southern Africa. It can also be found growing in Angola, Uganda, Botswana, Congo, Ethiopia and Sudan. The main stalk grows as high as 1 m with lots of side branches. The flowers produce a repulsive and pungent fragrance (Metafro, 2009). HO is used for the treatment of ailments like coughs and colds. The flower of HO is made into a paste for the treatment of acne and pimples. The plant is burnt and used to repel mosquitoes and other
biting insects (Hyde and Wursten 2007). The antidiabetic potential of this plant has also been reported (Erasto et al., 2005; Njagi et al., 2015).

![Picture of Helichrysum odoratissimum](source: www.plantzafrica.com)

**Figure 1.6: Picture of Helichrysum odoratissimum** (source: www.plantzafrica.com)

### 1.9.8 Helichrysum petiolare

*Helichrysum petiolare* (HP) is a plant which can be found in South Africa, Portugal and the United States of America. Leaves are hairy and dark green in colour (Hilliard, 1983). It is also called Imphepho in Zulu. The whole plant is crushed and boiled and taken as infusion. Its blood glucose lowering effect has been reported (Erasto et al., 2005).

### 1.9.9 Hypoxidaceae hypoxis hemerocallidea

*Hypoxidaceae hypoxis hemerocallidea* (HHH) is also called Inongwe. Pulverized fresh corms are soaked in hot water and taken to treat diabetes (Erasto et al., 2005). It is a medicinal plant which can be found growing in Lesotho, Mozambique, Zimbabwe, Limpopo, Guateng, Eastern Cape and KwaZulu-Natal provinces (van Wyk et al., 1997). This medicinal plant is edible and is taken for the treatment of various ailments such as asthma, tuberculosis, cancer, arthritis and hypertension (Watt and Breyer-Brandwijk, 1962; Hutchings, 1989; Pujol, 1990;
Albrecht, 1995; Hutchings 1996; van Wyk et al., 2002). Mahomed and Ojewole (2003) reported that the aqueous extract of this plant caused reductions in blood sugar levels in fasted normal and STZ-treated diabetic rats. The leaf and corm of this plant demonstrate an anti-inflammatory effect through the inhibition of COX-1 and COX-2 enzymes (Ncube et al., 2012).

1.9.10 Moringa oleifera

*Moringa oleifera* (MO) grows in Paraguay, Brazil, North-Eastern Pakistan, West Asia, the Arabian Peninsula, North-Eastern Bangladesh, Sri Lanka, East and West Africa and the West Indies, Central and South America from Mexico to Peru and Southern Florida, (Roloff et al., 2009). This plant is also called drumstick tree. MO is a deciduous, fast-growing tree which is 9 m high. It is highly nutritious and has been used for human medication. Leaves are about 30-45 cm long (Roloff et al., 2009). MO plant is highly nutritious and the health benefits derived from the consumption of MO are numerous. MO leaves, roots, flowers, seeds and gums are used for treating various ailments such as inflammation, liver and cardiovascular diseases (Faizi et al., 1994; Limaye et al., 1995; Rao and Misra, 1998). It has been reported that the methanolic extract of MO dried fruit powder can trigger insulin release from the rodent pancreatic beta cells (Francis et al., 2004). A recent report showed that the aqueous extract of MO leaves possesses antidiabetic and antioxidant effects (Hanan and Adel, 2014).
1.9.11 *Trigonella foenum graecum*

*Trigonella foenum graecum* (TFG) can be found growing in Afghanistan, France, Iran, Argentina, Nepal, Pakistan, Morocco, Bangladesh, Spain, India and Turkey. It is known to have a sweet smell and the seed is known to possess hypoglycaemic and hypocholesterolemic effects in type I and type II diabetics and diabetic animals (Khosla *et al.*, 1995; Puri *et al.*, 1995; Puri *et al.*, 2002; Kumar *et al.*, 2005a; Hamza *et al.*, 2012). It is also used for treating allergic and inflammatory diseases (Min-Jung *et al.*, 2012).

1.9.12 *Vernonia amygdalina*

*Vernonia amygdalina* (VA), is a plant known for its bitter taste, can be found growing in most countries of tropical Africa, and it can also be found growing in Limpopo, Mpumalanga, Eastern Cape and KwaZulu-Natal provinces of South Africa (Bonsi *et al.*, 1995; Awe *et al.*, 1999). This plant has been reported to elicit antioxidant and hepatoprotective effects (Nwanjo, 2005; Adaramoye *et al.*, 2008; Owolabi *et al.*, 2008; Adesanoye and Farombi, 2010). Previous studies reported the hypoglycaemic potential of aqueous and ethanolic
extract of the leaves of this plant (Erasto et al., 2005; Ekpo et al., 2007; Khang et al., 2011). The aqueous, ethanolic and methanolic extracts of the leaves of this plant have been reported to modulate serum lipid levels (Nwanjo, 2005; Ekpo et al., 2007 Adaramoye et al., 2008).

1.9.13 Vernonia oligocephala

Vernonia oligocephala (VO) is also called Umhlunguhlungu. It is widely distributed in Zimbabwe, Zambia and South Africa (South African National Biodiversity Institute, 2009). Twigs, leaves and roots of this plant are used for the treatment of ailments like diabetes, fever, abdominal pain, dysentery, constipation and rheumatism (Watt and Breyer Brandwijk, 1962; Pujol, 1990; Hutchings, 1996; Erasto et al., 2005; South African National Biodiversity Institute, 2009). The in vitro anti-plasmodial activity of this plant has been reported (Clarkson et al., 2004).

1.9.14 Psidium guajava

Psidium guajava (PG) originated from Mexico (Rios et al., 1977). It is also found growing in South America, Europe, Africa and Asia (Stone, 1970). PG is a small tree that grows up to 10 m high. The beneficial effects of PG on human health are numerous. The reported pharmacological activities include hepatoprotection, antispasmodic, anti-allergy, antiplasmodial, cardioactive, anticough, antimicrobial, antigenotoxic and anti-cancer effects (Gutierrez et al., 2008). Its antidiabetic potential in the regulation of blood glucose levels in STZ induced diabetic rats has also been reported (Chin Siu et al., 2011).

The roots, immature fruits, leaves and bark of PG can be used in the treatment of gastroenteritis, dysentery and diarrhoea. Leaves can also be used for the treatment of ulcers and rheumatic pain, while the consumption of PG relieves toothache (Heinrich et al., 1998). Treatment of diabetic patients with PG protected them against cytotoxicity induced by glucose, glyoxal, and methylglyoxal, showing the antiapoptotic potential of PG (Hsieh et al., 2007). Besides the medicinal uses, PG is employed as food: juice, pastes, concentrates, and jelly nectar etc. (Jimenez-Escrig et al., 2001). PG is also used to make hair combs (Morton, 1987). Previous studies have reported that the bark (Mukhtar et al., 2006), fruit (Chin Shiu et al., 2011) and leaves (Oh et al., 2005; Banu et al., 2013) of the common guava, P. guajava, exhibit an anti-diabetic effect. Previous authors have suggested that the antidiabetic effects of PG can be linked to polyphenolic compounds and triterpenes present in the plant, which ameliorates the deleterious effects of free radicals (Gutierrez et al., 2008).
Insulin responsive tissues such as liver, muscle, adipose tissue, kidney, brain and pancreatic β cells play important roles in the removal of excess glucose from the circulatory system (Thirone et al., 2006). There is little information on how oral PG supplementation might change the function of liver, muscle and adipose tissue in glucose homeostasis and pathogenesis of diabetes. It is with these considerations that this study was designed to investigate the anti-diabetic mechanism of action of PG, and also effects of PG on enzymes such as protein kinase B (PKB/Akt), glycogen synthase (GS) and glycogen phosphorylase (GP). The deposition of glycogen in liver and muscle following treatment was also investigated. The effect of PG on serum lipids; hormone sensitive lipase (HSL) enzyme and markers of liver and muscle dysfunction was also investigated. Compared with synthetic compounds, natural products are, less expensive, with minimal side effects and have antidiabetic potential (Verspohl, 2002; Neelesh et al., 2010). Therefore studies on edible plants with anti-diabetic properties will be valuable in the management of diabetes. In this study aqueous extracts of the medicinal plants listed below were investigated for their anti-diabetic potential in streptozotocin induced diabetic rats.

1. *Moringa oleifera*
2. *Psidium guajava*
3. *Helichrysum odoratissimum*

Figure 1.8: Picture of *Psidium guajava* (source: www.commons.wikimedia.org)
1.10 STREPTOZOTOCIN

The pancreas is made up of 1 million islets of Langerhans (Hellman et al., 2007). Most islets contain five types of cell: alpha cells (secrete glucagon), beta-cells (secrete insulin), delta cells (secrete somatostatin), epsilon cells (secrete ghrelin), and PP cells (secrete pancreatic polypeptide). Streptozotocin (STZ) is a glucosamine nitrosourea compound which is used to induce diabetes when administered to rats (Balamurugan et al., 2003) by targeting beta cells. STZ enters the beta cell via the glucose transporter GLUT2 and causes damage to the DNA by adding an alkyl group to DNA molecule (Melmed et al., 1973; Bennett and Pegg, 1981). DNA damage promotes activation of poly ADP-ribosylation, formation of superoxide radicals, leading to the destruction of beta cells (Szkudelski, 2001).

![Figure 1.9: Structure of streptozotocin (STZ) (Source: commons.wikimedia.org)](image)

1.11 AIM

To investigate the antidiabetic effects and mechanism of action of Psidium guajava in streptozotocin-induced diabetic rats.

1.12 OBJECTIVES

1. To determine the in vivo antidiabetic effects of aqueous extracts of Moringa oleifera, Helichrysum odoratissimum and Psidium guajava in streptozotocin induced diabetic rats.

2. To assess changes in the expression of p-Akt, GP, GS and HSL after treatment with PG using SDS-PAGE Western Blot analysis.

3. To measure the activities of glycogen phosphorylase and glycogen synthase enzymes in liver and skeletal muscle. This objective will also include an assessment of changes in levels of glycogen in liver and muscle following treatment of rats with PG.
4. To determine the effect of PG aqueous extract on the activity of hormone sensitive lipase enzyme in liver and adipose tissue and on serum triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, cardiac risk factor and atherogenesis.

5. Phytochemical characterization of the extract via Gas Chromatography Mass Spectroscopy (GCMS) to identify the bioactive compounds.

6. To investigate the effect of PG on ameliorating diabetic damage done to the pancreatic islets through histopathological analysis of the pancreas.

7. To determine the activities of serum lactate dehydrogenase, aspartate aminotransferase and alanine transaminase levels after treating rats with PG. These enzymes are used as biomarkers of tissue damage.
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

The following antibodies and chemicals used for this study were bought from South Africa and Germany. The antibodies for anti-phospho-Akt (Akt 1/PKB α), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) and anti-GS were purchased from Cell Signalling Technology, South Africa. The rabbit anti-GP and rabbit anti-HSL antibodies were purchased from ABCAM, Centurion, South Africa while the secondary goat anti rabbit antibody IgG alkaline phosphatase conjugate was purchased from BIORAD, Johannesburg, South Africa. The radionuclides cholesterol oleate, α-D-[U-14C]glucose-1-phosphate and U-14C-uridine diphosphoglucose (14C-UDPG) were purchased from BIOTREND, Cologne, Germany.

Sodium hydroxide (NaOH), hydrochloric acid (HCl), disodium hydrogen phosphate (Na₂HPO₄), potassium sodium tartarate (KNaC₄H₄O₄·H₂O), sodium citrate, potassium iodide (KI), sodium sulphate (NaSO₄), copper sulphate (CuSO₄·H₂O), potassium hydroxide (KOH), citric acid and sulphuric acid were purchased from MERCK, South Africa. Ammonium persulphate, 10X Tris/Glycine/SDS buffer, N, N, N, N-tetramethyl ethylenediamine (TEMED), sodium dodecyl sulphate (SDS) and Tris base were purchased from either SIGMA-ALDRICH or BIORAD, South Africa. Bovine serum albumin (BSA), streptozotocin (STZ), leupeptin hemisulphate, β-mercabromophenol blue, sodium pyrophosphate (Na₄P₂O₇), Nonidet P-40, sodium orthovanadate (Na₃VO₄), sodium fluoride (NaF), 3-(N-morpholino) propanesulfonic acid (MOPS), di-potassium hydrogen orthophosphate (K₂HPO₄), glycogen, 2-(N-morpholino) ethanesulfonic acid (MES), benzamidine hydrochloride hydrate, Triton-X 100, dithiothreitol (DTT), glucose-6-phosphate disodium salt, uridinediphosphoglucose (UDPG), α-D-glucose 1-phosphate disodium salt hydrate, N’N’- bis- methylene-acrylamide, phenol solution, acrylamide, adenosine monophosphate, methanol, phenylmethanesulfonylfluoride (PMSF), potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄), ethylene diaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Tween-20, glycerol, sodium chloride (NaCl) and glycine were purchased from SIGMA-ALDRICH and sourced through Capital Lab Supplies, South Africa.
2.2 ETHICAL CLEARANCE

Ethical clearance for animal studies was obtained from the Animal Ethics Committee of the University of KwaZulu-Natal (reference 105/12/Animal).

2.3 ANIMALS AND DIET

Male Sprague-Dawley rats 230-250g body weight were procured from the Biomedical Resource Unit (BRU) at University of KwaZulu-Natal (Westville Campus). The animals had free access to water and food (Meadows, Pietermaritzburg) throughout the experimental period. The experiments were performed and animals maintained according to guidelines stipulated in the University of KwaZulu-Natal (UKZN) Animal Ethics Committee Standard Protocol Booklet.

2.4 METHODS

2.4.1 Preparation of plant materials

Fresh leaves of *Psidium guajava* Linn (PG), *Moringa oleifera* (MO), *Helichrysum odoratissimum* (HO) were collected in Durban, KwaZulu-Natal province, South Africa. They were identified and authenticated by Prof Ashley Nicholas, Taxonomist, University of KwaZulu-Natal, South Africa, in the month of July 2012. A voucher specimen number TO 1-4/2012 has been submitted at the ward herbarium, University of KwaZulu-Natal, Westville Campus, Durban, South Africa. The leaves were washed with distilled water, air dried, crushed using a blender and extracted with distilled water for 48 hours at room temperature. The extract was filtered and the filtrate lyophilized using a freeze drier (Wirsam Scientific and Precision Equipment, (PTY) Ltd, Germany). The powder product was stored at room temperature for further analysis.

2.4.2 Induction of diabetes

After overnight fasting, a single dose of 40 mg/kg body weight of streptozotocin dissolved in 0.1 M citrate buffer pH 4.5 was administered to male Sprague-Dawley rats intraperitoneally for diabetes induction. Animals having blood glucose levels of 7-14 mmol/l after a week were considered to have a stable type II diabetic state.
2.4.3 Preliminary studies

The leaves of MO, HO and PG were dried under shade and ground into powder. The powder was extracted in water only at room temperature for 48h. Each of the three extracts was filtered and filtrates subsequently lyophilized using a freeze drier (Wirsam Scientific and Precision Equipment, (PTY) Ltd, Germany). The samples were stored at room temperature. The filtrate was used for subchronic studies.

After induction of diabetes, the route of administration for both the test substance and vehicle was via oral gavage. Animals were separated into eight groups of 6 rats each, NC (normal control), NPG (normal treated with Psidium guajava (PG)), NMO (normal treated with Moringa oleifera (MO)), NHO (normal treated with Helichrysum odoratissimum (HO)) and DC (diabetic control), DPG (diabetic animals treated with PG), DMO (diabetic animals treated with MO), DHO (diabetic animals treated with HO). The untreated normal and diabetic rats were given distilled water, while the treated normal and diabetic rats were given 400 mg/kg body weight of PG, MO and HO for 14 days after which animals were sacrificed.

Blood samples were collected into non-heparinized tubes and centrifuged at 3000 g for 10 min to obtain serum which was stored at -20°C pending analysis. A portion of adipose tissue (visceral side), liver and muscle was removed and stored at -20°C for subsequent analysis. The pancreas was also removed and fixed in 10% formalin. Animals having blood glucose levels of 7-14 mmol/l after a week were considered having a stable type II diabetic state before starting the experiment.

2.5 SUB-CHRONIC STUDIES

Male Sprague-Dawley rats were separated into 8 groups of 6 rats each: non-diabetic control (NC), non-diabetic PG (NPG), diabetic control (DC), diabetic PG (DPG). The NC and DC groups were given the vehicle (distilled water). The NPG and DPG groups were treated with aqueous extract of Psidium guajava (400 mg/kg body weight) daily for 14 days. Rats were euthanized after treatment. The adipose tissue, liver and skeletal muscle was collected, and stored at -20°C for subsequent analysis.

2.6 ORAL GLUCOSE TOLERANCE TEST (OGTT)

OGTT was conducted on both normal and diabetic rats. The animals were fasted for 12 hours after which glucose (1g/kg b.w.) was administered orally via oral gavage. Blood glucose
levels were measured at 0 (just before glucose ingestion), 15, 30, 60, 90 and 120 min after the ingestion of glucose using a glucometer (ACCU CHEK, Basel, Switzerland).

2.7 ACUTE STUDIES ON EFFECT OF *PSIDIUM GUAJAVA* AQUEOUS EXTRACT ON THE INSULIN SIGNALLING CASCADE

Male Sprague-Dawley rats of 230-250 g body weight were separated into 4 groups and in each group 2 rats were used at each time interval. The groups were as follows: non-diabetic control (NC), non-diabetic PG (NPG), diabetic control (DC), diabetic PG (DPG). The NC and the DC groups were given the vehicle (distilled water). The NPG and DPG were treated with *Psidium guajava* aqueous extract (400 mg/kg body weight). Rats were euthanized after treatment at the following time intervals: \((n = 2)\) for 15 and 30 minutes and \((n = 6)\) for 60 minutes. The adipose tissue, liver and muscle were collected and stored at \(-20^\circ\text{C}\).

2.8 TISSUE PROCESSING FOR WESTERN BLOTS

2.8.1 Skeletal muscle

Skeletal muscle samples were prepared for Western blotting by the method of Sakamoto *et al.* (2003). Samples were homogenized in a buffer comprising 20 mM Tris, pH 7.5, 10 mM Na_4P_2O_7, 100 mM NaF, 5 mM EDTA, 3 mM benzamidine, 1 % Nonidet P-40, 7 µM leupeptin, 2 mM Na_3VO_4 and 1 mM PMSF. Homogenates were transferred into centrifuge tubes, maintained on ice, and then centrifuged at 14,000 g for 10 mins at 4 \(^\circ\text{C}\). Supernatants were removed and kept for subsequent analysis. Thereafter, the protein concentrations in each sample were measured using the Biuret method.

2.8.2 Liver

The liver samples were processed according to the method of Feres *et al.*, (2010). Samples were homogenized in a solution containing 1 % Triton X-100, 2.0 mmol/L of PMSF, 100 mmol/L of Tris-HCl pH 7.4, 100 mmol/L of Na_4P_2O_7, 10 mmol/L of EDTA, 10 mmol/L of Na_3VO_4, 100 mmol/L of NaF and 3 µg/ml aprotinin. The homogenates were decanted into centrifuge tubes maintained on ice and then centrifuged at 12,000 g for 20 min at 4 \(^\circ\text{C}\). Supernatants were removed and stored for further analysis. The protein concentrations in each sample were measured using the Biuret method and BSA as a standard.
2.8.3 Adipose tissue

Adipose tissue samples were prepared using a method described by Alonso et al. (2005). Samples were homogenized in 3 ml of 3mM benzamidine, 50 mM Tris–HCl (pH 7.5), 0.05% sodium deoxycholate (C_{24}H_{39}NaO_4), 1% Nonidet P-40, 1 mM Na_3VO_4 and 150 mM NaCl. The homogenates were centrifuged at 12000 g at 4 °C for 10 min. Three different layers developed after centrifugation; the upper and bottom layers were discarded; only the middle layer (infranatant) was stored at -20 °C for subsequent analysis. The protein concentrations in each sample were measured using the Folin-Lowry method.

2.9 SDS-PAGE AND WESTERN BLOT ANALYSIS

Proteins in homogenates of samples from liver, skeletal muscle and adipose tissue were mixed with loading buffer with equal amounts of protein being loaded on each gel and separated by SDS-PAGE (10% gel) in Tris/Glycine/SDS buffer, pH 8.3 at 110 V using a Bio-Rad Mini – PROTEAN 3 Electrophoresis cell, BIORAD (South Africa). The proteins on gels were transferred to nitrocellulose membranes containing Tris/glycine/methanol buffer, pH 8.3 at 100 V for 2 hours using a Bio-Rad Electrophoretic Transfer Cell, BIORAD (South Africa). After transfer, the membranes were blocked in buffer containing 3% non fat dried milk, Tris buffered saline containing 0.1% Tween 20 and 20 mM Tris, and NaCl overnight at 4 °C for the three primary antibodies listed below except phospho-Akt. p-Akt was blocked in (Tris Buffer Saline-Tween 20 (TBS-T) only. The membranes were probed with primary antibodies (anti-glycogen synthase, anti-glycogen phosphorylase, anti-phospho Akt and anti-hormone sensitive lipase) diluted 1:1000 in TBS 0.1% Tween 20 with shaking for an hour at room temperature. Thereafter the membranes were washed (3×10 min) with TBS 0.1% Tween 20. After washing, membranes were probed with Goat anti-Rabbit IgG alkaline phosphatase conjugate (1:1500 in TBS 0.1% Tween 20 for 1 hour at room temperature after which the membranes were washed (4×5 min) with TBS 0.1% Tween 20. Proteins were viewed after exposure to 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium/nitro blue tetrazolium chloride (BCIP/NBT), thereafter, images were captured and the intensity of the bands was quantified using Gene Tools Analysis Software. Western blot analysis of GAPDH protein (loading control) was also performed according to the process mentioned above.
2.10 ANALYSIS OF ENZYME ACTIVITY

2.10.1 Glycogen synthase activity assay

Liver and skeletal muscle samples were prepared and processed for glycogen synthase activity assay using a radiochemical procedure as described by Mandarino et al. (1987). Samples were homogenized in a buffer containing 50 mM potassium phosphate buffer, pH 7.4, 20 mM EDTA, 2 mM DTT and 20 mM NaF. The homogenates were centrifuged, after which supernatants were collected and diluted in a buffer containing 50 mM Tris-HCl, pH 7.8, 25 mM NaF and 20 mM EDTA. The reaction was started by mixing 50 µl of the sample and 50 µl of reaction mixture containing 25 mM NaF, 10 mM glucose-6-phosphate (G6P), 20 mM EDTA, 50 mM Tris-HCl (pH 7.8), 1 µCi [U-14C]uridinediphosphate glucose (UDPG), 1 % glycogen and 5 mM UDPG. The reaction mixture was placed in a water bath for incubation at 30 °C for 20 minutes and the reaction was stopped by adding 50 µl of the incubated mixture on a filter paper, which was dropped in a vial containing cold 70 % (v/v) ethanol for glycogen precipitation. Filter papers were washed (2×30 minutes) in 70 % ethanol, allowed to dry and placed in scintillation vials containing scintillation fluid for 14C determination. A blank was prepared containing buffer only.

2.10.2 Glycogen phosphorylase activity (GP) assay

Glycogen phosphorylase activity was assayed using a radiochemical procedure adapted from Taylor et al. (2006). Liver and muscle samples were homogenized in a solution containing 50 mM NaF, 10 mM MOPS, 5 mM EDTA, and 1 mM DTT, pH 7.0. Homogenates were transferred into centrifuge tubes and maintained on ice. The homogenates were centrifuged at 4 °C for 10 minutes at 9000 g, after which supernatants were removed and stored for subsequent analysis. The reaction was started by mixing 50 µL of sample and 50 µL of assay buffer consisting 22.3 mM G-1-P, 33 mM MES, 0.34 % glycogen, and 1 µCi/ml D-[U14C]glucose-1-phosphate and the mixture was incubated inside a water bath at 30 °C for 20 mins. Thereafter the reaction was stopped by adding 50 µl of the incubated mixture on a filter paper which was dropped in a vial containing cold 70 % (v/v) ethanol for glycogen precipitation. The filter papers were washed (2×30 minutes) in 70 % ethanol, allowed to dry and placed in scintillation vials containing scintillation fluid for 14C determination. A blank was prepared containing buffer only.
2.10.3 HORMONE SENSITIVE LIPASE ACTIVITY (HSL) ASSAY

2.10.3.1 Adipose and liver tissue homogenization

Adipose tissue samples were prepared by the method described by Alonso et al. (2005). Samples were homogenized in a buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 0.25 mM sucrose, 1 mM EDTA, 5 μg/ml leupeptin, 1 mM PMSF and 3 mM benzamidine. The homogenates were transferred into centrifuge tubes and centrifuged at 12,000 g at 4°C for 10 mins using a Beckman Coulter centrifuge (Avanti J-26 XPI). Three layers developed after centrifugation, only the middle layer (infranatant) was collected and stored for subsequent analysis.

The liver was processed according to a method by Feres et al. (2010). Liver samples were homogenized in a buffer containing 1 % Triton X-100, 2 mM of PMSF, 100 mM of Tris-HCl (pH 7.4), 100 mM of Na₄P₂O₇, 100 mM of NaF, 10 mM of EDTA, 3 μg/ml aprotinin and 10 mM of Na₃VO₄. Homogenates were then centrifuged at 12,000 X g for 20 min at 4°C using Beckman Coulter J26-XPI centrifuge (USA) and the supernatant collected.

2.10.3.2 Preparation of the substrate solution

Two and a half (2.5 ml) substrate solution was prepared by emulsifying 1.8 mM cholesteryl (³H) oleate (S.A: 2.3 x 10⁻³ Ci/mmol) with 5.6 mg of phosphatidylcholine/phosphatidylinositol (3/1 by weight) in chloroform. (2 ml, 0.1 M) potassium phosphate (pH 7) was then added to the solution. The substrate solution was then subjected to a mild heat at 37°C to allow for chloroform removal. The substrate solution was sonicated (2 x 2 minutes) with 1 minute intervals. The sonication process was repeated, and thereafter, 0.5 ml of 0.5 g/ml BSA in 0.1 M potassium phosphate (pH 7) was added.

2.10.3.3 Activity assay

Hormone sensitive lipase activity was assayed by the procedure of Ylitalo et al. (2000) with some modifications. A mixture of 0.03 ml aliquot of substrate solution, 5 μl of 50 mM EDTA, 5 μl of 5 mM DTT and 100 μl of sample was incubated for 120 mins at 20°C with shaking at 100 cycles/min. The reaction was stopped by adding 800 μl of methanol/chloroform/heptane (1.41/1.25/1 by volume) and 250 μl of 0.1 M potassium carbonate/0.1 M boric buffer (pH 10.5). The released radioactively labeled oleate was retrieved by a one-step liquid-liquid partition system. The mixture was shaken for 20 sec and
centrifuged at 800 g for 20 min. A 0.25 ml aliquot taken from the upper phase was mixed with 1.8 ml scintillation fluid and placed in scintillation vials for counting.

2.11 SERUM ENZYMES

Blood samples were collected into non-heparinized tubes and placed in a container containing ice. Thereafter, serum was obtained by centrifuging the samples at 3000 rpm for 10 minutes. The serum was collected and stored at -20 °C for subsequent analysis. The activities of alanine aminotransferase (ALT), aspartate amino transferase (AST) and lactate dehydrogenase (LDH) were carried out using a Labmax Plano Chemistry analyzer from Brazil. The principles of the methods are described below.

2.11.1 Alanine Amino Transferase (ALT)

ALT activity was determined using the method described by Reitman and Frankel (1957).

**Principle**

ALT catalyzes the reaction between L-alanine and α-oxoglutarate, to yield L-glutamate and pyruvate. The pyruvate formed is reduced to lactate in a reaction catalysed by lactate dehydrogenase to form L-lactate and NAD⁺. The rate of the NADH oxidation is determined by measuring the absorbance of NADH at 340 nm.

\[
\alpha\text{-oxoglutarate} + \text{L-alanine} \quad \overset{\text{ALT}}{\longrightarrow} \quad \text{Pyruvate} + \text{L-glutamate}
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \quad \overset{\text{LDH}}{\longrightarrow} \quad \text{L-lactate} + \text{NAD}^+
\]

2.11.2 Aspartate Amino Transferase (AST)

AST activity was measured using the method described by Reitman and Frankel (1957).

**Principle**

The transfer of an amino group from L-aspartate to α-oxoglutarate is catalyzed by the enzyme aspartate amino transferase. The products of the reaction are L-glutamate and oxaloacetate. Oxaloacetate reacts with NADH in a reaction catalysed by malate dehydrogenase (MDH) to form NAD⁺. The catalytic AST activity is determined by measuring the absorbance of NADH at 340 nm.

\[
\alpha\text{-oxoglutarate} + \text{L-aspartate} \quad \overset{\text{AST}}{\longrightarrow} \quad \text{oxaloacetate} + \text{L-glutamate}
\]
Oxaloacetate + NADH + H⁺ → MDH → L-malate + NAD⁺

2.11.3 Lactate Dehydrogenase (LDH)

LDH assay was carried out using the method described by Wroblewski and La Due (1955).

Principle

The conversion of lactate to pyruvate, is catalyzed by the enzyme lactate dehydrogenase (LDH). The increase in absorbance of NADH at 340 nm is directly proportional to lactate dehydrogenase activity in the sample.

Lactate dehydrogenase (LDH) catalyzes the following reaction:

\[
\text{Lactate} + \text{NAD}^+ \rightarrow \text{Pyruvate} + \text{NADH}
\]

2.12 ESTIMATION OF LIVER AND SKELETAL MUSCLE GLYCOGEN CONTENT

Liver and skeletal muscle glycogen content was determined using a procedure adapted from Nader and Esser (2001). The tissues (0.25 g) were weighed and thereafter 1 ml of 30% KOH saturated with NaSO₄ was added to each sample. The samples were boiled for 30 minutes for complete digestion, removed from water bath and placed on ice. While on ice; 4 ml of 95% cold ethanol was added to the samples. Samples were vortexed immediately and incubated on ice for 30 minutes. After incubation, samples were centrifuged at 550 rpm using Beckman centrifuge 5810R for 30 minutes. The supernatants were discarded and this was followed by addition of 1 ml of H₂O and 5% phenol. Samples were vortexed and 5 ml of 36N sulphuric acid was added followed by incubation on ice for 30 minutes. Thereafter; the absorbances were measured using a UV-VIS spectrophotometer (Shimadzu, Corporation, Kyoto, Japan) at 490nm. A glycogen standard curve was constructed in the range of 0-100 µg/ml (see Appendix).

2.13 ESTIMATION OF SERUM LIPIDS

The blood samples were collected into non-heparinized tubes and placed in a container containing ice. Thereafter, serum was obtained by centrifuging the samples at 3000 rpm for 10 minutes using a centrifuge (Labofuge 200-Heraeussepatech). The serum was collected and stored at -20 °C for subsequent analysis. Serum lipid profiles (total cholesterol, triglycerides, LDL-c, cardiac risk factor, atherogenic index and HDL-c), were measured using a Labmax
Plano Chemistry analyzer, Brazil. The levels of low-density lipoprotein (LDL-c) were calculated using the Friedewald et al., (1972) formula, while atherogenic index (AI) and cardiac risk factor (CRF) were calculated using the formulae of Hostmark et al. (1991) and Kim and Sho (2007) respectively. The principles of the methods are described below.

2.13.1 Estimation of total serum cholesterol

This was carried out using the method described by Roschlau et al. (1974)

Principle

Cholesterol esters undergo enzymatic hydrolysis by an enzyme esterase to yield cholesterol and fatty acids, one of the products of hydrolysis. Cholesterol is further oxidized to give hydrogen peroxide and cholestene-3-one. The final process is the reaction of phenol with hydrogen peroxide and 4-aminoantipyrine to yield quinoneimine; this process is catalysed by peroxidase. The change in absorbance is measured at 520 nm. The color intensity is directly proportional to the concentration of cholesterol in the sample.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{- aminoantipyrine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4\text{H}_2\text{O}
\]

2.13.2 Estimation of serum HDL-Cholesterol

Serum HDL cholesterol analysis was performed using the method described by Loppes-Virella (1977).

Principle

In this assay, the first process is the addition of a precipitating reagent (20 % w/v polyethylene glycol in glycine buffer at pH 10 and 25 °C) to serum samples. Thereafter, the samples are vortexed, centrifuged and all beta lipoproteins (LDL and VLDL fractions) are precipitated. The resulting supernatant (which contains the HDL fraction) is used for the determination of HDL-cholesterol. The concentration of HDL-cholesterol is measured by hydrolyzing HDL-cholesterol esters to yield free HDL-unesterified cholesterol and fatty acid. The next process is the oxidation of cholesterol to yield cholestene-3-one and hydrogen
peroxide. After oxidation, the indicator of HDL-cholesterol is quinoneimine. Absorbance is measured at 600 nm and is directly proportional to HDL-cholesterol concentration.

\[
\text{HDL-cholesteryl esters} + \text{H}_2\text{O} \xrightarrow{\text{PEG-cholesteryl esterase}} \text{HDL-unesterified cholesterol} + \text{fatty acid}
\]

\[
\text{Unesterified cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholestene-3-one} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + \text{H}_2\text{O}
\]

**2.13.3 Estimation of serum triglyceride concentration**

The concentration of triglyceride in serum samples was determined using the method described by Fossati and Lorenzo (1982)

**Principle**

A lipase hydrolyses triglycerides into fatty acids and glycerol. Glycerol is converted to glycerol-1-phosphate and ADP in the presence of ATP and glycerol kinase. Thereafter, glycerol-1-phosphate is broken down by the enzyme glycerol phosphate oxidase to yield dihydroxyacetone, phosphate and hydrogen peroxide. Finally, the indicator of triglycerides (red colored quinoneimine dye) is formed when 4-chlorophenol reacts with 4-aminophenazone and hydrogen peroxide. The absorbance of the colored complex, which is measured spectrophotometrically at 546 nm, is directly proportional to the triglycerides concentration in the sample.

\[
\text{Triglycerides} + 3\text{H}_2\text{O} \xrightarrow{\text{lipases}} \text{fatty acids} + \text{glycerol}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-1-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-1-phosphate} + \text{O}_2 \xrightarrow{\text{glycerol phosphate oxidase}} \text{dihydroxyacetone} + \text{H}_2\text{O}_2 + \text{phosphate}
\]

\[
2\text{H}_2\text{O}_2 + \text{4-chlorophenol} + \text{4-aminophenazone} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\text{H}_2\text{O} + \text{HCl}
\]
2.14 PROTEIN DETERMINATION

2.14.1 Biuret assay for protein determination

The protein concentration in the liver was determined using the procedure described by Gornall et al., (1949) with modifications. The standard was prepared using bovine serum albumin (10 mg/ml BSA). To prepare the biuret reagent, 3 g of copper sulphate (CuSO₄·5H₂O) and 9 g of sodium potassium tartrate (NaKC₆H₄O₆·4H₂O) were dissolved in 500 ml of 0.2 M sodium hydroxide (NaOH), thereafter, 5 g of potassium iodide, (KI) was added and the solution made up to 1000 ml with 0.2 M NaOH. To 2 ml of protein sample, 3 ml of the biuret reagent was added. The solutions were vortexed and incubated for 10 minutes at 37 °C. Thereafter, solutions were read in a spectrophotometer at 540 nm. A standard curve of the absorbances against protein concentrations in the range of 0-10 mg/ml was plotted. The protein content of the samples was extrapolated from the standard curve.

2.14.2 Folin-Lowry assay for protein determination

Protein concentrations for muscle and adipose tissue were determined using a procedure described by Lowry et al. (1951). The samples were diluted using 0.1N NaOH, thereafter 5 ml of alkaline reagent (2 % Na₂CO₃ in 0.1 N NaOH) and copper sulphate-sodium in potassium tartrate solution (0.5 % CuSO₄·5H₂O in 1 % Na-K tartrate) were mixed together in the ratio of 50:1 (v/v). The resulting mixture was vortexed and incubated at 40 °C for 15 minutes. The tubes were cooled and 0.5 ml of Folin Ciocalteau reagent was added. The tubes were incubated at 25 °C for 30 minutes again and thereafter the absorbances of the resulting solutions were read in a spectrophotometer at 600 nm. The standard used was BSA and the standard curve of the absorbances against protein concentrations in the range of (20 – 100 µg) was plotted.

2.15 HISTOPATHOLOGY OF THE PANCREAS

Histopathological analysis of pancreatic tissues was carried out using a standard laboratory protocol by immersing the tissue in 10 % formalin after which it was dehydrated with (70 %, 80 %, 95 %, 100 %, 100 % 100 % alcohol) each for 1 hour. Thereafter, clearing was done with xylene (2×1 hour), and the sample was infiltrated with paraffin wax for (1.5 hour × 2) and placed in an oven overnight.
Specimens were then orientated for optimal sectioning and embedded in moulds. Moulds were placed on cold plate to settle wax and then the block was popped out. Specimens were sectioned at 4 μm on the HM 315 microtome and picked up on frosted slides. Sectioned tissue was then stained with haematoxylin and eosin as follows: xylene–3 min, 2 min, alcohol (100 %, 90 %, 70 % and 50 %) for 2 min each, after which it was immersed in distilled water, then haematoxylin for 5 min, the blue sections were put in water and then eosin for 3 min, after which the slides were dipped in 90 % alcohol and then 100 % alcohol. Excess alcohol was drained off and slides were placed in xylene before the coverslip was mounted permanently with DPX mountant. Coverslips were left overnight to dry thoroughly. The slides were then mounted in distyrene plasticizer xylene (DPX), cover-slipped and viewed with Leica slide scanner (SCN 4000, Leica Biosystems Germany).

2.16 GAS CHROMATOGRAPHY-MASS SPECTROMETRIC (GC-MS) ANALYSIS

Aqueous extract of Psidium guajava (PG) leaf was subjected to GC-MS analysis for identification of bioactive compounds present in PG. A standard laboratory protocol was used in the analysis of Psidium guajava. The analysis was done using Agilent Technologies 6890 Series GC coupled with an Agilent 5973 mass selective detector driven by Agilent chemstation software. A HP-5MS capillary column was used (30 m × 0.25 mm internal diameter, 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min and a linear velocity of 37cm/sec. The injector temperature was set at 250 °C. The initial oven temperature was 60 °C and was programmed to increase to 280 °C at the rate of 10 °C/min with a hold time of 4 min at each increment. The injection volume of 2 μL was made in the splitless mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 min and scan range 50-700 amu. The retention times, fragmentation pattern and mass spectral data were used in the identification of the unknown compounds by comparing them with those stored in the National Institute of Standards and Technology (NIST) library. This was done to confirm the name, molecular weight and structure of the bioactive compounds.
2.17 DATA ANALYSIS

All data analysis was done using GraphPadInStat Software (version 5.00, GraphPad Software, San Diego, California, USA). Statistical comparison between groups was done using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Values of $p < 0.05$ were taken as statistically significant.
CHAPTER 3
RESULTS

3.1 Preliminary studies on the effect of plant extracts on blood glucose levels

Table 3.1 Effect of plant extracts on blood glucose levels

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>NC</th>
<th>DC</th>
<th>NMO</th>
<th>DMO</th>
<th>NHO</th>
<th>DHO</th>
<th>NPG</th>
<th>DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEFORE TREATMENT</td>
<td>5.4±0.2</td>
<td>20.4±2.5</td>
<td>6.0±0.2</td>
<td>19.5±0.8</td>
<td>5.4±0.1</td>
<td>18.3±2.4</td>
<td>5.4±0.2</td>
<td>20.8±1.5</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFTER TREATMENT</td>
<td>5.2±0.05</td>
<td>28.6±1.0</td>
<td>5.1±0.3</td>
<td>22.9±0.8</td>
<td>5.5±0.2</td>
<td>24±0.7</td>
<td>5.6±0.2</td>
<td>19.7±1.6</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fasting blood glucose levels of normoglycaemic and hyperglycaemic male Sprague –Dawley rats n=6. The data is represented as mean ± SEM. Blood glucose levels were expressed in mmol/L. NC (normal control), NPG (normal treated with PG), NMO (normal treated with MO), NHO (normal treated with HO) and DC (diabetic control), DPG (diabetic animals treated with PG), DMO (diabetic animals treated with MO), DHO (diabetic animals treated with HO). MO – *Moringa oleifera*; HO – *Helichrysum odoratissimum* and PG – *Psidium guajava*.

The results in Table 3.1 show the fasting blood glucose levels of normal and diabetic animals after 14 days of treatment. Three different plants were used in this study (MO, HO and PG). There was an increase in blood glucose levels in both MO and HO diabetic treated animals after 14 days while PG treatment caused a slight decrease in blood glucose levels in diabetic animals, but this decrease was not significant.
3.1 Effect of *Psidium guajava* on blood glucose levels

Figure 3.1: Effects of PG on blood glucose levels after 14 days of treatment n = 5. * p < 0.05 values differ significantly from normal control (NC), normal treated (NPG) and diabetic treated (DPG) groups.

Figure 3.1 above shows the fasting blood glucose levels of normal and diabetic male Sprague-Dawley rats after 14 days of treatment with PG. PG reduced blood glucose levels in the diabetic treated group (DPG). In Figure 3.1, a significant p < 0.05 reduction in blood glucose levels was observed in normal control (NC), normal treated (NPG) and diabetic treated (DPG) animals as compared to untreated diabetic animals (DC).
3.2 Oral glucose tolerance test

Figure 3.2: Oral glucose tolerance tests (OGTT) were conducted on normal and diabetic 12-hr fasted rats treated with PG. Values are expressed as % of the initial glucose concentration which was taken to be 100%. The data is represented as mean ± SEM. (n = 4). NC = 4.76 ± 0.1208 NPG= 5.5 ± 0.1183 DC= 13.92 ± 0.3470 DPG= 9.280 ± 0.8434

Following a glucose load, fasting blood glucose levels of normal and diabetic male Sprague-Dawley rats were measured over a 2 hour period. The blood glucose levels are expressed as % variation in blood glucose levels from time zero. In Figure 3.2, there was a rapid decline in blood glucose levels in normal control (NC), normal treated (NPG) and diabetic treated (DPG) animals as compared to untreated diabetic animals at 120 minutes. This showed that better glucose tolerance abilities were seen among NC, NPG and DPG animals.
3.3 SDS-PAGE gels for liver, muscle and adipose tissue homogenates

Figure 3.3 Coomassie stained 10% SDS-PAGE gels of: liver and skeletal muscle supernatants with 20 μg of protein loaded in each lane, A. Lanes: 1 (molecular weight marker); 2 and 6 (NC) = normal control; 3 and 7 (NPG) = normal treated group; 4 and 8 (DC) = diabetic control; 5 and 9 (DPG) = diabetic treated group; and adipose tissue infranatant with 10 μg of protein loaded in each lane. B. Lanes: 1 (molecular weight marker); 2 (NC) = normal control; 3 (NPG) = normal treated group; 4 (DC) = diabetic control; 5 (DPG) = diabetic treated group.
The SDS-PAGE gels for liver, muscle and adipose tissue homogenates are shown in Fig 3.3 and differences in intensity of bands. The bands in the adipose tissue gel were faint due to low protein content in adipose tissue.

3.4 Acute studies on effects of PG on expression of enzymes of the insulin signalling pathway

An effect of PG on the activation of PKB/Akt was studied. The enzyme was phospho-Akt (Ser 473) with GAPDH being used as a loading control. Phospho-Akt was detected at one time point (30 minutes).

The result of AKT phosphorylation in muscle is shown in Figure 3.4. After thirty minutes of PG treatment, the expression of phosphorylated Akt was highest in diabetic treated animals, followed by the normal control group and then the untreated diabetic animals. Non-diabetic treated animals had the lowest expression of phospho-Akt. The bands at 15 and 60 minute intervals were poorly expressed.
3.5: Effect of PG treatment on Glycogen synthase and Glycogen phosphorylase activity in liver

Figure 3.5 A: Assessment of PG treatment on liver glycogen synthase activity. The results are presented as mean ± SEM for five rats. * p<0.05 significant when compared to normal control (NC) and diabetic control group (DC). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nanomoles of the reaction mixture).

Figure 3.5 B: Assessment of PG treatment on liver glycogen phosphorylase activity. The results are presented as mean ± SEM for five rats. NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nanomoles of the reaction mixture).
The results in Figure 3.5 A show liver glycogen synthase activity on day 14 post treatment with PG. In normal animals, PG caused an elevation of GS. Of note was the significant elevation of GS activity in treated diabetic animals compared to normal untreated animals. PG treatment enhanced GS activity in diabetic rats.

The result in Figure 3.5 B shows the liver GP activity on day 14 post treatment with PG. GP activity was slightly higher in untreated diabetic animals compared to diabetic treated animals. PG lowered GP activity in both NPG and DPG groups.

3.6 Effect of PG on expression of GP in liver

GP expression on day 14 of the subchronic study was lowest in untreated diabetic animals followed by diabetic treated animals. Non-diabetic control had the highest expression of GP amongst all groups.

Figure 3.6: Expression of (A) GP and (B) GAPDH in liver of diabetic and non-diabetic rats. Forty microgram (40µg) of protein was loaded for A and (20µg) for B. (C) are the normalized values showing the ratio of GP/GAPDH (loading control). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG).
3.7: Effect of PG treatment on Glycogen synthase and Glycogen phosphorylase activity in muscle

**Figure 3.7 A:** Assessment of PG treatment on muscle glycogen synthase activity. The values are presented as mean ± SEM for five rats. * p < 0.05 significant compared to normal control group (NC) and normal treated group (NPG). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nano moles of the reaction mixture).

**Figure 3.7 B:** Assessment of PG treatment on muscle glycogen phosphorylase activity. The results are presented as mean ± SEM for five rats. NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nano moles of the reaction mixture).
The results in Figure 3.7 A show muscle glycogen synthase activity on day 14 post treatment with PG. In normal animals PG caused an elevation of GS. Of note was the significant depression of GS activity in untreated diabetic animals compared to both normal control and normal treated animals. PG treatment enhanced GS activity in diabetic animals.

The results in Figure 3.7 B show that the muscle GP activity on day 14 post treatment with PG. GP activity was higher in untreated diabetic animals compared to diabetic treated animals. PG depressed GP activity in diabetic rats.

3.8 Effect of PG treatment on Glycogen synthase and Glycogen phosphorylase expression in muscle

The expressions of glycogen synthase and phosphorylase enzymes were investigated in liver and skeletal muscle on day 14 post treatment with PG. GAPDH was used as a loading control (Figures 3.8 A and 3.8 B).

![Figure 3.8 A: Expression of (A) GS and (B) GAPDH in muscle of diabetic and non-diabetic rats. 40 μg of protein was loaded for A and 20 μg for B. (C) are the normalized values showing the ratio of GS/GAPDH (loading control). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG).](image-url)
GS expression was depressed in untreated diabetic animals. Treatment of diabetic animals caused a dramatic rise in GS expression to levels beyond the control groups (Figure 3.8 A).

GP expression on day 14 of the subchronic study was highest in untreated diabetic animals followed by diabetic treated animals. Untreated normal animals had the lowest expression of GP. PG treatment reduced GP expression in diabetic animals (Figure 3.8 B).
3.9: Effect of PG treatment on glycogen content in liver

The results in Figure 3.9 show that untreated diabetic animals had the lowest amount of glycogen which was 60% of that in normal animals. The amount of liver glycogen was significantly higher by 123% in treated diabetic animals compared to untreated diabetic animals.
3.10: Effect of PG treatment on glycogen content in muscle

![Bar chart showing glycogen content in muscle](image)

Figure 3.10: Estimation of muscle glycogen content. The results are presented as mean ± SEM for five rats. * $p < 0.05$ significant when compared to diabetic control group (DC). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG).

The results in Figure 3.10 show that the amount of muscle glycogen was significantly higher in treated diabetic animals compared to untreated diabetic animals. Diabetic treated animals had the highest amount of glycogen.
3.11 Hormone sensitive lipase activity in liver

Figure 3.11: Assessment of PG treatment on liver hormone sensitive lipase activity. The results are presented as mean ± SEM for five rats. * p < 0.05 significant when compared to normal control group (NC), normal treated group (NPG) and diabetic treated group (DPG). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nano moles of the reaction mixture).

The result in Figure 3.11 shows the liver HSL activity on day 14 of the subchronic study. HSL activity in diabetic control animals was the highest being over 1000 % that in normal untreated animals. Treatment with PG depressed HSL activities for both diabetic and non-diabetic animals but 78 % depression in HSL activity in treated diabetic animals was more profound.
3.12 Hormone sensitive lipase activity in adipose tissue

The results in Figure 3.12 below show adipose tissue HSL activity on day 14 post treatment with PG. In normal animals PG treatment depressed HSL activity. Of note was the elevation of HSL activity in untreated diabetic animals which was significantly reduced by treatment with PG.

Figure 3.12: Assessment of PG treatment on adipose tissue hormone sensitive lipase activity. The results are presented as mean ± SEM for five rats. * p < 0.05 significant when compared to diabetic treated group (DPG). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG). nmol (nano moles of the reaction mixture).
3.13 Effect of PG on expression of HSL in liver

An effect of PG on the expression of hormone sensitive lipase enzyme was investigated in the liver after 14 days of treatment. Three isoforms of HSL were detected: 149, 90, 37 kDa (Fig 3.13 A). In Fig 3.13 A, the 149 kDa isoform was highly expressed in untreated normal animals, compared to untreated diabetic animals. PG treatment of diabetic animals reduced the expression of this isoform of HSL. The normal treated animals had the highest expression of this isoform.

The lowest expression for 90 kDa isoform was in diabetic treated animals. Treatment of normal animals with PG increased the expression of this isoform. The 37 kDa isoform was expressed more in non-diabetic animals when compared to untreated diabetic animals. The lowest expression was seen in untreated diabetic animals.
Figure 3.13: Expression of (A) HSL and (B) GAPDH in liver of PG-treated rats after 14 days. 60 μg of protein was loaded for A and 40 μg for B. (C) are the normalized values showing the ratio of HSL/GAPDH protein (loading control). NC (normal control), NPG (normal animals treated with PG), DC (diabetic control), DPG (diabetic animals treated with PG).

3.14 Serum lipid profile

Table 3.2

<table>
<thead>
<tr>
<th>LIPID PROFILE</th>
<th>NC</th>
<th>NPG</th>
<th>DC</th>
<th>DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mmol/l)</td>
<td>5.9±0.48</td>
<td>5.6±0.34</td>
<td>6.5±0.12</td>
<td>4.85±0.87</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>3.49±0.36</td>
<td>3.4±0.09</td>
<td>4.2±0.12</td>
<td>3.7±0.16</td>
</tr>
<tr>
<td>LDL-c (mmol/l)</td>
<td>0.5±0.15</td>
<td>0.6±0.07</td>
<td>1.5±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>HDL-c (mmol/l)</td>
<td>1.8±0.13</td>
<td>1.6±0.08</td>
<td>1.3±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4±0.19</td>
</tr>
<tr>
<td>CRF (mmol/l)</td>
<td>1.9±0.13</td>
<td>2.1±0.05</td>
<td>3.2±0.39</td>
<td>1.5±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AI (mmol/l)</td>
<td>0.9±0.13</td>
<td>1.10±0.05</td>
<td>2.2±0.39</td>
<td>0.5±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=5. Values of p<0.05 will be taken to imply statistical significance. NC (Normal Control); NPG (Normal treated group); DC (Diabetic Control); DPG (Diabetic treated group). <sup>a</sup> p < 0.05 compared to NC, NPG and DPG; <sup>b</sup> p < 0.05 compared to NC, NPG and DC; <sup>c</sup> p < 0.05 compared to NC, NPG and DC; <sup>d</sup> p < 0.05 compared to NC, NPG and DC.
The results in Table 3.2 show serum levels of triglycerides, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol cardiac risk factor and atherogenic index on day 14 post treatment with PG.

In treated diabetic animals, PG reduced the level of triglycerides but this was not statistically significant when compared to untreated diabetic animals. The level of total cholesterol was slightly higher in untreated diabetic animals compared to treated diabetic animals. Treatment with PG slightly reduced total cholesterol levels for both diabetic and treated non-diabetic animals.

LDL-c cholesterol levels were significantly higher in untreated diabetic animals compared to untreated normal animals. Treatment with PG reduced LDL-c in both treated diabetic and non-diabetic animals. In treated diabetic animals, PG caused an elevation of HDL-c. Of note was the significant elevation of HDL-c levels in treated diabetic animals compared to untreated diabetic animals.

Cardiac risk factor was significantly higher in untreated diabetic animals when compared to both non-diabetic and treated diabetic animals. In treated diabetic animals, PG depressed atherogenesis. AI was significantly higher in untreated diabetic animals compared to non-diabetic animals.
**3.15 HISTOPATHOLOGY OF THE PANCREAS**

![Figure 3.15: H&E staining for pancreatic tissues in normal and STZ induced diabetic rats. Rats were treated with *P. guajava* (PG) at 400 mg/kg body weight for 14 days. IL = islets of Langerhans, NC = Normal control group, NPG = Normal treated group, DC = Diabetic control group, DPG = Diabetic treated group.](image)

Histological examination was done on the pancreatic islets to assess the damage done by STZ and the protective effect of PG. The photomicrographs show the effect of PG on the pancreas of normal and diabetic rats. Photomicrographs of the NC and NPG animals showed that the morphology of the islet cells remained intact, whereas in the DC group extensive damage was done to the morphology of the islets of Langerhans which show a high degree of disintegration. In the DPG group, the deleterious effects of STZ on the morphology of the pancreatic islets were reversed by PG treatment.
3.16 Serum biomarkers of tissue damage

Table 3.3

Effect of PG treatment on serum ALT, AST and LDH

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>NPG</th>
<th>DC</th>
<th>DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/l)</td>
<td>80.40±6.80</td>
<td>68.00±3.93</td>
<td>123.6±8.50</td>
<td>80.60±10.88</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>247.4±15.35</td>
<td>260.0±17.66</td>
<td>208.2±14.34</td>
<td>283.8±33.61</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>283.4±18.34</td>
<td>279.6±29.06</td>
<td>230.6±16.33</td>
<td>344.2±52.57</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 5. Values of p < 0.05 will be taken to imply statistical significance. NC (Normal Control); NPG (Normal treated group); DC (Diabetic Control); DPG (Diabetic treated group).

p < 0.05 compared to NC, NPG and DPG

The results in Table 3.3 above show the levels of ALT, AST and LDH on day 14 of the sub-chronic study. The serum level of ALT was highest in diabetic control animals. PG treatment decreased ALT levels in treated diabetic animals. The level of AST was high in both normal control and normal treated animals compared to untreated diabetic animals. The highest level of AST was seen in treated diabetic animals. Untreated normal animals showed slightly higher LDH levels compared to normal treated animals. The treated diabetic animals had the highest LDH level.
3.17 Phytochemical characterization of aqueous extract of PG using GC-MS

Table 3.4: Identified bioactive compounds in the leaf of aqueous extract of Psidium guajava using GC-MS

<table>
<thead>
<tr>
<th>Identified compounds</th>
<th>Retention time (mins)</th>
<th>Molecular mass (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocatechuic acid</td>
<td>17.629</td>
<td>154</td>
</tr>
<tr>
<td>Guavanoic acid</td>
<td>13.634</td>
<td>529</td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td>14.029</td>
<td>456</td>
</tr>
<tr>
<td>2α-Hydroxyursolic acid</td>
<td>13.634</td>
<td>473</td>
</tr>
<tr>
<td>Benzene-1,2-diol</td>
<td>5.799</td>
<td>110</td>
</tr>
<tr>
<td>Guanosine 2’-O-methyl</td>
<td>5.858</td>
<td>297</td>
</tr>
<tr>
<td>5-Bromo-8-(5-nitrosalicylidene amino) quinoline hydrochloride</td>
<td>13.70</td>
<td>372.1</td>
</tr>
</tbody>
</table>

Aqueous extract of Psidium guajava leaves was subjected to GC-MS analysis in order to identify the bioactive compounds in the aqueous extract of the leaf. Seven compounds were identified in the extract by comparing their MS spectra to those of standard spectra from the NIST library. Guavanoic acid, oleanolic acid, protocatechuic acid, 2α-hydroxyursolic acid, benzene-1,2-diol, 2’-O-methyl guanosine and 5-bromo-8-(5-nitrosalicylideneamino)quinoline hydrochloride were found in the aqueous extract of the leaf.
Figure 3.18 GC-MS structure and spectrum of protocatechuic acid
Figure 3.19 GC-MS structure and spectrum of guavanoic acid
Figure 3.20 GC-MS structure and spectrum of oleanolic acid
Figure 3.2 GC-MS structure and spectrum of 2α-Hydroxyursolic acid
1,2-Benzenediol

Figure 3.22 GC-MS structure and spectrum of 1,2-Benzenediol
Figure 3.23 GC-MS structure and spectrum of 2'-O-methyl guanosine
5-Bromo-8-(5-nitrosalicylideneamino)quinolone hydrochloride

Figure 3.24: GC-MS structure and spectrum of 5-Bromo-8-(5-nitrosalicylideneamino) quinoline hydrochloride
CHAPTER 4

DISCUSSION

a. Introduction

Diabetes mellitus is characterized by impaired uptake and storage of glucose as well as reduced glucose utilization for energy purposes (Jacobsen et al., 2009; Sangeeta et al., 2010). Ethnomedical literature contains a large number of plants that have a potential in the treatment of diabetes, a disease in which reactive oxygen species and free radicals play a major pathophysiological role (Gutierrez et al., 2008).

The liver plays a major role in regulating blood sugar levels; it absorbs glucose and stores it in the form of glycogen. The absorption and release of glucose by the liver helps to maintain blood glucose homeostasis. Glucose homeostasis helps to keep blood glucose levels constant, and this is achieved by a balance in endogenous glucose production and utilization (Roden and Bernroider, 2003). Previous studies reported that skeletal muscle accounts for about 75% of glucose disposal (Jensen et al., 2011). The primary site for lipid storage is adipose tissue which also contains little amounts of glycogen. Glucose entering the fat cell can also be stored as glycogen (Leonards and Landau, 1960; Moody and Gliemann, 1968). High levels of plasma free fatty acids (FFA) impair insulin signaling processes and have been linked with type 2 diabetes mellitus and insulin resistance (Arner, 2002).

Various studies have shown that the bark (Leonti et al., 2001; Mukhtar et al., 2006), fruit (Chin-Shiu et al., 2011) and leaves (Mukhtar et al., 2004; Oh et al., 2005; Ojewole, 2005) of the common guava, P. guajava, exhibit anti-hyperglycaemic effect. In this study, we investigate the anti-diabetic mechanism of action of PG, and also effects of PG on the enzymes PKB/Akt, GS and GP. The deposition of glycogen in liver and muscle after treatment was also investigated. PG effect on markers of liver and muscle dysfunction, such as the activities of alanine amino transferases, aspartate amino transaminases and lactate dehydrogenase were measured in the serum. We also investigated the possibility that PG may exert its anti-hyperlipidemic effects by down-regulating the activity of hormone sensitive lipase enzyme, and also improve lipid profile reducing total cholesterol, triglycerides, cardiac risk factor, atherogenic index and LDL-cholesterol levels.
b. Oral glucose tolerance test (OGTT)

In the present study, there was a significant decrease in blood glucose levels of diabetic animals treated with PG compared to untreated diabetic animals (Figure 3.1), indicating its anti-diabetic potential. Furthermore, in the oral glucose tolerance test (OGTT) analysis, untreated diabetic animals showed high glucose intolerance while better glucose tolerance abilities were observed in diabetic animals treated with PG (Figure 3.2).

c. Histopathological analysis

Bioactive compounds in plants which possess antioxidant activity reduced the complications of diabetes mellitus (DM) by quenching the effects of free radicals (Yokozawa et al., 2002; Fenercioğlu et al., 2010). In our present study, phytochemical characterization of Psidium guajava aqueous extract revealed that the identified compounds might possess antioxidative activity which might be responsible for the observed antidiabetic effect. The above hypothesis was supported by histopathological examinations of the pancreatic islets as shown in Figure 3.16 above. Previous authors reported that markers of oxidative stress can be seen in diabetic rats, such as necrosis and degranulation in islets of Langerhans (Coskun et al., 2004; Amaral et al., 2008; Chin Siu et al., 2011). Our finding is similar to the work of the above mentioned authors. The improvement in the morphology of the pancreatic islets of diabetic animals treated with PG might be as a result of the bioactive compounds present in the plant; the treatment ameliorated the diabetic damage done to the pancreatic islets.

d. Glucose transport

Various studies have reported that the pathogenesis of diabetes can be linked to various signalling pathways, such as insulin signalling pathway (Huang and Chen, 2009). Regulation of enzymes in insulin, glycogenolytic or gluconeogenic pathways has presented promising sites therapeutic of interventions. For proper understanding of the mechanisms underlying the action of PG extract, the expression of an enzyme called Akt in skeletal muscle of normal and diabetic rats was determined. This protein was selected because it plays an important role in stimulating the movement of glucose transporter type 4 (GLUT 4) from internal membrane to the plasma membrane, thereby increasing glucose uptake. It also facilitates the synthesis of glycogen from glucose. Excessive production of reactive oxygen species (ROS) and impaired ROS clearance by antioxidant enzymes triggers oxidative stress, which promotes the activation of serine/threonine kinases such as Protein kinase C (PKC), Jun N-terminal kinase
(JNK), leading to the phosphorylation of insulin receptor or insulin receptor substrate (IRS) proteins (Hirosumi et al., 2002; Kim et al., 2004; Ragheb et al., 2009). This leads to a decline in their tyrosine phosphorylation and subsequent decrease in the activation of PI3-K and Akt, thereby promoting the development of type II diabetes. The function of many proteins may be affected by phosphorylation and dephosphorylation on specific tyrosine, serine and/or threonine residues, for example, if amino acid Serine -473 in the protein Akt is phosphorylated, Akt is active as a kinase, if not, it is an inactive kinase (Bononi et al., 2011).

In the present study, PG stimulated Akt phosphorylation was higher in diabetic treated animals compared with non-diabetic animals. The work of Guo and colleagues (2013) showed that guava leaf extract showed higher Akt Ser 473 and Thr 308 phosphorylation in skeletal muscle of SHRSP/ZF normal rats as compared with untreated SHRSP/ZF normal rats, thereby promoting glycogen synthesis. Our findings are somewhat similar to those reported in the work of the above mentioned authors. In the present study, PG treatment enhanced Akt phosphorylation in skeletal muscle of diabetic treated rats (Figure 3.4), thereby promoting glucose uptake and storage as glycogen.

e. Glycogen metabolism

Glycogen phosphorylase and glycogen synthase are the two important regulatory enzymes that catalyze the rate-limiting steps of glycogen breakdown and synthesis, respectively (Ferrer et al., 2003; Bouskila et al., 2010). Increased glycogen synthase activity has a stimulating influence on glycogen synthesis in the muscle. Previous studies have shown that skeletal muscle glycogen synthase activity is lowered in diabetic subjects studied under euglycaemic conditions (Kelley and Mandarino, 1990). In humans with type II diabetes, hyperglycaemia has been shown to reduce both GS activity (Thorburn et al., 1990) and glycogen synthesis (Shulman et al., 1990; Rothman et al., 1992). Evidence also suggests that GS activity is lowered in diabetic rats (Banu et al., 2013). Our findings mirrored those of the above mentioned authors. In the present study, the low activity of glycogen synthase enzyme in diabetic rats resulted in poor utilization of glucose as shown by the depressed synthesis of glycogen from glucose in the muscle (Figure 3.7 A and 3.10). PG significantly increased the activity of glycogen synthase enzyme in diabetic treated animals (Figure 3.7 A).

In the liver, GS activity was enhanced and the hepatic glycogen levels also increased after treatment (Figure 3.5 A and 3.9), this suggests that glycogen synthesis is impaired in
diabetes. Based on our present findings, it would seem appropriate to consider glycogen synthase activation and increased glycogen formation as one of the possible mechanisms by which PG exerts its hypoglycaemic effect. GP is an enzyme that occurs in two forms, phosphorylase a and b. It is responsible for the degradation of glycogen in a process called glycogenolysis. GP inhibition is one of the strategies that have been pursued for the development of new antidiabetic agents. The activity of glycogen phosphorylase a, which is expressed in human skeletal muscle correlated inversely with glycogen synthesis, (Munro et al., 2002). In the muscle, the activity of GP enhanced by diabetes was reduced after treating rats with PG (Figure 3.7 B). The same effects of PG on GP activity was observed in the liver (Figure 3.5 B).

Previous studies reported increased expression of the GP gene in the muscle of Type I diabetic humans and STZ-induced diabetic rats before treatment with insulin (Wallis et al., 1998). In the present study, PG reduced expression of GP in the muscle of treated diabetic animals (Figure 3.8 B) suggesting its hypoglycaemic potential through regulating glucose uptake into the muscle.

In the liver, the expression levels of GP were lower in untreated diabetic animals compared to non-diabetic and diabetic treated animals (Figure 3.6). It has been reported that the expression of phosphorylase mRNA was down-regulated in diabetic liver (Rao et al., 1995). It was also reported that the rate of degradation of phosphorylase mRNA was faster in diabetic hepatocytes as compared with normal hepatocytes (Rao et al., 1995). The authors show that diabetes causes a decrease in the half-life of glycogen phosphorylase mRNA from 14 h in normal hepatocytes to 6.5 h in diabetic hepatocytes (Rao et al., 1995). In the present study, treatment of diabetic animals with PG for 14 days showed a slight increase in GP expression. This may suggest that the effect of PG on the expression of hepatic glycogen phosphorylase is mediated through the stabilization of its mRNA levels.

Sub-chronic studies on GS expression in the muscle showed that the expression of GS was depressed in diabetic animals and treatment of diabetic animals with PG caused a dramatic rise in GS expression to levels beyond control levels. Some studies have provided evidence for the impairment in the activation of glycogen synthase in diabetes (Hers 1976; Nuttal, 1972). Our findings showed that following PG treatment in diabetic animals, GS activity was enhanced.
Storage of glucose as glycogen represents a major muscle of energy storage and also protects against hypoglycaemia (Newgard et al., 2000) and its rate of synthesis appears to be regulated by the enzyme glycogen synthase (Bogardus et al., 1984). Previous studies reported that for optimal glycogen deposition to occur, the active phosphorylated form of GP must decrease (Brau et al., 1997). Our findings revealed that PG was able to restore the depleted liver and muscle glycogen reserves in treated diabetic animals (Figures 3.9 and 3.10).

f. Lipid homeostasis

This study also investigated the anti-hyperlipidemic potential of PG in-vivo and its effect on hormone sensitive lipase enzyme followed by assessment of changes in levels of serum lipid profile. The adipocyte takes up dietary lipid which is assembled into triglyceride for long term storage. One of the disorders of lipid metabolism (Savage et al., 2007) is caused by inability of the adipose tissue to store lipid resulting in accumulation in other tissues (Bluher, 2009). Lipolysis in adipose tissue is controlled by HSL (Schweiger et al., 2006). Insulin plays a crucial role in lipid metabolism, increasing lipid synthesis in liver and fat cells, and attenuating fatty acid release from triglycerides in fat (Shulman, 2000). Insulin inhibits lipolysis in adipocytes, primarily through inhibition of the enzyme HSL (Anthonsen et al., 1998). The activity of the lipase can be inhibited through reductions in cAMP levels, which triggers a cAMP-specific phosphodiesterase in fat cells (Kitamura et al., 1999). Insulin reduced the phosphorylation of HSL.

Klannemark and colleagues (1998) suggest that HSL is involved in the polygenic background of obesity and type II diabetes. It also plays a critical role in regulating cellular lipid homeostasis. Increased mobilization of fatty acids from adipose tissue stores in diabetic patients leads to high levels of free fatty acid; this condition promotes the development of ketoacidosis, one of the complications of type I diabetes (Foster and McGarry, 1983).

Previous studies revealed that fat cells isolated from STZ-induced diabetic rats show increased maximal lipolytic response to agents acting to elevate HSL mRNA and protein levels (Sztalryd and Kraemer, 1995). Treatments of rat adipocytes with lipolytic agents (like noradrenaline) increased phosphorylation of HSL (Belfrage et al., 1980) but when treated with Insulin (anti-lipolytic agent), HSL showed a decline in phosphorylation (Nilsson et al., 1980).
Previous studies have shown that lack of HSL in liver causes cholesterol ester accumulation which leads to reduced cholesterol ester hydrolase activity (Sekiya et al., 2008). This suggests that by reducing HSL activity, enzymatic hydrolysis of cholesterol esters and their products such as cholesterol and fatty acids is reduced in the liver. On day 14, post treatment with PG, assessment of PG treatment on HSL activity in both liver and adipose tissue was carried out. In the liver, HSL activity in untreated diabetic animals was the highest (Figure 3.11). Treatment with PG depressed HSL activities for both diabetic and non-diabetic treated animals as shown in Figure 3.11. In adipose tissue, the result obtained mirrored that of the liver as shown in Figure 3.12.

Increases in cholesterol and triglyceride levels were reported in STZ-induced diabetic rats (Khan et al., 1995; Mitra et al., 1995; Sachdewa and Khemani, 2003; Steinberg, 2004), which may lead to the development of cardiovascular and cerebrovascular diseases. The results in Table 3.2 show the level of total cholesterol in the serum after the 14 day sub-chronic study. The level of total cholesterol was slightly higher in untreated diabetic animals compared to untreated normal animals. PG treatment slightly reduced total cholesterol levels for both diabetic and non-diabetic treated animals.

Carr and colleagues (2000) reported that elevated TG, LDL-c and TC with reduced HDL-c will promote the development of atherosclerosis and cerebrovascular disorders. The results in Table 3.2 show serum triglycerides levels following 14 days of treatment with PG. In diabetic treated animals PG reduced the level of triglycerides but the difference was not significant when compared to that of untreated diabetic animals.

Subjects with diabetes have a high risk for developing atherosclerosis and glycated LDL plays a vital role in promoting cardiovascular disease prevalence in this population (Calvo, 1997). LDL carries about 60-70% of serum cholesterol and transports it from the hepatocytes to other cells of the body (Antonopoulos, 2002).

Elevated levels of blood sugar increase the rate at which glycation reactions occur (Singh et al., 2014). Glycated LDL impairs endothelial function (Nivoit, 2003), enhances oxidative stress and inflammation in muscle cells (Toma, 2009) and also contributes to the atherogenic process (Calvo, 1997). Studies have reported that green tea leaves which contain oleanolic acid and 2α-hydroxyursolic acid reduce LDL (Tinahones et al., 2008). In the present study we also observed that PG treatment reduced LDL levels in diabetic rats.
The results in Table 3.2 show serum atherogenic index after the 14 day sub-chronic study. Atherogenesis is a process of developing atheromatous plaques in the walls of arteries. Increased levels of LDL and VLDL causes atherogenesis (National Institutes of Health; National Heart, Lung and Blood Institute, 2008). In diabetic treated animals, PG caused a reduction in atherogenesis. AI was significantly higher in untreated diabetic animals compared to non diabetic animals.

Studies have shown that oxidised LDL in particular plays an important role in the development of cardiovascular disease (Reaven and Witzim 1996; Steinberg, 2008). The results in Table 3.2 shows that the serum cardiac risk factor following 14 days of treatment with PG was significantly higher in untreated diabetic animals compared to non-diabetic, non-diabetic treated and diabetic treated animals.

HDLs are small, dense lipoprotein particles, and they reduce invasion and accumulation of white blood cells (WBC) within the walls of arteries that may lead to atherosclerosis (Ross and Ross, 1999). Previous studies reported that consumption of pomegranate polyphenols significantly reduced LDL-cholesterol levels while maintaining HDL levels in subjects with elevated cholesterol levels (Esmailzadeh, 2006). Our study identified triterpenoids and phenolic compounds in guava extract which reduced LDL-cholesterol levels and increased HDL levels. Thus phenolic compounds could be implicated in these effects.

Previous studies have also reported that chemicals in guava increased serum high density lipoprotein cholesterol level (Singh et al., 1992). In the present study, treatment of diabetic rats with PG decreased serum triglyceride, total cholesterol, cardiac risk factor, atherogenic index, and LDL-cholesterol with simultaneous increase in serum HDL-cholesterol results which suggest that PG can reduce risk of cardiovascular and cerebrovascular diseases.

g. Biomarkers of tissue damage

In a diabetic state, there is elevation in the activities of ALT, AST and LDH enzymes. The serum levels of ALT were significantly increased in the untreated diabetic animals, PG treatment of diabetic animals reduced serum levels of this enzyme. Ogueri et al. (2014) also reported the same effect of PG on serum ALT levels in alloxan induced diabetic rats. In our present study there was an increase in AST levels in the treated diabetic group compared to diabetic control group. An increase in AST levels has also been reported in diabetic animals treated with an acetone fraction of Senna singueana stem bark (Ibrahim and Islam, 2014) or
an aqueous extract of *Bridelia ferruginea* leaves (Aja et al., 2013). It was suggested that the increase may be due to cholastasis in which conjugated bilirubin is retained (Crook, 2006). A similar trend was observed for LDH levels in treated diabetic animals. In our present study, the beneficial effect of PG on ameliorating diabetic damage done to the pancreatic islets is evident from the improved morphology of pancreatic islets in PG treated diabetic animals. These results are similar to those observed with guava fruit extracts (Chin Siu et al. 2011).

**h. Phytochemical characterization**

Excessive production of free radicals within the living system can generate reactive oxygen and nitrogen species, leading to a condition called oxidative stress (Valko, 2006). Many diseases such as atherosclerosis, hypertension, and cancers have been shown to be mediated by oxidative stress (Tiwari, 2001). Previous studies reported that antioxidants protect the cells against the deleterious effects of free radicals (Lien et al., 2008). There are several ways by which the body protects itself against the toxic effects of free radicals. Firstly, through endogenous antioxidants (Halliwell, 2007) and secondly, through exogenous antioxidants provided through foods or supplements (Willcox et al., 2004).

Our findings showed that *Psidium guajava* aqueous extract contain free radical scavenging phytochemicals that might have the potential to inhibit free radical accumulation which usually build up due to chronic hyperglycaemia.

GC-MS analysis of the aqueous extract of the leaves of *Psidium guajava* was carried out in order to know the bioactive-compounds responsible for the observed antidiabetic effect. In the present study, guavanoic acid, oleanolic acid, protocatechuic acid, benzene-1,2-diol, 2'-O-methyl guanosine, 5-Bromo-8-(5-nitrosalicylideneamino)quinoline hydrochloride and 2α-Hydroxyursolic acid were identified.

Previous authors reported the presence of guavanoic acid, oleanolic acid, protocatechuic acid, 2α-Hydroxyursolic acid in PG leaf and fruit (Okuda et al., 1984; Begum et al., 2002a; Begum et al., 2002b; Begum et al., 2004).

Protocatechuic acid was reported by Thaipong et al. (2005) to exert antioxidative activity. Oleanolic acid also possesses anti-hyperglycaemic activity (Wang et al., 2011). Previous studies reported the presence of benzene-1,2-diol in the rhizomes of *Bergenia purpurascens* (Bajracharya et al., 2011), stem of *Ficus religiosa* (Manorenjitha et al., 2013) and stem bark
of *Annona senegalensis* (Awa et al., 2012). It has been reported to exert potent activities against food borne bacteria (Kim and Lee, 2014) and also known to possess antioxidant activity (Cervellati et al., 2001). To our knowledge this is the first report to show the presence of benzene-1,2-diol, 2'-O-methyl guanosine and 5-Bromo-8-(5-nitrosalicylideneamino) quinoline hydrochloride in the aqueous extract of PG leaves.

Therefore, from our findings we can speculate that the observed antidiabetic effect might be mediated through the identified bioactive compounds present in the plant.
CONCLUSIONS

1. PG increased expression of muscle GS and restored its activity which was depressed by diabetes in both liver and muscle thereby increasing glycogen storage. PG decreased expression of muscle GP and reduced its activity which had been elevated by diabetes in both liver and muscle. Thus glycogen breakdown would be expected to be slowed down. The effects of PG on these two enzymes could be one mechanism responsible for the observed anti-hyperglycaemic effect of PG.

2. PG treatment decreased HSL activity in liver and adipose tissue, which was accompanied by reduced serum triglycerides, total cholesterol, LDL-cholesterol, cardiac risk factor, atherogenic index and increased HDL-cholesterol.

3. PG protected pancreatic islets in STZ-induced diabetic rats and caused the restoration of serum ALT activity elevated by diabetes. These findings suggest that PG also protects the liver from diabetic damage.

4. The observed antidiabetic and hypolipidemic activities of PG may be due to the synergistic effects of the identified phenolic and triterpenoid bioactive compounds, highlighting the potential advantage of using whole extract.

5. PG may be a promising antidiabetic agent.

FUTURE STUDIES

The western blot analysis for p-Akt at 15 and 60 minutes did not come out well, so this will be repeated. Future studies will also involve looking at the role of glycogen synthase kinases (GSK-3) in glucose homeostasis and also other pathways that have been implicated in the pathogenesis of diabetes such as protein kinase C and c-Jun-N terminal kinase.
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PUBLICATIONS


2. “Anti-diabetic potential of *Psidium guajava* leaf in streptozotocin induced diabetic rats” by Toluwani Tella, Samson Mukaratirwa and Bubuya Masola to be submitted shortly to Food and Chemical Toxicology journal.
Appendix 1: Glycogen standard curve

Appendix 1: For materials and method see Figure 2.12
Appendix 2: Folin-Lowry assay standard curve

Appendix 2: For materials and method see Figure 2.14.1
Appendix 3: Biuret assay standard curve

Appendix 3: For materials and method see Figure 2.14.2