

The effect of plant-derived oleanolic acid on selected markers of lipid metabolism and insulin signalling pathway in streptozotocin- induced diabetic rats

BY

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DECLARATION

I, Sandile Victor Cele hereby declare that the dissertation entitled

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is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE**DECLARATION 1 - PLAGIARISM**

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycaemia; this condition is caused by lack of insulin secretion (Type 1) and/or insulin resistance (Type 2). In diabetic patients; carbohydrate, protein and lipid metabolism is disturbed due to the lack of the body's ability to utilise glucose efficiently. Management of type 1 diabetes involves insulin therapy which may be inconvenient for patients. Therefore alternative methods for management of type 1 diabetes involving medicinal products are being investigated. This study is aimed at investigating the effect of OA on markers of lipid metabolism and on proteins of the insulin signalling pathway in Type 1 diabetic rats as this plant product has anti-hyperglycaemic effects. Male Sprague-Dawley rats were divided into two groups (diabetic and normal). In both groups the rats were further divided into four groups and assigned to treatment as follows: vehicle, insulin, OA and OA plus insulin. Oral glucose tolerance test was performed in fasted and non-fasted diabetic rats for 2 hours. In acute studies the effect OA following treatment of rats was evaluated at 15, 30 and 60 minutes. In sub-chronic studies rats were treated daily for 14 days. OA did not improve glucose tolerance in diabetic rats after 2 hours of administration. However, it enhanced blood glucose lowering effect of insulin and this was statistically significant in fasted rats. In acute studies OA enhanced the effect of insulin in normal and diabetic animals as AKT phosphorylation was increased when insulin was used in combination with OA. OA reduced the expression and activity of HSL in liver tissue after 14 days of treatment in both normal and diabetic rats. In adipose tissue, OA reduced the expression of HSL in diabetic rats. However, OA alone did not reduce the activity of HSL but when it was combined with insulin, a reduction of HSL activity was observed. OA administration had no significant effect on TGA and HDL-c levels but significantly ($p < 0.05$) reduced total cholesterol and LDL-c in diabetic rats. It had no significant effect on total cholesterol, and increased LDL-c levels in normal rats. Serum AST and ALT levels in diabetic rats were reduced by OA administration but this reduction was not statistically significant. The results of this study suggest that OA enhances the hypoglycaemic effect of insulin, improves lipid profile and possesses hepatoprotective effects. Lastly, OA independently increases AKT phosphorylation and decreases HSL expression and activity.

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LIST OF ABBREVIATIONS

ACAT	Acyl-CoA: cholesterol O-acyltransferase
AKT	Protein kinase B
ALT	Alanine aminotransferase
Apo	Apo-lipoprotein
AST	Aspartate aminotransferase
b.w.	Body weight
BSA	Bovine serum albumin
cAMP	cyclic adenosine mono-phosphate
HDL	High-density lipoprotein
HMG-CoA	3 - hydroxyl - 3 – methylglutaryl-CoA reductase
HSL	Hormone sensitive lipase
IRS-1	Insulin receptor substrate-1
LCAT	Lecithin-cholesterol acyl transferase
LDL-c	Low-density lipoprotein cholesterol
LPL	Lipoprotein lipase
ox-LDL	Oxidised low-density lipoproteins
OA	Oleanolic acid
OGTT	Oral glucose tolerance test
PDE3B	Phosphodiesterase 3B
Rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STZ	Streptozotocin
Tc	Total cholesterol
TGA	Triglyceride
VLDL	Very low-density lipoprotein

PREAMBLE

Diabetes mellitus (DM) is a condition characterized by hyperglycaemia and increased risk of cardiovascular disease. In diabetic patients lipid metabolism is impaired due to the lack of the body's ability to utilize glucose efficiently. DM is associated with increased plasma cholesterol and triglyceride concentrations, which may lead to the development and progression of micro and macro vascular complications such as neuropathy, nephropathy, cardiovascular and cerebrovascular diseases. Management of DM involves the use of synthetic hypoglycaemic agents such as sulphonylureas, biguanides, α -glucosidase inhibitors and thiazolidinediones. However, insulin has remained the primary means of treating this disorder. Medicinal plants such as *Ficus thonningii* and *Syzygium cordatum* have been reported to have hypoglycaemic effect in normal hosts. Efforts have also gone into isolating bioactive agents by which these medicinal plants lower blood glucose. These bioactive agents include triterpenes-like oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA), which has been shown to have hypoglycaemic effects. There are various mechanisms by which OA can exert its hypoglycaemic effect such as the retardation of glucose absorption across the gastrointestinal membrane. Some studies have shown that OA increases glycogen synthesis in diabetic rats and this hypoglycaemic effect has also been observed with insulin treatment. This compound may therefore exert its effects via insulin signalling pathway. There is evidence which suggests that OA exerts its hypoglycaemic effect by activation of some proteins involved in the insulin signalling pathway. Recent studies have shown that OA increases phosphorylation of AKT in type 2 diabetic mice while some studies show that it enhances the activity of insulin tyrosine phosphorylation in cell lines. Of importance in this study is to investigate whether orally delivered OA affects lipid metabolism via the insulin signalling pathway in adipose tissue and liver in Type1 diabetes in which insulin is absent. The study will also assess whether OA delivered orally has any toxic effect on liver by assessing changes in serum levels of marker enzymes.

CHAPTER ONE

INTRODUCTION

1.1 Diabetes mellitus

Diabetes mellitus (DM) is a condition characterized by hyperglycaemia and increased risk of cardiovascular disease, which is a major cause of death and disability in most countries (Ai *et al.* 2009). The incidence rate of DM is increasing with increasing levels of obesity and also with aging of the general population over the world (Kim *et al.* 2008). Currently, an estimated 220 million people worldwide have diabetes and this will increase to 300 million by 2025 (Li *et al.* 2005; Kondeti *et al.* 2010). The most common types of diabetes mellitus are type 1, type 2 and gestational diabetes. Type 1 diabetes is due to pancreatic β cell destruction that results in insulin deficiency (insulin-dependent diabetes mellitus). Type 2 diabetes mellitus (non-insulin dependent diabetes mellitus) is the more prevalent of the two types of diabetes and is associated with both impaired insulin secretion and insulin resistance caused by a high calorie-nutrition and obesity. Globally, type 2 diabetes accounts for greater than 90% of the diabetes cases (Kim *et al.* 2008). Gestational diabetes mellitus is common amongst pregnant women due to insulin resistance (Schoenfelder *et al.* 2006). In diabetic patients lipid metabolism is altered due to the lack of the body's ability to utilize glucose efficiently resulting in a disease state referred to as dyslipidaemia. In type 1 diabetes, tight glucose regulation usually corrects dyslipidaemia. However, in type 2 diabetes tight regulation or reduction of blood glucose does not correct dyslipidaemia (Papadakis *et al.* 2001). Most of lipid disorders are associated with insulin resistance and failure of suppression of hormone-sensitive lipase in adipose tissue which leads to increased hepatic VLDL production and increased TG levels (Papadakis *et al.* 2001; Ai *et al.* 2009; Sorenson *et al.* 2009).

1.2 Role of lipoproteins in lipid homeostasis

Lipoproteins are spherical particles composed of a central core of non-polar lipids and a surface monolayer of phospholipids, free cholesterol and apolipoproteins; and their function is to transport non-water soluble cholesterol and triglyceride in plasma (Koren *et al.* 1996;

Verges, 2009). Lipoproteins differ in their content of proteins and lipids and are classified according to their density as chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Chung and Wasan, 2004). They are also classified based on their apolipoprotein content on their surface which governs the fate of the particle (Koren *et al.* 1996; Chung and Wasan, 2004). A detailed description of lipid metabolism is given in figure 1 below.

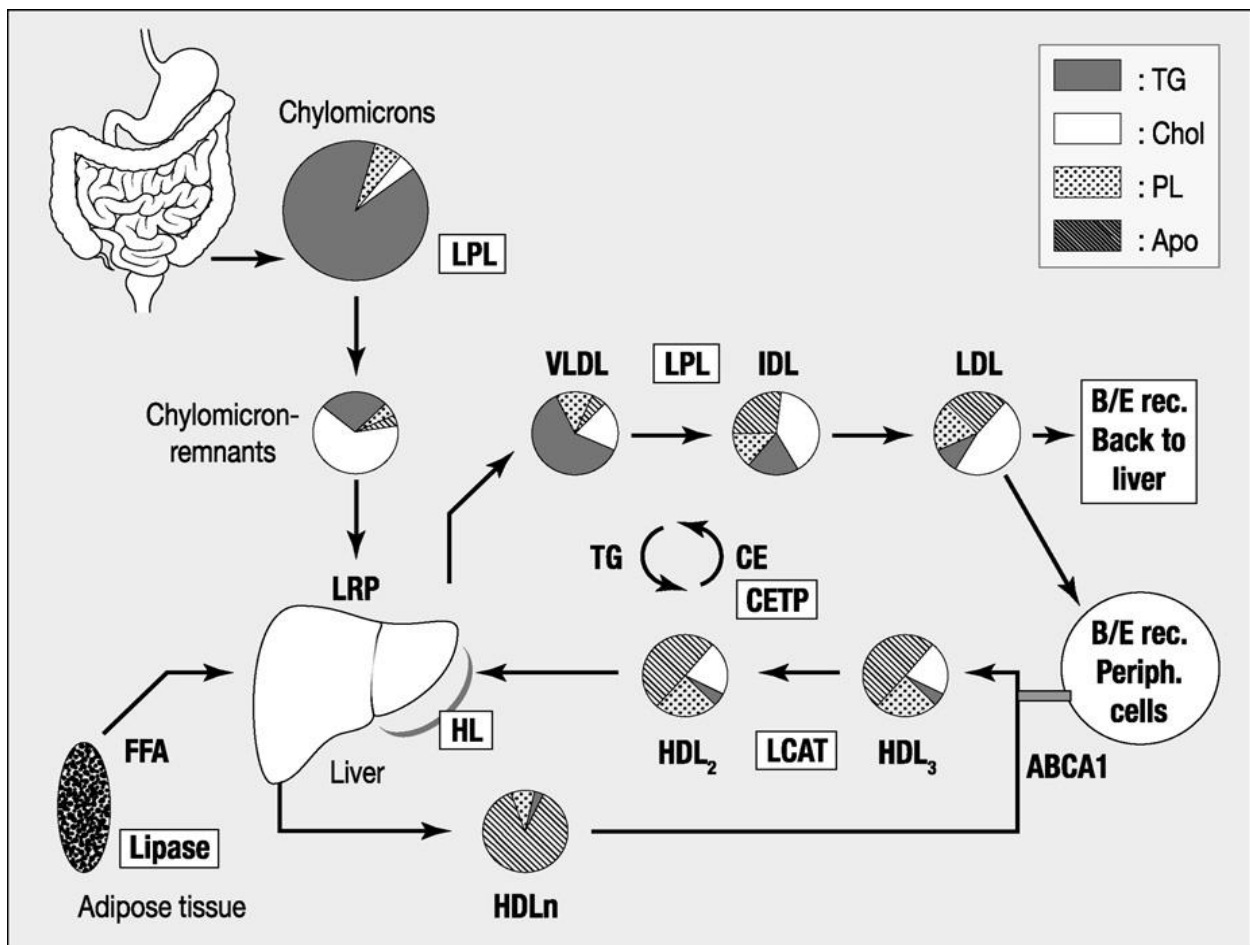


Figure 1: Lipoprotein metabolism. Lipoprotein lipase (LPL); very low-density lipoprotein (VLDL); intermediate lipoprotein (IDL); low-density lipoprotein (LDL); high-density lipoprotein (HDL); HL: hepatic lipase; cholesteryl ester transfer protein (CETP); lecithin-cholesterol acyl transferase (LCAT); B/E receptor (B/E rec.); LDL receptor related protein (LRP); free fatty acids(FFA); cholesterol (Chol); triacylglycerol (TG); phospholipids(PL); apolipoprotein (Apo); cholesterol esters (CE); ATP binding cassette A1 transporter (ABCA1). (Adapted from Verges, 2009)

1.2.1 Chylomicrons

Chylomicrons are the largest of the lipoprotein particles which are responsible for the transport of dietary triacylglycerol and cholesterol. They are composed of 85-90% triglyceride and cholesterol esters which are repackaged with apoB-48, apoA and phospholipids within the enterocytes (Chung and Wasan, 2004). Chylomicrons are then secreted by the intestinal mucosa into the lymphatic circulation before entering the bloodstream (Chung and Wasan, 2004; Verges, 2009). In the systemic circulation, they acquire cholesterol ester as well as apoC and apoE from HDL (Verges, 2009). The presence of apoC allows the particle to be recognised by the lipoprotein lipase (LPL) within the capillary beds of skeletal muscle and adipose tissue (Chung and Wasan, 2004). As a result, the triacylglycerols of chylomicrons are hydrolysed, releasing free fatty acids which are absorbed by nearby tissues for energy production and storage (Chung and Wasan, 2004). Residual particle known as chylomicron remnants return to the circulation and get cleared by the liver through receptor-mediated endocytosis; receptors on the surface of hepatocytes recognize apoE of the chylomicron remnant (Verges, 2009).

1.2.2 Very-low-density lipoprotein

VLDLs are triglyceride-rich plasma lipoprotein whose main function is to deliver lipids, mainly triglyceride from the liver to peripheral tissues (Barakat *et al.* 1996; Chung and Wasan, 2004; Verges, 2009). The lipid and protein components are synthesized in the liver, with apolipoproteins B100, C and E being the major protein components. Fatty acids used in triglyceride and phospholipid synthesis are derived from non-esterified fatty acids in the plasma, *de novo* synthesis from acetyl CoA and from hydrolysis of the lipids returned to the liver by other lipoproteins (Barakat *et al.* 1996). Triglyceride synthesis in the liver is influenced by insulin and glucagon levels and glycogen content in the liver (Barakat *et al.* 1996). In blood VLDL acquires cholesterol esters; apoC and apoE transferred from HDL to become mature VLDL particles (Chung and Wasan, 2004). These mature particles are hydrolysed by lipoprotein lipase (LPL) into VLDL remnants releasing apoE and apoC from the surface coat and transferring them back to HDL (Barakat *et al.* 1996; Chung and Wasan, 2004). This metabolic cascade leads to the formation of intermediate density lipoprotein

(IDL) particles, which can be removed from the circulation through interaction with the LDL receptor on hepatocytes or further metabolised to form LDL (Barakat *et al.* 1996; Verges, 2009).

1.2.3 Low-density lipoprotein

LDLs are plasma lipoproteins which are main carriers of unesterified and esterified cholesterol to tissues where they are internalized through the LDL-mediated pathway (Barakat *et al.* 1996). The cholesterol in the core of the LDL particle is esterified to fatty acids and this is delivered in a form of cholesterol esters (Chung and Wasan, 2004). LDL in the plasma arises from VLDL after it has lost the core triacylglycerides and some of the surface proteins except apoB-100 (Salter and Brindley, 1988; Barakat *et al.* 1996). ApoB-100s are essential for recognition of the LDL particle to its receptor and subsequent uptake into cells (Salter and Brindley, 1988). The majority of LDL is removed by the liver through the LDL receptor and other non-receptor-mediated pathways (Salter and Brindley, 1988).

The homeostasis of cholesterol concentration is due to three regulatory elements: 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA), the rate-limiting enzyme in cholesterol biosynthesis; acyl-CoA: cholesterol O-acyltransferase (ACAT) which re-esterifies excess intracellular cholesterol for storage; and expression of LDL receptors on the cell surface (Salter and Brindley, 1988). Furthermore, the regulation of serum LDL depends on the quantity of VLDL produced by the liver which then becomes converted to LDL and the proportion of VLDL remnants (IDL) that are removed by the LDL receptor in the liver (Salter and Brindley, 1988; Barakat *et al.* 1996). Therefore, the LDL receptor is the critical regulator of serum LDL concentrations both in rate of formation and rate of clearance of LDL particles.

1.2.4 High-density lipoprotein

HDL are heterogeneous lipoproteins containing several subclasses of lipoproteins, mainly HDL₂ and HDL₃ present in most species (Salter and Brindley, 1988). HDL is responsible for removal of cholesterol from peripheral tissues and for subsequent transport to the liver through a process known as 'reverse cholesterol' transport (Salter and Brindley, 1988; Chung and Wasan, 2004; Verges, 2009). HDL particles are secreted by the hepatocytes as small, lipid-poor lipoproteins that contain mostly apoA-I and receive phospholipids, apoCs and

apoE from chylomicrons and VLDL in the circulation (Chung and Wasan, 2004; Verges, 2009). Circulating HDL receives free cholesterol and phospholipids through the ATP-binding cassette A1 transporter from peripheral cells; this allows the transfer of free cholesterol and phospholipids from the cell cytoplasm to HDL particles (Barter *et al.* 2003). Within the HDL particles, free cholesterol is esterified by lecithin cholesterol acyltransferase (LCAT), which leads to the formation of HDL₃ particles (Chung and Wasan, 2004). Fusion of two HDL₃ particles leads to formation of one large HDL₂ particle. HDL₂ cholesterol ester-rich lipoproteins are degraded by lipases, leading to the formation of HDL remnant particles. These remnant particles get cleared by the liver after being recognised by scavenger receptors (Verges, 2009).

1.3 Lipid metabolism in diabetes

Alteration in lipid metabolism is more common in type 2 diabetes than in type 1 diabetes. The major abnormalities are high levels of TGAs, which are associated with reduced levels of HDL cholesterol (Batteridge, 1996; Maahs *et al.* 2005). Increased levels of TGAs also result in increased levels of circulating LDL cholesterol since both are derived from VLDL. High levels of circulating LDL cholesterol have been shown to reduce flow-mediated dilation of arterial vessels, resulting in endothelial dysfunction (Stratmann and Tschoepe, 2009) (refer to Fig 2). Endothelial dysfunction is considered as the early step in the development of atherosclerosis, and recruits inflammatory cells into the vessel wall and initiates plaque formation (Mehta *et al.* 2006). A dysfunctional endothelium facilitates oxidation of circulating LDL-c, monocyte entry, vasoconstriction, deposition of extracellular matrix and internalisation via expression of various adhesion molecules (Padakakis *et al.* 2001). Intravascular accumulation of LDL leads to its oxidation and induction of inflammation within the intima. Macrophage colony-stimulating factor causes monocytes to differentiate into macrophages which subsequently become foam cells which break down to form fatty streaks. These fatty streaks provide the beginnings of lipid core of the mature atherosclerotic plaque (Stratmann and Tschoepe, 2009). HDL is able to collect cholesterol from foam cells and transport it out of the artery wall for catabolism and excretion and it has antioxidant enzymes such as paraoxonase-1, which can inactivate pro-inflammatory phospholipids (Stratmann and Tschoepe, 2009). However, in diabetes mellitus there are low levels of

circulating HDL which perpetuates the formation of the plaque which leads to development of cardiovascular diseases (Williams, 2012).

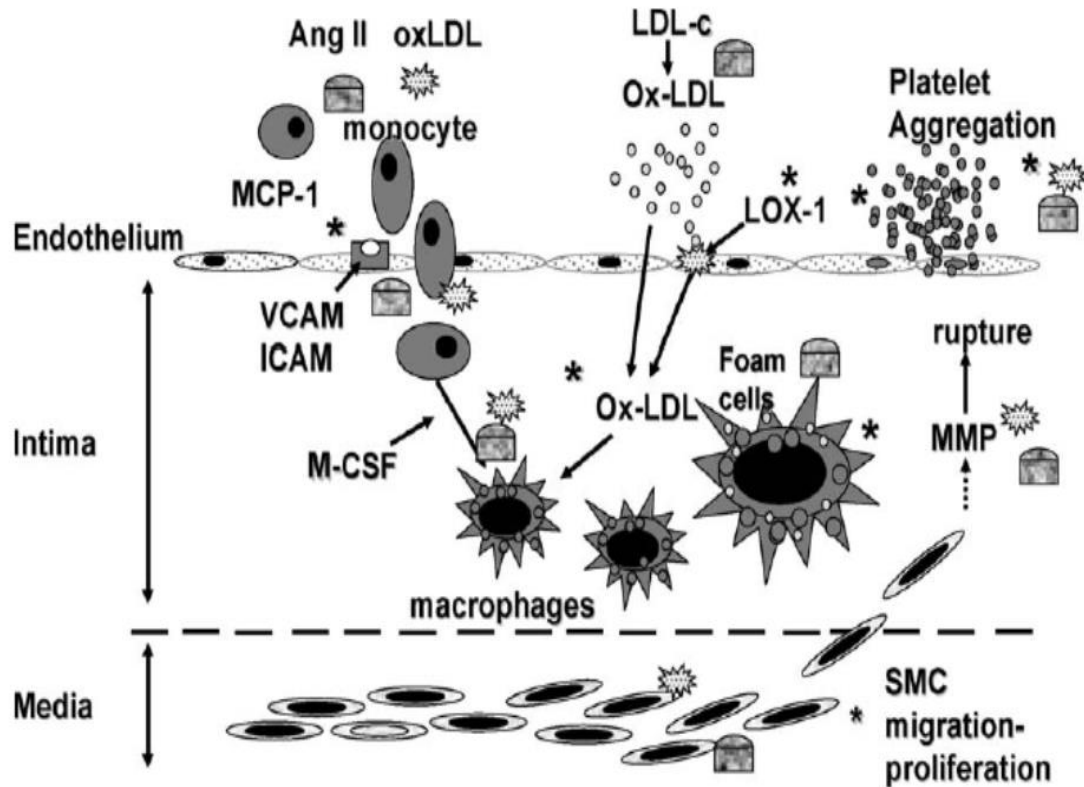


Figure 2: Postulated steps in atherosclerosis. Oxidised low-density lipoproteins (ox-LDL); Low-density lipoprotein cholesterol (LDL-c); leptin-like oxLDL receptor (LOX-1); Macrophage colony-stimulating factor (M-CSF); intracellular adhesion molecule (ICAM); Angiotensin II (Ang II); Monocyte chemoattractant protein-1 (MCP-1); Vascular cell adhesion molecule (VCAM); Matrix metalloproteinases (MMP) (Adapted from Mehta *et al.* 2006).

1.4 Management of diabetes mellitus

The main aim of treating diabetes is to lower elevated blood glucose levels to normal levels. Insulin, a hormone responsible for lowering blood glucose has been the primary means of treating diabetes since its discovery in 1922 (De Meytes, 2004). There are also different hypoglycaemic agents such as biguanides, sulphonylureas, thiazolidinediones and alpha-glucosidase inhibitors that have been developed to manage diabetes (Oiknine and Mooradian,

2003). Some of these hypoglycaemic agents are used in combination with insulin for maximum control of blood glucose. These hypoglycaemic agents use different mechanisms to lower blood glucose (Refer to Figure 3).

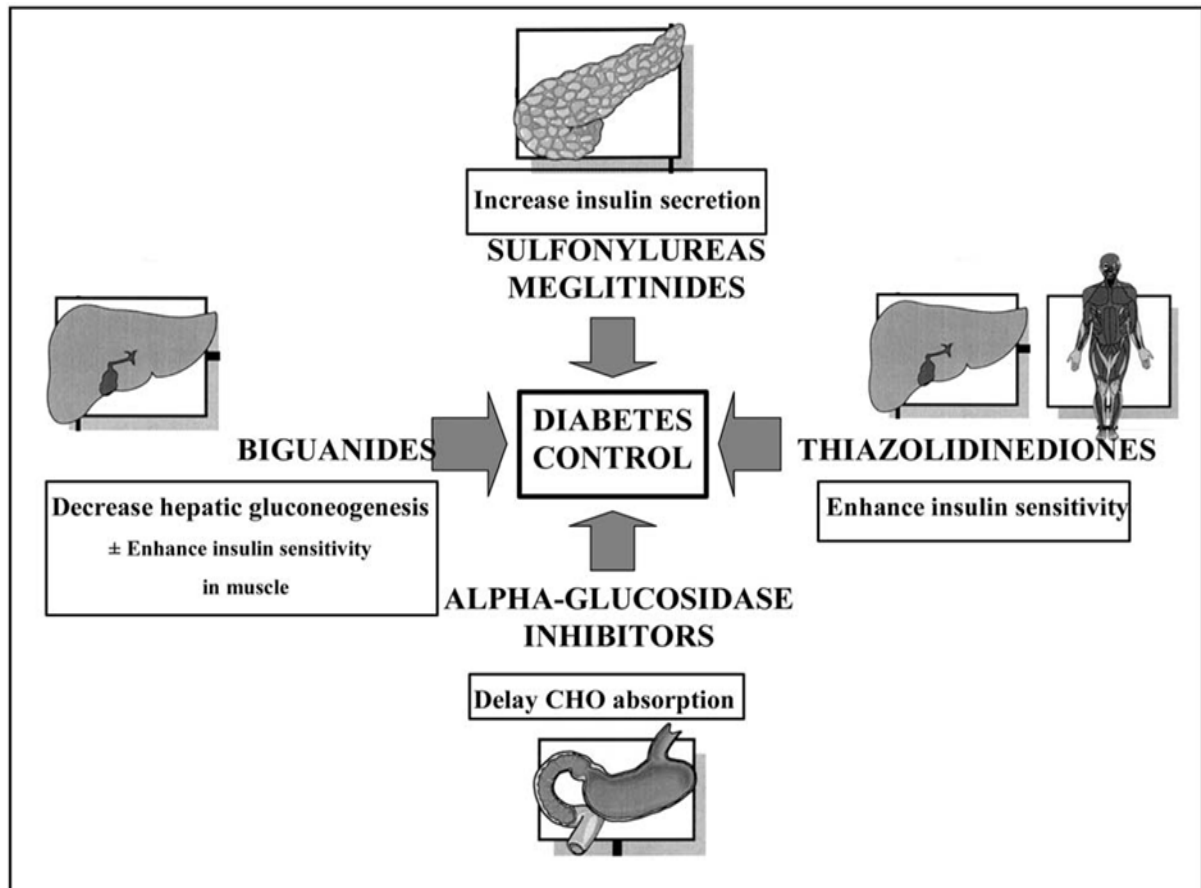


Figure 3: The mechanism of action of oral glucose-lowering agents. (Adapted from Oiknine and Mooradian, 2003).

1.4.1 Sulphonylureas

The first oral agents to be used for treatment of type 2 diabetes were the sulphonylureas (SFU) and were found to exert their hypoglycaemic effect by directly stimulating pancreatic β -cells (Anselmino, 2009; Donner, 2006). SFU bind to ATP-sensitive potassium channels and inhibit potassium efflux resulting in calcium influx through calcium channels and the rise in intra cytosolic calcium concentrations triggers the release of insulin (Anselmino, 2009; Oiknine and Mooradian, 2003). Thus SFUs increase basal and postprandial insulin secretion.

Administration of SFUs has been shown to lead to a decrease in glycosylated hemoglobin of 1% to 2% (Braunstein, 2003). However, these agents have been associated with weight gain and hypoglycaemia due to overstimulation of pancreatic β -cells and secondary failure due to beta cell exhaustion has been reported (Oiknine and Mooradian, 2003). There are also meglitinides drugs which have a SFU-like mechanism of action but they target a different binding site on pancreatic β -cells leading to a similar events triggering insulin release (Anselmino, 2009; Donner, 2006). The classes of drugs mentioned above are mostly used in type 2 diabetes. However, there are also commercially available drugs available for management or treatment of both type 2 and type 1 diabetes.

1.4.2 Biguanides

Currently, metformin is the only drug in this class available. The presence of insulin is necessary for this class of drugs to exert their hypoglycaemic effects (Anselmino, 2009; Donner, 2006). The molecular bases of how metformin decreases blood glucose are not well understood. It has been speculated that it enhances hepatic insulin response via inhibition of gluconeogenesis and to a lesser extent increasing the insulin sensitivity of peripheral tissues such as a muscle (Anselmino, 2009; Oiknine and Mooradian, 2003). The other effects of this drug include decrease appetite, food absorption and reduction in low-density lipoprotein (Setter *et al.* 2003). This drug is recommended in all patients with mild hyperglycaemia and overweight as it has been shown to decrease body weight at high doses (Anselmino, 2009). Clinical trials have demonstrated that metformin therapy reduces the risk of microvascular complication. However, side effects such as nausea and bloating have been reported (Oiknine and Mooradian, 2003).

1.4.3 Thiazolidinediones

Rosiglitazone and pioglitazone are representative of the thiazolidinediones (TZDs), which are a class of hypoglycaemic agents that decrease insulin resistance and improve insulin action (Braunstein, 2003). The mechanism of action of these drugs, like alpha-glucosidase inhibitors does not involve insulin secretion which makes them better candidates for type 1 diabetes treatment (Oiknine and Mooradian, 2003). TZDs enhance glucose uptake and utilization in peripheral tissues, especially skeletal muscle, liver and adipose tissue (Braunstein, 2003;

Oiknine and Mooradian, 2003). TZDs bind to and activate peroxisomal proliferator-activated receptor-gamma receptors, the activated receptors bind to DNA and increase transcriptional modulation of genes involved in carbohydrate and lipid metabolism (Oiknine and Mooradian, 2003). Some of the drugs in this class have been reported to increase HDL cholesterol and decrease triglyceride levels however their drawback is increasing LDL cholesterol which is associated with increased risk of coronary heart disease (Braunstein, 2003; Oiknine and Mooradian, 2003). TZDs are also associated with adverse effects such as weight gain and stimulation of adipogenesis (Setter *et al.* 2003).

1.4.4 α -Glucosidase inhibitors

One of the therapeutic approaches for preventing DM is to retard absorption of glucose via inhibition of α -glucosidase, a carbohydrate-hydrolysing enzyme in the digestive organ (Oiknine and Mooradian, 2003). α -Glucosidase inhibitors (AGIs) reversibly inhibit α -glucosidases, such as maltase and sucrase in the intestine, thus delaying the absorption of sugar from the gut and suppressing postprandial hyperglycaemia (Shai *et al.* 2010). α -Glucosidase inhibitors have other benefits, such as reducing triglycerides and postprandial insulin levels (Jong-Anurakkun *et al.* 2007). Acarbose and maglitol are known examples of competitive inhibitors of α -glucosidases. Some medicinal plants such as *Dillenia indica*, *Cratoxylum mangayi*, *Alstonia scholaris*, and *Cassia abbreviata* crude extracts have been shown to have an inhibitory effect on maltase and sucrase (Jong-Anurakkun *et al.* 2007; Shai *et al.* 2010).

1.4.5 Insulin

Insulin is the primary hormone responsible for controlling the transport, utilisation and storage of glucose in the body. Insulin is secreted by beta-cells of the pancreatic islets as a single chain, known as proinsulin (Pessin and Saltiel, 2000; Varshosaz, 2007). Proteolysis of proinsulin results in the removal of certain amino acids in the proinsulin and the connecting C-peptide providing the biological active polypeptide insulin (De Meytes, 2004; Varshosaz, 2007). This biologically active form of insulin is a monomer consisting of two chains, an A chain of 21 amino acids and a B chain of 30 amino acids linked by disulfide bonds (De Meytes, 2004). Insulin was first successfully isolated by Frederick Banting and Charles Best

in 1921 and was introduced into clinical practice in 1922, since then it has remained the primary means of treating type 1 diabetes (De Meytes, 2004; Varshosaz, 2007; Owens *et al.* 2003). Administration of insulin in the form of subcutaneous (s.c) injection has been the basis of insulin therapy since its introduction. Insulin formulated for s.c injection reaches its maximum activity at 2 to 3 hours following injection and the duration effect is said to be 6 to 8 hours (Varshosaz, 2007). Therefore treatment of diabetes with insulin requires regular injection of insulin and due to the inconvenience injections of insulin; various approaches have been attempted to administer insulin in non-injectable routes (Owens *et al.* 2003).

Insulin regulates glucose homeostasis in various ways, increasing the rate of glucose uptake primarily in skeletal muscle and adipose tissue and reducing hepatic glucose output via decreased gluconeogenesis and glycogenolysis (Pessin and Saltiel, 2000). In muscle and adipose tissue, insulin stimulates translocation of the glucose transporter GLUT4 to the surface thus clearing the circulating glucose. Insulin also affects lipid metabolism by increasing lipid synthesis in liver and adipose tissue, and inhibiting lipolysis in muscle and adipose tissue (Pessin and Saltiel, 2000). Patients on insulin treatment have shown non-compliance to medication due to discomfort of subcutaneous injection of insulin and insulin is not widely available in underdeveloped countries. On the other hand, plants which possess hypoglycaemic effect are available to all communities and are affordable.

1.4.6 Medicinal plant extracts

There has been a need to develop alternative methods to treat or manage diabetes such as medicinal plants due to their affordability by resource-poor people. Various medicinal plants with anti-diabetic properties have been used for several years worldwide (Drover *et al.* 2002; Ruzaidi *et al.* 2005; Musabayane *et al.* 2005). Prevalence of DM in India is estimated to be 1-5% with complications being the main cause of morbidity and mortality (Drover *et al.* 2002). Various indigenous plants have been used in the treatment of DM since the 6th century. These medicinal plants used in India include: *Aleo barbadensis*, *Artemisia pallens*, *Azadirachta indica*, and *Morus alba* and all have been shown to exert hypoglycaemic effect through different mechanisms (Drover *et al.* 2002). Ruzaidi *et al.* (2005) investigated the effect of Malaysian cocoa extract on blood glucose levels and lipid profiles in streptozotocin-induced diabetic rats. This extract was reported to increase body weight and decrease blood glucose concentrations through regeneration of β -cells damaged by streptozotocin (STZ) (Ruzaidi *et*

et al. 2005). Cocoa extract also improved lipid profiles by decreasing total cholesterol and triglyceride levels in hyperglycaemic group and these lipids have previously been shown to contribute to the development of vascular disease (Ruzaidi *et al.* 2005). The cocoa extract also increased levels of HDL cholesterol which suggest that it has some protective effect against hypercholesterolemia risks in diabetes (Ruzaidi *et al.* 2005). However, the exact mechanisms by which this extract lower blood glucose and improve lipid profile need further studies. Some medicinal plants have been shown to have effects on carbohydrate metabolic enzymes (Kondeti *et al.* 2010; Ngubane *et al.* 2010).

Pterocarpus santalinus bark has been used for treatment of diabetes and it has also been used for treatment of skin irritation, helminths, ulcers and eye disease (Kondeti *et al.* 2010). The effect of bioactive agent of *Pterocarpus santalinus* bark, ethyl acetate: methanol extract has been shown to decrease blood glucose concentration by improving insulin secretion, increasing liver glycogen levels in diabetic rats and enhancing hepatic hexokinase activity (Kondeti *et al.* 2010). Hexokinase is an insulin dependent key enzyme in the glycolytic pathway. Increased activity of hexokinase causes the increase in glycolysis, glycogenesis and glucose utilisation for energy production (Kondeti *et al.* 2010; Ngubane *et al.* 2010). In diabetic patients, there is increased activity of gluconeogenic enzymes due to activation or increased synthesis of the enzymes contributing to increased hepatic glucose production. Activation of gluconeogenic enzymes may also be due to decreased insulin levels given that under normal conditions, insulin suppresses these enzymes (Kondeti *et al.* 2010). Treatment of diabetic rats with *Pterocarpus santalinus* extract reduced the activity of these enzymes and this reduction can result in decreased concentration of glucose in the blood (Kondeti *et al.* 2010). This plant extract also increases glucose utilisation by the pentose phosphate pathway by increasing the activity of glucose-6-phosphate dehydrogenase (Kondeti *et al.* 2010).

Plants of the genus *Syzygium* have been used in the treatment of diabetes mellitus in rural communities of Asia, South America and South Africa. *Syzygium cordatum* leaf extract has been shown to decrease plasma glucose levels and hepatic glycogen in STZ-induced diabetic rats (Musabayane *et al.* 2005). Hypoglycaemic effect observed with *Syzygium cordatum* leaf extract did not involve insulin secretion since pancreatic β -cells were destroyed with STZ and this indicated that the extract may have increased glucose uptake via other mechanisms

(Musabayane *et al.* 2005). Spectroscopic analysis of *Syzygium cordatum* leaf extract has been shown to have oleanolic acid (OA), ursolic acid, methyl maslinate and methyl corosolate which can be involved in this plant's hypoglycaemic properties (Musabayane *et al.* 2005).

1.4.6.1 Oleanolic acid

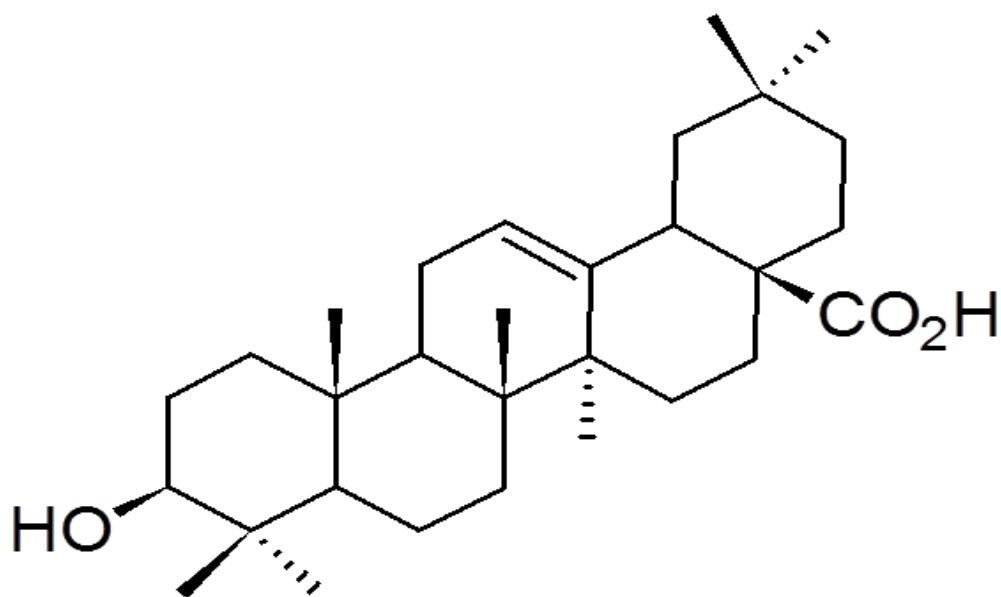


Figure 4: Chemical structure of oleanolic acid (International Union of Pure and Applied Chemistry, IUPAC).

Oleanolic acid (OA) (3 β -hydroxy-olea-12-en-28-oic acid) is triterpene found in a variety of plant species, this compound and its isomer ursolic acid have been found to have numerous biological properties with therapeutic potential. These compounds have been reported to possess anti-inflammatory, anti-tumourigenic, anti-viral and hepatoprotective effects in a dose dependant manner (Liu, 1995; Gao *et al.* 2009). Recently, OA has been shown to possess antimycobacterial activity against six drug sensitive and drug resistant strains and a synergistic effect was observed when OA was used in combination with isoniazid, rifampicin or ethambutol (Ge *et al.* 2010). Teodoro *et al.* (2008) reported that OA exerts its hypoglycaemic effects by directly stimulating pancreatic β -cells to improve insulin secretion

in isolated INS1 832/13 pancreatic β -cells. However, it was not shown whether OA uses the same mechanism to lower blood glucose *in vivo*. Great effort has gone into studying the mechanism by which OA exerts its hypoglycaemic effect. Recent studies done on plant-derived OA have given evidence that its hypoglycaemic effect does not involve insulin secretion and this bioactive agent has been shown to have a positive effect on kidney function (Mapanga *et al.* 2009). For bioactive agents to be used for diabetes treatment, they should have a positive effect on complications associated with DM. Mapanga *et al.* (2009) assessed the effect of *S. cordatum* leaf derived OA in renal function by evaluating its ability to increase urinary sodium outputs parameters and creatine clearance of STZ-induced diabetic rats as a measure of glomerular filtration rates, which determines the degree of electrolytes retention. In acute and chronic studies, OA significantly increased urinary sodium output as it increased glomerular filtration rate in STZ-induced diabetic rats in comparison to non-diabetic rats and this compound also decreased blood glucose and urinary glucose in STZ-induced diabetic rats (Mapanga *et al.* 2009). Recent studies have shown that OA increased hepatic and muscle glycogen concentrations of both non-diabetic and STZ-induced diabetic rats, and an additive effect was observed when OA was combined with insulin which showed that OA has the ability to exert its hypoglycaemic effects by enhancing insulin effect (Ngubane *et al.* 2010). The ability of OA to stimulate muscle glycogen synthesis in diabetic rats indicates that it has insulin mimetic effects as muscle glycogen is dependent on insulin.

OA has antioxidant activity as it has been shown to decrease malondialdehyde and increase superoxide dismutase and glutathione peroxidase activities of the liver and kidney in alloxan-induced diabetic rats (Gao *et al.* 2009). These enzymes function together to scavenge free oxygen species which can cause tissue damage. This compound has also been shown to reduce levels of serum cholesterol, triacylglycerols, LDL-c and increases HDL-c in type 2 diabetic rats; this could reduce the risk of diabetic patients developing cardiovascular diseases (Gao *et al.* 2009; de Melo *et al.* 2010). In diabetes, there is an increase in serum levels of enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase which are found elevated when there is hepatic damage. However, treatment of type 2 diabetic rats with OA reduces the levels of these enzymes (Gao *et al.* 2009; Ge *et al.* 2010). Jung *et al.* (2007) have shown that OA and UA reduces hyperglycaemia by enhancing the activity of insulin on tyrosine phosphorylation on insulin sensitive cell lines with the latter compound showing greatest enhancement and also enhancing the effect of insulin on GLUT 4 translocation. This evidence suggests that these

compounds may act as insulin receptor activators. Other mechanism by which OA reduces hyperglycaemia is by inhibition of α -glucosidase, an enzyme involved in the absorption of glucose from the small intestine (Ali *et al.* 2002). OA and UA diminish renal aldose reductase and glyoxalase activity, and down regulates their mRNA expression in diabetic rats (Wang *et al.* 2010). This is beneficial to diabetic patients by preventing or delaying progression of diabetic nephropathy by reduction of advanced glycation end product (Wang *et al.* 2010). Recently OA has been shown to reverse hyperglycaemia in type 2 diabetic mice four weeks post treatment, indicating that it may be a potential drug for sustained control of hyperglycaemia (Zeng *et al.* 2012). This sustained control of hyperglycaemia was associated with suppression of gluconeogenesis in the liver which is mediated by the AKT/FoxO1 axis. FoxO1 is a key transcriptional factor regulating hepatic gluconeogenesis and its phosphorylation by AKT leads to its degradation. Therefore increased levels of phosphorylated AKT suppress the expression of glucose 6-phosphatase a key enzyme for hepatic glucose production (Zeng *et al.* 2012). Thus, literature evidence suggests that the hypoglycaemic effect of OA involves phosphorylation of some of the proteins of the insulin signalling pathway.

1.5 Insulin signalling pathway

Insulin receptor (IR) is a tetrameric protein which consists of two identical extracellular α -subunits and two transmembrane β -subunits which have tyrosine kinase activity (Chung *et al.* 2004; Jung *et al.* 2007; Daisy *et al.* 2009). In response to increased blood glucose levels insulin is secreted by pancreatic β -cells. Insulin binds to the α -subunit and causes conformational change and stimulation of the receptor kinase activity via auto-phosphorylation of tyrosine residues in the β -subunits (Jung *et al.* 2007). The activated IR kinase phosphorylates substrate proteins, including the IRS family of proteins (Jung *et al.* 2007). This triggers the activation of phosphatidylinositol-3 kinase (PI3K) kinase signalling cascade which is one of the earliest steps in the insulin signalling pathway (Jung *et al.* 2007). The end product of the PI3K cascade [phosphatidylinositol (3,4,5) trisphosphate] activates protein kinase B (AKT) which stimulates glucose uptake in skeletal muscle, adipocytes and cardiac muscle tissue via translocation of GLUT-4 vesicles to the plasma membrane (Jung *et al.* 2007; Daisy *et al.* 2009). Activated AKT also inhibit lipolysis via activation of phosphodiesterase 3B (PDE3B) which inhibits cAMP accumulation and this inhibition results

in down regulation of the hormone sensitive lipase (HSL) which is the key in fat breakdown (Jung *et al.* 2007; Daisy *et al.* 2009).

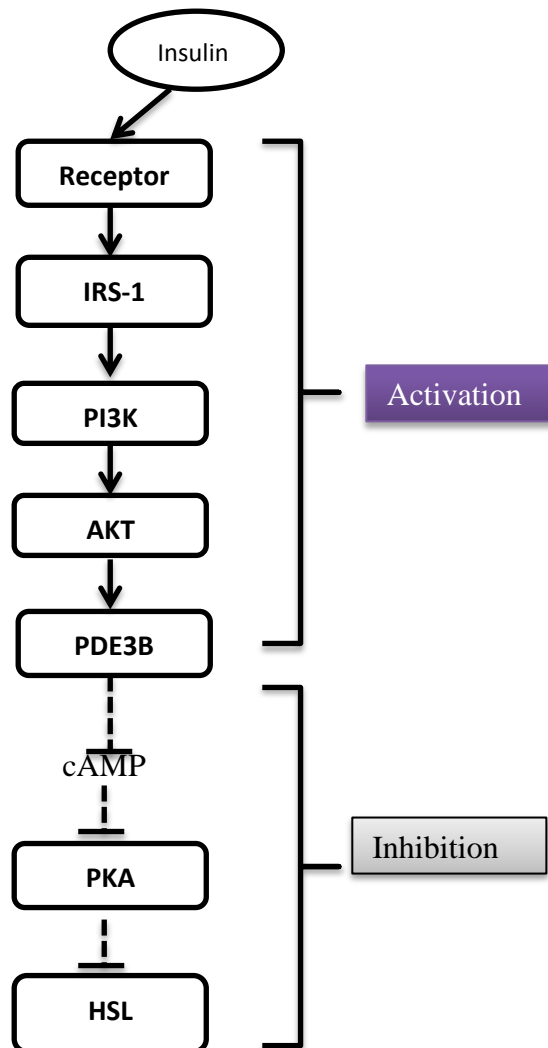


Figure 5: Schematic representation of phosphorylation cascade affecting lipid metabolism.

1.5.1 Insulin receptor substrate-1

Insulin receptor substrate-1 (IRS-1) is a phospho-protein which has a molecular weight of ~160-185 kDa and is a major substrate for insulin receptor kinase (Smith *et al.* 1996). IRSs (IRS-1 to IRS-4) contain pleckstrin homology (PH) domains, phosphotyrosine binding (PTB) domains at the amino-terminus, and several tyrosine phosphorylation sites at the carboxyl-terminus (Ye *et al.* 2002). IRS proteins can dock on the intracellular domain of the IR and mediate its signalling. Activation of the IR leads to recruitment and phosphorylation of IRS proteins which in turn recruit and activate other downstream signalling molecules, such as Shc and src-homology 2 (SH2) domain-containing proteins, including PI-3 kinase (Ye *et al.* 2002). IRS-1 is required in insulin stimulated mitogenesis and perhaps in insulin-stimulated glucose uptake (Smith *et al.* 1996).

1.5.2 Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinases (PI3Ks) have been classified into three major subfamilies according to their structure and the substrate specificity. Class I PI3Ks are activated by cell surface receptors and consist of two subfamilies, Class IA and Class IB, based on the associated adaptors (Jiang and Liu, 2008). Class IA PI3Ks are composed of heterodimers of a p110 catalytic subunit and a p85 regulatory subunit. There are three different isoforms of p110 catalytic subunit: p110 α , p110 β and p110 δ that are encoded by PIK3CA, PIK3CB and PIK3CD genes, respectively (Jiang and Liu, 2008). The p85 regulatory subunit also has three major isoforms: p85 α , p85 β and p55 γ that are encoded by PIK3R1, PIK3R2 and PIK3R3 genes, respectively (Jiang and Liu, 2008). The PIK3R1 gene codes for two shorter isoforms, p55 α and p50 α through alternative splicing. Class IB PI3Ks are composed of heterodimers of a p101 regulatory subunit and a p110 γ catalytic subunit. There are two other p101 homologues, p84 and p87PIKAP (PI3K γ adaptor protein of 87 kDa). Class IA PI3Ks can be activated by receptor tyrosine kinases (RTKs), while Class IB PI3Ks by G-protein coupled receptors (GPCRs) (Donahue and Fruman, 2004). Phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃] are the major products of class I PI3Ks generated from phosphatidylinositol-4,5-bisphosphate (Jiang and Liu, 2008; Donahue and Fruman, 2004). These end products activate protein kinase B.

1.5.3 Akt/Protein kinase B

Akt proteins, also known as protein kinases B (PKBs), comprises of 3 proteins, Akt 1, 2, and 3 have emerged as central control nodes in the regulation of an array of fundamental cellular functions such as cell proliferation, survival and programmed cell death, glucose metabolism, ribosomal function, transcription, and cell migration via phosphorylation of a multitude of substrates (Shtilbans *et al.* 2008). The Akt (PKB) pathway is activated by phosphatidylinositol 3-kinase (PI3-K) in response to insulin, growth factors, cytokines and cell stress, causing the recruitment of Akt to the plasma membrane (Timmons *et al.* 2009). Phosphatidyl inositol-3 kinase converts phosphatidyl inositol-4,5-bisphosphate (PIP₂) at the 3' position of the inositol ring to PIP₃. The presence of PIP₃ is required for activation of Akt, which occurs at the plasma membrane via Akt phosphorylation, mediated by 2 phosphatidyl inositol-3-phosphate dependent kinases, PDK1, which phosphorylates Akt 1 at Serine (Ser) 473 and PDK2, which phosphorylates Akt 1 at Threonine (Thr) 308. Phosphorylation at Thr 309 and Ser 474 activates Akt 2 (Hay, 2011). Phosphorylation at Thr 305 activates Akt 3. Phosphorylated Akt dissociates from the membrane and phosphorylates phosphodiesterase in the cytosol (Shtilbans *et al.* 2008).

1.5.4 Phosphodiesterase 3B

Phosphodiesterase 3B (PDE3B) belongs to a super family of cAMP and cGMP degrading enzymes and is expressed in cells important for glucose and lipid metabolism such as adipocytes, hepatocytes and pancreatic β -cells (Nilsson *et al.* 2006, Degerman *et al.* 2011). The PDE3 family consist of two members, PDE3A and PDE3B (Zmuda-Trzebiatowska *et al.* 2006, Chung *et al.* 2003). PDE3B role and regulation have been extensively studied in adipocytes where it has been demonstrated to have a key role in insulin-mediated inhibition of lipolysis. Insulin-induced phosphorylation and activation of PDE3B leads to increased hydrolysis of cAMP, lowering of protein kinase A (PKA) activity and thereby a decrease in PKA-dependent phosphorylation and activation of hormone sensitive lipase and lipolysis (Zmuda-Trzebiatowska *et al.* 2006). PDE3B has also been suggested to be involved in the regulation of insulin-induced glucose uptake, GLUT-4 translocation to the plasma membrane, and lipogenesis in adipocytes (Nilsson *et al.* 2006).

1.5.5 Hormone sensitive lipase

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that is highly expressed in adipose and steroidogenic tissues. The enzyme has broad substrate specificity, displaying hydrolytic activity against triacylglycerol, diacylglycerol and cholesteryl ester (Shen *et al.* 2001). Observations from HSL-null mice have shown that HSL is responsible for 50% of the neutral triglyceride lipase activity and all of the neutral cholesteryl ester hydrolase activity in white adipose tissue. Thus HSL plays an important role in regulating lipolysis and the release of fatty acids from adipose tissue (Shen *et al.* 2001). Hydrolysis of adipocyte triacylglycerols occurs through three consecutive reactions and is catalysed by two enzymes: HSL and monoglyceride lipase. HSL alone catalyses the hydrolysis of triacylglycerols and diglycerides, whereas the participation of monoglyceride lipase is required to obtain complete hydrolysis of monoglycerides (Holm, 2003).

Lipolytic stimuli increase lipolysis by activating adenylate cyclase and raising intracellular concentrations of cyclic AMP, with resultant activation of cyclic AMP-dependent protein kinase (PKA), which phosphorylates both perilipins and HSL. The phosphorylation of HSL is associated with an increase in hydrolytic activity of the enzyme and the translocation of HSL from the cytosol to the lipid droplet in some physiological settings (Shen *et al.* 2001; Greenberg *et al.* 2001). PKA has been shown to phosphorylate HSL at residues Ser563, Ser659, and Ser660, all of which reside in a 150-amino acid stretch, termed the regulatory module. The regulatory module is found within the C-terminal domain of HSL, which also contains the catalytic triad (Holm, 2003; Greenberg *et al.* 2001). *In vitro* PKA phosphorylation of HSL can be monitored as an increased activity against triglyceride and cholesteryl ester substrates. *In vivo*, PKA phosphorylation is known to result in translocation of HSL from a cytosolic location to a location at the lipid droplet (Holm, 2003) (Refer to figure 6).

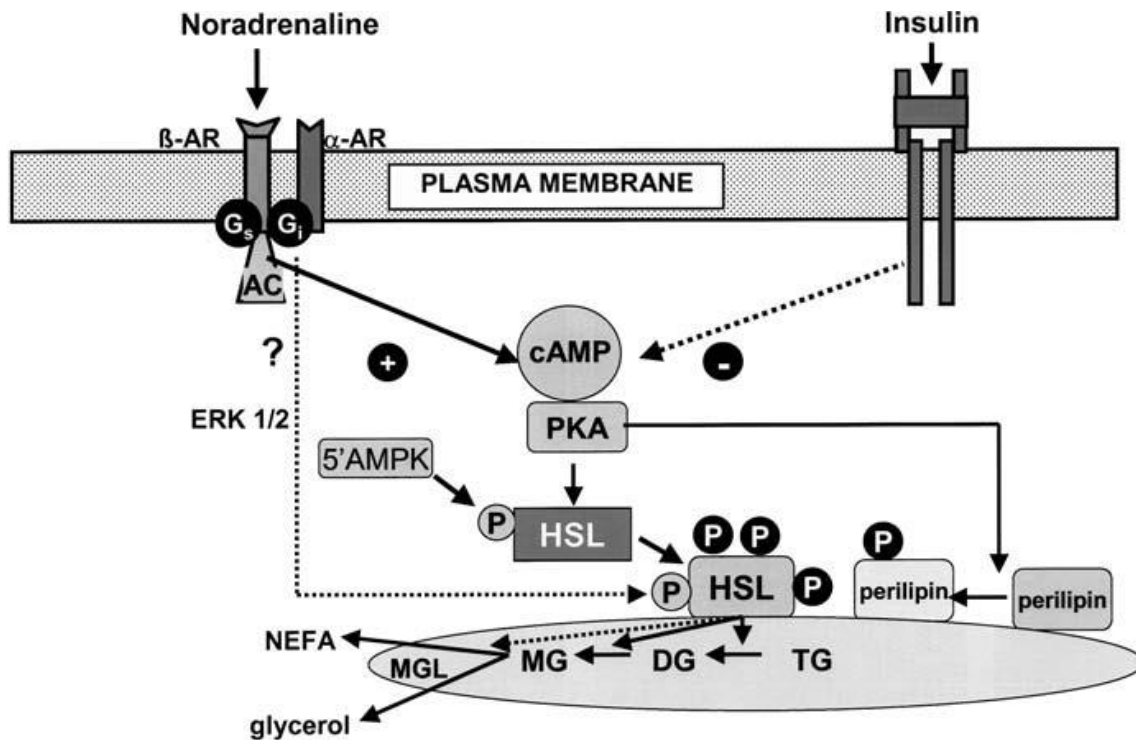


Figure 6: Hormonal control of adipocyte hydrolysis. α -adrenergic receptors (α -AR); β -adrenergic receptors (β -AR); G-protein (G); Adenylate cyclase (AC); Extracellular-signal-regulated kinase 1/2 (ERK1/2); cyclic adenosine mono-phosphate (cAMP); protein kinase A (PKA); 5'-AMP-activated protein kinase (5'AMPK); Hormone-sensitive lipase (HSL); Triglyceride (TG); Diglyceride (DG); Monoglyceride (MG); Monoglyceride lipase (MGL); Non-esterified fatty acids (NEFA) (Adapted from Holm, 2003)

1.6 Justification of the study

Previous studies have reported that the hypoglycaemic effects of *Syzygium cordatum* (Hochst.) [Myrtaceae] extracted mixtures of oleanolic acid (OA) and ursolic acid (UA) in streptozotocin-induced diabetic rats are in part mediated via increased hepatic glycogen synthesis (Musabayane *et al.* 2005). There are other possible mechanisms by which OA can exert its hypoglycaemic effect such as the retardation of glucose absorption across the gastrointestinal membrane (Ali *et al.* 2002). Ngubane *et al.* (2010) have also shown that OA increases glycogen synthesis in diabetic rats and this hypoglycaemic effect has also been observed with insulin treatment. This compound may therefore exert its effects via insulin signalling pathway. There is literature evidence which suggests that OA exerts its hypoglycaemic effect by activation of some proteins involved in the insulin signalling pathway. Recent studies have shown that OA increases phosphorylation of AKT in type 2

diabetic mice (Zeng *et al.* 2012) while some studies have shown that it enhances the activity of insulin tyrosine phosphorylation in cell lines (Jung *et al.* 2007). Therefore this study will investigate whether OA exerts its hypoglycaemic effect by increasing phosphorylation of IRS-1, AKT and HSL in type 1 diabetic rats. The study will also assess changes in the expression of HSL over the treatment period. This compound has also been shown to reduce levels of serum cholesterol, triacylglycerols, LDL-c and increase HDL-c in diabetic rats, suggesting that it has antihyperlipidemic properties (Gao *et al.* 2009; de Melo *et al.* 2010). However, these studies were conducted over a long period of time and were done on type 2 diabetic models. Therefore this study will also assess the effect of OA on lipids over a short period of time in type 1 diabetic rats. Diabetes is known to induce hepatic dysfunction which is evidenced by increase liver enzymes following its induction. This study will also investigate the effect of OA on aspartate aminotransferase (AST) and alanine aminotransferase (ALT), enzymes used as markers of liver damage.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Male Sprague-Dawley rats (250-300g body weight) bred and housed in the Biomedical Research Unit (BRU) of University of KwaZulu–Natal were used in this study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), 12 h light/dark cycles and had free access to water and standard rat chow (Meadows, Pietermaritzburg, South Africa). All experiments were performed with approval of the University of KwaZulu-Natal Research Ethics Committee guidelines for experimental animals (reference 080/11/Animal and 019/12/Animal).

2.1.2 Reagents

All chemicals were of analytical grade and were purchased from Sigma Aldrich chemicals and Bio-Rad laboratories except for tween 80, chloroform, heptane, methanol and diethyl ether which were purchased from Merck, South Africa. Phosphorylated antibodies (pAKT Ser473 and pHSL Ser563) were obtained from Cell Signalling Technology, Laboratory Specialist Services cc, South Africa whereas non-phosphorylated HSL (ab45422) was purchased from ABCAM, Biocom Biotech, South Africa. Secondary antibodies: Goat anti-rabbit IgG dylight 650 conjugated was purchased from Thermoscientific, Pierce Biotechnology (U.S.A) and anti-rabbit alkaline phosphatase conjugated IgG was obtained from Bio-Rad laboratories (S.A). Humalog kwikpen insulin from Lilly (S.A.), radioactively labelled cholesterol oleate [9,10-3H] from BIOTREND Chemikalien GmbH , Germany and oleanolic acid (OA) from Shaanxi King Stone Enterprise Company Ltd (China) were used in this study.

2.2 Methods

2.2.1 Dissolution of OA

Three different concentrations of dimethyl sulphoxide (DMSO) [10 %; 30 %; 50 %] in physiological saline were used to determine the solubility of OA. 100 mg of OA was first suspended in different concentrations of DMSO and diluted down with saline in a glass vial, after vigorous stirring the vials were left undisturbed for 2 hours at room temperature. The suspension of OA was then assessed after the 2 hour incubation period. The concentration of DMSO that gave the best OA suspension was used in subsequent studies.

2.2.2 Experimental design

Male Sprague-Dawley rats were divided into two groups (diabetic and normal). In both groups the rats were further divided into four groups and assigned to treatment as follows:

Group 1: Normal control (NC) treated with vehicle (50 % DMSO in saline)

Group 2: Normal insulin (NI) treated with 4 unit/kg b.w. insulin

Group 3: Normal OA (NO) treated with 80 mg/kg b.w. OA

Group 4: Normal OA + insulin (NOI) treated with OA plus insulin

Group 5: Diabetic control (DC) treated with vehicle (50 % DMSO in saline)

Group 6: Diabetic insulin (DI) treated with 4 units/kg b.w insulin

Group 7: Diabetic OA (DO) treated with 80 mg/kg b.w. OA

Group 8: Diabetic OA + insulin (DOI) treated with OA plus insulin

2.2.3 Induction of diabetes mellitus

Diabetes mellitus was induced as previously described by Daisy *et al.* (2009), male Sprague-Dawley rats were given a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg) dissolved in cold freshly prepared 0.1 M citrate buffer (pH 4.5). Animals that exhibited blood glucose concentration of ≥ 18 mmol/L after one week were considered as stable diabetic animals and were used in the study. Control animals were injected with the vehicle citrate buffer.

2.2.4 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed on diabetic rats. Rats that were fasted overnight for 18 hours were divided into three groups of six animals each. Group 1: diabetic control; Group 2: diabetic rats treated with insulin; Group 3: diabetic rats treated with OA. Prior to the beginning of the experiment, blood was collected from the tip of the tail (time 0). And then rats in all groups were orally administered glucose (0.86 g/kg b.w). In groups in which the effect of insulin and OA was to be examined, glucose load was followed by subcutaneous and oral administration of insulin (4 units/kg b.w.) and OA (80 mg/kg b.w), respectively. Thereafter blood was collected at 15, 30, 60 and 120 minutes; and blood glucose was measured using a glucometer (Accu-Check Perfoma, Roche diagnostics, New Zealand). Another OGTT was performed as described above but rats were not starved and were not given a glucose load prior to receiving treatment.

2.2.5 Treatment and terminal studies

In the acute study, animals were treated once as indicated in the experimental design. The animals were then rapidly sacrificed after 15, 30 and 60 minutes. Immediately after being sacrificed, blood samples were collected into thrombin-coated tubes to obtain serum. The rats were then quickly dissected to collect epididymal adipose tissue and liver samples which were immediately snap frozen in liquid nitrogen and stored at -20°C . In sub-chronic study, the animals were treated once on a daily basis for 7 and 14 days. These animals were sacrificed at the end of the treatment period with blood; adipose and liver tissue being harvested and processed as described in the acute study.

2.2.6 Tissue homogenization for western blot analysis

2.2.6.1 Liver

Liver samples (8ml/g tissue) were prepared as described by Feres *et al.* (2010). Samples were homogenized in freshly prepared ice-cold buffer (100 mM Tris-HCl [pH 7.4], containing 1% Triton X-100, 100 mM of sodium pyrophosphate, 100 mM of sodium fluoride, 10 mM of ethylenediaminetetraacetic acid, 10 mM of sodium orthovanadate, 2.0 mM of phenylmethylsulfonyl fluoride, and 3 µg/ml aprotinin). Insoluble material was removed by centrifugation for 20 min at 12 000 x g at 4 °C using Beckman Coulter centrifuge (Avanti J-26 XPI). The resulting supernatant was collected into eppendorf tubes and kept at - 20 °C for further analysis.

2.2.6.2 Adipose tissue

The samples of adipose tissue (100 mg) were prepared as described by Alonso *et al.* (2005). The samples were homogenized at 4°C in 3 ml of lysis buffer (50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 1% Nonidet P40, 0.05% sodium deoxycholate, 1 mM sodium orthovanadate and 3 mM benzamidine). The extracts were centrifuged at 17 418 x g at 4°C for 20 min using Beckman coulter centrifuge (Avanti J-26 XPI). There were three layers observed after centrifugation; upper fatty layer, middle infranatant and bottom pellet. The infranatant was collected into eppendorf tubes and kept at - 20 °C for further analysis.

2.2.7 Western blot analysis

Similar-sized aliquots 40 and 120 µg (liver and adipose tissue) were subjected to reducing SDS-PAGE (10% gel). After loading the molecular weight marker (7 µl) and the samples, the proteins were subjected to electrophoresis at a voltage of 120 V for 90 minutes. The proteins were electro-blotted from the gel onto nitrocellulose membranes for 120 min at 4°C. Nonspecific protein binding to the nitrocellulose membranes was reduced by incubating the membrane overnight with blocking buffer (1 % BSA in PBS) at 4°C. The primary antibody (anti-pAkt Ser473 or anti-HSL ab45422) was diluted 1:1000 in PBS 0.1% tween 20 and overlaid on nitrocellulose membrane with shaking for 1 hour at room temperature. Thereafter,

the membrane was washed four times for 5 minutes each with PBS 0.1% tween 20. After the first washes, a secondary antibody (goat anti-rabbit IgG, dylight 650, Thermo scientific) was diluted in PBS 0.1% tween 20 and overlaid on the nitrocellulose membrane with shaking for 1 hour at room temperature. The membrane was washed four times for 5 minutes with PBS 0.1% tween 20. The membrane was then rinsed with PBS before viewing and capturing the image under fluorescence using a G:BOX-XR5 and Syngene Tools, Vrsn 4.03.00 software (Vacutec, S.A.). Western blot analysis of GAPDH protein (loading control) was performed as explained above except that 20 µg of liver tissue was loaded.

For phosphorylated HSL (pHSL Ser563), 120 µg of liver was subjected to SDS-PAGE (10% gel). Tween buffered saline – tween (TBST) containing 1% BSA was used as a blocking agent and to dilute the antibodies. The secondary antibody was anti-rabbit IgG conjugated to alkaline phosphatase. The secondary antibody binds specifically to the first antibody and the conjugated enzyme allows for detection of the protein of interest on the blot. TBST was used to wash the membrane after incubation with the antibodies. A substrate BCIP/NBT solution was used to develop colour. After colour had developed, a membrane was washed with water prior to capturing the blot image with a G:BOX-XR5.

2.2.8 Hormone sensitive lipase activity assay

2.2.8.1 Liver and adipose tissue homogenization

Hormone sensitive lipase activity assay was performed as described by Ylitalo *et al.* (2000) with minor modifications. Pieces of adipose tissue and liver (200 mg and 8 g, respectively) were homogenised in (3 ml and 8 ml) of a 50 mM Tris–HCl (pH 7.5) buffer containing 0.25 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol and protease inhibitors (10 µg/ml antipain and 3 mM benzamidine). The samples were then centrifuged at 17 418 x g at 4°C for 45 min using Beckman coulter centrifuge (Avanti J-26 XPI). There were three layers that resulted after centrifugation; upper fatty layer, middle infranatant and bottom pellet. The infranatant was collected into eppendorf tubes and kept at - 20 °C for further analysis.

2.2.8.2 Preparation of the substrate solution

To prepare 2.5 ml substrate solution, 1.8 mM cholesteryl[³H]oleate (S.A: 2.3×10^{-3} Ci/mmol) was emulsified with 5.6 mg of phosphatidylcholine/phosphatidylinositol (3/1 by weight) in chloroform. Thereafter, 2 ml of 0.1 M potassium phosphate (pH 7) was added and the solution was heated to 37°C to allow for evaporation of chloroform. The substrate suspension was sonicated 2 times for 2 minutes with 1 minute intervals using a sonic ruptor ultrasonic homogenizer (Omni international company, USA) at 40 % power. After the second sonication, 0.5 ml of 0.5g/ml BSA in 0.1 M potassium phosphate (pH 7) was added.

2.2.8.3 Activity assay

The activity of lipase was assayed as follows; a 30 µl aliquot of substrate solution was mixed with 5 µl of 50 mM EDTA, 5 µl of 5 mM DTT and 100 µl of sample and then incubated for 2hrs at 20°C with shaking at 100 cycles/min. The reaction was then terminated by addition of 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), and the radioactively labelled oleate was recovered by a one-step liquid-liquid partition system. The mixture was shaken vigorously for 20 sec and the centrifuged at 800 x g for 20 min. A 250 µl aliquot from the upper phase was used for scintillation counting. To 250 µl aliquot 1.8 ml of Fluka liquid scintillation fluid counter was added before counting.

2.2.9 Measurement of serum lipids and liver enzymes

The blood samples were left in ice for 2 hours after collection to allow them to clot. Thereafter, the samples were centrifuged at 3 000 rpm for 10 minutes to obtain serum using a bench centrifuge (Labofuge 200 – Heraeussepatech). Serum was used to determine the levels of lipids (Total cholesterol, triacylglycerols and high-density lipoprotein-cholesterol) and serum liver enzymes (AST and ALT) using a Labmax Plano Chemistry analyzer (Labtest Av. Paulo Ferreira da Costa, 600, Brasilia, Brazil). Low-density lipoprotein (LDL-c) levels were calculated using the Friedewald formula:

$$\text{LDL-c} = \text{Total cholesterol} - \text{HDL-c} - (\text{TGA}/5) \text{ (Friedewald, 1972).}$$

Principle of total cholesterol assay

A cholesterol reagent supplied by the manufacturer is used to measure the concentration of total cholesterol in a sample. In the reaction, cholesterol esters are hydrolysed by cholesterol esterase to yield free cholesterol and fatty acids. An enzyme cholesterol oxidase oxidises free cholesterol to cholestene-3-one and hydrogen peroxide. Finally, peroxidase catalyses the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a coloured quinoneimine product.

Principle of triglyceride assay

In this assay, a triglyceride reagent is used to measure the concentration of triglyceride in a sample. A lipase hydrolyses triacylglycerols in the sample into glycerol and free fatty acids. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase, horseradish peroxidase causes the oxidative coupling of 3, 5-dichloro-2-hydroxybenzenesulfonic acid with 4-aminoantipyrine to form a red quinoneimine dye.

Principle of HDL-c assay

In the measurement of HDL-c, the samples are first spun to precipitate the cholesterol. HDL cholesterol reagent is used to measure concentration of cholesterol in a sample. In the reaction, cholesterol esters are hydrolysed by cholesterol esterase to yield free cholesterol and fatty acids. An enzyme cholesterol oxidase oxidises free cholesterol to cholestene-3-one and hydrogen peroxide. Finally, peroxidase catalyses the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a coloured quinoneimine product.

Principles of aspartate and alanine aminotransferases assay

AST: catalyses the transfer of an amino group from L-aspartate to 2-oxoglutarate to form oxaloacetate and L-glutamate. The produced oxaloacetate is then quantitatively by the MDH – NADH reaction which oxidises NADH. The decrease in absorbance due to the oxidation of NADH to NAD is monitored at 340 nm. The rate of decrease in absorbance of the reaction mixture is directly proportional to the AST enzyme in the sample.

ALT: catalyses the transfer of an amino group from L-alanine to 2-oxoglutarate to form pyruvate and L-glutamate. The produced pyruvate is then determined quantitatively by the LDH – NADH reaction which oxidises NADH. The decrease in absorbance due to the

oxidation of NADH to NAD is monitored at 340 nm. The rate of decrease in absorbance of the reaction mixture is directly proportional to the ALT enzyme in the sample.

2.2.10 Protein determination

Protein determination was carried out using the biuret method for liver samples and the Lowry method for adipose tissue samples. The biuret assay for protein concentration was as described by Gornall *et al.* (1949) with modifications. To 2 ml of sample in a test tube, 3 ml of biuret reagent [12 mM copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 32 mM potassium sodium tartrate ($\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$), and 30 mM potassium iodide (KI) in 0.1 N NaOH] was added. The tubes were vortexed before incubation at 37 °C for 10 minutes. The tubes were then allowed to cool and absorbance was read at 540 nm against a blank containing buffer. Bovine serum albumin (BSA) of 0 – 100 µg was used to construct a standard curve from which protein concentrations of samples were extrapolated.

In adipose tissue, protein concentration was determined by the method of Lowry *et al.* (1951) with BSA as standard (0-100 µg). Samples were diluted using 0.1 N NaOH to 0.5 ml final volume, and 5 ml of alkaline reagent (100 volumes of 4% sodium carbonate in 0.1 N NaOH and 4% copper sulphate in 8% of sodium potassium tartrate) were then added and the mixture was incubated at 40°C for 15 minutes. The tubes were allowed to cool before adding 0.5 ml of Folin Ciocalteu reagent (diluted 1:2 with deionised water). The tubes were left standing at room temperature for 30 minutes and the absorbance was read at 600 nm.

2.3 Statistical analysis

All data were expressed as means \pm standard error of means (SEM). Statistical 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 Turkey-Kramer multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

CHAPTER THREE

RESULTS

This chapter presents the results as follows:

- a) Dissolution of OA
- b) Glucose tolerance
- c) Acute studies
- d) Chronic studies

3.1 Dissolution of OA

Oleanolic acid suspended better in 50 % DMSO in physiological saline. Therefore, 80 mg/kg b.w. of OA suspended with 50 % DMSO in physiological saline was used in subsequent studies.

3.2 Oral glucose tolerance test

The OA treatment did not decrease acute blood glucose levels in rats (Figure 7 below). This trend was observed in both starved and non-starved rats. The blood glucose levels started to decrease after 15 minutes following insulin treatment in non-starved rats. This decrease became significant after 60 minutes in rats that were not starved when compared to diabetic controls and OA treated rats. In rats that received glucose, insulin decreased glucose after 15 minutes and this decrease was only significant after 2 hours when compared to control animals and OA treated rats. Treatment of non-starved rats with insulin and OA showed blood glucose decrease that was similar to rats treated with insulin only. In starved animals, the effect of OA plus insulin was greater than that of treatment with insulin. This difference became significant after 30 minutes following treatment.

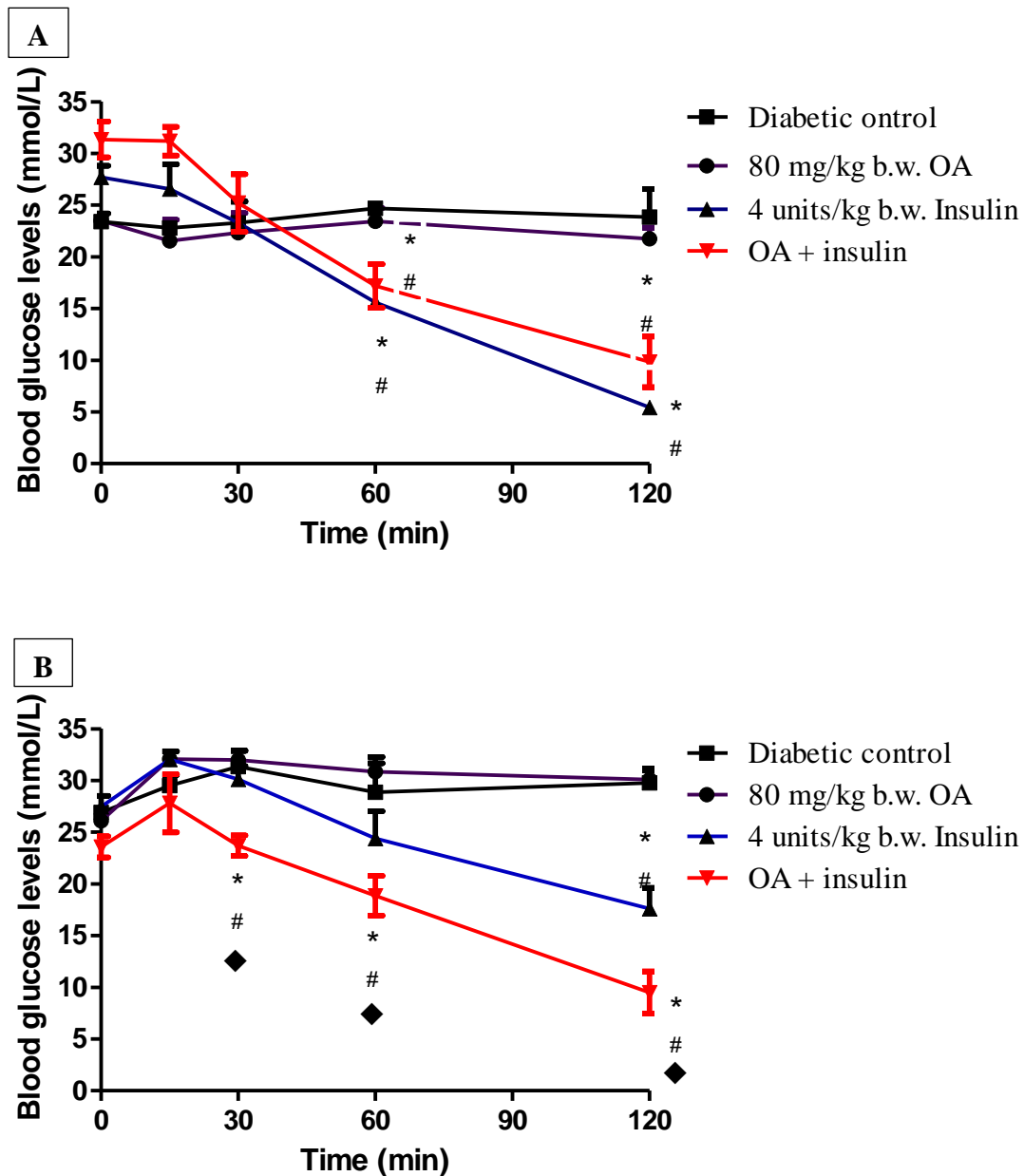


Figure 7: Acute effect of OA on blood glucose levels in diabetic rats. A: Non-fasted rats not given glucose load; B: Fasted rats given glucose load. Values are presented as means \pm SEM for four rats per group. * $p < 0.05$ by comparison diabetic control, # $p < 0.05$ by comparison with OA treated rats, ♦ $p < 0.05$ by comparison to insulin treated rats.

3.3 Acute studies

3.3.1 SDS-PAGE of liver and adipose tissue samples

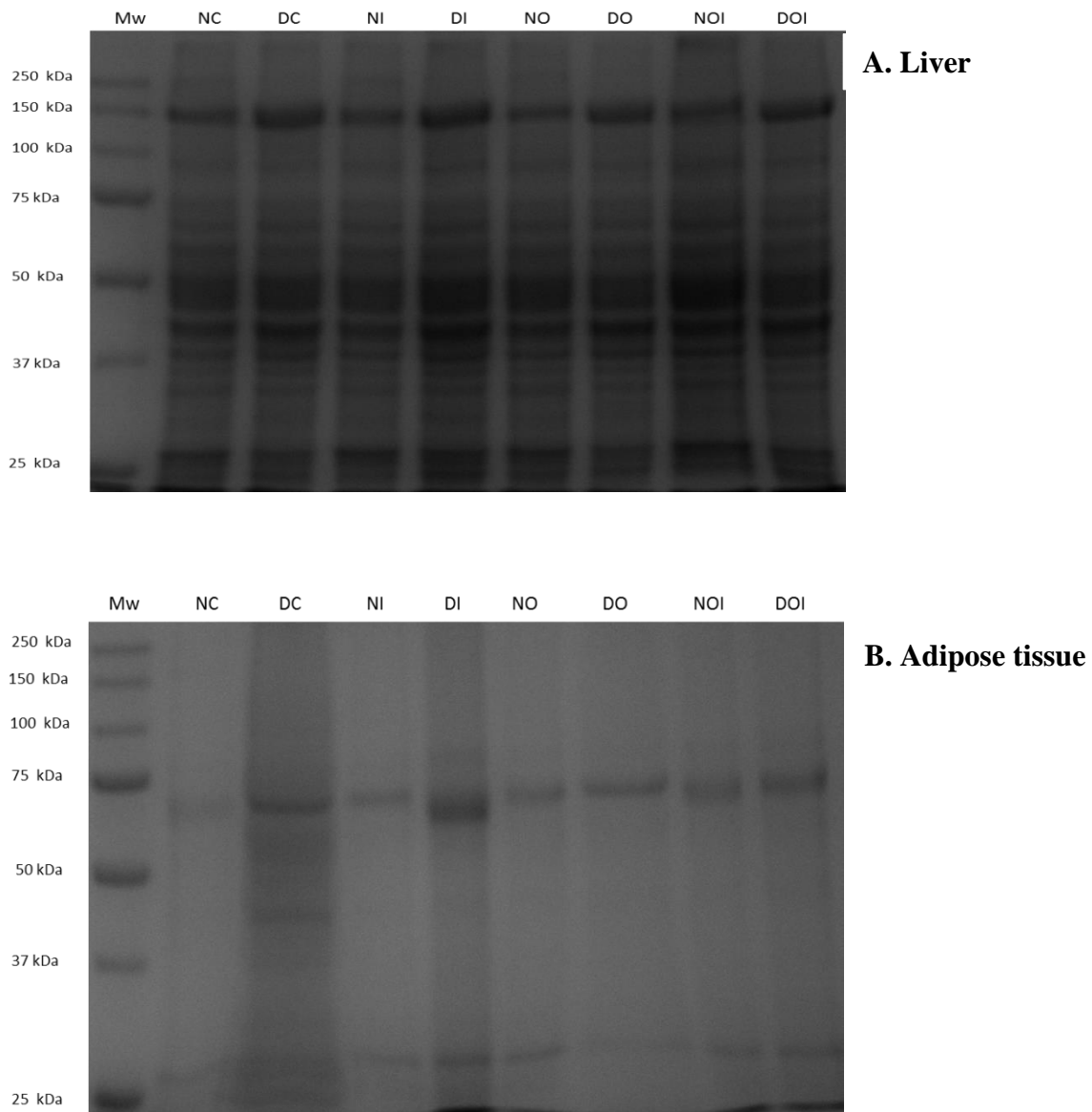


Figure 8: 10 % reducing SDS-PAGE gels of liver supernatant and adipose tissue infranatant (40 μ g and 120 μ g, respectively). Mw = Molecular weight; NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin.

The results in Fig 8 show the proteins that were present in liver and adipose tissue after the gels were stained with a coomassie stain. There were low proteins in the infranatant of adipose tissue when compared to the supernatant of liver tissue. In subsequent studies similar gels were prepared and probed with different specific antibodies of interest.

3.3.2 Expression of AKT and HSL liver and adipose tissue samples

Two samples from each group were randomly chosen and mixed for western blot analysis.

3.3.2.1 Effect of OA on phosphorylation of AKT

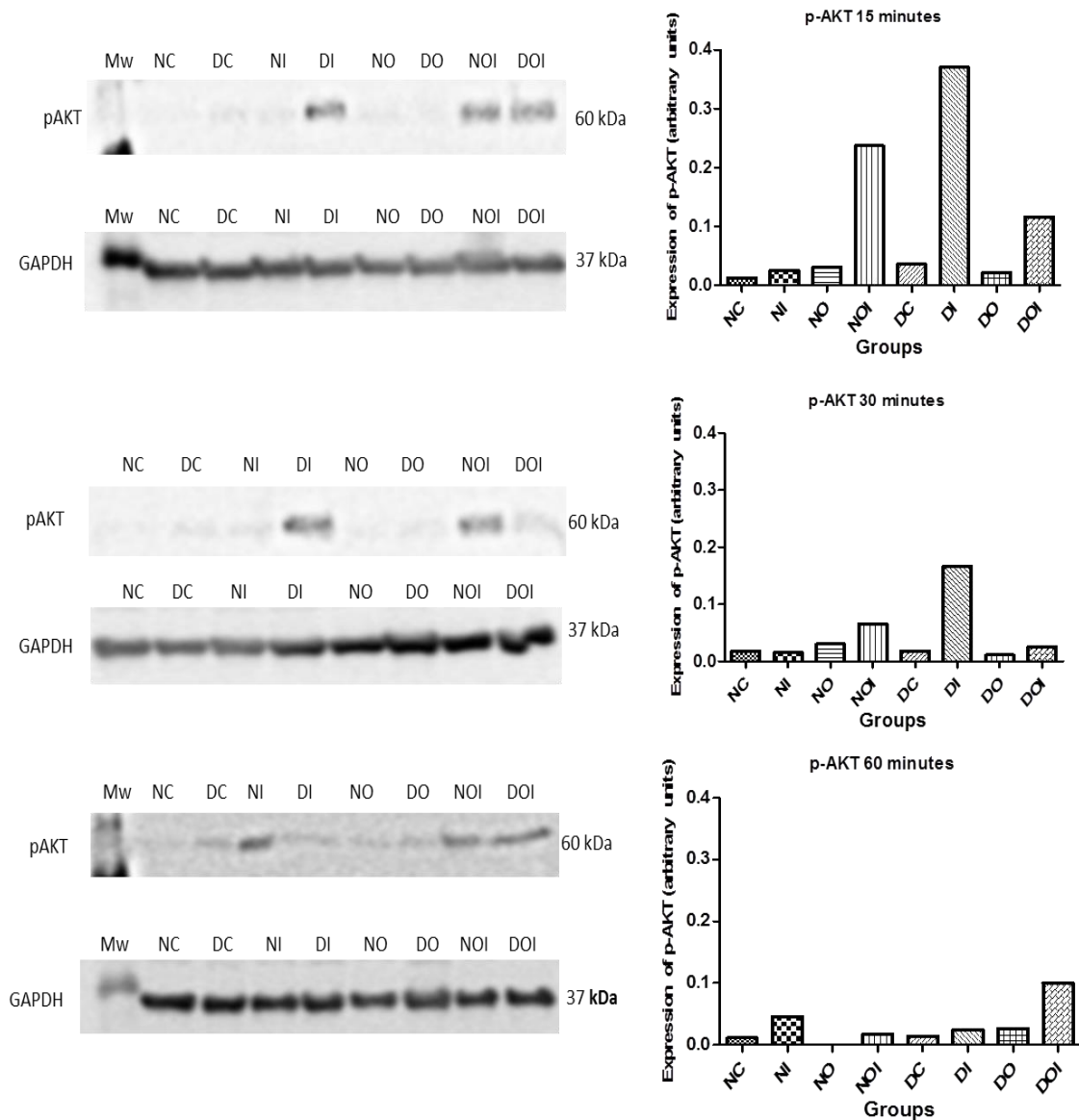


Figure 9: Effect of OA on phosphorylated AKT in liver 15, 30 and 60 minutes following treatment (40 μ g and 20 μ g protein was loaded for p-AKT and GAPDH, respectively). The values from densitometry of p-AKT were normalised to the levels of GAPDH protein (loading control) and were expressed as the mean-fold increase. Mw = Molecular weight; NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin. On the normalised data, non-diabetic and diabetic animals are grouped separately for simplicity.

The results of AKT phosphorylation in liver are shown in Fig 9. Fifteen minutes after treatment, the expression of phosphorylated AKT was high in diabetic animals treated with insulin, normal animals treated with OA plus insulin and in diabetic animals treated with OA plus insulin. This trend in AKT phosphorylation was observed 30 minutes after treatment but expression levels were lower. It was not observed in the DOI group in which the AKT band is distorted. In all other groups, the expression of phosphorylated AKT was very low. Sixty minutes after treatment, phosphorylated AKT expression was elevated in normal animals treated with insulin only and in diabetic animals treated with OA plus insulin; there was low expression of this protein in other groups. Of note is the gradual decrease in AKT phosphorylation in diabetic animals treated with insulin following 15, 30 and 60 minutes of treatment. Interestingly, the expression of AKT in diabetic animals treated with OA did not show any significant change in all treatment times.

The results of phosphorylation of HSL are shown in Fig 10 below. Fifteen minutes after treatment, phosphorylation of HSL was high in normal control animals when compared to diabetic control animals. In diabetic rats, expression of phosphorylated HSL decreased following treatment with insulin and a slight decrease was observed following treatment with OA plus insulin. However, treatment of diabetic rats resulted in an increase in the amount of phosphorylated HSL. In normal animals, treatment of animals with insulin and OA plus insulin resulted in a slight decrease in the amount of phosphorylated HSL. In normal animals treated with OA there is a smeared band which the system could not quantify. Thirty minutes after treatment, phosphorylation of HSL was high in normal control animals when compared to diabetic control animals. In diabetic rats, the expression of phosphorylated HSL was elevated following treatment with insulin whereas in animals treated with OA and OA plus insulin smeared bands were observed but could not be quantified by the system. In normal animals, treatment with insulin, OA and OA plus insulin resulted in decrease in the expression of phosphorylated HSL. Sixty minutes after treatment, phosphorylation of HSL was high in normal control animals when compared to diabetic control animals. In diabetic animals, expression of phosphorylated HSL was elevated following treatment with insulin and OA. However, following treatment with OA plus insulin a reduction in the expression of phosphorylated HSL was observed. In normal animals, treatment of animals with insulin seems to have no effect on phosphorylation of HSL.

3.3.2.2 Effect of OA on phosphorylation of Hormone sensitive lipase

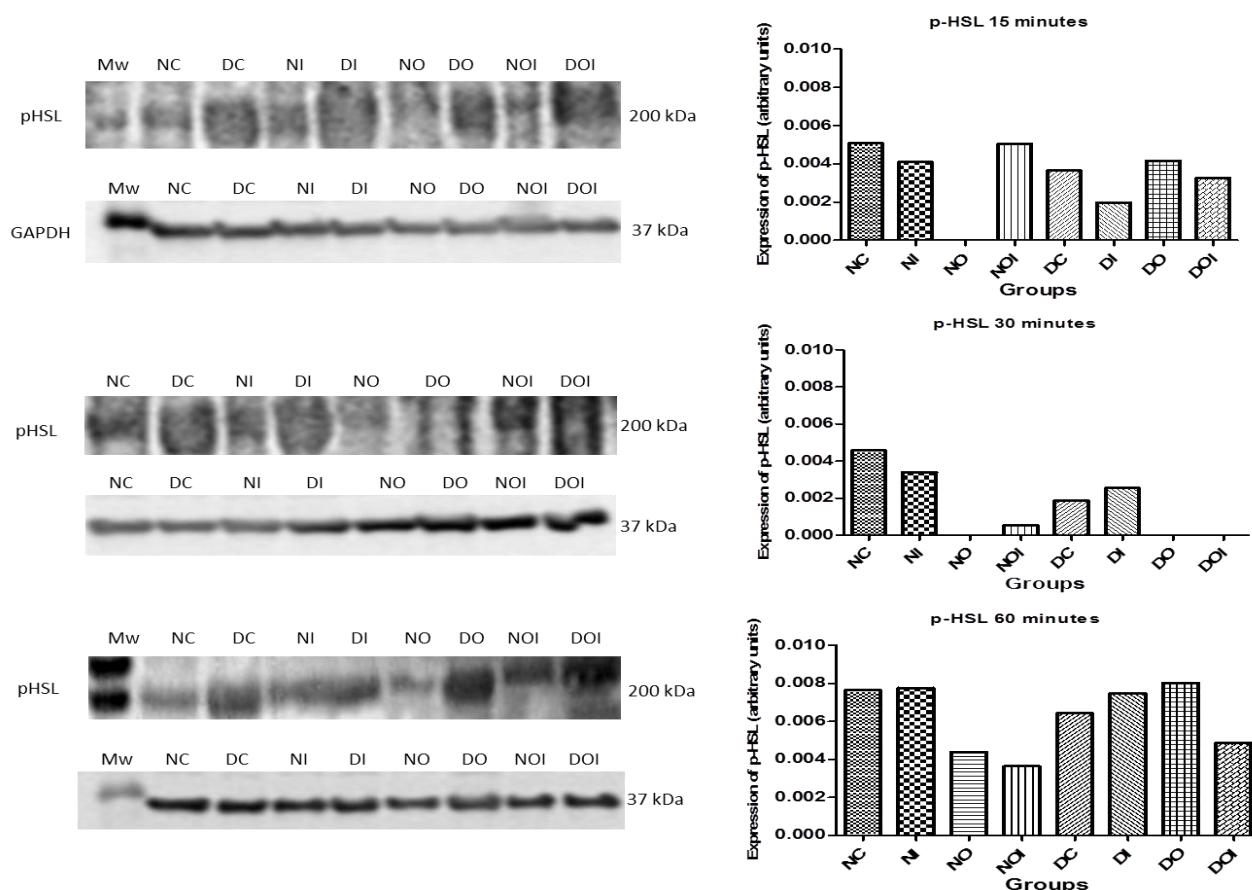


Figure 10: Effect of OA on phosphorylated HSL in liver following 15, 30 and 60 minutes of treatment (120 μ g and 20 μ g protein was loaded for p-HSL and GAPDH, respectively). The values from densitometry of p-HSL were normalised to the levels of GAPDH protein (loading control) and were expressed as the mean-fold increase. Mw = Molecular weight; NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin. On the normalised data, non-diabetic and diabetic animals are grouped separately for simplicity.

However, following treatment of animals with OA and OA plus insulin there was a decrease in the expression of phosphorylated HSL.

3.4. Sub-chronic studies

3.4.1 Effect of OA on total Hormone Sensitive Lipase in liver tissue

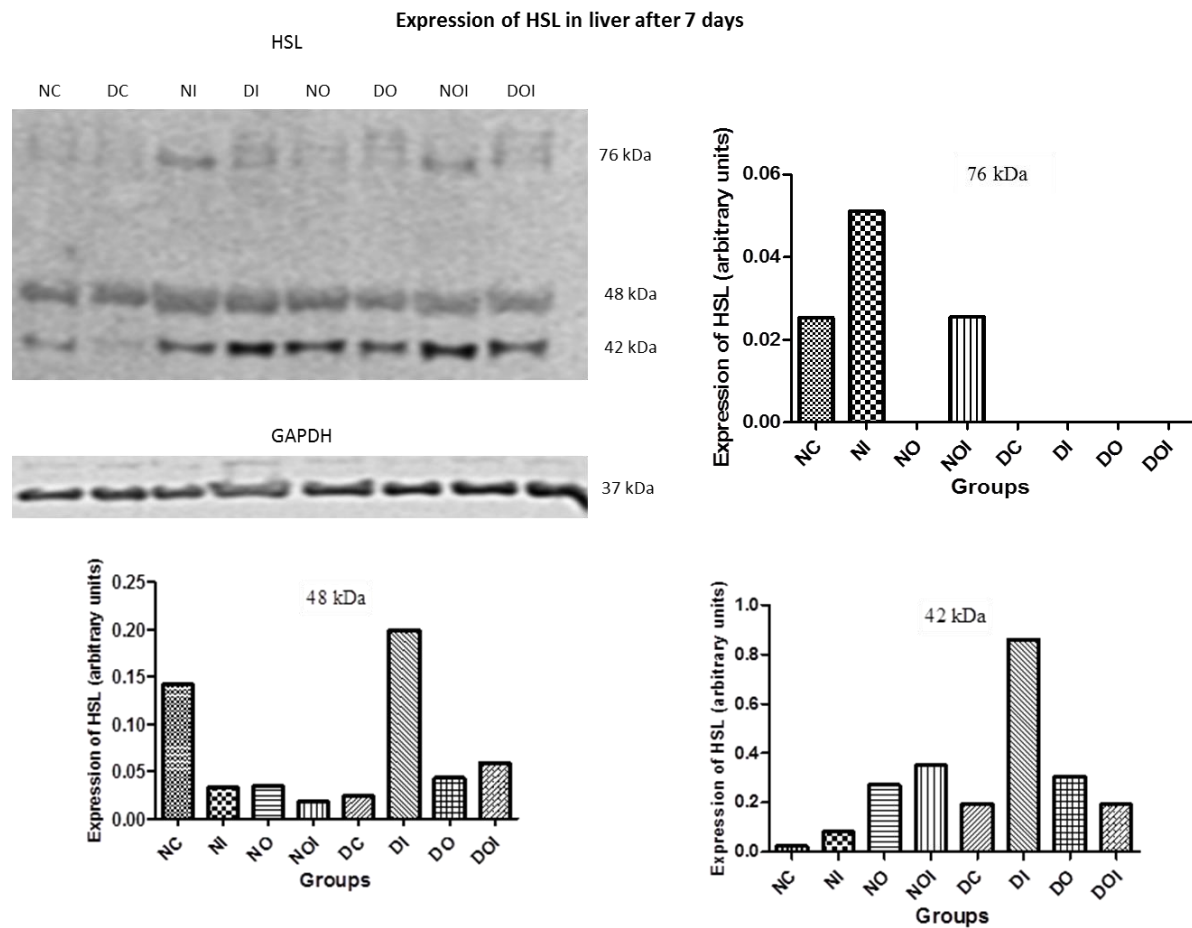


Figure 11: Effect of OA on HSL expression in liver following 7 days of treatment (40 µg and 20 µg protein was loaded for HSL and GAPDH, respectively). The values from densitometry of HSL were normalised to the levels of GAPDH protein (loading control) and were expressed as the mean-fold increase. NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin. On the normalised data, non-diabetic and diabetic animals are grouped separately for simplicity.

The results of HSL expression in liver after 7 days of treatment are shown in Fig 11. There were 3 bands of HSL observed in liver tissue after 7 days of treatment (76, 48, 42 kDa). The 76 kDa isoform was only quantified in three experimental groups: normal control, normal animals treated with insulin only and normal animals treated with both insulin and OA. There does, however, seem to weak and diffuse bands in other groups. The 48 kDa isoform was highly expressed in two experimental groups, normal control and in diabetic animals treated with insulin only. In all the other groups, the expression of this isoform was very low. The expression of 42 kDa HSL isoform was high in diabetic controls when compared to normal controls. However, treatment of normal animals with insulin, OA and insulin plus OA increased the expression of this isoform. Treatment of diabetic animals with insulin dramatically increased the expression of this isoform while OA treatment caused a slight increase in the expression. A combination of OA and insulin seemed to have no effect on the expression of this isoform when compared to diabetic control animals.

The results of HSL expression in liver after 14 days of treatment are shown in Fig 12 below. There were three isoforms of HSL detected (80, 50, 45 kDa) following a 14 day treatment of rats. The 80 kDa isoform was highly expressed in the diabetic control animals compared to normal control animals after the experimental period. Following 14 day treatment with insulin, OA and OA plus insulin expression of this HSL isoform decreased in diabetic animals especially in animals treated with insulin only. Treatment of normal animals with insulin increased the expression of this isoform of HSL while treatment with OA only resulted in a decrease in the expression. Treatment of normal animals with OA plus insulin seemed to have no effect on the expression of this isoform. The 50 kDa isoform was expressed more in normal controls when compared to diabetic control animals. Treatment of normal animals with insulin increased the expression of this isoform whereas treatment of normal animals with OA and OA plus insulin decreased its expression. The treatment of diabetic animals with insulin decreased the expression while treatment with OA increased the expression of this isoform. Insulin plus OA treatment seemed to have no effect. The expression of a 45 kDa isoform of HSL was low in both control groups. However, following 14 day treatment of both normal and diabetic animals had elevated expression of this isoform in all the treated groups, and this expression was more prominent in normal animals.

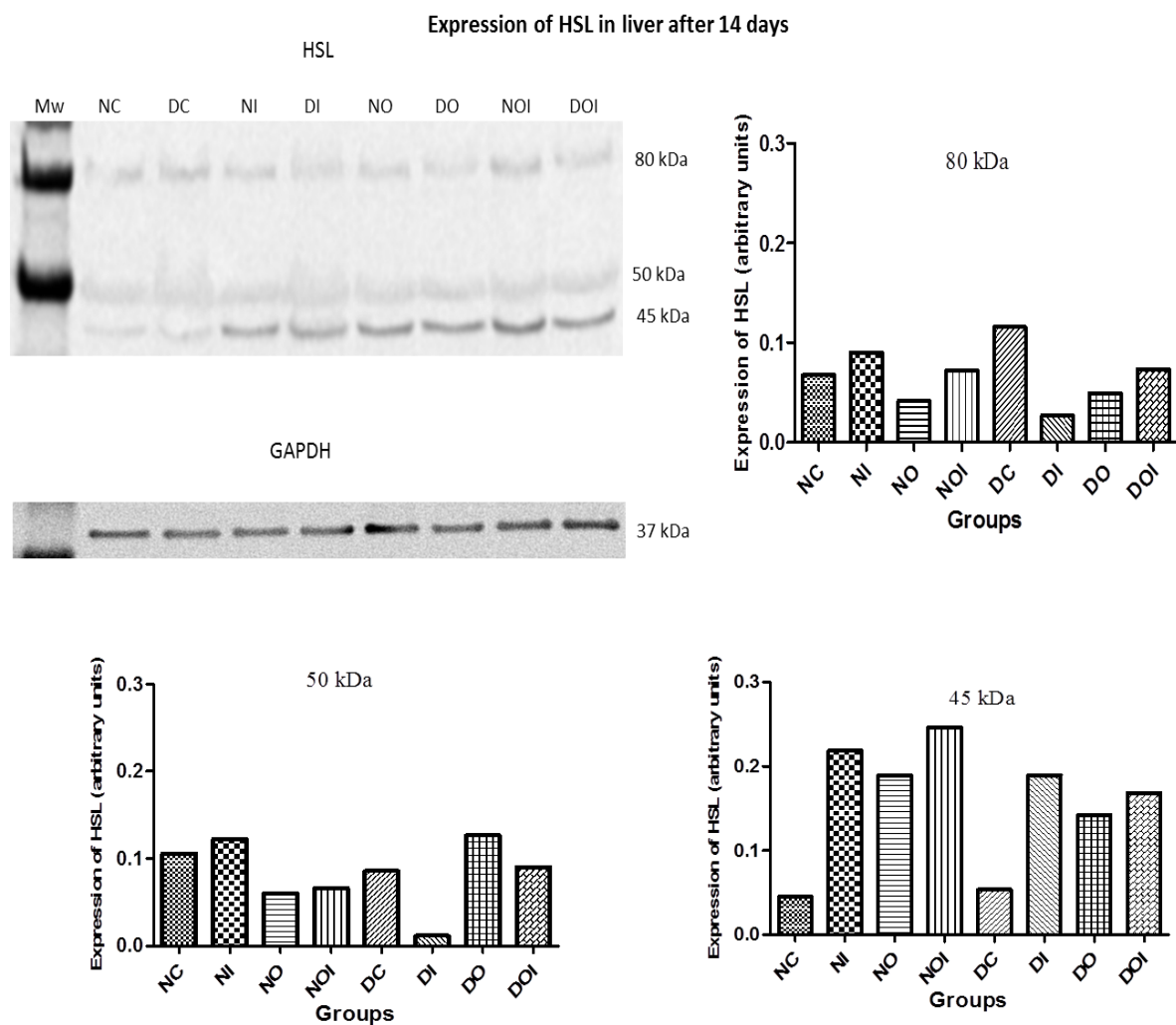


Figure 12: Effect of OA on HSL expression in liver following a 14 day treatment (40 µg and 20 µg protein was loaded for HSL and GAPDH, respectively). The values from densitometry of HSL were normalised to the levels of GAPDH protein (loading control) and were expressed as the mean-fold increase. Mw = Molecular weight; NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin. On the normalised data, non-diabetic and diabetic animals are grouped separately for simplicity.

3.4.2 Effect of OA on total Hormone Sensitive Lipase in adipose tissue

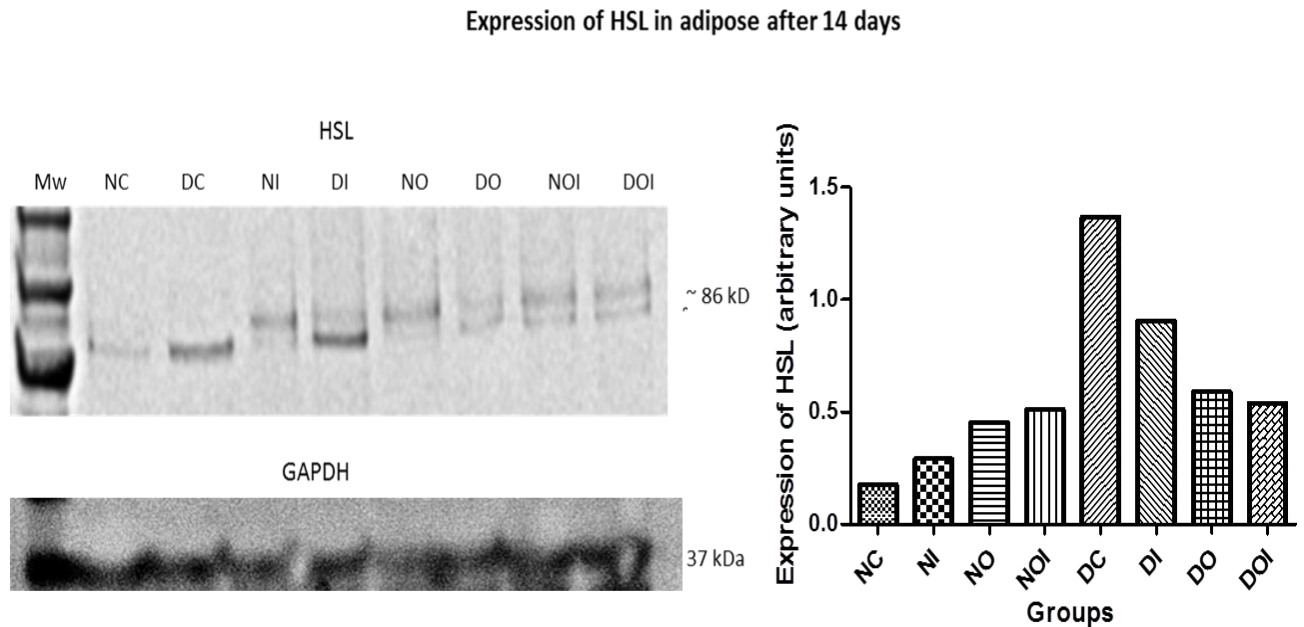


Figure 13: Effect of OA on HSL expression in adipose tissue following a 14 day treatment (120 μ g protein was loaded for both HSL and GAPDH). The values from densitometry of HSL were normalised to the levels of GAPDH protein (loading control) and were expressed as the mean-fold increase. Mw = Molecular weight; NC = normal control; DC = diabetic control; NI = normal insulin; DI = diabetic insulin; NO = normal OA; DO = diabetic OA; NOI = normal OA + insulin; DOI = diabetic OA + insulin. On the normalised data, non-diabetic and diabetic animals are grouped separately for simplicity.

The results of HSL expression in adipose tissue are shown in Fig 13. HSL of a molecular weight of approximately 86 kDa was detected in adipose tissue following 14 day treatment. HSL was highly expressed in diabetic control animals when compared to normal control animals. Following treatment of diabetic animals with insulin and OA, the expression of HSL decreased and this decrease was more prominent when diabetic animals were treated with OA plus insulin. Treatment of normal animals with insulin, OA and OA plus insulin resulted in an increase in HSL expression.

3.5 Hormone sensitive lipase activity

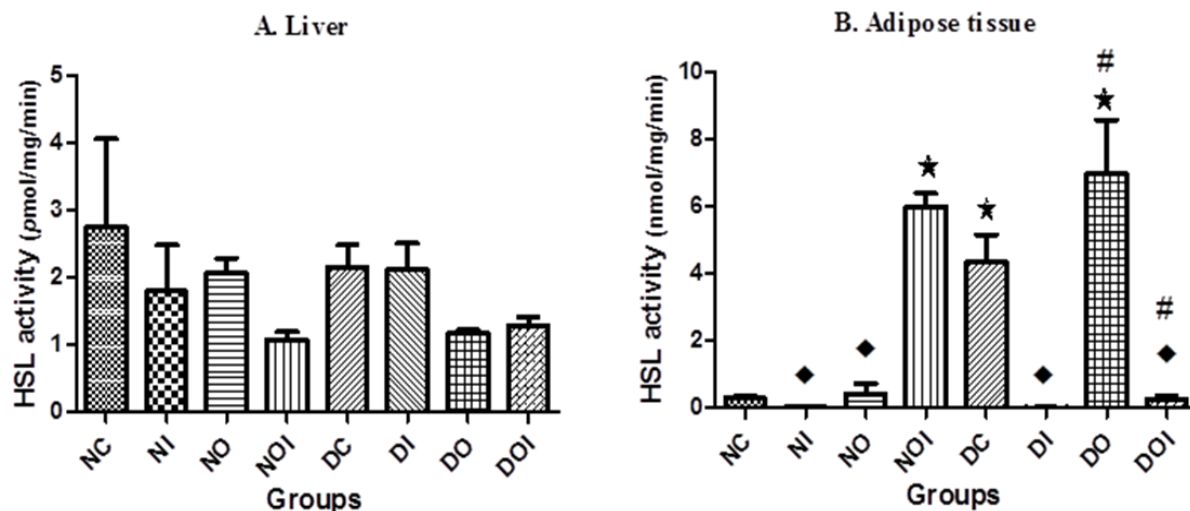


Figure 14: Effect of OA on HSL activity in liver (A) and adipose tissue (B) following a 14 day treatment (n= 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC). ♦ $p < 0.05$ by comparison with diabetic control animals (DC). # $p < 0.05$ by comparing diabetic to normal rats of the same treatment.

Figure 14 shows the results of HSL activity in liver and adipose tissue. In liver, there was no significant difference in HSL activity across all treatment groups. However, normal animals treated with insulin or oleanolic acid had low HSL activity when compared to normal control animals. A combination of insulin plus OA lowered HSL activity further in normal animals. In diabetic animals, diabetic control and insulin treated animals seems to have higher HSL activity when compared to animals treated with OA and OA plus insulin. Adipose tissue had high HSL activity compared to liver. In normal animals, the HSL activity in animals treated with insulin plus OA was very high when compared to that in control animals and those animals treated with insulin or OA. In diabetic animals, control animals and OA treated animals had high HSL activity whereas those treated with insulin or insulin plus OA had very low activity.

3.6 Serum lipid profile

3.6.1 Total cholesterol

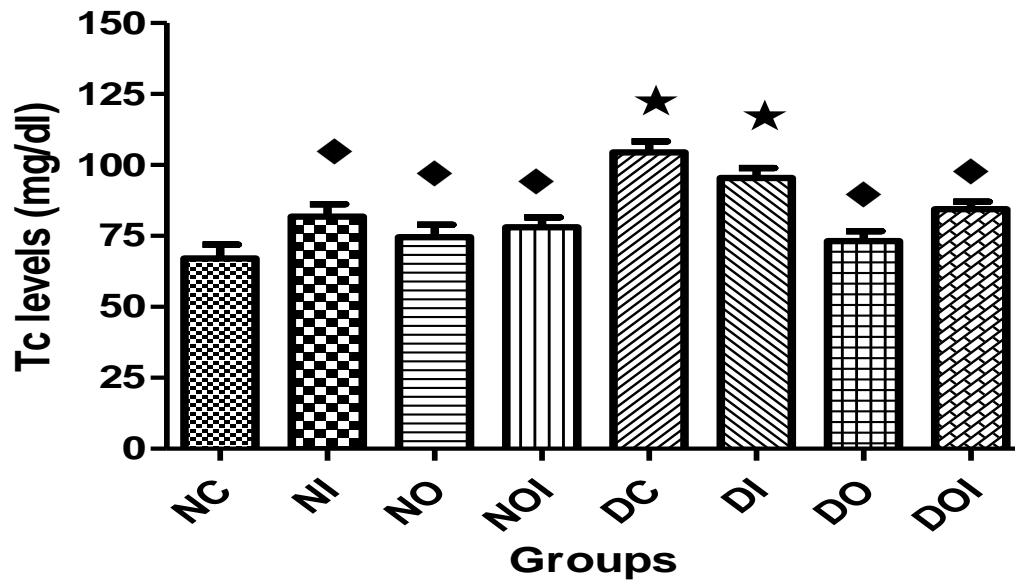


Figure 15: The effects of OA on total cholesterol (TC) levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC). ♦ $p < 0.05$ by comparison to diabetic control animals (DC).

The levels of total cholesterol were significantly high in diabetic control group when compared to normal control group (Fig 15). Following a 14 day treatment of diabetic animals with insulin, there was a reduction in levels of total cholesterol even though this was not statistically significant. Treatment of diabetic animals with OA and OA plus insulin resulted in a significant reduction in total cholesterol levels. Treatment of normal animals seemed to have no effect of the levels of total cholesterol.

3.6.2 Triacylglycerols

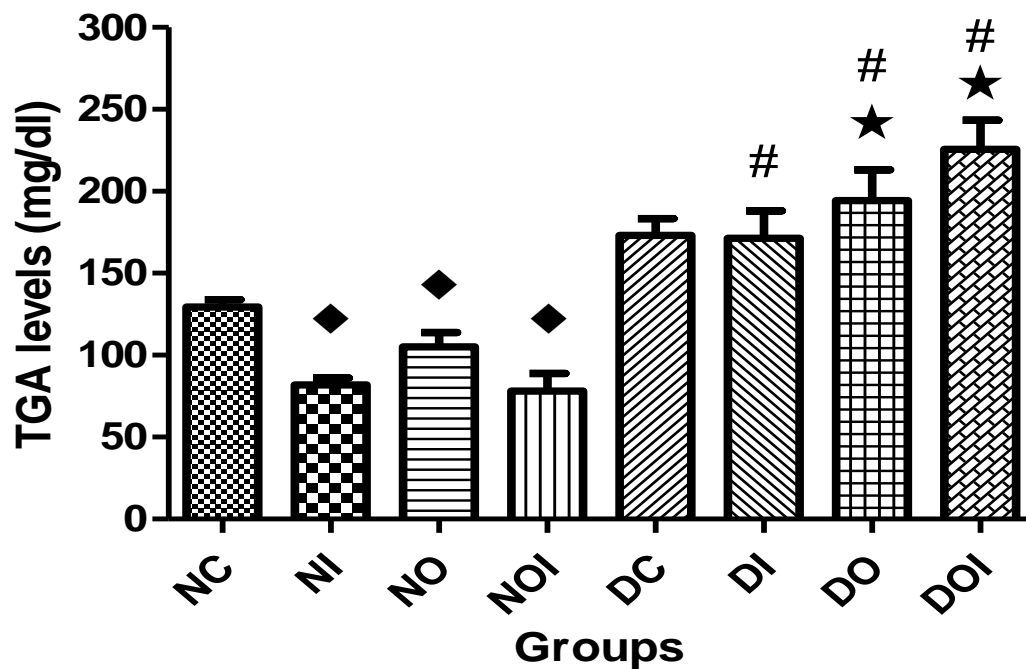


Figure 16: The effects of OA on triacylglycerols (TGA) levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC). ♦ $p < 0.05$ by comparison to diabetic control animals (DC). # $p < 0.05$ by comparing diabetic to normal rats of the same treatment.

The levels of triacylglycerols were high in diabetic control animals when compared to normal control animals even though this was not statistically significant (Fig 16). Treatment of normal animals with insulin, OA and OA plus insulin decreased the levels of triacylglycerols. However, treatment of diabetic animals with OA and OA plus insulin significantly elevated triacylglycerols while treatment with insulin alone seemed to have no effect. Levels of TGA were high in all diabetic animals when compared with normal animals of the same treatment.

3.6.3 High-density lipoprotein cholesterol

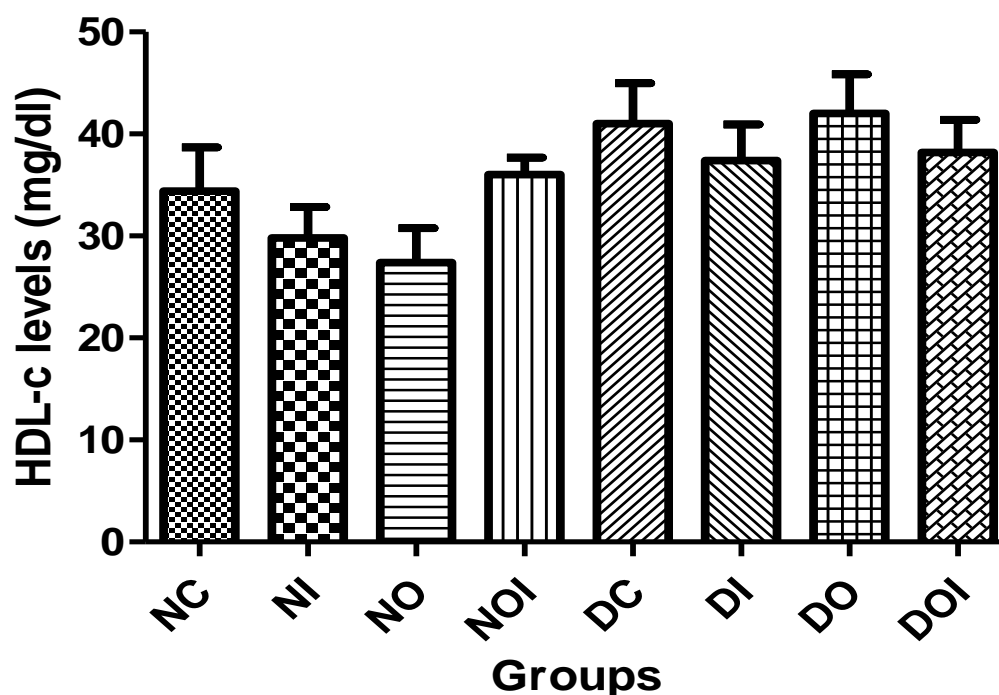


Figure 17: The effects of OA on HDL-c levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM.

There was no statistical difference observed in levels of HDL-c across all groups, but it can be seen that in normal animals treated with OA or insulin only, there was a slight decrease (15% and 23%, respectively) in levels of HDL-c when compared to normal controls (Fig 17). However in normal animals treated with OA plus insulin no change was observed following treatment. There was also a slight decrease in HDL-c levels of 9 % and 7 % observed in diabetic animals treated with insulin and OA plus insulin, respectively and a 2.4 % increase in diabetic animals treated with OA when compared to diabetic control group. Treatment with OA and insulin only seemed to have a greater effect in normal animals.

3.6.4 Low-density lipoprotein cholesterol

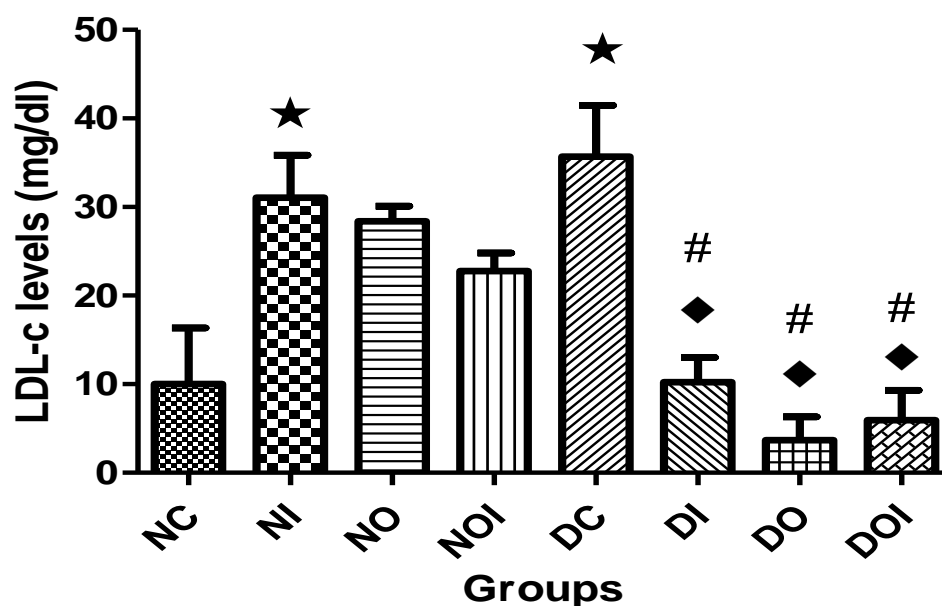


Figure 18: The effects of OA on LDL-c levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC). ♦ $p < 0.05$ by comparison diabetic control animals (DC). # $p < 0.05$ by comparing diabetic to normal rats of the same treatment.

Diabetic control animals had higher levels of LDL-c levels when compared to normal control animals. However, following 14 day treatment with insulin, OA and OA plus insulin the levels of LDL-c drop significantly to levels of normal control animals (Fig 18). In fact LDL-c levels of diabetic animals treated with OA or OA plus insulin are below those in control animals. The levels of LDL-c increased following treatment in all treated normal animals and this increase was only statistically significant in animals treated with insulin only.

3.7 Serum liver enzymes

3.7.1 Aspartate aminotransferase (AST)

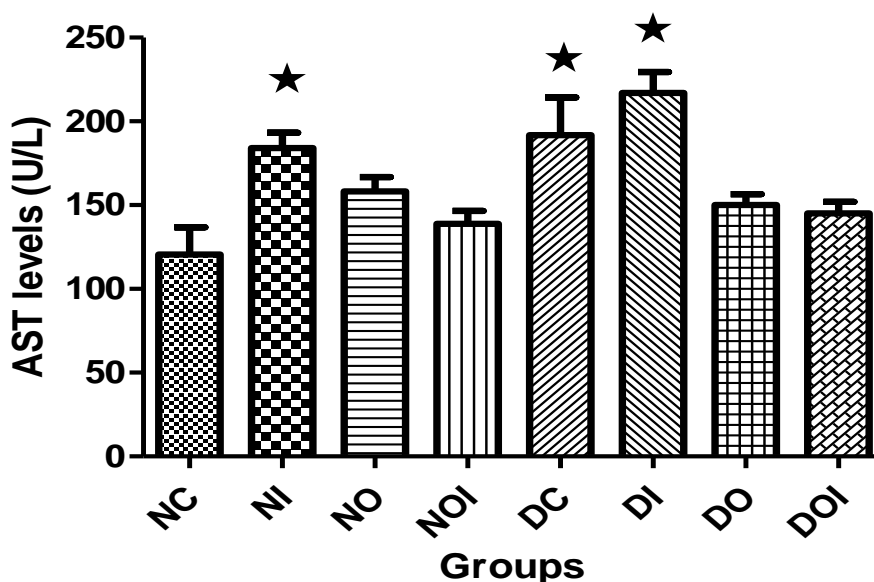


Figure 19: The effects of OA on aspartate aminotransferase (AST) levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC).

The results of AST levels in serum are shown in Fig 19. The AST levels were high in diabetic control group compared to normal control group, and this difference was statistically significant. The treatment of both normal and diabetic animals with insulin elevated the levels of AST even though this increase was statistically significant in normal animals only. In normal animals this elevation of AST levels was also observed in animals treated with OA and OA plus insulin but was not as high as that of animals treated with insulin only. Treatment of diabetic animals with OA and OA plus insulin decreased the levels of AST.

3.7.2 Alanine aminotransferases (ALT)

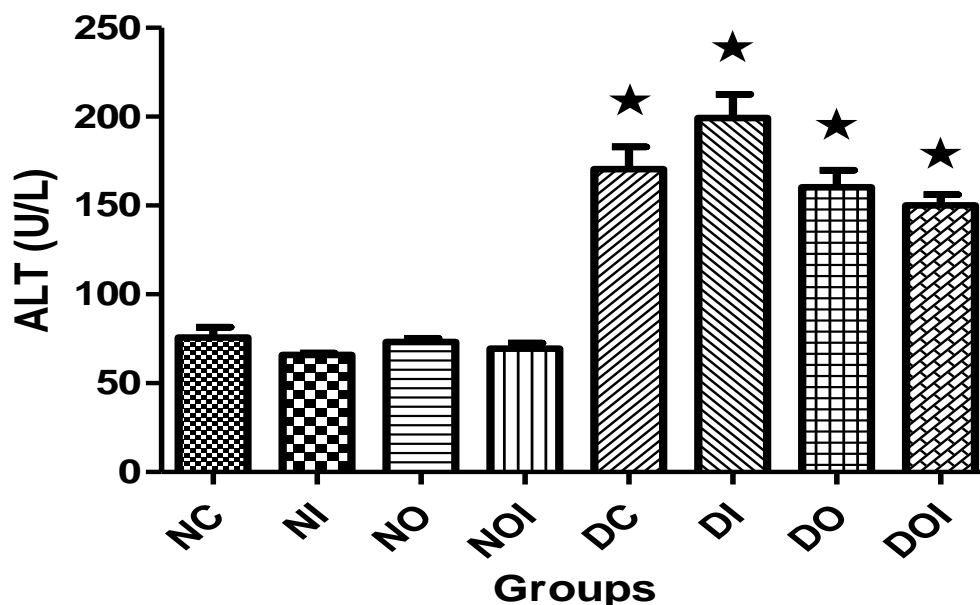


Figure 20: The effects of OA on alanine aminotransferase (ALT) levels in normal and diabetic male Sprague-Dawley rats following 14 day treatment (n = 6 in each group). Values are presented as means \pm SEM. ★ $p < 0.05$ by comparison with normal control animals (NC).

The results of ALT levels in serum are shown in Fig 20. The ALT levels were high in diabetic control animals when compared to normal control animals and this difference was statistically significant. Treatment of normal animals did not have any significant effect on ALT levels. However, treatment of diabetic animals with insulin increased ALT levels while treatment with OA and OA plus insulin decreased them. The changes of ALT in diabetic animals following treatment were not statistically significant. The levels of ALT were reduced by 6% and 12% in diabetic animals treated with OA and OA plus insulin when compared to diabetic control animals, respectively. Levels of ALT were high in all diabetic animals when compared with normal animals of the same treatment.

CHAPTER FOUR

DISCUSSION

The aim of this study was to investigate the effect of plant-derived oleanolic acid on insulin signalling pathway, lipid profile and serum liver enzymes (aspartate and alanine aminotransferase) in streptozotocin-induced diabetic male Sprague-Dawley rats. This study was conducted in an effort to better understand the mechanism by which oleanolic acid exerts its hypoglycaemic and other metabolic effects in the type 1 diabetic rat model. Streptozotocin was used to induce diabetes; this compound is the commonly used diabetogenic agent in both insulin-dependent and non-insulin dependent diabetes depending on the dose used and route of delivery (Szkudelski, 2001). Streptozotocin enters through glucose transporter GLUT2 also expressed in pancreatic beta cells where it causes DNA damage and subsequent death of beta cells and it has also been reported that it causes production of reactive oxygen species (Szkudelski, 2001; Gao *et al.* 2009). In this study 60 mg/kg b.w. of streptozotocin was used to induce type 1 model of diabetes and only animals with ≥ 18 mmol/L blood glucose after 18 hours of fasting were used (Refer to fig. 7). The two-hour oral glucose tolerance tests results show that OA had no acute effect on blood glucose levels as there was no statistical difference observed between diabetic control and OA-treated rats blood glucose levels in both fasted and non-fasted rats. Insulin treated rats which acted as positive control showed a significant decrease in blood glucose levels after 60 minutes in non-fasted rats. In fasted rats given a glucose load, the significant decrease in blood glucose with insulin was only observed after 120 minutes. The results from the OGTTs suggest that OA on its own does not exert hypoglycaemic effects two hours after its oral administration. When OA was combined with insulin in non-fasted animals, it did not have any effect as the results were similar to those of treatment with insulin only. However, administration of OA plus insulin to fasted rats loaded with glucose significantly decreased blood glucose after 30 minutes when compared to rats given insulin only. These findings are in agreement with those of Gao *et al.* (2009) and Zeng *et al.* (2012) which showed that OA lowers blood glucose levels in the presence of insulin. Zeng *et al.* (2012) also showed that OA does not decrease blood glucose in type 1 diabetic rats treated for 2 weeks. These results suggest that OA enhances the effect of insulin in fasted animals loaded with glucose.

This study also investigated the sub-chronic effect of oleanolic acid on lipid profile (Total cholesterol, triacylglycerols, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol) following a 14 day daily treatment regime. This was of importance as it has been shown that the most common lipid abnormalities in diabetes are associated with increase in cholesterol and triacylglycerols; increase in these lipids leads to development of micro and macro vascular complications associated with diabetes (Mitra *et al.* 1995; Ruzaidi *et al.* 2005). The results of this study show that diabetic control animals have high levels of total cholesterol when compared to normal control animals. Following treatment, total cholesterol decreased in diabetic animals treated with OA and OA plus insulin but remained high in diabetic animals treated with insulin only. These results suggest that OA possess anti-hypercholesterolemic effect in type 1 diabetes. In normal animals, the levels of total cholesterol did not change suggesting that treatment of normal animals had no effect on total cholesterol. The levels of triacylglycerols were also high in diabetic control animals when compared to normal control animals. After treatment of normal animals, the levels of triacylglycerols dropped. However, it remained the same in diabetic animals suggesting that treatment did not have any effect on diabetic treated animals. de Melo and colleagues (2010) have previously reported that OA improves dyslipidemia after high fat diet by decreasing total cholesterol and triglyceride levels in normal male Swiss mice. In this study OA also decreased the levels of triacylglycerols in normal rats but increased them in diabetic rats. However, the opposite was true with total cholesterol in normal animals in which its levels increased following treatment with OA in contrast to diabetic animals in which the total cholesterol levels dropped significantly. Zeng *et al.* (2012) have reported that OA lowers liver and plasma triacylglycerols in type 2 diabetic rats. However, in this study OA did not reduce triglyceride levels in diabetic rats but it did so in normal animals. These findings suggest that OA activity requires the presence of insulin.

HDL is responsible for transport of cholesterol from peripheral tissues and subsequent transport to the liver through a process known as ‘reverse cholesterol’ transport (Salter and Brindley, 1988; Chung and Wasan, 2004). LDLs are lipoproteins which are main carriers of unesterified and esterified cholesterol to tissues where they are internalized through the LDL receptors (Barakat *et al.* 1996). In diabetes, levels of HDL-cholesterol are increased and those of LDL-cholesterol are increased and this poses a threat in diabetic patients as these changes have been associated with development of atherosclerosis (Gao *et al.* 2009; de Melo *et al.*

2010). In this study, the results showed that diabetic animals had high levels of HDL-c compared to normal animals but this difference was not statistically significant. However, the levels of HDL-c decreased following treatment with insulin, OA and insulin plus OA. This decrease was more prominent in normal animals even though this decrease was not statistically significant. The minor changes that were observed could be due to a fact that the animals were exposed to treatment for a short period of time. These results suggest that treatment for 14 days did not have any significant effect on HDL-c levels. The levels of LDL-c were higher in diabetic control animals than in normal control animals after 14 days. Following treatment of diabetic animals, levels of LDL-c were reduced to levels of normal levels. However, treatment of normal animals seemed to increase LDL-c levels even though this increase was not statistically significant. These results suggest that treatment for 14 days has a positive effect on LDL-c levels only in diabetic animals. Studies done on type 2 diabetic animals have shown that OA improves lipid profile in diabetic rats by decreasing total cholesterol, LDL-c and TGA and increasing HDL-c levels (Gao *et al.* 2009; de Melo *et al.* 2010)

This study also had acute experiments to investigate the effects of OA on phosphorylation of enzymes involved in the insulin signalling pathway such as AKT and HSL. Protein kinase B/AKT is a central protein in the insulin signalling pathway and it is activated by phosphatidyl-4, 5-bisphosphate in response to binding of exogenous growth factors such as insulin to cell surface receptors (Shtilbans *et al.* 2008). The results of this study show that after 15 and 30 minutes of treatment the expression of phosphorylated AKT was high in diabetic animals treated with insulin only, normal animals treated with OA plus insulin and diabetic animals treated with OA plus insulin. After treating the animals for 60 minutes, expression of phosphorylated AKT was high in normal animals treated with insulin only and, normal and diabetic animals treated with OA plus insulin. These results suggest that when diabetic animals are treated with insulin they are more sensitive when compared to normal animals which only showed a significant response after 60 minutes of treatment. Since diabetic animals lack insulin, administration of it causes a quick response as there will be elevated levels of glucose in the blood. However, in normal animals there will be normal levels if glucose and insulin in the blood therefore administration of insulin will not cause much change as it does in diabetic animals. The increased sensitivity in diabetic rats could be due to increase in insulin receptors on insulin dependent cells, as there will be little or no

glucose within the cells. In normal animals treated with OA, there was a lower amount of phosphorylated AKT suggesting that OA on its own has little effect on AKT phosphorylation. However, in diabetic animals treated with OA only there was low expression of phosphorylated AKT. Interestingly, after 15 and 30 minutes following treatment there was very low expression of phosphorylated AKT in normal animals treated with insulin only, high expression was only observed in normal animals treated with OA plus insulin and a similar trend was observed with diabetic animal after 60 minutes of treatment. These results suggest that OA does have enhancement effect on phosphorylated AKT when used in combination with insulin. These findings are in agreement with those of Zeng and colleagues (2012) who showed that OA increases expression of AKT in liver tissue in type 2 diabetic animals. Other pentacyclic triterpenoids have been shown to stimulate insulin-mediated tyrosine phosphorylation in 3T3-L1 adipocytes (Jung *et al.* 2007).

This study also aimed in investigating the effect of OA on phosphorylated levels of hormone sensitive lipase (p-HSL) after 15, 30 and 60 minutes after treatment. This was done to investigate whether OA mimics the activity of insulin which suppresses or dephosphorylates HSL thus inhibiting lipolysis. The HSL isoforms that have been reported have a molecular weight ranging from 84 – 130 kDa but in this study a 200 kDa band was detected in a phosphorylated state of HSL. It is possible that phosphorylated HSL dimerises and bind to a fatty acid binding protein (Krintel, 2010). Shen and colleagues (2000) have shown that an 84 kDa HSL isoform dimerises into a 160 kDa fragment in a phosphorylated state. Diabetes mellitus is accompanied with increased lipolysis in adipose tissue due to lack of insulin, this is followed by elevated levels of serum free fatty acids (FFA) and subsequent increased gluconeogenesis in the liver (Clause *et al.* 2005; Nishino *et al.* 2007). The elevated levels of FFAs induce insulin resistance in insulin sensitive tissues. Thus inhibition of FFAs release may result in amelioration of diabetes (Voshol *et al.* 2003; Nishon *et al.* 2007). Treatment of normal animals with insulin resulted in decrease in pHSL levels after 15 and 30 minutes of treatment; and this decrease in phosphorylation was reversed after 60 minutes of treatment when compared to control animals. However, treatment of diabetic animals resulted in decrease in pHSL levels after 15 minutes but after 30 and 60 minutes of treatment the phosphorylation of HSL increased. These observations suggest that insulin on its own suppress the phosphorylation of HSL after 15 and 30 minutes following treatment of normal animals with OA only, there was no pHSL observed. After 15 and 30 minutes following

treatment some of the bands were smeared and were not detected by the system for quantification. However, after 60 minutes following treatment all bands were visible and were quantified, thus giving a clear indication of treatment on the expression of phosphorylated HSL. In normal animals, treatment with insulin seems to have no significant effect whereas treatment with OA and OA plus insulin reduced the levels of phosphorylated levels of HSL. In diabetic animals, treatment with insulin and OA slightly increased the levels of phosphorylated HSL and the combination of OA plus insulin reduced levels of phosphorylated HSL. These results suggest that OA in combination with insulin may reduce HSL phosphorylation.

In this study, effect of OA was also investigated on expression and activity of HSL in liver and adipose tissue after a 14 day daily treatment regime. HSL has been reported to be the key enzyme which regulates the most essential energy source and this enzyme is present in many tissues such as: adipose, heart, pancreatic beta cells and liver (Lampidonis *et al.* 2011). HSL in various tissues has been shown to hydrolyse a variety of carboxyl esters (Quiroga and Lehner, 2012). Streptozotocin-induced diabetes has been shown to cause a two-fold increase in HSL activity in adipose tissue and this change in activity parallels with an increase in HSL mRNA and HSL immunoreactive protein (Sztalryd and Kraemer, 1995). The results of this study also showed that untreated STZ induced diabetic rats had increased HSL expression when compared to normal untreated rats after 14 days of treatment. Unsurprisingly, treatment of diabetic animals with insulin resulted in a decrease in HSL expression and activity in adipose tissue. These results were in agreement with those of Sztalryd and Kraemer (1995) who showed that insulin treatment inhibits or decreases lipolysis in adipose tissue. In adipose tissue, treatment of normal animals with insulin, OA and OA plus insulin resulted in an increase in HSL expression but the activity of the enzyme was decreased except for animals treated with OA plus insulin. OA treatment caused a decrease in HSL expression and activity of the enzyme in diabetic animals. Insulin and OA treatment reduced both expression and activity of HSL; this suggests that OA enhances the activity of insulin. The results of this study suggest that OA plus insulin does inhibit lipolysis in STZ-induced diabetes in adipose tissue following sub-chronic treatment.

There has been three HSL isoforms that have been reported with molecular weight ranging from 84 – 130 kDa (Holm, 2003) and indeed in adipose tissue the HSL that was detected in this study ranged from 80 – 100 kDa. However, Langfort and colleagues (1999) reported HSL with a molecular weight of 55 kDa in muscle. In this study; three isoforms of HSL with molecular weight of approximately 76, 48 and 42 kDa were detected in the liver. Very little has been reported on HSL in the liver but some studies have shown that deficiency of HSL in liver causes cholesterol ester accumulation which leads to decreased cholesterol ester hydrolase activity (Sekiya *et al.* 2008). This indicates that HSL plays a major role in the liver. A 76 kDa isoform of HSL was only expressed in control normal animals and normal animals treated with insulin and OA plus insulin. The 48 kDa isoform was highly expressed in normal control animals when compared to diabetic control. Following 7 day treatment, in normal animals the expression of this isoform decreased. However, treatment of diabetic animals resulted in the increased expression of this isoform and this increase was more prominent in animals treated with insulin only. The 42 kDa isoform was highly expressed in diabetic control animals when compared to normal control animals. Treatment of both normal and diabetic animals resulted in an increase in the expression of this isoform and this increase was more pronounced in diabetic animals treated with insulin only.

The effect of OA on the expression of HSL was also investigated in liver tissue after 14 days treatment. The three isoforms observed after 7 days of treatment were also observed after 14 days of treatment. However, the approximate molecular weights of these isoforms were a slightly higher than what there were at 7 days (45, 50 and 80 kDa) and this could be attributable to experimental variation. In this study, an 80 kDa isoform was highly expressed in diabetic control animals compared to normal animals which suggest that this isoform mainly functions to hydrolyse triacylglycerols. All treated diabetic animals showed a decrease in the expression of this isoform suggesting that OA does have an inhibitory effect on HSL. Interestingly, in normal animals a decrease in expression was observed only in animals treated with OA. An increase in animals treated with insulin was observed whilst no change was seen in OA plus insulin treatment group. The 50 kDa isoform seemed to be expressed constantly across all experimental groups. The expression of a 45 kDa isoform was low in both control animal groups. Following treatment, the expression of this isoform was elevated in all animal groups with greater effects observed in normal animals and more so in normal animals treated with OA plus insulin. This isoform may be associated with hydrolysis

of cholesterol esters that has been reported in liver. Interestingly, the increase in the expression of a 45 kDa isoform of HSL parallels with an increase in HDL-c in treated diabetic animals which also suggest that this isoform may be responsible for hydrolysis of cholesterol esters in the liver. After 14 days of treatment, the effect of OA was also assessed on the activity of HSL. In normal animals HSL activity was high in control animals and those treated with insulin and OA whereas a reduction in activity was observed in animals treated with OA plus insulin. In diabetic animals, HSL activity was high in control and insulin treated animals when compared to OA and OA plus insulin treated animals. These results on HSL activity further show that OA does enhance the activity of insulin as both normal and diabetic animals treated with OA plus insulin showed a reduction in HSL activity in liver.

Diabetes has been shown to induce liver damage as evidenced by increase in levels of serum aminotransferases in diabetic rats (Gao *et al.* 2009). In diabetes, the elevated levels of circulating glucose results in production of free radicals (Al-Faris *et al.* 2010). These radicals are generated by glycosylation of proteins and oxidation of glucose. Free radicals also exert cytotoxic effects on cellular lipids and this tissue damage has been observed in the development of diabetes mellitus (Lee, 2006). The results of this study demonstrate that in untreated diabetic animals levels of both AST and ALT are elevated when compared to untreated normal animals as has been reported in other studies (Jeong, 1999; Gao *et al.* 2009). Following treatment of diabetic animals for 14 days, there was a decrease in levels of AST and ALT in animals treated with OA and OA plus insulin. This suggests that OA possess hepatoprotective effect as has been reported by other researchers (Liu, 1995; Jeong, 1999; Gao *et al.* 2009). In diabetic animals that were treated with insulin only, the levels of AST and ALT increased. Interestingly, there has been a reported case of a 45 year old man who was diagnosed with type 2 diabetes and his aminotransferases began to increase after starting insulin treatment (Tawata *et al.* 2000). Even in normal animals, treatment with insulin significantly elevated the levels of AST but the levels of ALT did not change. However, treatment with OA and OA plus insulin did not have any effects on AST and ALT levels in normal animals. These results suggest that OA does have hepatoprotective properties as it reduces AST and ALT levels that were elevated in diabetic animals. The reduction of these aminotransferases was more prominent in animals treated with both insulin and OA, suggesting that insulin enhances the activity OA. The results of this study indicate that OA in absence of insulin can increase phosphorylation of AKT and decrease expression of HSL in

liver and adipose tissue in diabetic rats. OA can improve lipid profile in diabetic rats as it reduces total cholesterol and LDL-c levels after 14 days of treatment.

CHAPTER FIVE

CONCLUSION

The results that were obtained from this study suggest that OA does not possess hypoglycaemic effect two hours following administration but does enhance the hypoglycaemic effect of insulin. OA improves the lipid profile as it reduces total cholesterol and TGA levels; OA also protects liver from damage as it reduces AST and ALT levels in serum. The activity of OA involves phosphorylation of AKT. OA has the ability to decrease the expression and activity of HSL in liver and adipose tissue. OA may reduce the risk of developing cardiovascular complications associated with diabetes and may be a potential drug for the management of type 2 diabetes as it enhances the activity of insulin.

5.1 Limitations and future studies

The limitations to this study were that the effect OA was only investigated on AKT and HSL protein of the insulin signalling pathway. Future studies should also investigate protein such as PDE3B and PKA as they are in between AKT and HSL in the insulin signalling pathway, as the activity of HSL may be affected by other hormones such as adrenaline and glucagon. Future studies should include chronic studies to investigate the long term effect of OA on lipid profile and insulin signalling pathway proteins.

CHAPTER 6 – REFERENCES

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CHAPTER SEVEN

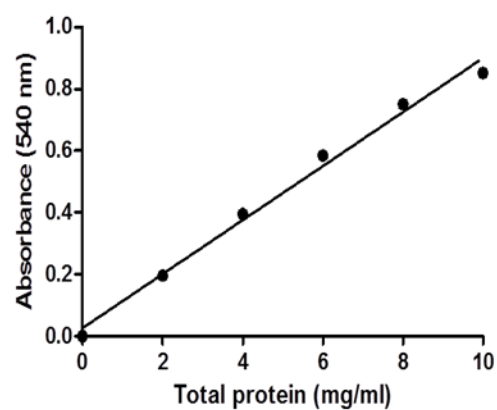
APPENDICES

Appendix 1: Biuret and Lowry assay standard curve

Total protein (mg/ml) Absorbance (540nm)

0.	0.0000
2.	0.1970
4.	0.39515
6.	0.5849
8.	0.75105
10.	0.85235

Biuret assay standard curve



Total protein (µg) Absorbance (600 nm)

0.	0.00000
20.	0.04000
40.	0.08300
60.	0.12900
80.	0.17000
100.	0.20200

Lowry assay standard curve

