UNIVERSITY OF KWAZULU-NATAL

Effects of oleanolic acid and *Centella asiatica* on enzymes of carbohydrate metabolism in muscle and skin of streptozotocin-induced diabetic Sprague-Dawley rats

SILVANA PAULA OCTAVIO LANGA

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Effects of oleanolic acid and *Centella asiatica* on enzymes of carbohydrate metabolism in muscle and skin of streptozotocin-induced diabetic Sprague-Dawley rats

BY

SILVANA PAULA OCTAVIO LANGA (210522040)

Submitted in fulfilment of the requirements for the degree of Master of Science in the Discipline of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science

Supervisor: **Dr. B. Masola**
Discipline of Biochemistry
School of Life Sciences
College of Agriculture, Engineering and Science

Co-Supervisor: **Prof. S. Mukaratirwa**
Discipline of Biological Sciences
School of Life Sciences
College of Agriculture, Engineering and Science

Declaration

I, Silvana Paula Octavio Langa hereby declare that the dissertation entitled

"Effects of oleanolic acid and *Centella asiatica* on enzymes of carbohydrate metabolism in muscle and skin of streptozotocin-induced diabetic Sprague-Dawley rats" is the result of my own investigation and that it has not been submitted in part or in full to any other degree or to any other university. Where the use of the work of others was made, it so duly acknowledged in the text.

Student S.P.O. Langa Signature _ _ _ _ _ _ _ _ _ _ Date_ _ _ _ _ _ _

Supervisor Dr B. Masola Signature _ _ _ _ _ _ _ _ _ _ Date_ _ _ _ _ _ _

Co-Supervisor Professor S. Mukaratirwa Signature_ _ _ _ _ _ _ _ _ _ _Date _ _ _ _ _ _ _

DECLARATION 1 - PLAGIARISM

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I, …….…… declare that

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2. This thesis has not been submitted for any degree or examination at any other university.

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DECLARATION 2 – PUBLICATIONS AND PRESENTATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

In the publication listed below, I contributed by determining the activity of hexokinase. The supervisor and co-authors made materials and analytical facilities available, wrote and submitted the manuscript. The results in this manuscript are not part of this thesis but are relevant to discussions therein.

PUBLICATION

Mukundwa A., **Langa S. O.,** Mukaratirwa S., Masola B. (2016a). *In vivo* Effects of Diabetes, Insulin and Oleanolic Acid on Enzymes of Glycogen Metabolism in the Skin of Streptozotocin-Induced Diabetic Male Sprague-Dawley Rats. *Biochemical and Biophysical Research Communications.* 471(2):315-31

Signed: ……Date: ……..

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List of abbreviations

α	Alpha
β	Beta
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
APS	Ammonium persulphate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CA	*Centella asiatica*
CDC	Centre for Disease Control
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase IV.
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
F2,6BP	Fructose-2,6-bisphosphate
FBPase	Fructose-1,6-bisphosphatase
G6Pase	Glucose-6-phosphatase
GTP	Guanosine triphosphate
GLP1	Glucagon-like peptide 1
GLUT1	Glucose transporter-1
GLUT2	Glucose transporter-2

GLUT4	Glucose transporter-4
GP	Glycogen phosphorylase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase-3
HCl	Hydrochloric acid
H and E	Hematoxylin and Eosin
HK	Hexokinase
HLA	Human leukocyte antigen
i.p	Intraperitoneal injection
ITP	Inosine triphosphate
KCl	Potassium chloride
KI	Potassium iodide
KOH	Potassium hydroxide
MES	2-(N-Morpholino) ethanesulfonic acid
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MOPS	3-(N-Morpholino) propanosulfonic acid
Na_3VO_4	Sodium orthovanadate
Na_2CO_3	Sodium carbonate
NAD^+	β-Nicotinamide adenine dinucleotide sodium salt
NADH	β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate
$NADP^+$	β-Nicotinamide adenine dinucleotide phosphate reduced
NADPH	β-Nicotinamide adenine dinucleotide-2- phosphate
NaF	Sodium fluoride
NaOH	Sodium hydroxide

$NaSO_4$	Sodium sulphate
Nrf-2	Nuclear factor (erythroid-derived)-like 2
OA	Oleanolic acid
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	Peroxisome proliferator activated receptor-γ
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STZ	Streptozotocin
UDPG	Uridine diphosphate glucose
TEA	Triethyl amine
TEMED	N, N, N, N-Tetramethyleylenediamine

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Abstract

Diabetes prevalence increases yearly with an expected increase to 642 million people by the year 2040. Conventional treatment of diabetes involves insulin therapy and other synthetic drugs. However, drawbacks of these forms of treatments include high cost and adverse side effects. There is therefore, a renewed focus on the use of plants and plant derived products for treatment due to their safety and limited side effects. Oleanolic acid (OA) is a plant derived triterpenoid compound that has been shown to suppress expression of genes encoding hepatic gluconeogenic enzymes and in so doing inhibits hepatic glucose production. *Centella asiatica* (L.) Urban (CA), an herbaceous plant belonging to the family Umbelliferae, has been traditionally used for the management of diabetes. Thus, this study was aimed at investigating the effects of OA and CA on selected enzymes of carbohydrate metabolism in muscle and skin of streptozotocin- or 10% fructose-streptozotocin-induced diabetic Sprague-Dawley rats. Non-diabetic and diabetic rats were treated with OA, 500 mg/kg of CA, 1000 mg/kg of CA and metformin over 14 days. OA increased HK and PFK activity of diabetic animals by 82% and 432% ($p < 0.05$) in muscle and 17.7% and 71% in skin respectively. OA also lowered FBPase activity by 24%. Similarly, 500 mg/kg of CA increased HK and PFK activity of diabetic animals by 4.4% and 624% ($p < 0.05$) in muscle and 11% and 35% in skin respectively. OA and CA reduced PFK expression in both muscle and skin of diabetic animals. CA also increased GS activity by 346% ($p < 0.05$) in skin of diabetic animals. Treatment with CA decreased ALT and AST levels and reduced damage to muscle fibre and skin epidermis of diabetic animals in histological studies. The findings of this study indicate that hypoglycaemic effect OA and CA may occur through modulation of glycolysis, gluconeogenesis and glycogen synthesis. Tissue damage and structural changes in muscle and skin caused by diabetes may be reduced by treatment with CA.

CHAPTER 1

Introduction

1.1 Overview of diabetes

The prevalence of diabetes increases yearly with an estimated 415 million people living with diabetes worldwide in 2015 and this figure being expected to increase to 642 million people by the year 2040 (IDF, 2015). South Africa alone had approximately 2.3 million people living with diabetes in 2015 (IDF, 2015). Diabetes is a chronic metabolic disorder characterized by hyperglycaemia due to absolute or relative impairment of insulin secretion and /or action (ADA, 2012). Common symptoms of diabetes include polyuria, polydipsia, polyphagia and sometimes results in susceptibility of certain infections. The economic burden of diabetes is highlighted by the fact that it accounted for 11% of the global health care expenditure with 80% of the patients being from low and middle income countries (IDF, 2013).

Various studies suggest that hyperglycaemia of diabetes is associated with long term complications of diabetes which include vision loss, renal failure, cardiovascular diseases, progression of skin diseases (Demirseren *et al.*, 2014) and other macro and microvascular complications (Stratton *et al.*, 2000; Singleton *et al.*, 2003; Fowler, 2008). The primary goal of treatment of diabetes is therefore to maintain glycaemic control thus reducing hyperglycaemia. Deficiency of insulin or impairment of its action can affect the regulation of glycogenic, glycolytic and gluconeogenic enzymes exacerbating hyperglycaemia. Conventional treatment of diabetes involves insulin therapy and other synthetic drugs which have an anti-hyperglycaemic effect. However, the drawbacks of these forms of treatments include high cost, poor compliance and adverse side effects (Lebovitz, 2011). There is therefore, a renewed focus on use of plants and plant derived products for treatment due to their safety and limited side effects.

Diabetes is mainly classified into 3 categories: Type-I diabetes, type-II diabetes and gestational diabetes. Type-I diabetes is the less common type identified by the absolute lack of insulin secretion due to destruction of β-cells of the pancreas. The most common form, Type-II diabetes is classified by insulin resistance and reduced insulin secretion. Gestational diabetes is defined as various degrees of glucose intolerance mainly common during pregnancy (Horvath *et al.*, 2010)

1.1.1 Type-I diabetes

Type-I diabetes or insulin dependent diabetes is prevalent in 5-10% of diabetic patients (ADA, 2012). It is an autoimmune disease in which pancreatic β-cells are unable to produce insulin or pancreatic cells are defective leading to lack of or deficient insulin secretion. Genetic predisposition, autoimmunity, viral infections and environmental factors are some etiological aspects involved in the development of the disease (Muthukrishnan *et al.*, 2007). The human leukocyte antigen (HLA) region on chromosome 6 is the major site susceptible to genetic variation that contributes to the risk of development of this disease (Brorsson *et al.*, 2010). Some patients present with ketoacidosis when the disease first manifests itself (Rother, 2007). Most of type-I diabetes is classified as immune mediated where the loss of insulin producing β-cells is mediated by T-cells involved in autoimmune attack (Rother, 2007). The destruction of β-cells occurs over long periods of time but peak prevalence typically occurs during puberty. There is currently no known method available to prevent this chronic disorder thus lifelong treatment with exogenous insulin is required for survival.

1.1.2 Type-II diabetes

Type-II diabetes, also known as non-insulin dependent diabetes, is the most common form of diabetes that comprises of 90% of diabetes cases worldwide (Xing *et al.*, 2009). It is characterized by elevated blood glucose levels due to reduced secretion of insulin (Stumvoll *et al.*, 2005) and by tissue specific resistance to insulin resulting in inefficient absorption of glucose into liver, muscle and adipose tissues. The precise cause of type-II diabetes is not yet known but may be associated with genetic susceptibility (Kahn *et al.,* 1996) and environmental factors (Figure 1.1) which include obesity, physical inactivity, diet and age. These features amplify the chances of individuals developing the disease (Ling and Groop, 2009). Although commonly affecting adults, over the years, type-II diabetes has become more prevalent in children and young adults. The most prevalent form, type-II diabetes can be reversed when in its early stages (Lim *et al.*, 2011), or be effectively managed.

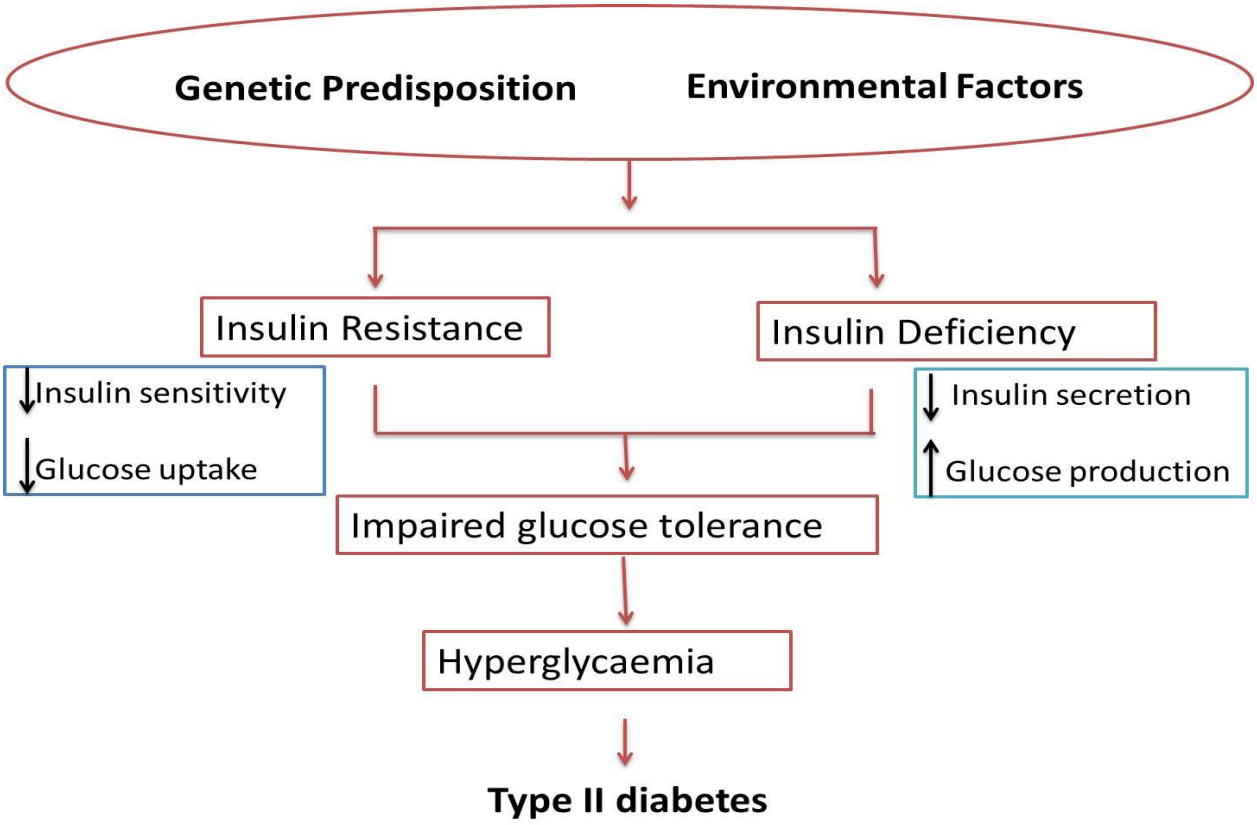


Figure 1. 1: Pathogenesis of type-II diabetes

1.1.3 Gestational diabetes

Gestational diabetes is defined as a glucose intolerance of varying degrees with onset or first manifestation during pregnancy (Horvath *et al.*, 2010). Statistics show that gestational diabetes presents itself in almost 14% of pregnancies (Kim *et al.*, 2002). This form of diabetes not only causes health complications for the parent, but also for the child during pregnancy and birth (Metzger *et al.*, 2008). These complications include bone fractures and hypoglycaemia, caesarian section, pre-eclampsia, birth injuries, need for neonatal care and hyperbilirubinemia (Metzger *et al.*, 2008). Long term complications of these infants includes the risk of impaired glucose control (Plagemann *et al.*, 1997a) which may later result in obesity (Plagemann *et al.*, 1997b).

Some patients with gestational diabetes continue to be hyperglycaemic even after delivery and are at risk of developing type-II diabetes years later (Kim *et al.*, 2002; Bellamy *et al.*, 2009). Studies have shown that pregnant women who are obese are more at risk of developing gestational diabetes than normal weight women (Chu *et al.*, 2007; Hedderson *et al.*, 2010). Treatment of gestational

diabetes significantly reduces the risk of mortality in infants and improves the quality of life of the women (Crowther *et al.*, 2005).

1.1.4 Complications due to diabetes

Although originating from different pathogenic causes, both type-I and II diabetes are characterized by hyperglycaemia leading to microvascular and macrovascular complications. Microvascular complications include diabetic retinopathy, nephropathy and neuropathy. Diabetic retinopathy is known to be the leading cause of new cases of blindness in adults (CDC, 2011) while nephropathy is the leading cause of kidney failure in diabetic patients (CDC, 2011). In 2011, 44% of new cases of kidney failure occurred in those with diabetes in USA (CDC, 2011). Neuropathy is defined as a group of diseases or a disease that affects nerves with more than half of patients with diabetes being affected in their lifetime resulting in loss of or altered sensation and pain in their legs and feet. This contributes to the potential risk of diabetes-related foot problems that are difficult to treat and in extreme cases may lead to amputation. It is estimated that 60% of lower limb amputations occur in patients with diabetes (CDC, 2011). Macrovascular complications arise from changes in larger blood vessels which result in cardiovascular and cerebrovascular disorders leading to myocardial infarctions and strokes, respectively. Patients with diabetes present a 2 to 4 times higher increase in strokes and increase in mortality due to cardiovascular diseases than patients without diabetes (CDC, 2011). In 2010, in USA, heart disease and stroke were noted to be 1.8 and 1.5 times higher, respectively, amongst patients 20 years or older diagnosed with diabetes (CDC, 2014).

1.2 Glucose metabolism

1.2.1 Glucose metabolism in liver

The liver plays a central role in glucose homeostasis (Wu *et al.*, 2001; Wu *et al.*, 2005) by contributing towards hepatic glucose utilization and hepatic glucose production depending on the available plasma glucose levels. The liver is responsible for disposing up to 25-35% of the oral glucose load (Meyer *et al.*, 2002) and achieves glucose homeostasis by producing glucose through gluconeogenesis and glycogenolysis or storing excess glucose as glycogen (Nuttall *et al.*, 2008).

Hepatic glucose homeostasis is mainly controlled by hormones insulin and glucagon. Insulin increases activity of glycolysis and glycogenesis while inhibiting gluconeogenesis and glycogenolysis in the liver (Aronoff *et al.*, 2004; Nuttall *et al.*, 2008). Glucagon on the other hand has the opposite effect. Epinephrine is mainly used when glucagon response is impaired (Aronoff *et al*., 2004). In the fasting state, the liver provides energy to the body through glycogenolysis and gluconeogenesis to restore blood glucose concentrations (Moore *et al.*, 2012). The breakdown of glycogen is catalyzed by glycogen phosphorylase. The product of glycogen phosphorylase, glucose-1-phosphate is further converted. by phosphoglucomutase, to glucose-6-phosphate which in turn is converted to glucose (Manabe *et al.*, 2013). After feeding, hepatic glucose production is suppressed and glucose is utilized through glycolysis and stored as glycogen in the liver. Animals with type-II diabetes show increased hepatic glucose production due to suppressed insulin levels (Bogardus *et al.*, 1984; Rizza, 2010). Administration of insulin decreases glucose levels of STZ-induced diabetic rats in liver (Bogardus *et al.*, 1984). Studies have demonstrated reduction of hepatic blood glucose as a potential treatment of diabetes (Morral, 2003).

1.2.2 Glucose metabolism in skeletal muscle

Skeletal muscle is one of the major tissues responsible for carbohydrate and lipid metabolism in the body (Kelley and Goodpaster, 2001) and is responsible for 75% of glucose disposal under insulin stimulated conditions (Saltiel and Kahn, 2001). It therefore plays a major role in regulation of blood glucose and storage of glycogen.

Insulin stimulates translocation of GLUT4 glucose transporters from the intracellular pool to the cell surface of skeletal muscle which then facilities glucose entry into the cell (Rowland *et al.*, 2011). This rate limiting step controls the rate at which glucose is utilized in skeletal muscle (Dimitriadis et al., 2011). After transport, glucose is introduced into the glycolytic cycle through phosphorylation by hexokinase. The product of hexokinase, glucose-6-phosphate is further metabolized downstream in glycolysis or channeled towards glycogen synthesis (Mizgier *et al.*, 2014). In glycogenesis, Glucose-6-phosphate is converted to glucose-1-phosphate by phosphoglucose isomerase. Glucose-1-phosphate is converted to UDP-glucose which is subsequently added to glycogen by glycogen synthase (Jensen *et al.*, 2006). In glycolysis, glucose-

6-phosphate is further metabolized, providing substrates for energy production (Guo *et al.*, 2012). Both glycolysis and glycogenesis are stimulated by insulin in skeletal muscle (Dimitriadis *et al.*, 2011). The rate of glycolysis and glycogenesis is impaired in skeletal muscle of STZ -induced diabetic animals through inhibition of hexokinase, phosphofructokinase (Da Silva *et al.*, 2010) and glycogen synthase (Jensen *et al.*, 2012).

1.2.3 Role of skin in glucose metabolism

The skin functions as a biological boundary between the inner and outer environment of the body. This organ is responsible for various protective functions and is also involved in absorption, secretion of electrolytes, and regulation of heat and water. Since all these functions require energy, various pathways are likely to be involved in the generation of energy. Although literature suggests that little is known about such pathways, there has been evidence of amino acid metabolism, oxygen uptake and carbon dioxide production (Decker, 1971).

The presence of glycolysis has also been partly recognized through the presence of hexokinase (Halprin and Ohkawara, 1966b) and phosphofructokinase (Kondo and Adachi, 1971) in the epidermal layer of the skin, while others showed the presence of glucose metabolism by showing the presence of intermediate products in skin slices (Cruickshank *et al*., 1957; Bernstein and Sweet, 1959). Gluconeogenic enzymes have also been demonstrated in rat skin although the activities of fructose-1,6-bisphosphatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase were twenty times lower compared to liver on wet-weight basis (Peters and White, 1976). Diabetes results in several skin related complications including disturbances in wound healing properties (Knas *et al.*, 2013). Alloxan-induced diabetic animals have been shown to have decreased skin epithelium thickness (Tanga *et al.*, 2003). Diminished hexokinase activity has been observed in skin of STZ-induced diabetic animals (Mukundwa *et al.*, 2016a).

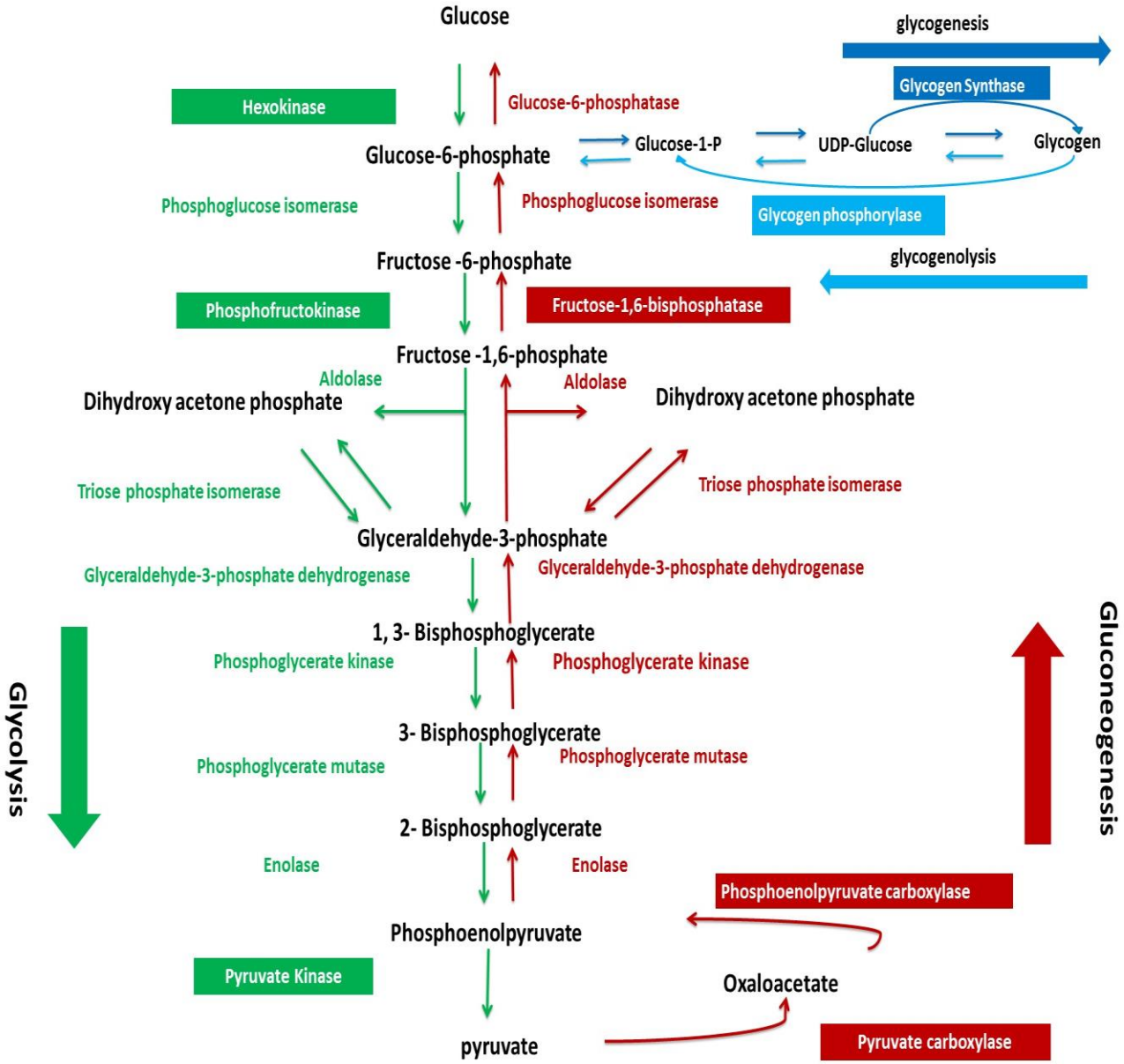


Figure 1.2: Summary of pathways involved in glucose metabolism including glycolysis, gluconeogenesis, glycogenesis and glycogenolysis

1.2.3 Glycolytic enzymes

Glycolysis is a metabolic pathway that involves the breakdown of glucose after being taken up by cells. The pathway provides substrates for energy production via ATP formation (Xiong *et al.*,

2011). The pathway also provides substrates for other pathways such as polyol pathway, pentose phosphate pathway, glycogenesis and lipogenesis. Regulatory steps in glycolysis include glucose uptake by glucose transporters, glucose phosphorylation by hexokinase, conversion of fructose-6-phosphate into fructose-1,6-phosphate by phosphofructokinase and pyruvate production by pyruvate kinase (Figure 1.2) (Guo *et al.*, 2012). Regulation of these rate limiting enzymes is often linked to regulation of glucose metabolism and therefore glycaemic control (Guo *et al.*, 2012).

1.2.3.1 Hexokinase

Hexokinase (HK) catalyzes the activation of glucose through phosphorylation in an ATP-dependent reaction (Wilson, 2003). The product of this enzyme can be channeled into various pathways including the pentose phosphate pathway depending on tissue specific metabolic requirements (Figure 1.3).

HK exists as multiple isoenzymes each with similar properties but differentiated from one another through ion exchange chromatography or electrophoresis (Katzen and Schimke, 1965). Genetic evolution suggests that the enzyme evolved through duplication and fusion of ancestral HK resulting in its dimeric form found in multicellular organisms. The isoenzymes are distinguished as Type- I, -II, and -III and type-IV. Type-I is dominant in brain and kidney, type-II in muscle and type-III is mainly found in the liver (Grossbard and Schimke, 1966). Similar properties shared by these three isoenzymes include a molecular weight of approximately 100 kDa each comprised of two 50 kDa subunits, a high affinity for glucose, allosteric inhibition by the reaction product, glucose-6-phosphate and optimum pH of between 7.8 and 8.8 (Grossbard and Schimke, 1966). The three isoforms can however be distinguished from each other on the basis of different stabilities in heat and proteolytic activation; different K_m values for glucose and ATP and also different K_i values for ADP and glucose-6-phosphate (Grossbard and Schimke, 1966). Type-IV also known as Glucokinase is however predominantly found in the liver and has a molecular weight of 50 kDa, low affinity for glucose and is not subject to feedback regulation by glucose-6-phosphate (Katzen and Schimke, 1965; Grossbard and Schimke, 1966).

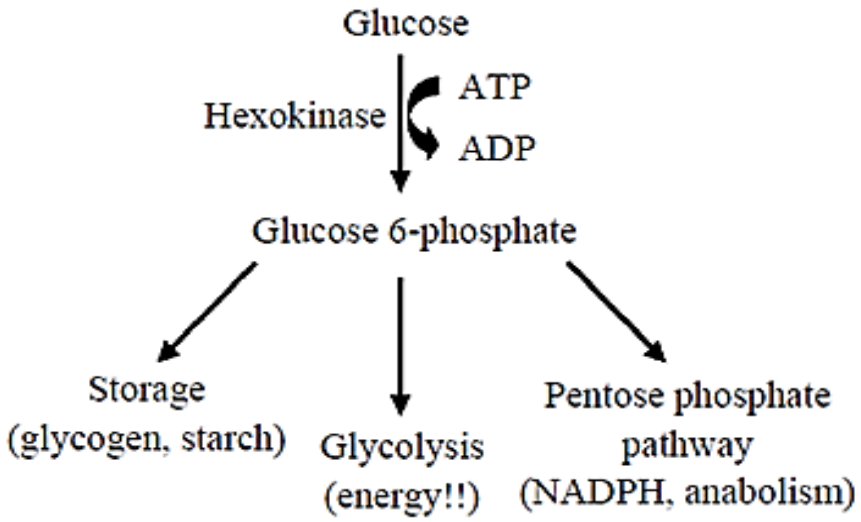


Figure 1.3: Channeling of the product of hexokinase as substrate for a variety of pathways. Adapted from (Wilson, 2003).

Glucose-6-phosphate, the product of the enzyme serves as primary precursors for different metabolic pathways such as glycolysis, glycogenesis and pentose phosphate pathway (Figure1.3).

Since HK plays an integral role in glucose homeostasis, various studies have looked at its modulation as a means of overall glycolysis regulation in diabetes. Several studies have shown that HK activity is diminished in diabetic animals (Katzen *et al.*, 1970; Gardiner *et al.*, 2007; Da Silva *et al.*, 2010). Katzen *et al.* (1970) attributed this due to loss of hexokinase type-II protein rather than inhibition by glucose-6-phosphate. The activity of HK type-II returned to normal following administration of insulin suggesting that synthesis of HK type-II may be regulated by insulin (Katzen *et al.*, 1970). Da Silva *et al.* (2010) showed that metformin could reverse the decreased HK activity in STZ-induced diabetic mice. Increased HK activity has been shown after treatment using several hypoglycemic agents such as asiatic acid (Ramachandran and Saravanan, 2013).

1.2.3.2 Phosphofructokinase

The reaction catalyzed by phosphofructokinase (PFK) is a rate determining reaction that plays a key role in glucose metabolism. PFK phosphorylates fructoses-6-phosphate at the first carbon atom using ATP as the phosphate donor to form a diphosphate, fructose-1, 6-bisphosphate. This reaction is an ATP and Mg^{2+} dependent reaction that is irreversible under intracellular conditions (Schöneberg *et al.*, 2013). The PFK reaction is considered the pacemaker of glycolysis by forcing the cell to metabolize glucose rather than converting to another sugar or storing it (Schöneberg et al., 2013).

PFK exists as 3 different polymeric isoforms PFK M (muscle), L (liver) and P (platelets) which are located on chromosome 1, 21 and 10, respectively, with a molecular weight of approximately 85 kDa in humans (Vora and Francke, 1981; Dunaway *et al.*, 1988; Sola-Penna *et al.*, 2010). The PFK gene is a derivative of the prokaryote gene that has undergone mutations such as gene duplication, in tandem fusion and divergence. As a result of these mutations, the mammalian gene contains an allosteric site for inhibitory ATP and an activator site for fructose-2,6-bisphosphate not found in the bacterial gene (Li *et al.*, 1999; Sträter *et al.*, 2011). Expression of the enzyme isoforms is tissue specific with PFKM the only isoform expressed in all tissue types while skeletal muscle is the only tissue that expresses one form of the enzyme (Dunaway *et al.*, 1988).

PFK is known to be a complex oligomeric structure that contains different forms of the enzyme, mainly dimers and tetramers as the monomers tend to be highly unstable. The balance between the dimers and tetramers correlate with the regulation of the enzyme. Dimers correlate with low catalytic activity however when polymerized form fully active tetramers, displaying high catalytic activity (Luther *et al.*, 1986; Zancan *et al.*, 2008). Equilibrium between the different forms of the structure is stimulated by the change in physical and chemical conditions such as pH, temperature, concentration of the enzyme and allosteric ligands (Luther *et al.*, 1986).

Fructose-2,6-bisphosphate (F2,6BP), the product of PFK-2 is a powerful activator of PFK and overcomes ATP inhibition of the enzyme (Hers and Van Schaftingen, 1982; Zancan *et al.*, 2008). The enzyme may also be allosterically regulated by metabolites such as citrate, drugs and intracellular proteins (Sola-Penna *et al.*, 2010). Insulin stimulates the phosphorylation of PFK which activates glucose consumption in skeletal muscle hence activating glycolysis (Da Silva *et*

al., 2010). Studies have shown that PFK activity decreases in liver, muscle and heart of diabetic animals (Da Silva *et al.*, 2010; Da Silva et al., 2012).

1.2.3.3 Pyruvate kinase

The reaction catalyzed by pyruvate kinase (PK) is an irreversible (under physiological conditions) rate determining reaction in glycolysis. The enzyme catalyzes the reaction of phosphoenolpyruvate and ADP to form pyruvate and ATP, requiring monovalent (K^+) and divalent (Mg^{2+}) cations for its activity (Yamada and Noguchi, 1999). The product, pyruvate, feeds into various metabolic pathways downstream making it an important control point in glycolysis (Muñoz and Ponce, 2003).

PK is a 60 kDa tetramer, evolutionarily conserved enzyme that exists as four isozymes in mammalian tissues, each with different kinetic properties depending on tissue specific requirements. The isozymes have similar structure but differ in their gene expression and regulation. The L type is predominantly expressed in liver, R type in red blood cells, M1 type in skeletal muscle as well as brain and M2 type in embryonic tissue. These four isozymes are encoded by two genes that are present in humans, PKLR and PKM genes. The PKLR gene encodes both the L and R isoenzymes through the use of different promoters (Noguchi *et al.*, 1987). The PKM gene encodes both M1 and M2 isoenzymes which differ by splicing at position 9 or 10 of exon, respectively (Figure 1.4) (Noguchi *et al.*, 1986; David *et al.*, 2010).

Each isoenzyme has different kinetic properties specific for different metabolic requirements of each specific tissue. The M1 type seems to lack any allosteric control and the presence of hormones and diet seem to have no influence on the quantity of enzyme present hence it is constitutively expressed (Muirhead *et al.*, 1986) while the M2, L and R types are allosterically regulated through hormonal and dietary control. Pyruvate kinase is regulated by its substrate phosphoenolpyruvate, by feedforward activation of upstream intermediate Fructose-1,6-bisphosphate and feedback inhibition by ATP (El-Maghrabi *et al.*, 1982; Yamada and Noguchi, 1999; Muñoz and Ponce, 2003).

Pyruvate kinase activity has been shown by various studies to be decreased in alloxan- and STZ-induced diabetes animals (Raju *et al.*, 2001; Anand *et al.*, 2010; Rasineni et al., 2010;Majd *et al.*,

2014). This has been attributed to diminished glucose metabolism and ATP production. The activity of the enzyme increased upon administration of insulin (Yamada and Noguchi, 1999).

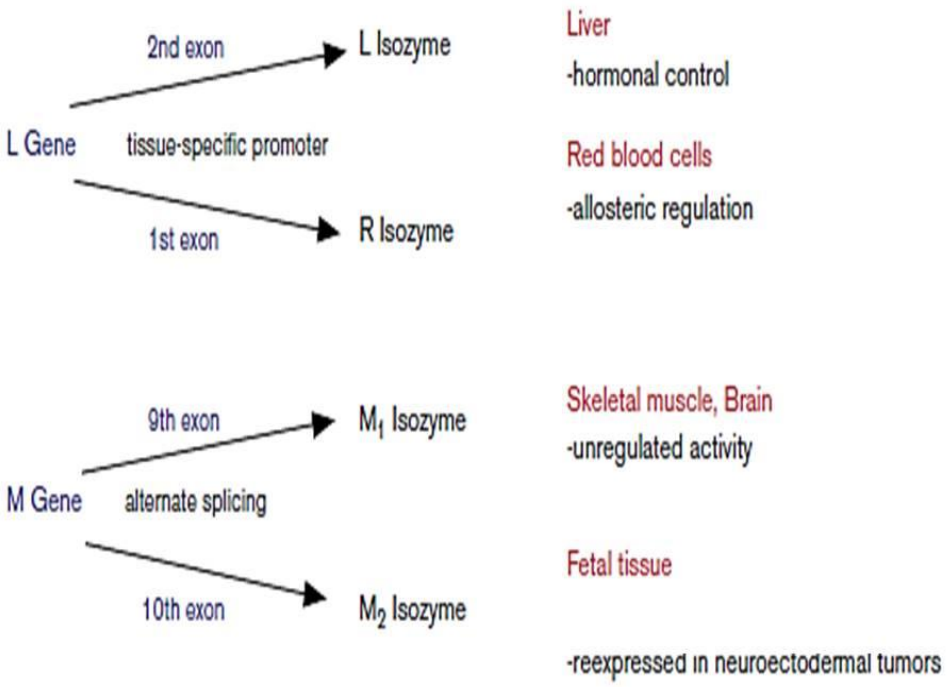


Figure 1. 4: Isoenzymes of pyruvate kinase encoded for by two different genes, adapted from (Jurica et al., 1997)

1.2.4 Gluconeogenesis enzymes

Gluconeogenesis is the anabolic pathway of glucose production from precursors such as lactate, pyruvate and alanine. Key regulatory enzymes include phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) (Figure 1.2). These enzymes are regulated by insulin at the transcriptional level. Hence involvement of gluconeogenesis in blood glucose production is recognized as a target for treating hyperglycaemia.

1.2.4.1 Phosphoenolpyruvate carboxykinase

Phosphoenolpyruvate carboxykinase (PEPCK) is the first rate limiting enzyme in gluconeogenesis (Rognstad, 1979). The enzyme catalyzes the conversion of oxaloacetate into phosphoenolpyruvate with release of carbon dioxide (Croniger *et al.*, 2002). It requires the divalent manganese ion for activity and GTP or ITP as a phosphate donor (Croniger *et al.*, 2002). This reaction is important in gluconeogenesis because it is thought to be a rate limiting reaction since it can bypass the thermodynamically unstable reaction catalyzed by pyruvate kinase in glycolysis (Burgess *et al.*, 2007).

PEPCK exists as both mitochondrial (PEPCK-M) and cytosolic (PEPCK-C) forms with both isoenzymes showing 63% sequence identity. The two isoforms have molecular weights of approximately 69 kDa (Zimmer and Magnuson, 1990). PEPCK-M exists in liver before birth while PEPCK-C is only present at birth where it is thought to be linked to the beginning of gluconeogenesis and glucose homeostasis in the liver (Ballard and Hanson, 1967). Transcription of the enzyme is stimulated by glucagon (Lamers *et al.*, 1982) and glucocorticoids while it is inhibited by insulin (Sasaki *et al.*, 1984).

Diet and hormones such as glucocorticoids and insulin control only PEPCK-C gene transcription while PEPCK-M is constitutively expressed (Tilghman *et al.*, 1974). Apart from its role in gluconeogenesis, PEPCK-C is also involved in three more major pathways including glyceroneogenesis (Reshef *et al.*, 2003), synthesis of serine (Kalhan and Hanson, 2012) and conversion of glutamine and glutamate to phosphoenolpyruvate (Montal *et al.*, 2015). A role in cataplerosis has also been suggested where PEPCK-C breaks down carbon skeletons from amino acids which are then used in biosynthetic pathways such as gluconeogenesis in liver and kidney (Owen *et al.*, 2002).

The role of PEPCK-C in glucose metabolism highlights its importance in diabetes as one of the potential therapeutic targets for anti-diabetic drugs. PEPCK-C activity is increased in diabetic animals (Dayer *et al.*, 2009; Dayer and Dayer, 2010). Various studies have explored the use of inhibitors as a pharmacological potential of managing diabetes. Some have investigated post transcriptional gene silencing using vectors (Gómez-Valadés *et al.*, 2006) and others have

investigated the use of amino acids such as glutamate (Dayer and Dayer, 2010) or tryptophan (Alvares and Ray, 1974) as inhibitors of PEPCK-C activity.

1.2.4.2 Fructose-1, 6-bisphosphatase

Fructose-1, 6-bisphosphatase (FBPase) is a 37 kDa homotetramer enzyme involved in dephosphorylation of fructose-1,6-phosphate yielding fructose-6-phosphate and an inorganic phosphate (Zhang *et al.*, 2010). The enzyme requires divalent cations such as Mg^{2+}, Mn^{2+} and Zn^{2+} for its activity and monovalent cations such as K^{+} may further enhance enzyme activity. When coupled with PFK, FBPase is able to control the recycling between fructose-6-phosphate and fructose-1, 6-bisphosphate to aid in regulation of glycolytic/gluconeogenic cycle (Rashid *et al.*, 2002).

FBPase is regulated directly by AMP and F2,6BP, and indirectly by glucagon and insulin (Iversen *et al.*, 1997). F2,6BP (a powerful activator of PFK) is a competitive inhibitor of the FBPase substrate, fructose-1,6-bisphosphate while AMP inhibits FBPase at its allosteric site (Van Schaftingen and Hers, 1981; Rakus *et al.*, 2005). AMP inhibition is thought to be a mechanism of protecting the muscle against the loss of energy due to cycling between PFK and FBPase.

FBPase is a rate-limiting enzyme in the gluconeogenic pathway that plays a key role in the overall regulation of this pathway. Studies have shown that FBPase is involved in glucose-stimulated insulin secretion in β-cells (Kebede *et al.*, 2008; Zhang *et al.*, 2010). FBPase is expressed in β-cells where it acts as a regulator by controlling glucose sensing and insulin secretion. Overexpression of β-cells resulted in reduced insulin secretion (Kebede *et al.*, 2008). This suggests that the enzyme might play a role in β-cell dysfunction during pathogenesis of diabetes. Studies suggest that FBPase activity is increased in liver, kidney and skeletal muscle of alloxan-induced diabetic rats (Alam *et al.*, 2014). Administration of hypoglycaemic agents such as eugenol (Srinivasan *et al.*, 2014) and fraxetin (Murali *et al.*, 2013) also resulted in decrease in enzyme activity in diabetic rats. FBPase is regarded as a target for drugs that would inhibit endogenous glucose production without directly affecting glycogenolysis, glycolysis, and the tricarboxylic acid cycle (Erion *et al.*, 2005).

1.2.5 Glycogen metabolism

Glycogen is a form of stored glucose and glycogen metabolism involves two pathways, glycogenesis and glycogenolysis (Figure 1.2). Glycogen structure is characterized by straight chains of glucose linked by α-1,4 glycosidic bonds that branch every 10 to 13 residues by α-1, 6 linkages. Glycogen is stored in liver and skeletal muscle with the latter being a major site of glucose disposal (Jensen and Lai, 2009). The main function of glycogen is to maintain physiological blood glucose concentrations with the liver being the only major tissue that contributes directly towards the release of glucose in the blood (Nuttall *et al.*, 2008). Glycogen in skeletal muscle fails to directly contribute to free glucose in the blood due to the lack of the enzyme glucose-6-phosphatase. Instead, skeletal muscle contributes indirectly by the breakdown of glycogen through conversion into lactate which can be transported to the liver therefore contributing towards glycaemic control. Glycogen concentrations are regulated by glycogen synthase and glycogen phosphorylase activity.

1.2.5.1 Glycogen synthase

Glycogen synthase (GS) is the final enzyme in the synthesis of glycogen that catalyzes the α-1, 4-glycosydic linkage of UDP-glucose to glycogen and is a major rate limiting enzyme in glycogen synthesis (Jensen *et al.*, 2006). The enzyme is regulated allosterically and by covalent modifications. Phosphorylation of GS occurs at nine sites leading to deactivation of the enzyme during post translational modifications. Two of the sites (2 and 2a) are located at the amino terminal while the remaining (3a-c,4,5,1a-b) are located in the carboxyl terminal of GS. Phosphorylation at sites 2,2a,3a and 3b are reported to be the major sites involved in regulating enzyme activity *in vitro* (Skurat *et al.*, 1994) and *in vivo* (Skurat *et al.*, 2000; Højlund *et al.*, 2009). Insulin activates GS through protein kinase B-mediated phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3). Phosphorylation of GSK-3 decreases its activity resulting in decreased phosphorylation activation of GS (McManus *et al.*, 2005; Bouskila *et al.*, 2008). GS is allosterically activated by glucose-6-phosphate (Bouskila *et al.*, 2010) while ATP acts as an inhibitor by lowering the enzyme's affinity for UDP-glucose (Solling, 1979). Studies have shown that glycogen synthase is impaired in type-II diabetes (Thorburn *et al.*, 1990; Jensen *et al.*, 2012).

1.2.5.2 Glycogen phosphorylase

Glycogen phosphorylase (GP) is a rate limiting enzyme that catalyzes the breakdown of the terminally linked glucose residues on glycogen into glucose-1-phosphate which subsequently leads to the formation of glucose (Manabe *et al.*, 2013). Three isoforms that are known to exist in skeletal muscle; liver and brain are respectively located on chromosomes 11, 20 and 14. All 3 isoforms having a molecular weight of approximately 97 kDa made up of 842 residues (Monod *et al.*, 1965)and pyridoxal-5-phosphate acts as a cofactor of the enzyme.

GP is regulated through allosteric control by AMP or IMP and through phosphorylation and dephosphorylation (Johnson, 1992). This allows the enzyme to exist as two interconvertible forms. GPa is the phosphorylated form with high activity and high affinity for the substrate. This is known as the relaxed (R) state. GPb is its unphosphorylated form that has low activity and low affinity for its substrate. This is known as the tense (T) state (Johnson, 1992). Both forms are required for balance between inactive T state and active R state. In the resting muscle, GP exists as GPb which can be activated through phosphorylation by phosphorylase kinase on ser14 or through allosteric binding of AMP or IMP converting it to GPa (Villar-Palasi and Wei, 1970). Dephosphorylation of GPa is catalyzed by phosphorylase phosphatase (Villar-Palasi and Wei, 1970). GP is allosterically inhibited by glucose, glucose-6-phosphate, fructose-1-phosphate, ADP, xanthines and ATP (Figure 1.5). These hinder the degradation of glycogen by disabling balance between the T state and R state (Nuttall *et al.*, 2008).

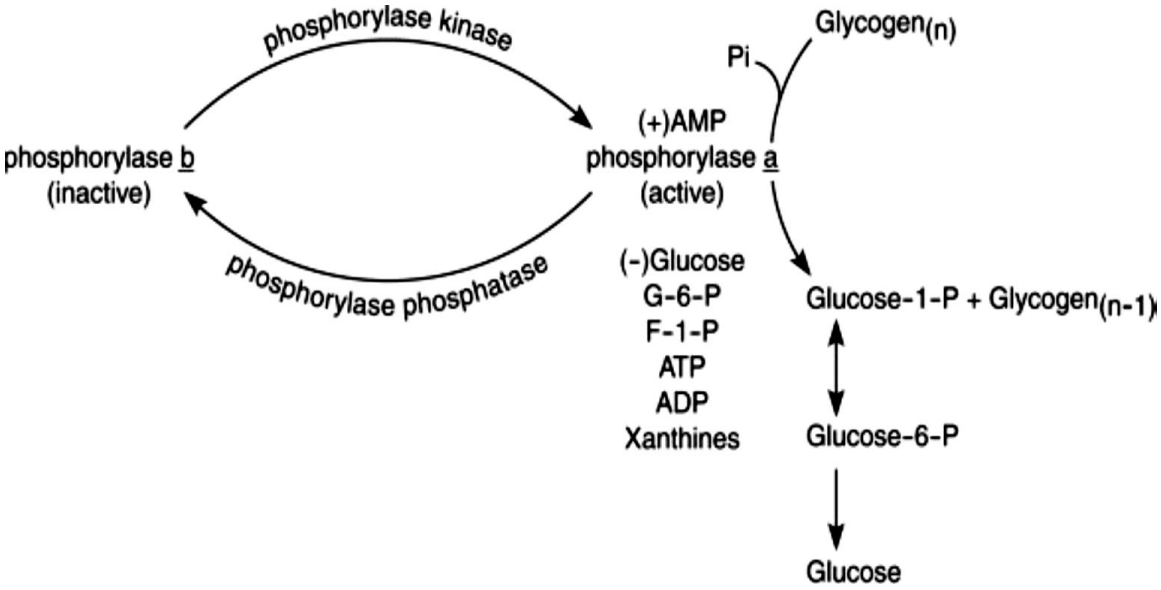


Figure 1. 5: Regulation of glycogen phosphorylase, (Adapted from Nuttall et al., 2008).

Studies have shown that STZ- induced diabetic animals are characterized by increased levels of GPa (Docsa *et al.*, 2011). The use of GP inhibitors has been explored as possible drugs for controlling type-II diabetes since they may improve glucose tolerance, prevent glycogen breakdown and stimulate glycogen synthesis (Oikonomakos *et al.*, 2000; Baker *et al.*, 2005; Nagy *et al.*, 2013)

1.3 Treatment of Diabetes

1.3.1 Conventional treatment of diabetes

Although diabetes cannot be cured, it can be managed through tight glycaemic control thus reducing complications associated with the disease. This is achieved mainly through medication, lifestyle changes or a combination of both (Norris *et al.*, 2002). At present, the management of Type-I diabetes involves the use of insulin therapy, diet and exercise. Dietary modifications, physical exercise, insulin therapy and oral drugs such as sulphonylureas, biguanides, alpha-glucosidase inhibitors are used for the management of type-II diabetes (Norris *et al.*, 2002).

Insulin therapy

Type-I diabetes is conventionally treated with insulin therapy. Insulin is a 6 kDa peptide that is secreted by pancreatic β-cells in response to an increase in blood glucose levels. Insulin or insulin analogs are required to stimulate uptake or entry of glucose into tissues such as liver and skeletal muscle where glucose is converted into energy or it is stored as glycogen thus clearing any excess glucose (Stapleton, 2000). Insulin also stimulates glycolysis and glycogenesis but inhibits gluconeogenesis and glycogenolysis thus reducing glucose production in hepatic tissue (Cummings, 2006). Insulin also regulates transcription of genes of several key enzymes associated with carbohydrate and fatty acid metabolism (O'Brien *et al.*, 1990).

The use of insulin however has its own shortcomings, which makes it less than ideal for management of diabetes. The most common side effect of insulin therapy is hypoglycaemia (Friedewald *et al.*, 2008) and weight gain (Mäkimattila *et al.*, 1999). Weight gain may result in obesity which is known to cause insulin resistance and one of the risk factors of cardiovascular diseases and hypertension (Landsberg *et al.*, 2013). This makes insulin a less than ideal form of treatment as it may lead to more complications than the original problem of diabetes. Other side effects include insulin allergy (Wu *et al.*, 2013), auto antibodies (Yu *et al.*, 2012), insulin resistance (Gambineri *et al.*, 2012) and altered metabolic control (Nolan *et al.*, 2015).

Figure 1.6 summarizes the oral drugs used as glucose lowering agents in the management of type -II diabetes. These drugs include sulphonylureas, meglitinides, α-glucosidase inhibitors, thiazolidinediones and metformin.

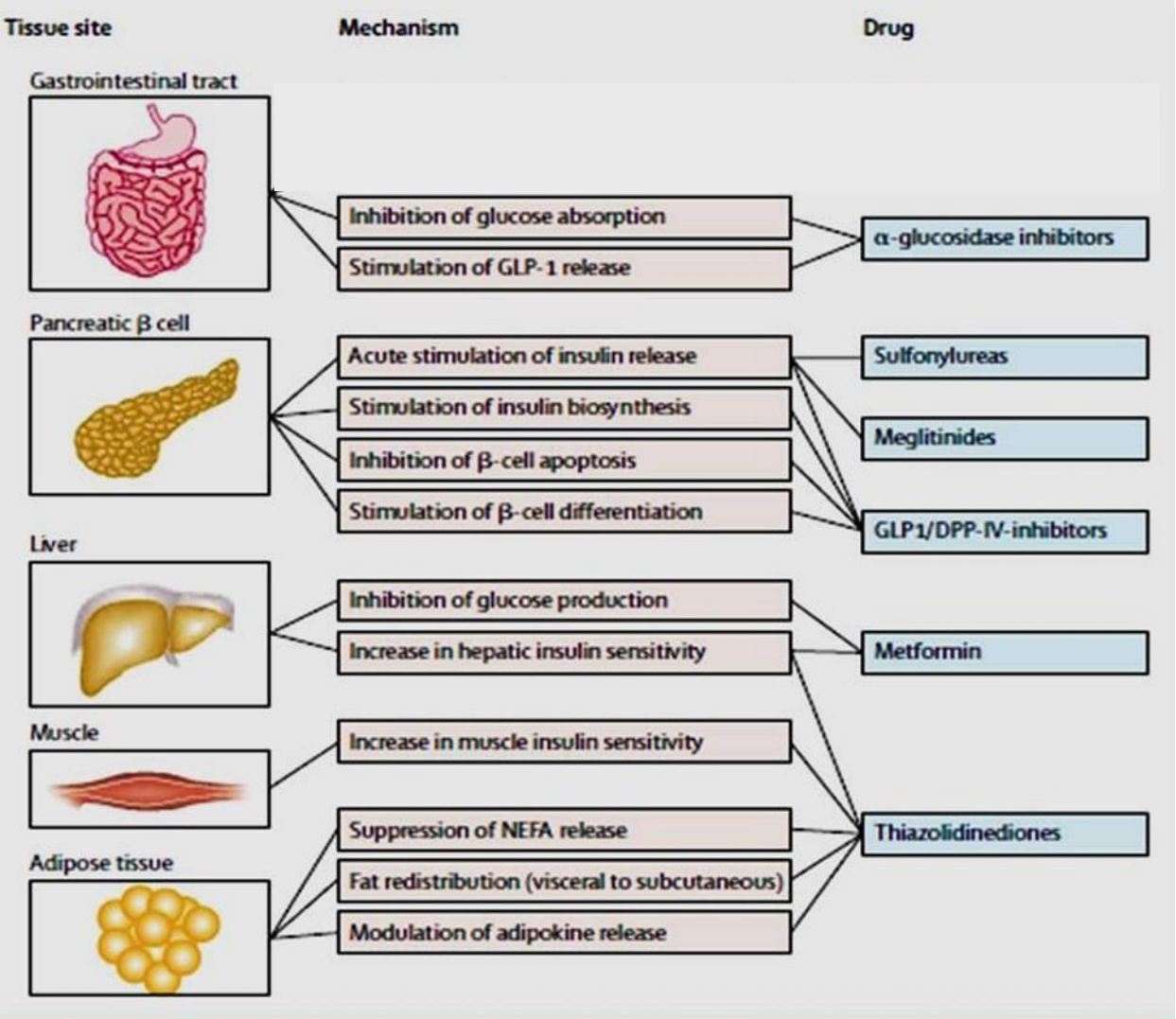


Figure 1. 6:Treatment of diabetes using conventional drugs, (Adapted from Stumvoll *et al.*, 2005). GLP1 = glucagon-like peptide 1. DPP-IV = dipeptidyl peptidase IV.

Sulphonylureas and meglitinides

Sulphonylureas and meglitinides stimulate the secretion of insulin in β-cells (Figure 1.6) (Sunaga et al., 2001). This occurs by binding to a sulphonylurea SUR receptor on pancreatic β-cells leading to closing of ATP-sensitive potassium channels. The closing of these channels results in decrease in potassium influx and depolarization of β-cell membrane which in turn results in an uptake of calcium ions into β-cells. The increased intracellular calcium ions activates the translocation of

secretory granules containing insulin to the cell surface and release of insulin through exocytosis (Proks *et al.*, 2002). Meglitinides act on potassium dependent ATPase enzyme leading to the closure of the ATP sensitive potassium channel similarly to sulphonylureas (Sunaga *et al.*, 2001).

Sulphonylureas are classed into first and second generations based on when they became available. First generation sulphonylureas include acetohexamides, chlorpropamides, tolazamides and tolbutamides. Second generation sulphonylureas include glimepirides, glipizides and glyburides (Proks *et al.*, 2002). Both generations of drugs have the same level of efficacy but second generation sulphonylureas are considered more potent and safer than first generation making them more commonly prescribed (Philippe and Raccah, 2009). Meglitinides consist of repaglinide and nateglinide (Sunaga *et al*., 2001).

The advantages of using sulphonylureas include relative affordability and their ability to reduce microvascular complications (UKPDS, 1998). The adverse effects however may outweigh the advantages of these drugs. The side effects of sulphonylureas and meglitinides include hypoglycaemia and weight gain (UKPDS, 1998; Nauck *et al.*, 2007) Since sulphonylureas are able to act at low concentrations of plasma glucose and over a long period of time, risks of potential hypoglycaemia has been shown in a number of studies (Bodmer *et al.*, 2008; Ragia *et al.*, 2014). The loss of insulin secretory capacity over time leading to the eventual exhaustion of β-cells often referred to as secondary failure also occurs when using these drugs (Polo *et al.*, 1998; Jiang *et al.*, 2016).

Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors include acarbose and miglitol. They w act by decreasing the rate of carbohydrate absorption and digestion by competitively inhibiting the action of enzymes, primarily α-glucosidase in the small intestine (van de Laar *et al.*, 2005). An α -glucosidase is an enzyme located in the brush border of the epithelium of the small intestine where it breaks down disaccharides and other carbohydrates into absorbable glucose. Alpha-amylase hydrolyses complex starch. Alpha-glucosidase inhibitors competitively inhibit these enzymes by binding to the carbohydrate binding region of the enzymes causing a delay in carbohydrate absorption and reducing postprandial plasma glucose levels (Holman *et al.*, 1999).

The advantages of α-glucosidase inhibitors include their ability not to cause hypoglycaemia or weight gain (Holman *et al.*, 1999) as seen is other oral hypoglycaemic agents. Studies have shown that acarbose could reduce the risk of progression from impaired glucose tolerance to type-II diabetes (Chiasson *et al.*, 2004). It has also been shown to reduce the risk of cardiovascular disease and hypertension events in patients with impaired glucose tolerance (Chiasson *et al.*, 2003; Chiasson *et al.*, 2004; Hanefeld *et al.*, 2004). The side effects of these drugs however include gastro-intestinal symptoms such as diarrhoea and abnormal dysfunction and flatulence (Hsiao *et al.*, 2006). Abnormal liver function has also been reported in some cases (Hsiao *et al.*, 2006).

Thiazolidinediones

There are three types of drugs found in this class of anti-diabetic agents commercially available which include troglitazone, pioglitazone and rosiglitazone. Thiazolidinediones are antidiabetic agents that enhance insulin sensitivity. They increase glucose uptake and utilization in peripheral tissues and reduce hepatic glucose production (Miyazaki *et al.*, 2001). Thiazolidinediones are ligands that act on the peroxisome proliferator activated receptor (PPAR-γ) that are found in skeletal muscle and hepatic cells and in predominantly adipose cells (Lehmann *et al.*, 1995). Activation of PPAR-γ in adipocytes induces expression of genes involved in insulin signaling cascade such as GLUT4 therefore improving insulin sensitivity (Hammarstedt *et al.*, 2005).

Thiazolidinediones have been shown to achieve glycaemic control in patients with type-II diabetes when used as a monotherapy or in combination with other drugs such as sulphonyureas or metformin. The side effects however outweigh the benefits of using thiazolidinediones. These include weight gain, hepatotoxicity (Marcy *et al.*, 2004), increase in myocardial infarctions, risk of heart failure (Nissen and Wolski, 2007) and bone fracture incidences (Kahn *et al.*, 2008). The risk of bladder cancer has also been linked to the use of thiazolidinediones over a 2 year period (Lewis *et al.*, 2011).

Dipeptidyl peptidase-4 inhibitors

Dipeptidyl peptidase-IV (DPP-IV) inhibitors are a class of antidiabetic agents that increase the half-life of incretin hormones, glucagon-like peptide-1 (GLP1) and glucose dependent insulinotropic polypeptide (GIP) by prolonging their degradation (Mentlein *et al.*, 1993; Mari *et*

al., 2005). GLP-1 and GIP are produced in response to meals, improves β- cell function by stimulating insulin secretion and suppressing the release of glucagon (Ahrén *et al.,* 2004). There is evidence of DPP-IV delaying gastric emptying and increasing mass of islet cells (Egan *et al.,* 2003; Aschner *et al.,* 2006). Common available DPP-IV inhibitors include sitagliptin and vildagliptin (Thornberry and Gallwitz, 2009).

DPP-IV inhibitors are used as a monotherapy or as a combination with other agents such as metformin or sulphonylureas (Aschner *et al.*, 2006; Charbonnel *et al.*, 2006). DPP-IV inhibitors however, exhibit side effects on the upper respiratory tract causing infections (Lambeir *et al.*, 2003).

Biguanides

This class of drug includes burformin, phenformin and metformin. The biguanide metformin is the most common drug used for treatment of type-II diabetes (Boussageon *et al.*, 2012) although the mode of action is unclear. Metformin enhances insulin sensitivity in hepatic and peripheral tissue (Stumvoll *et al.*, 1995). It inhibits gluconeogenesis and stimulates glycolysis by lowering glucose production in liver and increasing glucose disposal in peripheral tissues respectively (Musi *et al.*, 2002). Metformin also decreases absorption of glucose in the intestine (Ikeda *et al.,* 2000). Metformin lowers glucose levels without increasing insulin secretion.

Since insulin secretion is not affected, metformin does not cause hypoglycaemia when used as a monotherapy treatment (Bodmer *et al.*, 2008). Another benefit of using metformin is that it does not cause weight gain (Mäkimattila *et al.*, 1999) and also improves lipid profile . The efficacy of metformin diminishes over time although why this happens is still unclear. The use of phenformin has since been discontinued because it caused lactic acidosis (Bergman *et al.,* 1978). Nausea, diarrhea and abdominal pains are the most common side effects associated with the use of metformin (Moghetti *et al.*, 2000).

Although these anti-diabetic agents can exhibit glycaemic control, unfortunately none of them can alleviate all complications associated with diabetes without causing any major side effects.

1.3.2 Treatment using herbal extracts

The diverse use of plants in traditional medicine has been practiced by various countries, cultures and tribal groups since ancient times. Herbal medications have been used in the treatment of a wide variety of ailments all over the world (Adbullahi, 2011). A large population of developing countries still depend on traditional medicine derived from plants (Adbullahi, 2011). However, the efficacy and mechanism of action of some of these extracts are yet to be elucidated. Many plants contain phytoconstituents such as glycosides, saponins, flavonoids and terpenoids which have been shown to have antidiabetic potential**.** Over 800 plants have been identified for their potential antidiabetic ability (Daisy and Eliza, 2007), but only a few of these plants have been proven to have significant effect (Jia *et al.*, 2009).

Some proposed mechanisms of plant active ingredients include the ability to stimulate insulin secretion (Keller *et al.*, 2011), antioxidant effects (Ravi *et al.*, 2004), regulation of enzymes, increasing the expression of insulin receptors (Gao *et al.*, 2007) or acting to stimulate the secretion of insulin. Their mode of action however is limited by factors which may include that plant extracts are made up of different constituents and only a few of those may be therapeutically effective. Also, different parts of a plant have different ingredient profiles thus upon extraction may yield different active ingredients. Regardless of these factors, these plants need to be investigated for their advantages. The use of plants for treating diseases is becoming popular due to their safety, effectiveness, minimal side effects, cheap cost and availability.

1.3.2.1 Oleanolic acid

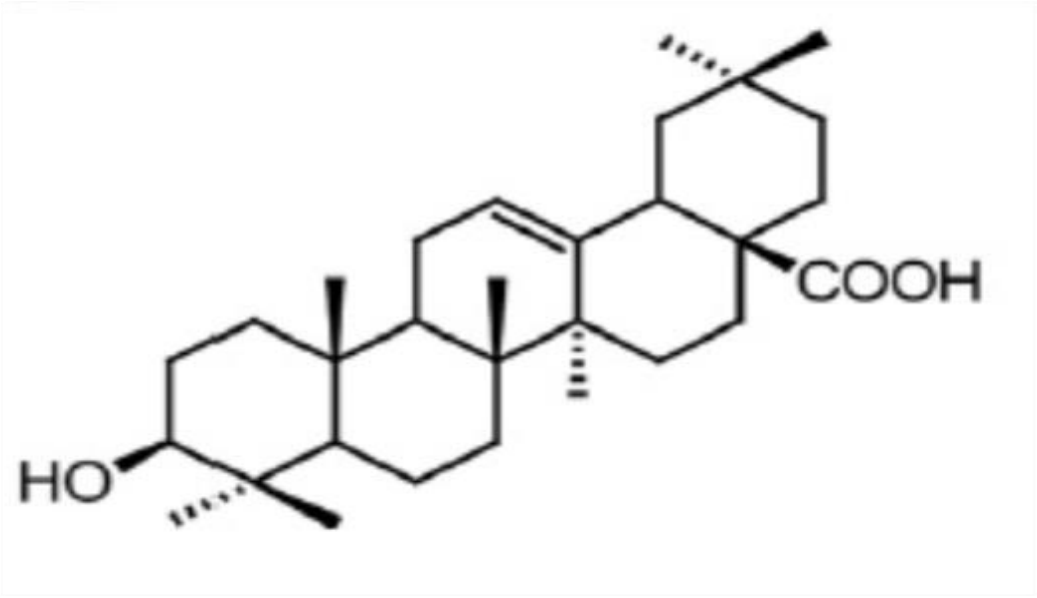


Figure 1. 7: Chemical structure of oleanolic acid (Pollier and Goossens, 2012).

Oleanolic acid (OA) is a pentacyclic triterpenoid compound (Figure 1.7) prevalent in plants belonging to the Oleaceae family, especially olives which serve as the main commercial source of oleanolic acid (Sporn *et al.*, 2011). The biologically active compound is usually found with its isomer ursolic acid with which it shares its pharmacological properties (Liu, 1995). Common functions of OA include protection from pathogens and prevention from water loss (Heinzen *et al.*, 1996). An example of this is seen in olive leaves where OA protects against fungal attack (Kubo and Matsumoto, 1984). In plants, OA may become conjugated and serve as a defense compound against pathogens (Szakiel *et al*., 2003).

1.3.2.1.1 Pharmacological properties of OA

OA has been used as an over the counter drug for liver diseases such as viral hepatitis due to its hepatoprotective effect. Even though its mechanism of action has not yet been fully elucidated, OA has been shown to be effective in protecting the liver against chemically induced liver injury due to diseases such as fibrosis and cirrhosis (Wang *et al.*, 2010). OA has been reported to increase

nuclear accumulation of a key regulator of transcription and detoxifying enzyme known as nuclear respiratory factor 2 (Nrf2). This in turn leads to the induction of Nrf2-dependent genes that play a role in the protection of the liver (Reisman *et al.*, 2009). Other properties of OA include cardioprotective (Mapanga *et al.*, 2012), anti-inflammatory, anti-oxidant (Somova *et al.*, 2003) and anti-cancer (Liby *et al.*, 2007; Laszczyk, 2009) effects.

1.3.2.1.2 Hypoglycaemic effects of oleanolic acid

Previous studies have shown that OA has potential hypoglycaemic effects *in vitro* and *in vivo*. OA has been reported to enhance insulin secretion in pancreatic islet cells *in vitro* comparable to the standard drug, tolbutamide (Teodoro *et al.*, 2008). *Syzygium aromaticum*, a plant rich in oleanolic acid was shown *in vitro* to limit the expression of hepatic gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase enzymes involved in glucose production thus indicating hypoglycaemic activity (Prasad *et al.*, 2005). STZ-induced diabetic animals treated with 100 mg/kg b.w. OA were shown to have lowered blood-glucose comparable to those treated with the standard drug metformin (Wang *et al.*, 2011).

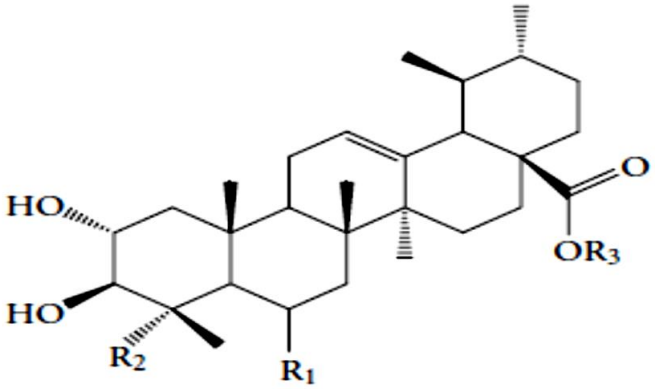
1.3.2.2 *Centella Asiatica* (L.) Urban

Figure 1. 8: Picture of *Centella asiatica*

Centella Asiatica (L.) Urban (CA) is a herbaceous, mildly scented plant belonging to the family *Umbelliferae* known by different vernacular names such as "Gotu kola" in India," Pohekula" in Hawaii, "Indian pennywort" in USA or "Icudwane" in South Africa. The plant has ovoid shaped leaves that emerge in clusters and thrives in shady, moist and marshy areas (Figure 1.8). CA is native to many tropical and subtropical regions such as India, Australia, Madagascar, Japan, Colombia, China, South America as well as South Africa (Verma *et al.*, 1999). The plant is used worldwide for its nutritional purposes where it is ingested in food and beverages providing needed proteins, minerals and vitamins such as ascorbic acid, thiamine and β-carotene (Gupta *et al.*, 2005; Odhav *et al.*, 2007). It may either be consumed raw as a salad or ingested along with sweet potatoes or coconut milk to tolerate its bitter taste (Huda-Faujan *et al.*, 2009).

1.3.2.2.1 Phytochemistry of CA

The phytochemistry of CA reveals a rich chemical composition including triterpenes, saponins, flavonoids, glycosides, alkaloids essential oils and more (Ling et al., 2000). The triterpene acids which include asiatic acid and madecassic acid and their respective triterpene ester glycosides, asiaticosides and madecassosides (Figure 1.9) are known to be some of the main biologically active components of the plant (Ling *et al.*, 2000). These triterpenes are used as biomarkers of quality assessment of the plant (Ling et al., 2000). CA has been revealed to also possess a high degree of polyphenols contributed mostly by various flavonoids such as quercetin, kaempferol, catechin and naringin which are thought to be involved in the antioxidant activity of CA (Zainol *et al.*, 2003; Huda-Faujan *et al.*, 2009; Reihani and Azhar, 2012). The plant is also known to be rich in essential oils, saponins, alkaloids and amino acids such as serine, alanine, threonine, aspartic acid, glutamic acid, histidine, lysine and threonine (Zainol *et al.*, 2003).



	R_1	R_2	R_3
Asiatic acid	H	CH_2OH	H
Madecassic acid	OH	CH_2OH	H
Madasiatic acid	OH	CH_3	H
Asiaticoside	H	CH_2OH	Glucose-glucose-rhamnose
Madecassoside	OH	CH_2OH	Glucose-glucose-rhamnose

Figure 1. 9: Main biologically active components of CA, (Adapted from Orhan 2012).

Due to its rich chemical composition, CA has been used in folk medicine for the treatment of various illnesses and described by some as a "herbal cure all" (Arora *et al.*, 2002). These illnesses include jaundice, vomiting, scabies, leprosy, tonsils, diarrhea, skin disorders, asthma, stomach disorders, cholera, elephantiasis nerve tonic, hepatitis and many more. Apart from aiding in treatment of these illnesses, CA has gained much of its popularity in wound healing, and skin disorders as well as memory enhancement (Arora *et al*., 2002)

1.3.2.2.2 Pharmacological properties of CA

CA has been reported to improve memory and learning in mice during postnatal brain development (Rao *et al.*, 2005). In another study, CA was found to ameliorate colchicine- induced memory impairment and associated oxidative stress that lead to difficulties in learning and memory ability of male Wistar rats. This suggested CA as a prospective treatment of neurodegenerative disorders such as Alzheimer's disease (Kumar *et al.*, 2009). The antioxidant effect of CA also showed great

potential in neurological disorders by protecting rat brain from age related oxidative damage when administered at those of 300 mg/kg b.w. daily for 60 days (Subathra *et al.*, 2005).

Asiaticosides are involved in wound healing and are known to have vascular effects while brahmosides are thought to have effects on the central nervous system (Gohil *et al.*, 2010). Some properties of CA include hepatoprotection (Antony *et al.*, 2006) where CA was shown to potentially protect the liver against carbon tetrachloride-induced liver damage in male Sprague Dawley rats. This was shown through the decrease in the levels serum marker enzymes; aspartate amino transferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) and increase in serum proteins and albumin levels.

1.3.2.2.3 Hypoglycaemic effect of CA

CA has been traditionally used for the management of diabetes in Asia, Indian Ayurvedric medicine (Grover *et al.*, 2002) and Africa, specifically Tanzania (Mutayabarwa *et al.*, 2005). Both methanol and ethanol extracts of CA showed hypoglycaemic potential in diabetic rats by lowering glucose levels in glucose tolerance test. However the methanol extract showed higher hypoglycaemic potential than the ethanol extract, suggesting that methanol extraction may enrich the active constituents of the plant (Chauhan *et al.*, 2010). Another study showed that diabetic rats treated with 50, 100 and 200 mg/kg b.w. of CA daily for 22 days had lower blood glucose levels when compared to the standard drug metformin (Rahman *et al.*, 2012). Diabetic rats treated with 50 mg/ kg b.w. Asiatic acid, a biologically active constituent of CA, has been shown to lower plasma glucose levels of STZ- induced diabetic rats. It was also shown to influence the activity of glucose-6-phosphatase, fructose-1,6-bispospatase, hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase as well as glycogen content in liver of diabetic rats (Ramachandran and Saravanan, 2013).

1.4 Rationale of study

Diabetes is characterized by hyperglycaemia and treatment mainly focuses on glycaemic control. Several natural plants and plant-derived compounds including CA and OA respectively have been investigated and shown to have anti-diabetic potential (Teodoro *et al.*, 2008; Rahman *et al.*, 2012) Skeletal muscle accounts for about two thirds of total glucose uptake under insulin stimulated conditions (Saltiel and Kahn, 2001). Skin being the largest organ in the body plays a significant role in the body's total glucose metabolism. Glycolysis, gluconeogenesis, glycogenolysis and glycogenesis play roles important role in glucose metabolism mainly through regulatory enzymes that catalyze the rate limiting reactions in each pathway. An understanding of how OA and CA affect the rate limiting enzymes in glucose metabolism is yet to be fully elucidated. Thus, the study investigated the effects of OA and CA on the rate limiting enzymes of glucose metabolism.

1.5 Aim and objectives

Aim

To determine the effect of OA and CA on key carbohydrate metabolizing enzymes in skeletal muscle and skin of streptozotocin-induced type-II diabetic rats.

Objectives

1. To determine the activities of hexokinase, phosphofructokinase and fructose-1,6-bisphosphatase in diabetic rats after treatment with OA and CA extract using enzyme assays.

2. To measure the expression of phosphofructokinase and glycogen phosphorylase using SDS-PAGE and western blot analysis.

3. To determine glycogen phosphorylase and glycogen synthase activities; and glycogen content in muscle and skin after treatment with OA and CA.

4. To assess amelioration of tissue damage by carrying out a histological analysis of both muscle and skin tissue following treatment with CA.

5. To assess amelioration of tissue damage by determining serum aspartate transaminase (AST) and alanine transaminase (ALT) levels after treatment with CA.

1.6 Outline of the thesis

This dissertation is comprised of six chapters. The first chapter above is an introductory chapter that is composed of an in-depth literature review, rationale of the study and research aim and objectives. The literature review encompasses all relevant and recent information about diabetes and current oral treatments and their limitations. It includes a review of the known anti-diabetic effects of Oleanolic acid and *Centella asiatica*.

Chapter 2, the Materials and Methods chapter, describes all reagents and methods used to acquire the results. This chapter covers the induction of insulin insufficiency as well as insulin resistance and insulin insufficiency models of type-II diabetes in male Sprague-Dawley rats obtained from the Biomedical Research unit, Westville campus. Enzyme activity assays, western blots and histological examinations were carried out on harvested muscle and skin tissues while alanine amino transferase and aspartate amino transferase activities were determined in serum Statistical analysis was performed using Graphpad software.

Chapter 3 comprises of results obtained from experiments described in the previous chapter. These results include enzyme activities, SDS-PAGE, enzyme expressions and histological examinations of both muscle and skin samples.

Chapter 4 is the discussion which evaluates the anti-diabetic effects of oleanolic acid and *Centella asiatica* in muscle and skin of streptozotocin-induced diabetic animals. A comparison is also made between their effects in the two types of diabetic models used in this study.

Finally, Chapter 5 contains the conclusion, limitations and possible future aspects of the study while Chapter 6 comprises of the references and appendices.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Animals

Male Sprague-Dawley rats weighing (250-300 g) were obtained from Biomedical Research Unit at University of KwaZulu-Natal, Westville campus. The animals were fed a standard rat chow diet *ad libitum* (Meadows, Pietermaritzburg, South Africa) and allowed free access to drinking water for the entire experimental period. Animals in which diabetes was to be induced to determine the effects of *Centella asiatica* extract were only allowed to drink 10% fructose solution prior to induction. Procedures involving animals and their care were conducted per the guidelines of the University of KwaZulu-Natal (UKZN) Research Ethics Committee which approved the study (Ethical Clearance 112/14/Animal).

2.1.2 Chemicals

The following reagents were purchased from Sigma Aldrich (through Capital Lab Supplies, New Germany, South Africa): Streptozotocin (STZ), dimethyl sulphoxide (DMSO), potassium chloride, phenol solution, fructose, glucose, trizma base, ethylene diaminetetraacetic acid (EDTA), benzamidine, phenylmethanesulfonylfluoride (PMSF), sodium orthovanadate (Na_3VO_4), aprotinin, triethyl amine (TEA), magnesium chloride($MgCl_2$), adenosine triphosphate (ATP) ,β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$), β-Nicotinamide adenine dinucleotide (reduced) (NADH), 3-(N-morpholino) propanesulfonic acid (MOPS), 3-(N-morpholino) ethanesulfonic acid (MES), uridine diphosphate glucose (UDPG), glucose-6-phosphate, radiolabeled glucose-1-phosphate disodium salt hydrate Sodium fluoride (NaF), dithiothreitol (DTT), bovine serum albumin (BSA), glycogen, glucose-6-phosphate dehydrogenase, phosphoglucomutase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, aldolase, bromophenol blue, Glycerol, acrylamide.

Methanol, ethanol, sodium potassium tartatrate ($KNaC_4H_4O_4.H_2O$), potassium iodide (KI), potassium hydroxide (KOH), sodium sulphate ($NaSO_4$), hydrated copper sulphate ($CUSO_4.H_2O$),

sulphuric acid, and Folin-Ciocalteu phenol reagent were purchased from Merck, Modderfontein, South Africa. Sodium dodecyl sulphate (SDS), tris, N, N, N, N-Tetramethlyethylenediamine (TEMED),10X Tris/Glycine/SDS buffer, ammonium persulphate, Immun-star ™ alkaline phosphate substrate, goat anti rabbit-IgG alkaline phosphate conjugate, immun-star ™ enhancer were purchased from BIORAD, Johannesburg, South Africa. The antibodies anti-Glyceraldehyde -3-phosphate dehydrogenase (anti-GAPDH), anti-glycogen phosphorylase (anti-GP) and anti-phosphofructokinase were purchased from ABCAM (through Biocom Biotech, Centurion, South Africa). Deionized water was used to prepare all reagents.

2.2 Methods

2.2.1 Preparation of oleanolic acid

An oleanolic acid (OA) suspension was prepared in a vehicle containing dimethyl sulphoxide (DMSO): physiological saline solution (1:2). The OA was first suspended in DMSO and then diluted by an equal volume of saline.

2.2.2 Preparation of *Centella asiatica* extract

Centella asiatica leaves were freshly harvested from the University of KwaZulu-Natal, Westville Campus, Durban, South Africa. A photographic voucher specimen (Dladla 2) containing all characteristics of the leaves was deposited at the Ward Herbarium at University of KwaZulu-Natal for authentication. The leaves were authenticated by Professor Ashley Nicholas at the Department of Biology, University of KwaZulu-Natal. Leaves were air-dried and the dried leaf material was then ground to fine powder form using a grinder and thereafter soaked in 1L methanol at room temperature for 4 days. The extracts were collected in pre-weighted glass bottles and then filtered using Whatman no.1 filter paper. Filtrates were evaporated at 60°C in a water bath to remove methanol. A 10% yield was obtained from 500 g of dried leaves which yielded 50 g after extraction. CA leaf extract was dissolved in deionised water before being administered to the animals.

2.2.3 Induction of diabetes

Diabetes was induced by a single intraperitoneal (i.p) injection of streptozotocin (STZ) 40 mg/kg body weight dissolved in freshly prepared cold 0.1 M citrate buffer (pH 4.5). This gives insulin insufficiency "type-II" diabetic rats that are not insulin resistant. These animals were treated with OA for comparison with previous studies (Mukundwa *et al.*, 2016a; Mukundwa *et al.*, 2016b) in which STZ at 60 mg/kg body weight was used to induce type-I diabetes. Diabetes was also induced by having animals drink 10% fructose for 14 days followed by a single intraperitoneal (i.p) injection of streptozotocin (STZ 40 mg/kg body weight) dissolved in freshly prepared cold 0.1 M citrate buffer (pH 4.5). This gives type-II diabetic rats that have both insulin resistance and insufficiency (Wilson and Islam, 2012). These animals were used for the CA study. Animals with a fasting blood glucose concentration of 7 mM to 16 mM after a week of induction were considered stable diabetic animals. Fasting blood glucose was measured using a glucometer (Accu-Check perfoma, USA).

2.2.4 Treatment of animals

Male Sprague-Dawley rats (250-300 g) used to determine the effects of OA were randomly divided into non-diabetic (N) and diabetic (D) groups (Figure 2.1). Non- diabetic animals were further subdivided into non-diabetic control animals (NC) and non-diabetic treated with 80 mg/kg b.w. of OA (NO). Diabetic rats were subdivided into diabetic control (DC), diabetic rats treated with 80 mg/kg b.w. of OA (DO) and diabetic groups treated with metformin 300 mg/kg b.w. of metformin dissolved in distilled water (DM). The control groups were orally dosed with 3 ml/kg b.w. of DMSO: Physiological saline (1:2) daily. Groups treated with OA were orally dosed with 80 mg/kg b.w. of OA (Ngubane *et al.*, 2011) while groups being treated with metformin were given 300 mg/kg b.w. of metformin dissolved in distilled water. All the groups were treated daily for 14 days.

Rats used to determine the effects of CA extract were divided into non-diabetic (N) and diabetic rats (D) (Figure 2.2). Non-diabetic rats were grouped as non-diabetic control (NC) while diabetic rats were further divided into diabetic control (DC), diabetic rats treated with 500 mg/kg b.w. (D+500) and 1000 mg/kg b.w. (D+1000) of CA extract and diabetic rats treated with 300 mg/kg

b.w. of metformin (DM). The control groups were orally dosed with 3 ml/kg b.w. of DMSO: Physiological saline (1:2) daily. All the groups were treated daily for 14 days.

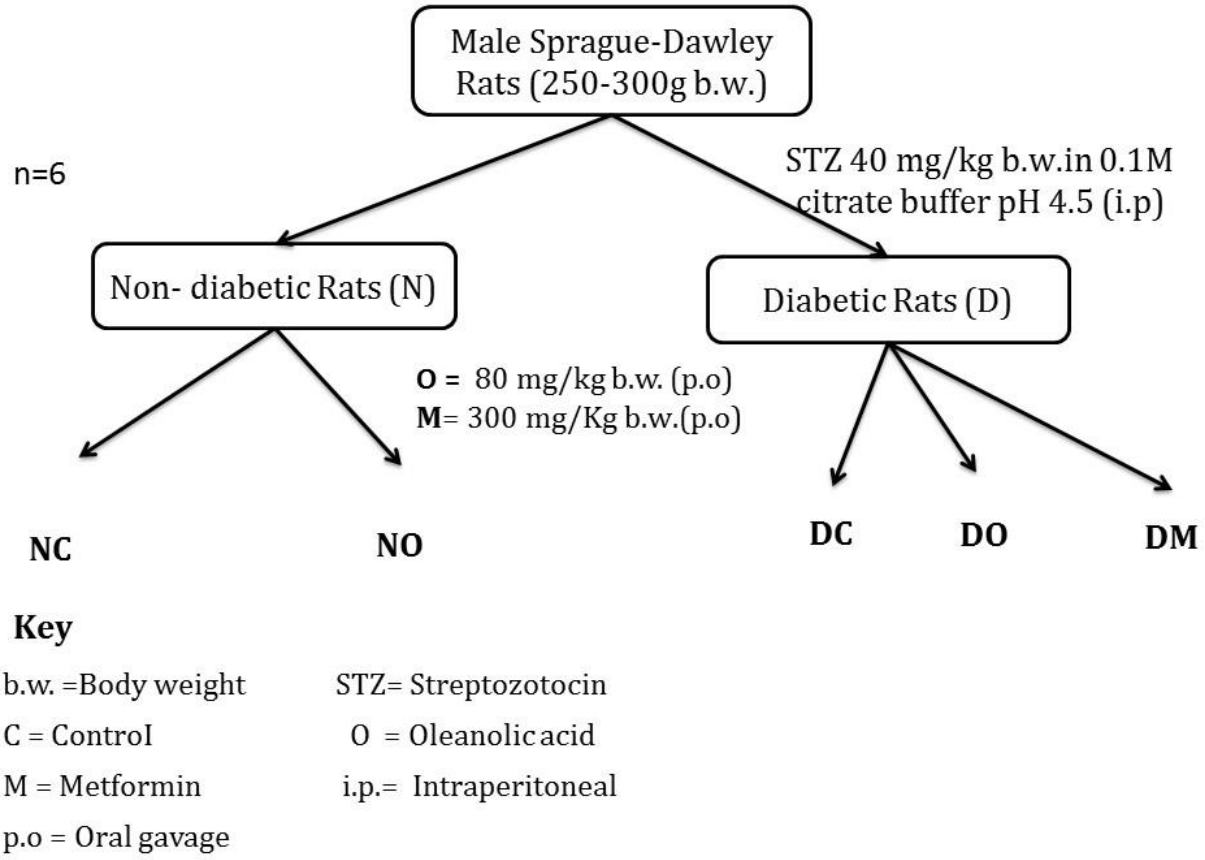


Figure 2. 1: Experimental design of OA treatment study

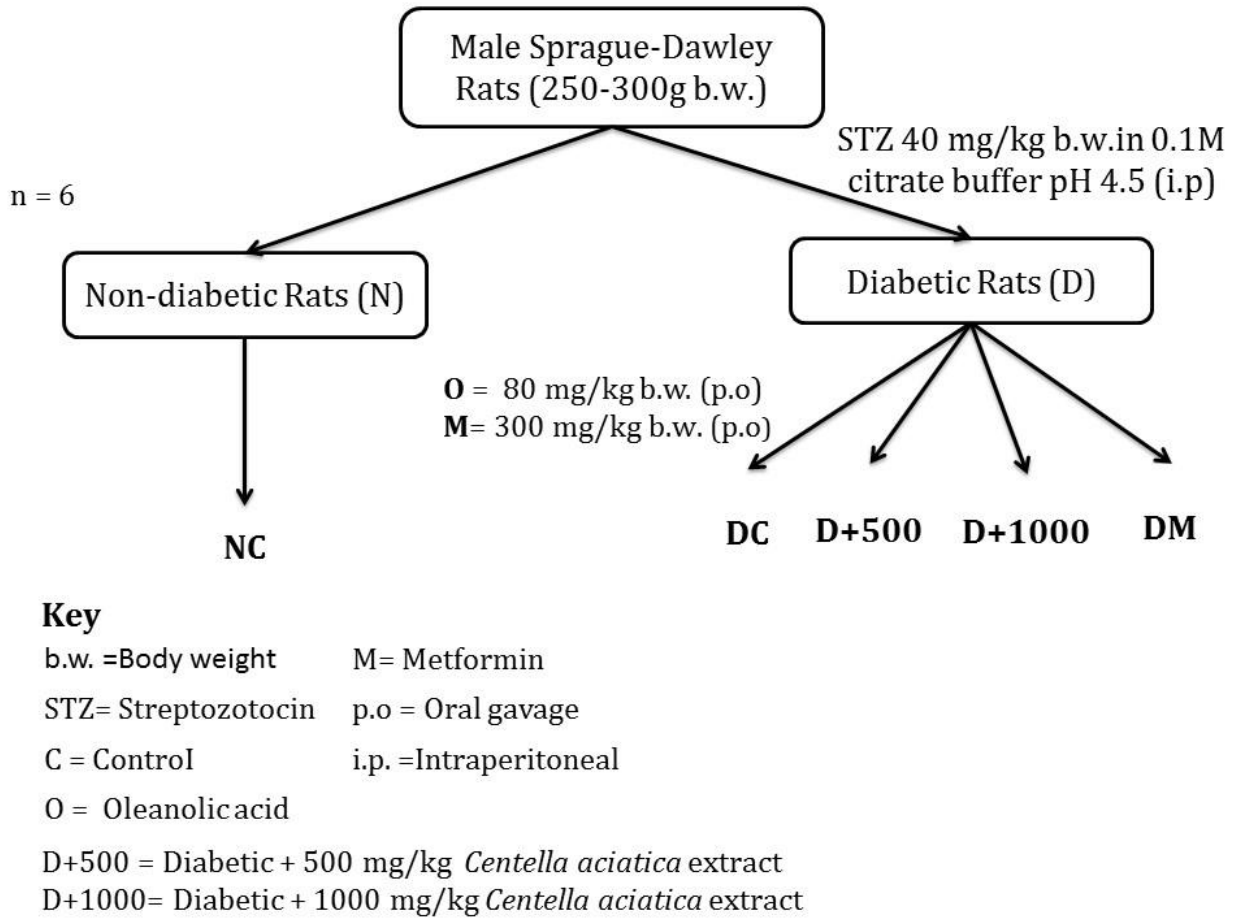


Figure 2. 2 Experimental design of CA treatment study. Rats drank 10% fructose for 14 days before STZ treatment.

2.2.5 Harvesting of blood and tissues

On the 15th day, rats were euthanized by inhalation of an overdose of Isofor anaesthetic (Safeline Pharmaceuticals, South Africa). Rats were bled by cardiac puncture (Code: RCBP) and blood collected into thrombin-coated tubes and stored in ice to be processed to obtain serum. A Heraeus Labofuge 200 Centrifuge (Thermoscientific, USA) operated at 3000 rpm for 10 minutes was used to obtain serum from blood. Skeletal muscle and skin tissue were harvested, snap frozen in liquid nitrogen and stored at -20°C for further analysis. For histological analysis both skeletal muscle and skin tissue were collected and fixed in 10 % (v/v) formalin until use.

2.2.6 Tissue processing

2.2.6.1 Tissue processing for enzyme activity assays

Tissues were processed by methods described by Ngubane *et al.* (2011) with modifications. An OMNI TH2 tissue homogenizer (NW Kennesaw, GA, USA) operated at 35 000 rpm was used to homogenise skeletal muscle (125 mg/ml) and skin (100 mg/ml) tissue in ice cold buffer containing 50 mM Tris/HCl (pH 7.4), 100 mM KCl and 1mM EDTA. Thereafter skeletal muscle homogenates were centrifuged for 30 minutes at 4 ^{0}C in an 8 x 50 ml rotor at 7741 *g* and skin at 27216 *g* for 10 minutes at 4 °C using a Beckman Coulter J26-XPI centrifuge (USA). Supernatants were then transferred into Eppendorf tubes and frozen at -20 °C until further use. The protein concentration in supernatants was determined in skeletal muscle by the Biuret method (Gornall *et al.*, 1949) and in skin by the Folin-Lowry method (Lowry *et al.*, 1951), using Bovine serum albumin BSA as a standard.

2.2.6.2 Tissue processing for Western blots

Skeletal muscle and skin were processed by randomly selecting 3 samples from each group and homogenizing 100 mg tissue/ml in ice cold buffer containing 50 mM Tris, 1 mM EDTA, 1mM benzamidine, 1mM PMSF, 2 mM Na_3VO_4 and 0.5 µg/ml of aprotinin using an OMNI TH2 tissue homogenizer operated at 35000 rpm. Thereafter skin homogenates were centrifuged for 45 minutes at 4 ^{0}C in an 8 x 50 ml rotor at 27000 *g* and skeletal muscle at 7741 *g* for 10 minutes using a Beckman Coulter J26-XPI centrifuge (USA). Supernatants were then transferred into eppendorf tubes and frozen at -20 ^{0}C until further use. The protein concentration in supernatants was again determined in skeletal muscle by the Biuret method and in skin by the Folin-Lowry method using BSA as a standard.

2.2.7 Enzyme assays

2.2.7.1 Hexokinase activity assay

Hexokinase activity in skeletal muscle and skin samples was assayed as described by Ngubane *et al.* (2011) with modifications. Hexokinase activity was measured as illustrated in Figure 2.3 below

in a coupled reaction where glucose is phosphorylated using ATP as a phosphate donor to glucose-6-phosphate in a reaction catalysed by hexokinase. The glucose-6-phosphate produced was converted to gluconate-6-phosphate while $NADP^+$ is reduced to form NADPH. Hexokinase activity is directly proportional to NADPH produced. The rate of increase in absorbance due to NADPH is measured at 340 nm and 25°C for 8 minutes following 2 minutes of equilibration using a Varian *Cary 50 UV/Vis* Spectrophotometer (Australia).

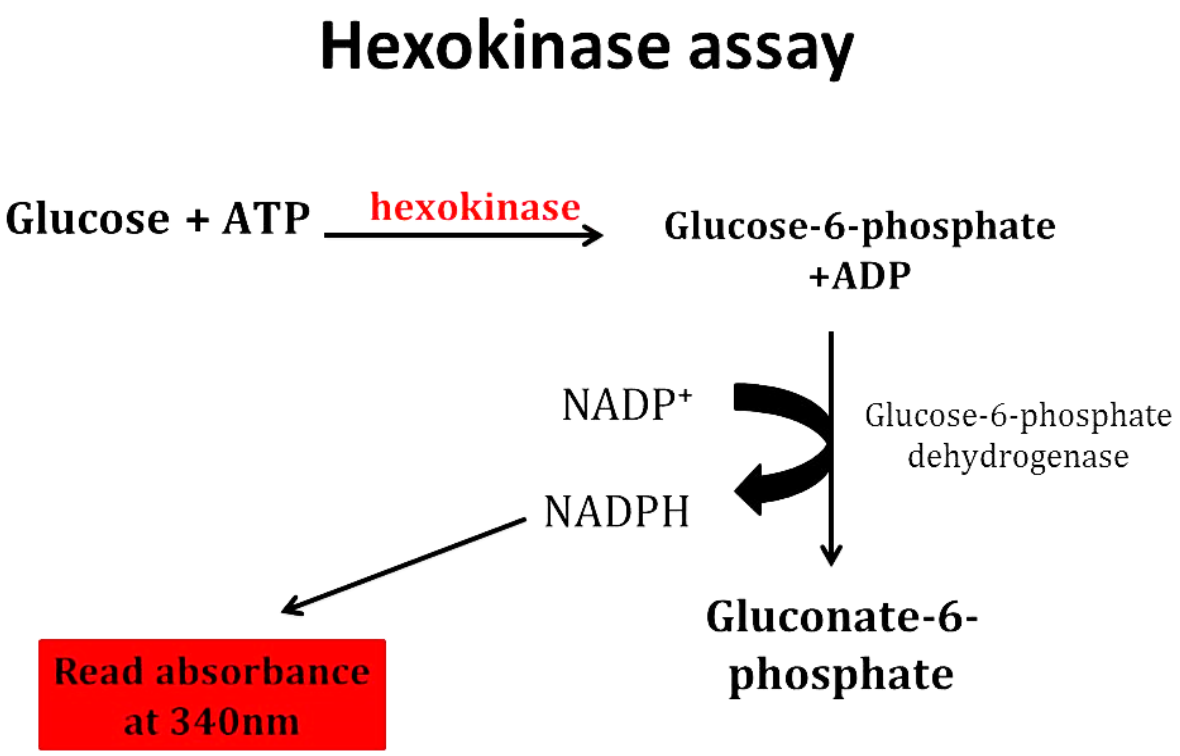


Figure 2. 3: Hexokinase activity assay

The reaction mixture (2.8 ml) contained the following (final concentrations): 20 mM triethanolamine-HCl buffer (pH 7.6), 222 mM glucose, 8 mM $MgCl_2$, 0.91 mM $NADP^+$, 0.64 mM ATP (Na salt), 5 mM mercaptoethanol, 0.55 U/ml Glucose-6-phosphate dehydrogenase. 100 µL of the sample was added to start the reaction. The activity was expressed as nmol/min/mg protein.

2.2.7.2. Phosphofructokinase activity assay

The activity of phosphofructokinase in skeletal muscle and skin was assayed according to methods described by Mandarino *et al.* (1987) with modifications as illustrated in Figure 2.4. Fructose-6-

phosphate was phosphorylated by phosphofructokinase to yield fructose-1,6-bisphosphate. The product is acted upon by aldolase to yield glyceraldehyde-3-phoshate which was further isomerized by triose phosphate isomerase into dihydroxyacetone phosphate. Dihydroxyacetone phosphate was catalyzed by glceraldehyde3-phosphate dehydrogenase while NADH was oxidized to NAD^+. Phosphofructokinase activity was directly proportional to NADH consumed. The rate of decrease in absorbance by NADH was measured at 340 nm and 25 °C for 6 minutes following 2 minutes of equilibration in a Varian *Cary 50 UV/Vis* Spectrophotometer

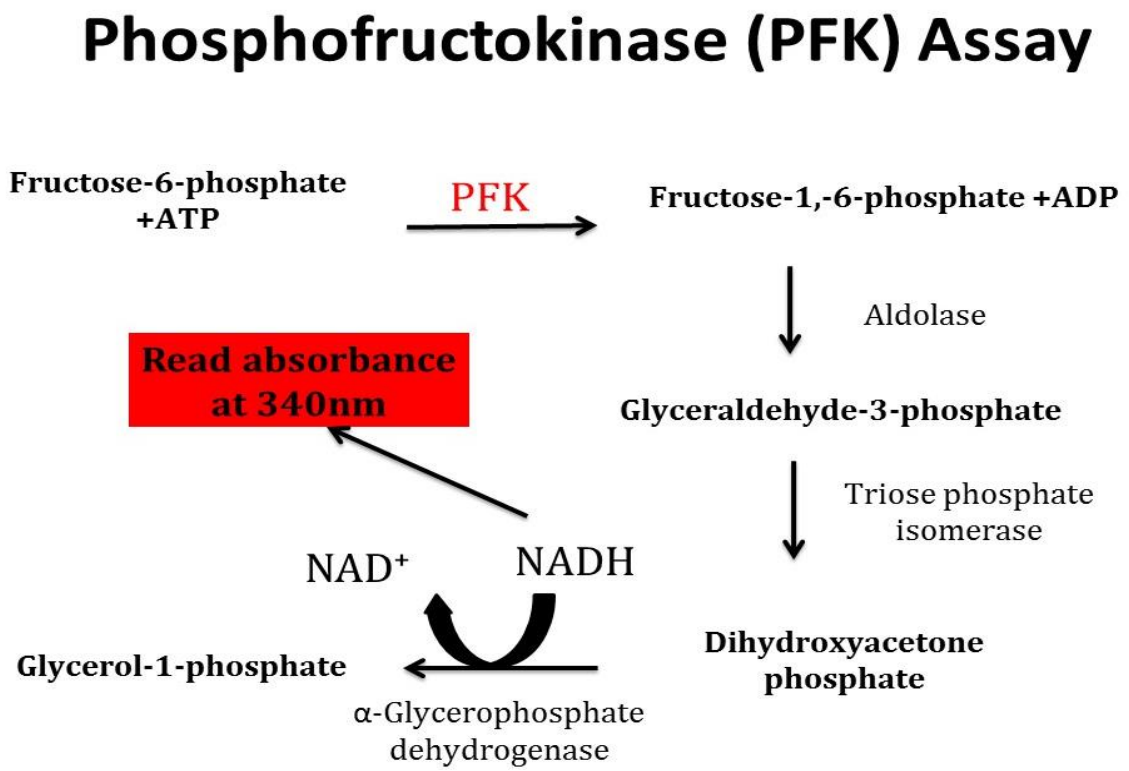


Figure 2. 4: Phosphofructokinase activity assay

The reaction mixture (1 ml) consisted of the following concentrations: 50 mM Tris (pH 8.0) containing 2 mM ATP, 5 mM $MgCl_2$, 0.2 mM NADH, 2 mM Fructose-6-phosphate, 5U each of auxiliary enzymes (aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase). The reaction was started by addition of 30 µL of the sample. The activity was expressed as nmol/ min/ mg protein.

2.2.7.3 Fructose-1, 6-bisphosphatase activity assay

The activity of fructose-1,6-bisphosphatase in skeletal muscle and skin samples was measured in a coupled enzyme assay as described by Opie and Newsholme (1967) with modifications as illustrated in Figure 2.5. Fructose-1, 6-bisphosphate is dephosphorylated by fructose-1, 6-bisphosphatase to yield fructose-6-phosphate. This product is isomerized by phosphoglucose isomerase to produce glucose-6-phosphate. The glucose-6-phosphate produced is converted to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase while $NADP^+$ is reduced to NADPH. Fructose-1, 6-bisphosphatase activity is directly proportional to NADPH produced. The rate of increase in absorbance due to NADPH was measured at 340 nm and 25 °C for 6 minutes following 2 minutes of equilibration in a Varian *Cary 50 UV/Vis* Spectrophotometer.

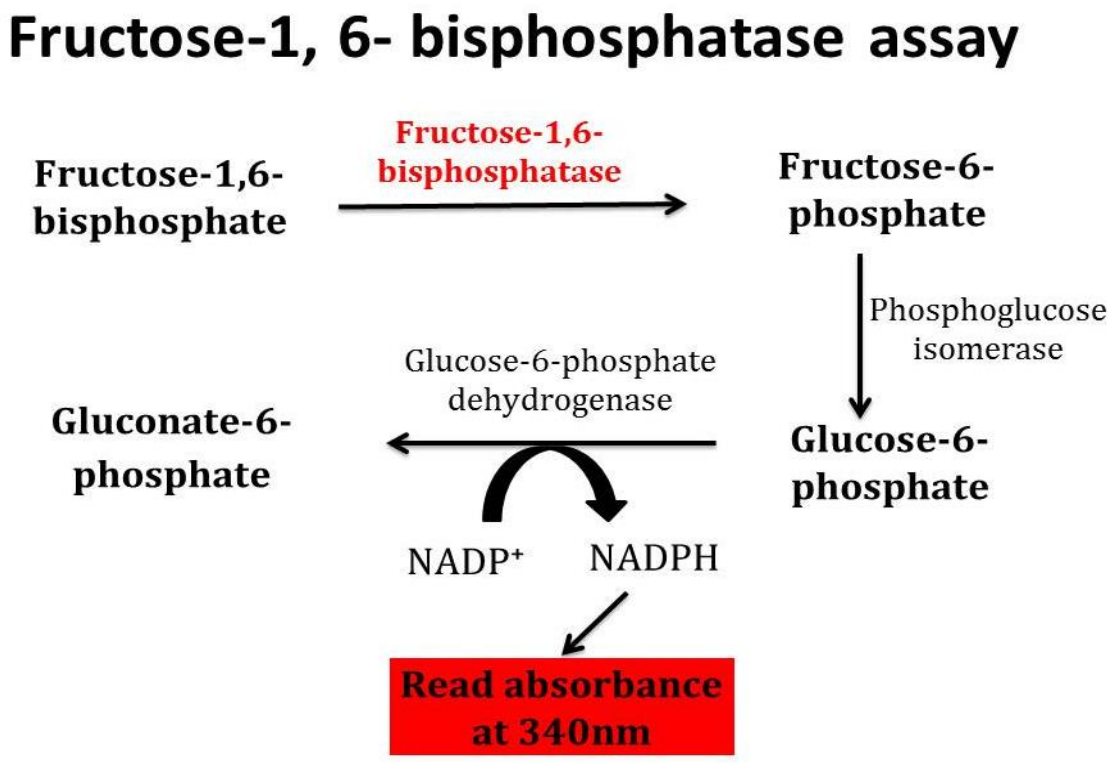


Figure 2. 5: Fructose-1, 6-bisphosphatase activity assay

The reaction medium consisted of the following in final concentrations: 50 mM-Tris-HCl buffer (pH 7.5), 20 mM mercaptoethanol, 6 mM $MgSO_4$, 1 mM EDTA, 0.2 mM $NADP^+$ and 0.1 mM

fructose-1, 6-diphosphate. To 640 µL of assay medium in a microcuvette, 550 U/mg protein each of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase and 20 µL of tissue sample was added to start the reaction. The activity was expressed as nmol/ min/ mg protein.

2.2.7.4 Glycogen phosphorylase activity

Glycogen phosphorylase activity was assayed according to method described by Taylor *et al.* (2006). Samples (100 mg/ml) were homogenized in ice cold buffer containing 10 mM MOPS, 5 mM EDTA, 50 mM NaF and 1m M DTT (pH7.0) using an OMNI TH2 tissue homogenizer operated at 35 000 rpm. The homogenates were then centrifuged at 9000 *g* for 10 minutes at 4 °C in a Beckman coulter J26-XPI centrifuge.

The reaction was started by incubating a mixture of 50 µL of sample and 50 µL buffer containing 33 mM MES, 0.34 mM glycogen, 22.3 mM Glucose-1-phosphate and 1µCi/ml radiolabeled D[U^{14}C]-glucose-1-phosphate at 30°C for 20 minutes. Termination of the reaction was performed by adding 50 µL of reaction mixture onto a 2 x 2 cm filter paper then immediately submerging in 70 % (v/v) ethanol to precipitate the glycogen. The filter paper was rinsed twice in ethanol for 30 minutes then left to dry. The dried filter paper was then placed in scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C was determined using a PACKARD 1900 TR liquid scintillation counter. Water was used as blanks to determine background counts. The activity was expressed as nmol/ min/ mg protein.

2.2.7.5 Glycogen synthase activity

Glycogen synthase activity was assayed as described by Taylor *et al.* (2006) with modifications. 50 mg/ml of tissue was homogenized in ice cold buffer containing 2 mM DTT, 20 mM EDTA, 20 mM NaF and 50 mM potassium phosphate buffer using an OMNI TH tissue homogenizer operated at 35000 rpm. The homogenates were centrifuged at 20 000 *g* for 30 minutes at 4 °C using a Beckman coulter J26-XPI centrifuge. Supernatants were diluted (1:5) in buffer containing 50 mM Tris (pH 7.8), 25 mM NaF, and 20 mM EDTA.

The reaction was started by addition of 50 µL of sample to 50 µL buffer containing 50 mM Tris (pH 7.8), 25 mM NaF, 20 mM EDTA, 1% (w/v) glycogen, 1 µCi/ml [$U^{14}C$] uridine diphosphate glucose, 5 mM Uridine diphosphate glucose and 10 mM glucose-6-phosphate. The reaction mixture was vortexed and incubated for 20 minutes at 30 °C. Termination of the reaction was performed by adding 50 µL of reaction mixture onto a 2 x 2 cm filter paper then immediately submerging in 70% (v/v) ethanol to precipitate the glycogen. The filter paper was rinsed twice in ethanol for 30 minutes then left to dry. The dried filter paper was then placed in scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C was determined using a PACKARD 1900 TR liquid scintillation counter. Water was used as blanks to determine background counts. The activity was expressed as nmol/ min/ mg protein.

2.2.8 Protein determination

2.2.8.1 Folin- Lowry method

Protein concentrations in skin were determined by the method of Lowry *et al.* (1951). The following reagents were prepared: sodium carbonate solution (20 g/L Na_2CO_3 in 0.1 M NaOH) and copper sulphate–sodium potassium tartarate solution (5 g/L $CuSO_4.5H_2O$ in 10 g/L Na, K tatarate). On the day of the experiment alkaline reagent was prepared by mixing alkaline sodium carbonate solution and copper sulphate-sodium potassium tartrate solution 50:1 and the Folin Ciocalteu reagent was prepared by diluting stock 1:2 with deionized water.

25 µL of sample and 475 µL of 0.1 N NaOH were added to each tube plus 5 ml of alkaline reagent. This solution was vortexed and incubated at 40˚C for 15 minutes. Samples were allowed to cool then add 0.5 ml Folin reagent was added to each tube. The solution was vortexed again and incubated at room temperature for 30 minutes and absorbance was read at 600 nm. Protein standards were prepared using BSA and these were used to construct a standard graph in the range 20 - 100 µg. The concentration of each sample was extrapolated from the standard curve graph.

2.2.8.2 Biuret assay method

Protein concentrations were determined following methods described by Gornall *et al.* (1949) with modifications. The biuret reagent was prepared by dissolving 3 g of cupric sulphate pentahydrate

($CuSO_4.5H_2O$) and 9 g of sodium potassium tetrahydrate ($KNaC_4H_4O_4.H_2O$) in 500 ml of 0.2 M sodium hydroxide (NaOH). Thereafter 5 g of potassium iodide (KI) was added. The volume was subsequently made up to 1 L by addition of 0.2 M NaOH.

2 ml of diluted sample and 3 ml biuret reagent were added in each test tube. Afterwards the solution was vortexed and incubated at 37°C for 10 minutes. Thereafter samples were cooled and absorbance was read at 540 nm in a Varian Cary 50 UV/Vis Spectrophotometer. A standard curve using BSA was prepared by plotting absorbances of protein standards against their known protein concentrations in the range of 0-10 mg/ml. Protein concentrations in samples were calculated using this standard graph.

2.2.9 Determination of glycogen content

Glycogen content was determined following methods described by Nader and Esser (2001) with modifications. 0.5 g of tissue was boiled for 30 minutes in 1ml of 30% KOH saturated with $NaSO_4$ until completely digested. Thereafter glycogen was precipitated in 4 ml of ice cold 95% ethanol followed by centrifugation at 20000 *g* for 30 minutes. The pellet was then re-suspended in 1ml of deionised water. 1 ml of 5% Phenol was added to the glycogen suspension followed by 5 ml of sulphuric acid and incubation for 30 minutes in ice. Absorbance was read at 490 nm using an UVmini-1240, UV-VIS spectrophotometer. The standards were prepared using glycogen concentrations in the range of 0-100 µg/ml and water was used as a blank. The glycogen content in the samples were calculated using this standard graph.

2.2.10 Histological studies

Samples previously fixed in formalin were dehydrated in ascending concentrations (70%, 90% and 100%) of ethanol and then in xylene. Thereafter tissues were positioned longitudinally and cross-sectionally and embedded in wax of paraffin overnight. Once tissues were embedded they were cut into 4µm sections using a microtome and placed in a 60°C water bath before being picked up in glass microscope slides. They were allowed to dry overnight. The slides containing sectioned tissues were then deparaffinised with xylene and rehydrated in decreasing concentrations (v/v) of ethanol (100%, 80%, 70% and 50% respectively) and finally rinsed in water. Slides were then stained with haematoxylin for 5 minutes, rinsed in water, followed by counter staining in eosin for

3 minutes. The slides were then mounted with DPX, closed with a cover slip and left to dry for 2 days. Slides were scanned using Leica SCN400, Germany (Software version: SlidePath Gateway Client viewer 2.0).

2.2.11 Serum alanine amino transferase and aspartate amino transferase

Blood samples were centrifuged at 3000 rpm for 10 minutes in a benchtop Labfuge 200 - Heraeussepatech Centrifuge to obtain serum. Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a *Labmax Plano* Chemistry analyser (Labtest Av, Paulo Ferreira do Costa, Brasilia, Brazil)

2.2.12 SDS-PAGE and western blot analysis

Proteins were resolved by SDS-PAGE using the SDS-PAGE (Laemmli) reducing buffer system (Laemmli, 1970). The following reagents were used to make 10% and 15% SDS PAGE gels: Acrylamide/Bis-acrylamide containing 29.2 g/100 ml of acrylamide and 0.8 g/100 ml of N'N'-bis-methylene-acrylamide, 10% (w/v) SDS, 1.5 M Tris-HCl pH 8.8, 0.5 M Tris -HCl pH 6.8, running buffer, 10% (w/v) ammonium persulphate (APS) and sample buffer. Stock solution of running buffer consisted of 30.3 g Tris-base, 144 g glycine and 10 g SDS pH 8.3 made up to 1 L with deionized water. The sample buffer (SDS reducing buffer) was made up of a total volume of 9.5 ml composed of 3.55 ml deionized water, 1.25 ml of 0.5 M Tris -HCl, pH 6.8, 2.5 ml of glycerol, 2 ml of 10% (w/v) SDS and 0.2 ml of 0.5% (w/v) bromophenol blue. 50 µL of mercaptoethanol was added to 950 µL sample buffer before use. The samples were diluted 1:2 with sample buffer and heated at 95°C for 4 minutes.

To formulate 10% gels, 4.1 ml deionized water, 3.3 ml of 30% degassed acrylamide/bis-acrylamide 2.5 ml gel buffer and 0.1 ml of 10% (w/v) SDS were mixed together. To prepare 15% gels the same ingredients were mixed together except 2.4 ml of deionized water and 5 ml of acrylamide/bis-acrylamide were used. In the resolving gel, 1.5 M Tris- HCl, pH 8.8 was used as gel buffer while in the stacking gels, 0.5 M Tris-HCl, pH 6.8 was used as the gel buffer. Before pouring the gels 50 µL of 10% APS and 5 µL TEMED were added to resolving gel and 50µL of

10% APS and 10 µL TEMED were added to the stacking gel. The buffers were swirled gently to initiate polymerization.

Resolving gel was poured below comb teeth on glass of gel cassette with a layer of 1:1 butanol: water and allowed to polymerize for 45 to 60 minutes. Thereafter, the top of the resolving gel was rinsed and dried and stacking gel buffer was poured on top of resolving gel with desired comb to form wells. Stacking gel buffer was allowed to polymerize for 15-30 minutes. After the gel was formed, the comb was removed gently and wells were thoroughly rinsed with distilled water before use.

Proteins of equal amounts and 3 µL of molecular weight marker were loaded into wells and resolved using a Bio-Rad mini PROTEIN 3 electrophoresis cell from BIORAD, South Africa. The samples and molecular weight marker were subjected to electrophoresis in running buffer at 110 V for 90 minutes. The proteins on the gels were transferred to PVDF membranes in Tris/Glycine/Methanol buffer, pH 8.3 using a Bio-Rad Trans-Blot Electrophoretic Transfer Cell, (BIORAD, South Africa) and ran at 100 V for 2 hours. The PVDF was soaked in 100% methanol before transfer for activation of the membrane.

After transferring, the membranes were blocked at 4 °C overnight in blocking buffer containing 3% low fat milk in Tris-buffered saline with Tween 20 (TBS-Tween) to reduce protein non-specific binding. The TBS-Tween buffer was made up of 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.4. The primary antibody was added to blocking buffer (1:1000), overlaid on the PVDF membranes and shaken for 1 hour at room temperature. Membranes were then washed three times for 5 minutes with TBS-Tween buffer and probed with Goat anti-Rabbit IgG Alkaline phosphatase conjugate (diluted 1:1000 in PBST) for 1 hour at room temperature with gentle agitation. PVDF membrane was washed again with TBS-Tween for 5 minutes. Thereafter excess liquid was removed without letting the membrane dry and the immun-Star chemiluminescent substrate from BIORAD, (Johannesburg, South Africa) was added and incubated for 5 minutes. Thereafter signal was detected through chemiluminescence. The antibody-bound proteins were visualized using a Chemidoc imaging system, (BIORAD, South Africa). Images were captured using the Lab image software and the bands were quantified using Gene Tools analysis software.

2.2.13 Statistical analysis

Data are expressed as means ± standard error of means (SEM). Statistical comparison of the differences between the control means and experimental groups was performed with GraphPad InStat Software (version 5.0, GraphPad Software, San Diego, California, USA), using one-way analysis of variance, followed by Tukey– Kramer multiple comparison test. A p value of < 0.05 was considered significant.

CHAPTER 3

Results

This chapter represents the results of experiments performed to evaluate the effects of oleanolic acid and *Centella asiatica* on selected glycolytic and gluconeogenic enzymes in skeletal muscle and skin diabetic rats by determining enzyme activity and enzyme expression. The activities and expression of key enzymes of glycogen metabolism as well as results of histological examination of skeletal muscle and skin of non-diabetic, diabetic and treated animals are also reported.

3.1 Enzyme activity in tissues of non-diabetic and diabetic animals treated with OA

Figures 3.1 to 3.4show the effect of OA on the activities of hexokinase, phosphofructokinase and fructose-1,6-bisphosphatase in skeletal muscle and skin.

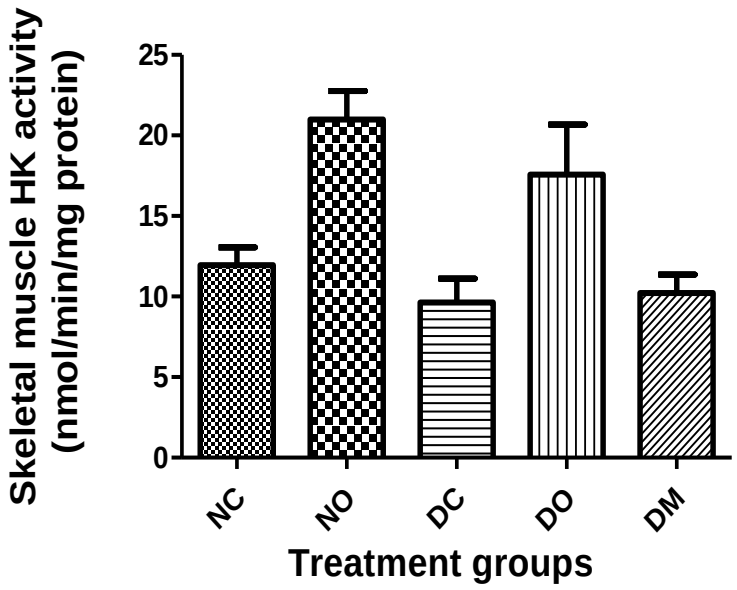


Figure 3.1: Effects of OA on hexokinase activity in skeletal muscle of non-diabetic and diabetic rats following 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). HK = hexokinase; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

Figure 3.1 depicts the effects of OA on hexokinase activity in skeletal muscle of non-diabetic and diabetic rats. At the end of the 14-day treatment study, there were no significant differences in hexokinase activity amongst the control groups and within non-diabetic and diabetic animal groups. However, amongst the non-diabetic animals, treatment with OA increased hexokinase activity by 75% compared to the control animals. Hexokinase activity slightly decreased in diabetic control animals by comparison to non-diabetic control animals. Amongst the diabetic animals, treatment with OA increased hexokinase activity while treatment with metformin did not alter the hexokinase activity compared to the diabetic controls.

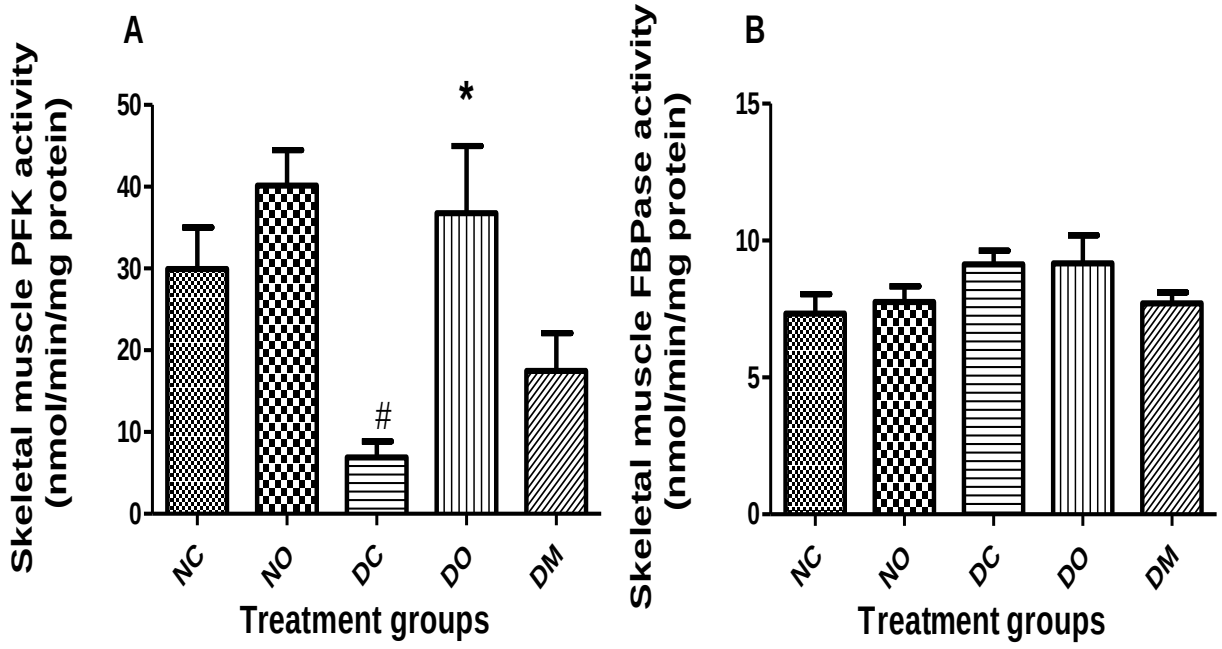


Figure 3.2: Effects of OA on (A) phosphofructokinase and (B) fructose-1,6-bisphosphatase activity in skeletal muscle of non-diabetic and diabetic rats following 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). # = $p < 0.05$ by comparison with NC and * = $p < 0.05$ by comparison with DC. PFK = phosphofructokinase; FBPase = fructose-1,6-bisphosphatase; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

The results in Figure 3.2 show the effect of OA on (A) phosphofructokinase and (B) fructose-1, 6-bisphosphatse activities in skeletal muscle after 14 days of treatment. Non-diabetic OA treated animals did not show significant difference in phosphofructokinase activity compared to non-diabetic control animals. However, phosphofructokinase activity significantly ($p < 0.05$) decreased by 77% in diabetic animals compared to non-diabetic control animals after the 14-day treatment period. Diabetic animals treated with OA showed significantly ($p < 0.05$) increased (432 %) activity compared to the diabetic control animals. Animals treated with metformin also showed higher phosphofructokinase activity than diabetic controls although this was not statistically significant.

There were no significant differences in fructose-1, 6- bisphosphatase activity between non-diabetic control and diabetic control animals and within non-diabetic and diabetic treatment groups. However, fructose-1, 6-bisphosphatase activity increased in diabetic control animals by 25% compared to untreated control animals. Treatment of diabetic animals with OA resulted in no significant difference compared to the diabetic control animals. Diabetic animals treated with metformin had slightly lower enzyme activity compared to the diabetic control although this decrease was not significant.

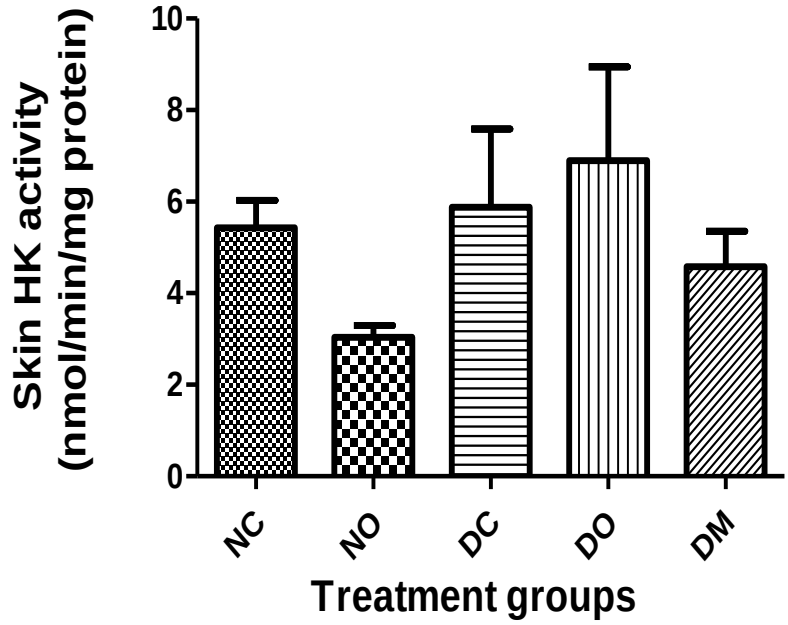


Figure 3.3: Effects of OA on hexokinase activity in skin of non-diabetic and diabetic rats after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). HK = hexokinase; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

The results in Figure 3.3 depicts the effects of OA on hexokinase activity in skin after a 14-day study period. Hexokinase activity in non-diabetic animals treated with OA was reduced compared to the non-diabetic control animals. The diabetic controls showed higher hexokinase activity than non-diabetic control animals although this was not significantly different. Diabetic animals treated with OA had slightly increased activity compared to the diabetic control animals. Treatment of diabetic animals with metformin resulted in lower hexokinase activity by 22% compared to diabetic control animals.

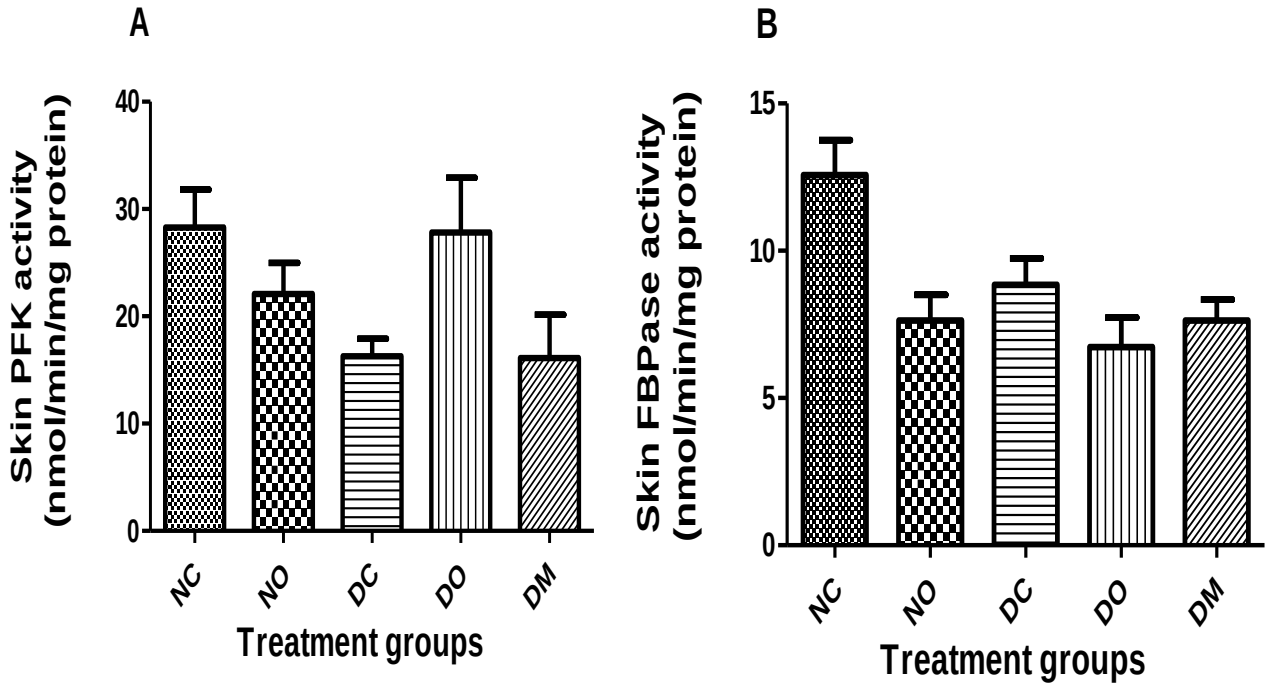


Figure 3.4: Effects of OA on (A) phosphofructokinase and (B) fructose-1, 6-bisphosphatase activity in skin of non-diabetic and diabetic rats after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). PFK= phosphofructokinase; FBPase = fructose-1,6-bisphosphatase; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

Figure 3.4 represents the effects of OA on (A) phosphofructokinase and (B) fructose-1, 6-bisphosphatase activity in skin of non-diabetic and diabetic animals after 14 days of treatment. Amongst the non-diabetic animals, OA treated animals showed 22% lower phosphofructokinase activity compared to the controls although this was not statistically significant ($p > 0.05$). Diabetic control animals also demonstrated low phosphofructokinase activity compared to the non-diabetic control animals. OA treated diabetic animals had increased phosphofructokinase activity by 71% while metformin treated diabetic animals showed no significant differences in activity compared to diabetic control animals.

Amongst the non-diabetic animals, those treated with OA had lower fructose-1,6-bisphosphatase activity compared to the control animals although this was not statistically significant. Although

non-diabetic control and diabetic control animals showed no significant difference in their enzyme activities, the enzyme activity was lower in diabetic animals. Treatment of diabetic animals with OA and metformin slightly lowered enzyme activity although not significantly.

Table 3.1: Effects of OA of glycogen levels in skeletal muscle and skin after 14 days of treatment of non-diabetic and diabetic animals.

Treatment groups	Muscle (mg/g tissue)	Skin (mg/g tissue)
NC	0.54 ± 0.05	0.75 ± 0.07
NO	1.2 ± 0.07#	0.73 ± 0.10
DC	0.63 ± 0.01	0.75 ± 0.09
DO	0.89 ± 0.01*	0.64 ± 0.04
DM	0.67 ± 0.05	0.81 ± 0.10

Values are expressed as means ± SEM, (n=6 in each group). # = $p < 0.05$ by comparison with NC and * = $p < 0.05$ by comparison with DC. NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

Table 3.1 depicts the effects of OA on glycogen levels in muscle and skin after 14 days of treatment. In muscle, the amount of glycogen was 122% higher non-diabetic animals treated with OA compared to the non-diabetic control animals, this increase being significant ($p < 0.05$). This NO group had the highest amount of glycogen amongst all the groups. Diabetic animals treated with OA and metformin had higher glycogen levels compared to diabetic controls with those treated with OA being 41% higher ($p < 0.05$).

In skin, there were no significant differences in glycogen levels amongst the groups. OA treated non-diabetic animals had slightly lower glycogen levels than the non-diabetic control animals. Diabetic animals treated with OA had a lower amount of glycogen while those treated with metformin had a higher amount of glycogen compared to diabetic controls. Diabetic animals

treated OA had the lowest amount of glycogen while those treated with metformin had the highest amount of glycogen.

3.2 Enzyme activity in tissues of non-diabetic and diabetic animals treated with CA

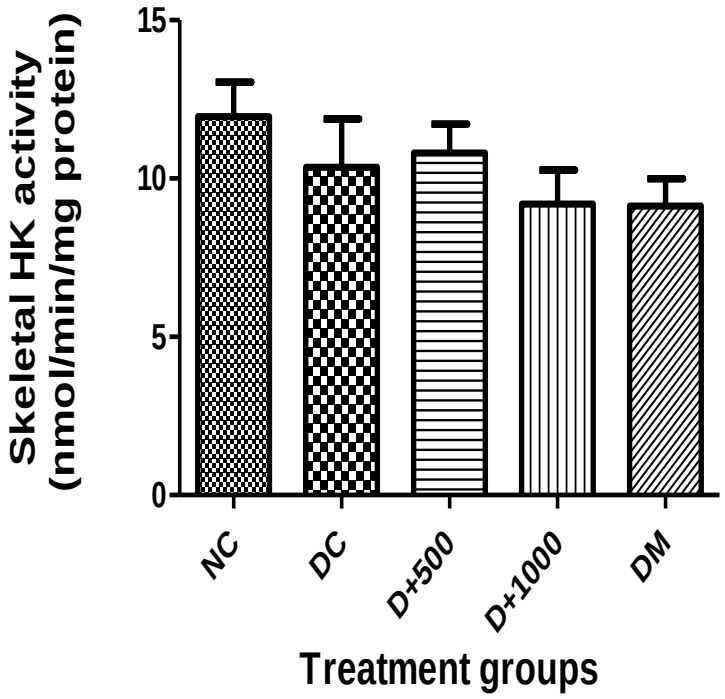


Figure 3.5: Effects of CA on skeletal muscle hexokinase activity in of non-diabetic and diabetic animals after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). HK = hexokinase; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The results of effects of CA on hexokinase activity in skeletal muscle following 14 days of treatment are shown in Figure 3.5. There were no statistical differences in hexokinase activity amongst all treatment groups. However, diabetic control animals showed somewhat lower activity than non-diabetic untreated animals. Diabetic animals treated with 500 mg/kg b.w. of CA showed an increase in enzyme activity when compared to the diabetic controls. This activity was slightly lowered in animals treated with 1000 mg/kg b.w of CA and metformin.

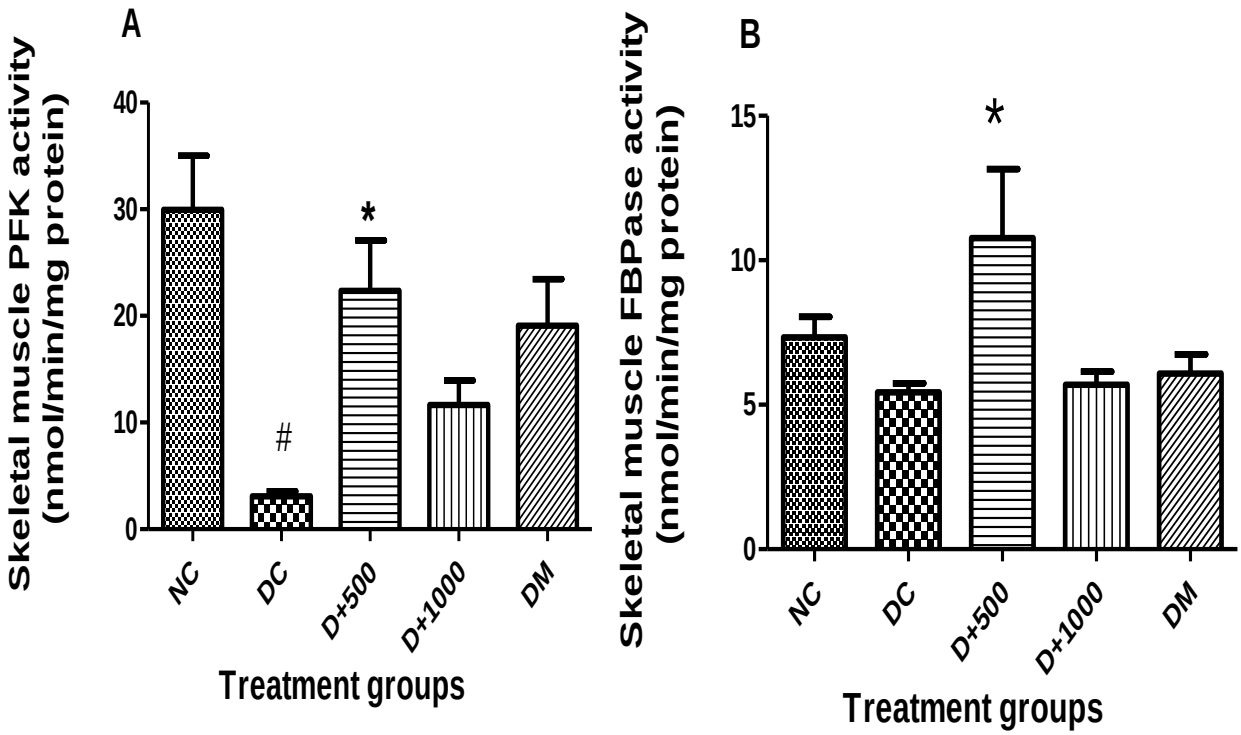


Figure 3.6: Effects of CA on skeletal muscle (A) phosphofructokinase and (B) fructose-1, 6-bisphosphatase activity of non-diabetic and diabetic rats after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). # = $p < 0.05$ by comparison with NC and * = $p < 0.05$ by comparison with DC. PFK = phosphofructokinase; FBPase = fructose-1,6-bisphosphatase; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

Figure 3.6 depicts the effects of CA on skeletal muscle (A) phosphofructokinase and (B) fructose-1, 6- bisphosphatase activity following 14 days of treatment. Diabetic animals had reduced ($p < 0.05$) the activity of phosphofructokinase by 90% compared to non-diabetic animals. Diabetic animals treated with 500 and 1000 mg/kg b.w of CA extract had increased ($p < 0.05$) phosphofructokinase activity amounting to 624% and 278% respectively compared to diabetic control animals. Treatment of diabetic animals with metformin also resulted in an increase phosphofructokinase activity compared to control animals.

Diabetic control animals had lower fructose-1, 6-bisphosphatase activity compared to non-diabetic control animals. Treatment of diabetic animals with 500 mg/kg b.w of CA resulted in a 98% increase ($p < 0.05$) in enzyme activity compared to the diabetic control animals. Diabetic animals

treated with 1000 mg/kg b.w of CA and those treated with metformin showed no significant difference in enzyme activity compared to the diabetic control animals.

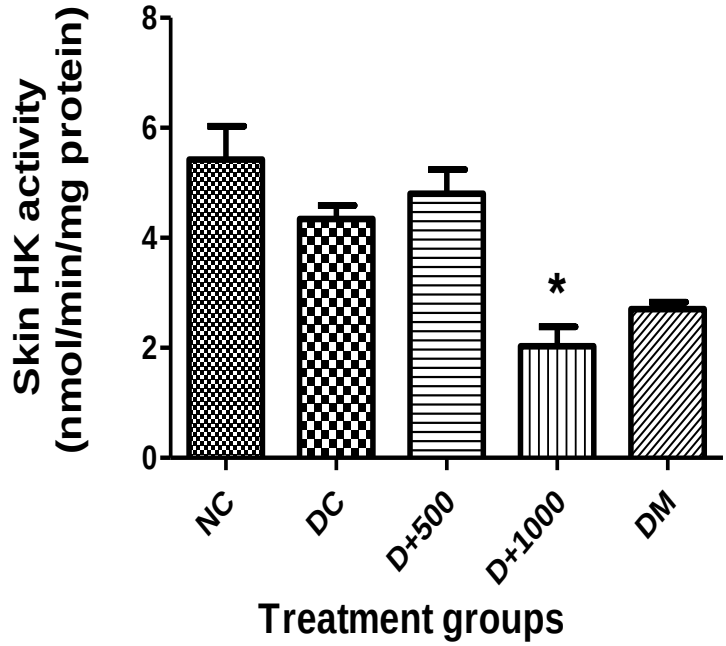


Figure 3.7: Effects of CA on hexokinase activity in skin of non-diabetic and diabetic animals after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). * = $p < 0.05$ by comparison with DC. HK = hexokinase; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The results of effects of CA on hexokinase activity in skin following 14 days of treatment are shown in Figure 3.7. Diabetic control animals showed lower hexokinase activity than non-diabetic control animals. Amongst the diabetic animals, those treated with 500 mg/kg b.w. of CA showed higher activity than the control animals although this was not statistically significant ($p > 0.05$). Diabetic animals treated with 1000 mg/kg b.w. of CA had 53% lower hexokinase activity than diabetic control animals, this difference being significant ($p < 0.05$). Metformin treated diabetic animals also had lower enzyme activity than the diabetic controls, although this was not statistically significant ($p > 0.05$).

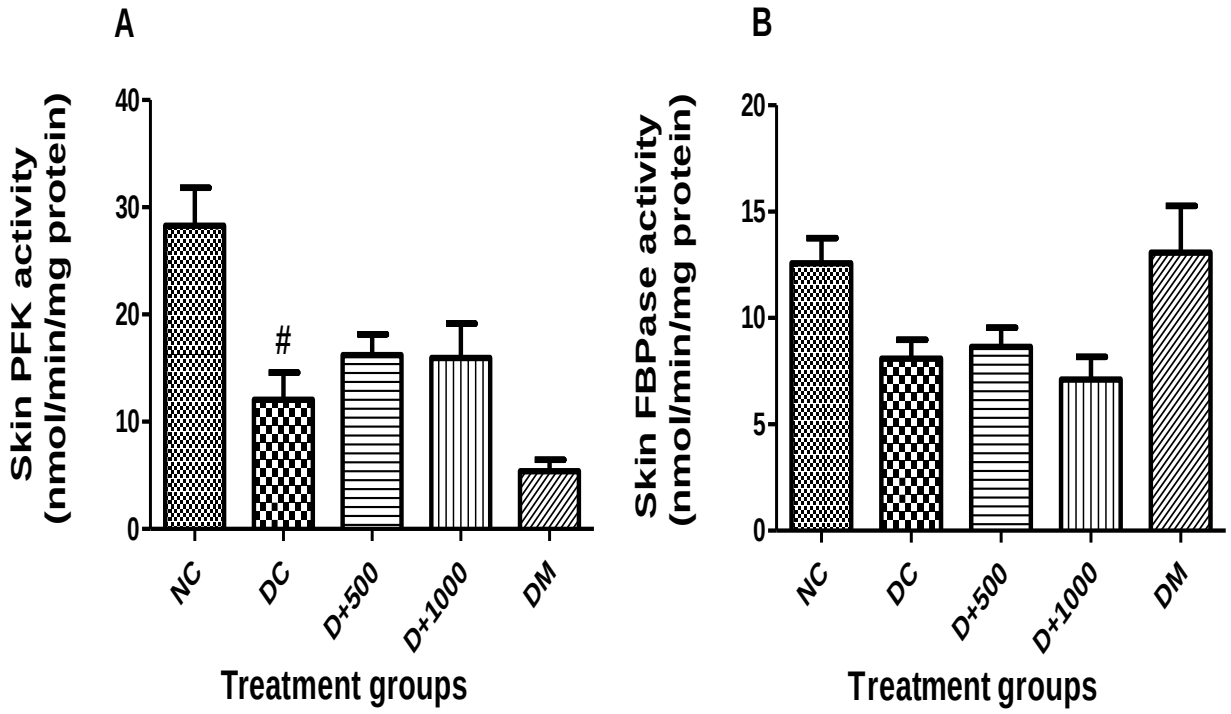


Figure 3.8: Effects of CA of (A) phosphofructokinase (PFK) and (B) fructose-1, 6-bisphosphatase (FBPase) activity in skin of non-diabetic and diabetic rats after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). # = p < 0.05 by comparison with NC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

Figure 3.8 depicts the effects of CA on (A) phosphofructokinase and (B) fructose-1,6-bisphosphatase activity following 14 days of treatment. Phosphofructokinase activity in diabetic control animals was 75% lower compared to non-diabetic control animals, this decrease being significant ($p < 0.05$). Treatment of diabetic animals with 500 and 1000 mg/kg b.w. of CA increased activity of the enzyme compared to the diabetic control animals. Treatment of diabetic animals with metformin significantly lowered ($p > 0.05$) phosphofructokinase activity by 55% compared to the diabetic control animals.

The fructose-1,6-bisphosphatase activity of the diabetic control animals was lower than that of the non-diabetic control animals. Amongst the diabetic animals, treatment with 500 mg/kg b.w. of CA and those treated with metformin had increased fructose-1,6-bisphosphatase activity while animals

treated with 1000 mg/kg b.w. of CA extract had slightly decreased enzyme activity compared to the control animals although though this decrease was not statistically significant.

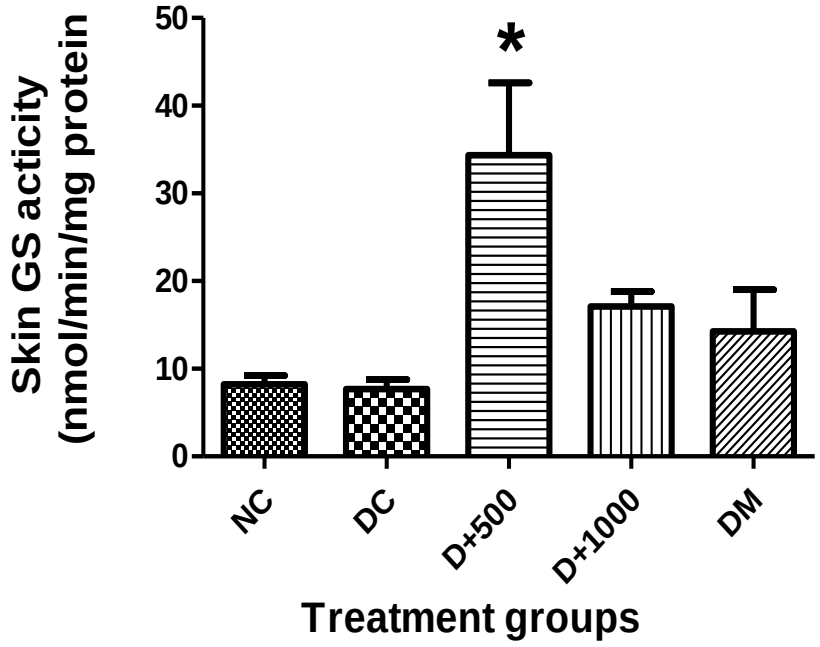


Figure 3.9: Effects of CA on glycogen synthase (GS) activity in skin of non-diabetic and diabetic animals after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM, (n = 6). * = p < 0.05 by comparison with DC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The effects of CA on glycogen synthase activity in skin is depicted in Figure 3.9. Non-diabetic and diabetic control animals had similar enzyme activity. Diabetic animals treated with 500 mg/kg b.w. of CA showed a dramatic 346% higher ($p < 0.05$) enzyme activity than the diabetic control animals. Diabetic animals treated with 1000 mg/kg b.w. and those treated with metformin also had higher glycogen synthase activity compared to the diabetic control animals but the increases were not statistically significant.

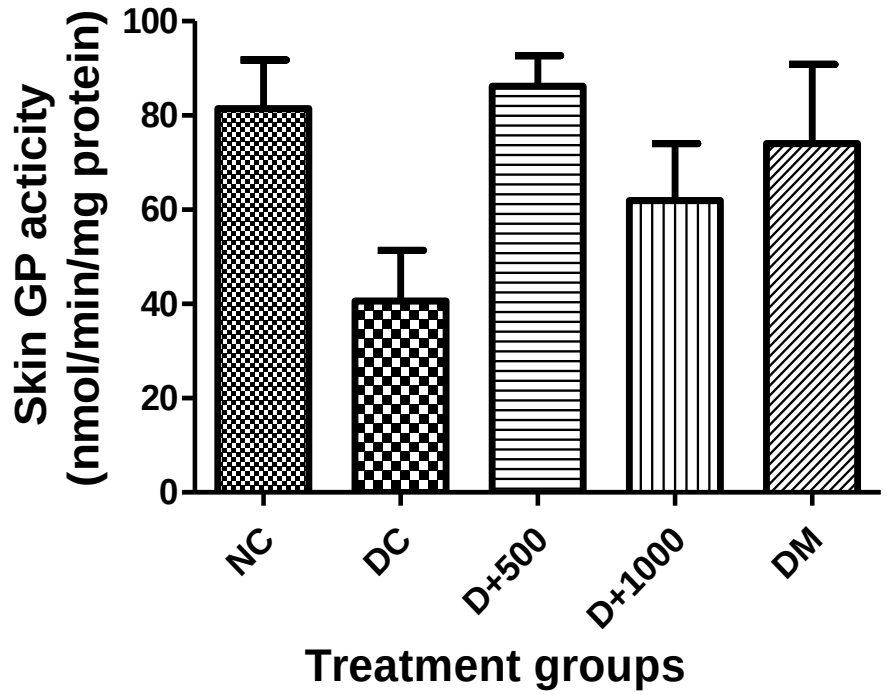


Figure 3. 10: Effects of CA on glycogen phosphorylase (GP) activity in skin of non-diabetic and diabetic animals after 14 days of treatment. Values are expressed as means and vertical bars indicate SEM (n = 6). * = p < 0.05 by comparison with DC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The effects of CA on glycogen phosphorylase activity in skin is shown in figure 3.10. There were no statistical differences amongst all the treatment groups. However, diabetic control animals showed lower GP activity than non-diabetic control animals. Amongst the diabetic animals, those treated with 500 mg/kg b.w. of CA, 1000 mg/kg b.w. of CA and metformin showed elevated enzyme activity compared to the diabetic control animals. The 500 mg/kg b.w. of CA restored GP activity back to non-diabetic control values.

Table 3.2: Effects of CA on glycogen levels in skin after 14 days of treatment.

Treatment groups	NC	DC	D+500	D+1000	DM
Skin (mg/g) tissue	0.74 ± 0.06	0.85 ± 0.05	0.80 ± 0.06	0.76 ± 0.05	0.80 ± 0.061

Values are expressed as mean ± SEM, (n = 6). NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

Table 3.2 shows the effects of CA on glycogen levels in skin of non-diabetic and diabetic animals following 14 days of treatment. There were no statistically different values in glycogen in levels amongst the groups ($p > 0.05$). Diabetic control animals presented 15% higher amounts of glycogen compared to the non-diabetic control animals. Amongst the diabetic animals, those treated 500 mg/kg b.w. of CA, 1000 mg/kg b.w. of CA and metformin showed 6 and 11% lower glycogen levels than control animals, respectively. Animals treated with 1000 mg/kg b.w. of CA had the lowest amount of glycogen content compared to diabetic control animals and the animals treated with 500 mg/kg b.w. of CA. Metformin treated animals had almost the same amount of glycogen as diabetic control animals.

Table 3.3: Effects of CA on serum aspartate aminotransferase (AST) and alanine amino transferase (ALT) following 14 days of treatment**.**

Serum enzymes	NC	DC	D+500	D+1000	DM
AST (U/L)	49.17 ± 2.33	57.83 ± 3.08	47.83 ± 4.28	47.00 ± 1.65	49.50 ± 1.78
ALT (U/L)	44.40 ± 2.12	47.80 ± 2.71	28.20 ± 5.08*****	33.83 ± 5.21	33.60 ± 1.50

Values are expressed as mean ± SEM, (n = 6). * = p < 0.05 by comparison with DC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

Table 3.3 shows the effects of CA on serum enzymes, aspartate aminotransferase and alanine amino transferase, in non-diabetic and diabetic rats following 14 days of treatment. The diabetic control animals showed higher AST activity than that of the normal animals. The diabetic animals treated with 500 mg/kg b.w of CA, 1000 mg/kg b.w. of CA and metformin had lower AST activities compared with the diabetic control animals. Both groups treated with CA had almost very similar activities of AST

With respect to ALT, the diabetic control animals had a higher activity of ALT enzyme than the non-diabetic control animals. Diabetic animals treated with 500 mg/kg b.w. of CA had significantly lower ($p < 0.05$) activity of ALT compared to the diabetic control animals. Diabetic animals treated with 1000 mg/kg b.w. of CA and metformin also had similar enzyme activities ($p > 0.05$).

3.3 SDS-PAGE of muscle and skin proteins

SDS-PAGE gels were run using 10% reducing polyacrylamide gels for skeletal muscle and skin to show their distribution pattern in each of the treatment groups. Figure 3.11 represents results of proteins of those animals treated with OA while Figure 3.12 represents results of animals treated with CA. These types of gels were further used for western blot expression studies using specific antibodies.

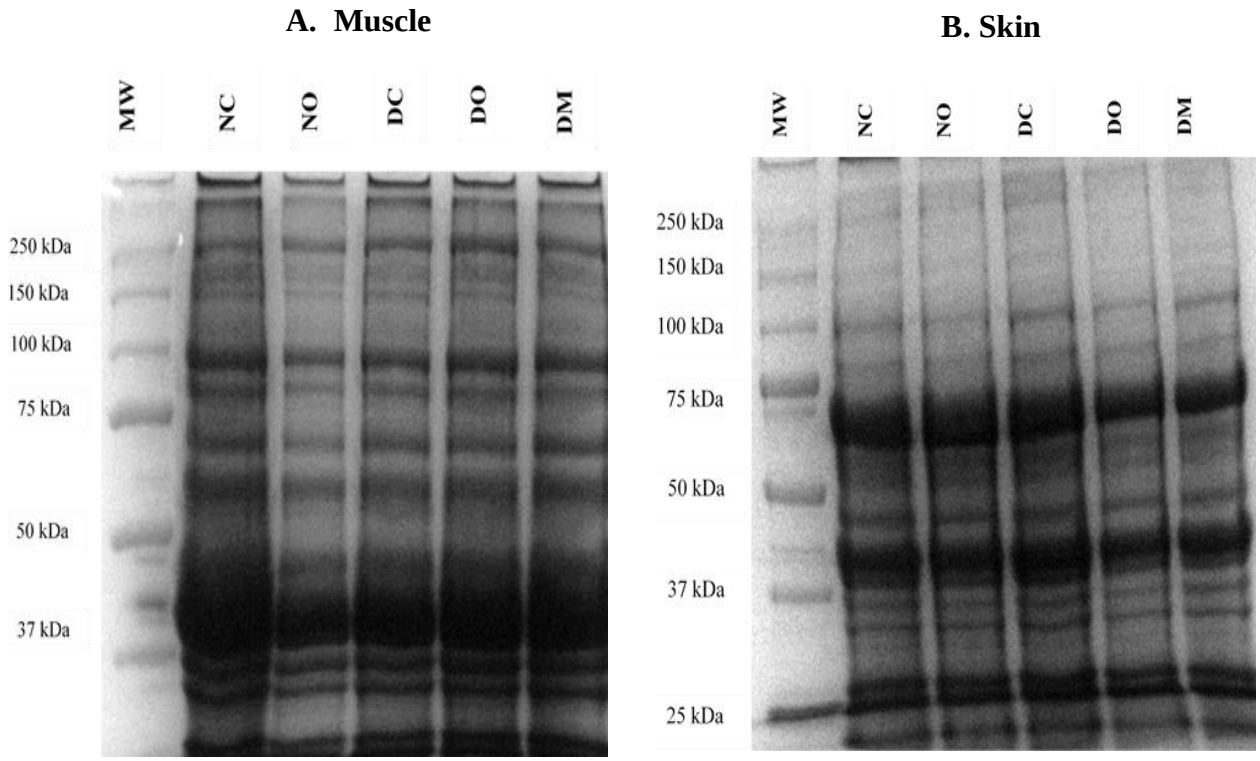


Figure 3. 11: 10% reducing SDS-PAGE gel of (A) skeletal muscle and (B) skin tissue homogenates in the OA study. 40 µg of protein were loaded in each lane. MW= Molecular Weight; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

The results in Figure 3.11 shows 10% reducing SDS-PAGE gels of skeletal muscle and skin showing the distribution of proteins between the different treatment groups after gels were stained with Coomassie Blue. In muscle, NC group appeared to show highest intensity of proteins

compared to the rest of the treatment groups. There was also a high intensity of protein around 37 kDa. In skin, the highest intensity of proteins was seen below 75 kDa and above 37 kDa.

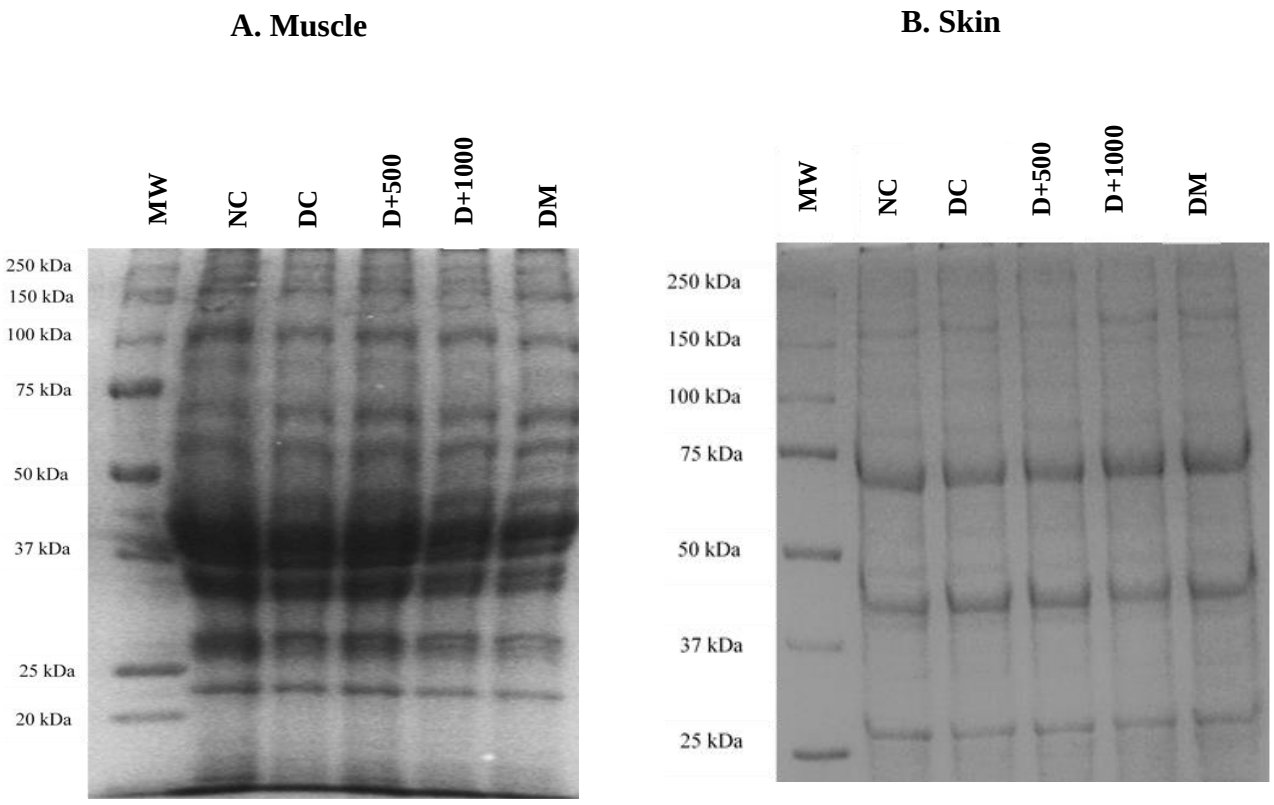


Figure 3.12: 10% reducing SDS-PAGE gel of skeletal muscle (A) and skin (B) tissue homogenates in the CA study.40 µg of protein was loaded in each lane. MW= Molecular Weight; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

Figure 3.12 shows 10% reducing SDS-PAGE gels indicating protein distribution in skeletal muscle and skin tissue homogenates stained with coomassie blue stain. Muscle tissue homogenates seemed to show a higher amount of protein than skin. In muscle, the proteins appeared most intense around 37 kDa. In skin proteins appeared more intense above 25 kDa, below 50 kDa and below 75 kDa.

3.4 Enzyme expression studies in muscle and skin

For good resolution, 10% reducing polyacrylamide gels were used for expression of PFK and GP while 15% reducing polyacrylamide gels were used for expression of GAPDH in both skeletal muscle and skin.

3.4.1 Expression of PFK and GP proteins in muscle and skin.

3.4.1.1 Effects of OA on expression of PFK in muscle and skin

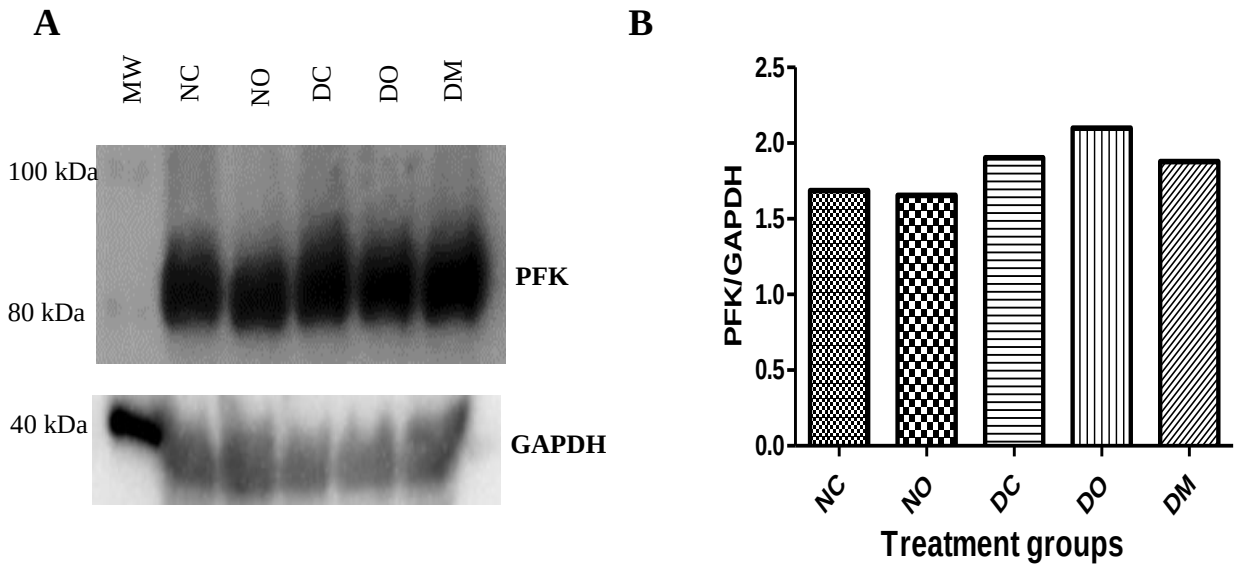


Figure 3. 13: (A) Western blots of PFK (85 kDa) and GAPDH (37 kDa) in skeletal muscle following 14 days of treatment with OA. 20 and 40 µg was loaded for PFK and GAPDH respectively. (B) PFK was normalized against GAPDH and were expressed as PFK/GAPDH ratio. MW= Molecular Weight; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic +OA and DM = Diabetic + Metformin.

The results of the effects of OA on expression of PFK and GAPDH in muscle are shown in Figure 3.13. Amongst the non-diabetic animals, control and OA-treated animals with showed almost similar amounts of expression of PFK. The diabetic control animals expressed a higher level of PFK compared to non-diabetic control animals. Amongst the diabetic animals, PFK expression

was highest in OA treated animals than in the control animals. Diabetic control metformin treated animals showed almost the same amount of enzyme expression.

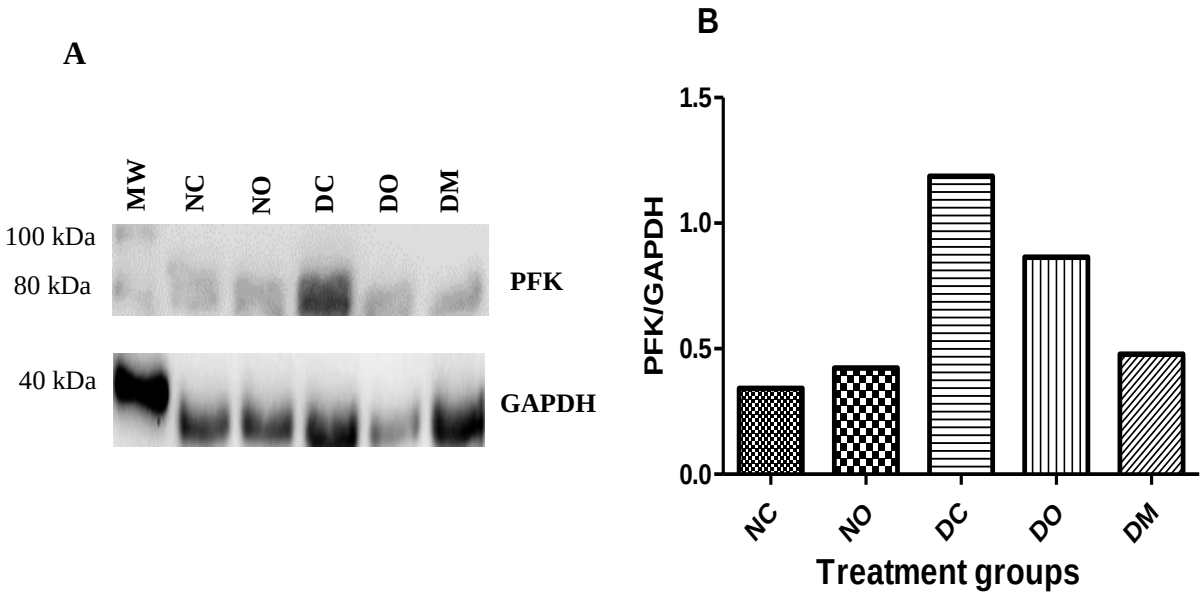


Figure 3.14: (A) Western blots of PFK (85 kDa) and (B) GAPDH (37 kDa) in skin following 14 days of treatment with OA. 30 and 40 µg was loaded for PFK and GAPDH respectively. PFK was normalized against GAPDH and were expressed as PFK/GAPDH ratio. MW = Molecular Weight; NC = Non-diabetic control; NO = Non-diabetic + OA; DC = Diabetic control; DO = Diabetic + OA and DM = Diabetic + Metformin.

The results of the effects of OA on expression of PFK and GAPDH in skin are shown in Figure 3.14. Non-diabetic animals treated with OA had a slightly higher amount of expression ratio than the control animals. The diabetic control animals had 3-fold higher expression of PFK compared non-diabetic control animals. Amongst the diabetic animals, treatment with OA and metformin reduced PFK expression by 27% and 60%, respectively compared to diabetic control animals. The highest expression ratio is seen in the diabetic control animals and the lowest in the non-diabetic control animals.

3.4.1.2 Effects of CA on expression of PFK in skeletal muscle and skin

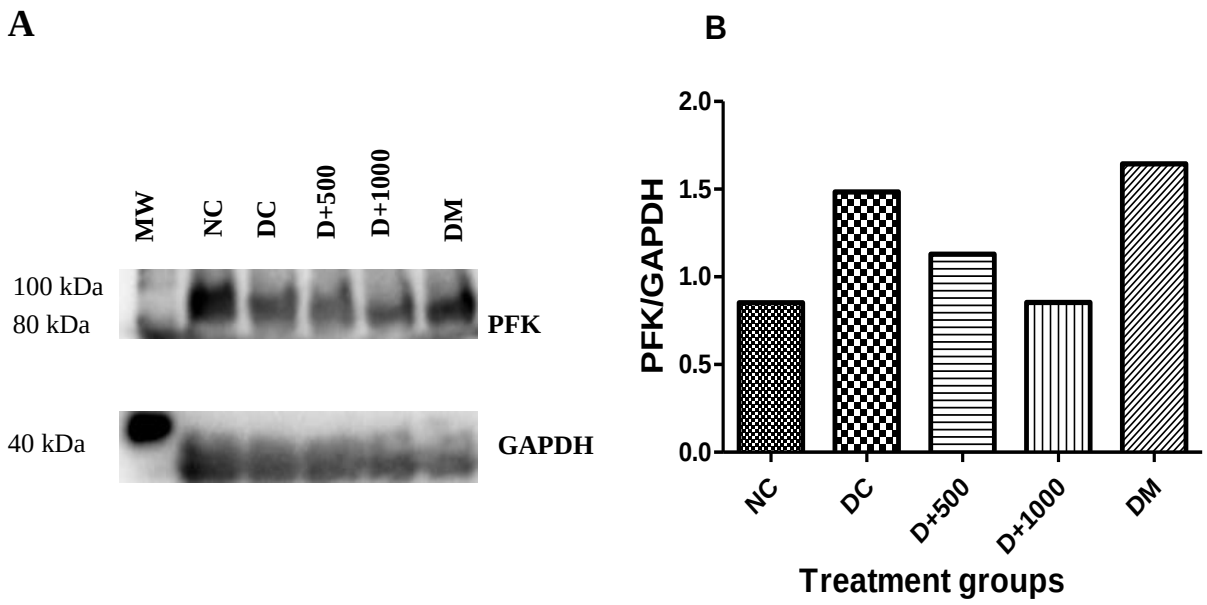


Figure 3. 15: (A) Western blots of PFK (85 kDa) and GAPDH (37 kDa) in muscle following 14 days of treatment with CA. 30 µg and 40 µg was loaded for PFK and GAPDH respectively. (B) PFK was normalized against GAPDH and were expressed as a PFK/GAPDH ratio. MW = Molecular Weight; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The effects of CA in expression of PFK in muscle is depicted in Figure 3.15. Diabetic control animals showed higher PFK expression than the non-diabetic control animals. Diabetic animals treated with 500 mg/kg b.w. and 1000 mg/kg b.w. of CA had lower PFK expression compared to the diabetic control animals with those treated with 1000 mg/kg b.w. having the lowest. Diabetic animals treated with metformin had higher expression compared to the diabetic control animals. Diabetic animals treated with metformin showed the highest amount of expression in muscle.

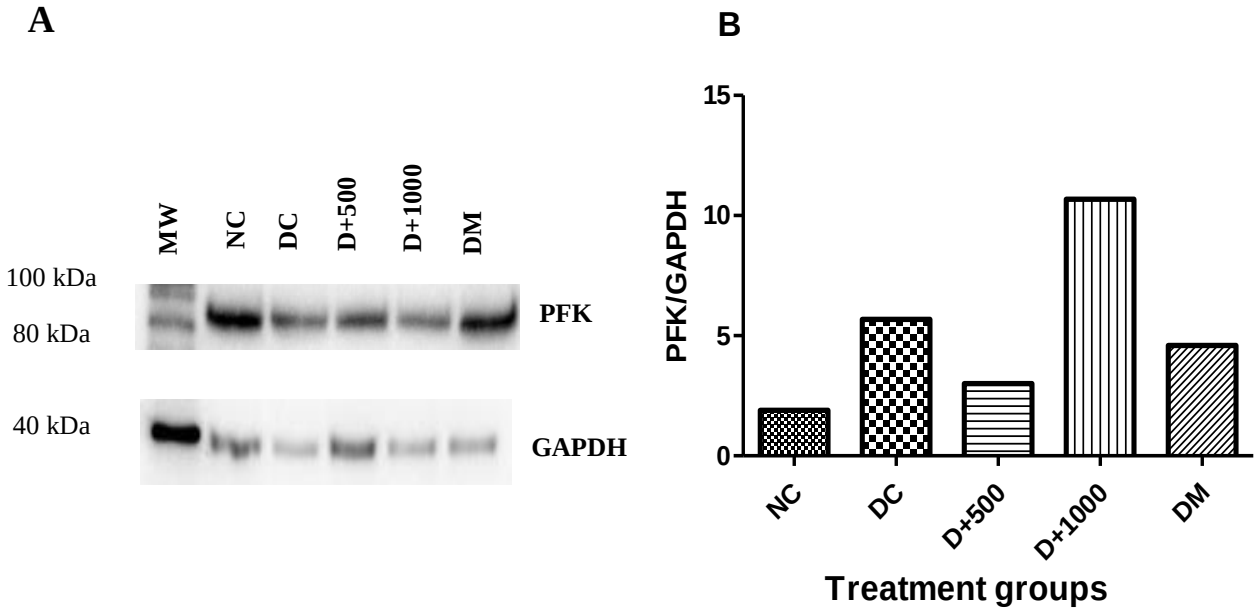


Figure 3.16: (A) Western blots of PFK (85 kDa) and GAPDH (37 kDa) in skin following 14 days of treatment with CA. 30 µg and 40 µg was loaded for PFK and GAPDH respectively. (B) PFK was normalized against GAPDH and were expressed as a PFK/GAPDH ratio MW= Molecular Weight; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The effects of CA on PFK expression in skin of non-diabetic and diabetic animals are shown in Figure 3.16. Diabetic control animals showed a higher PFK expression than the non-diabetic control animals. Diabetic animals treated with 500 mg/kg b.w. of CA had decreased enzyme expression levels than the diabetic control animals. Treatment of diabetic animals with 1000 mg/kg b.w. of CA resulted in the highest expression of PFK among the diabetic animals. Diabetic animals treated with metformin showed lower expression ratio when compared to the diabetic control animals.

3.4.2 Effects of CA on expression of glycogen phosphorylase in skin

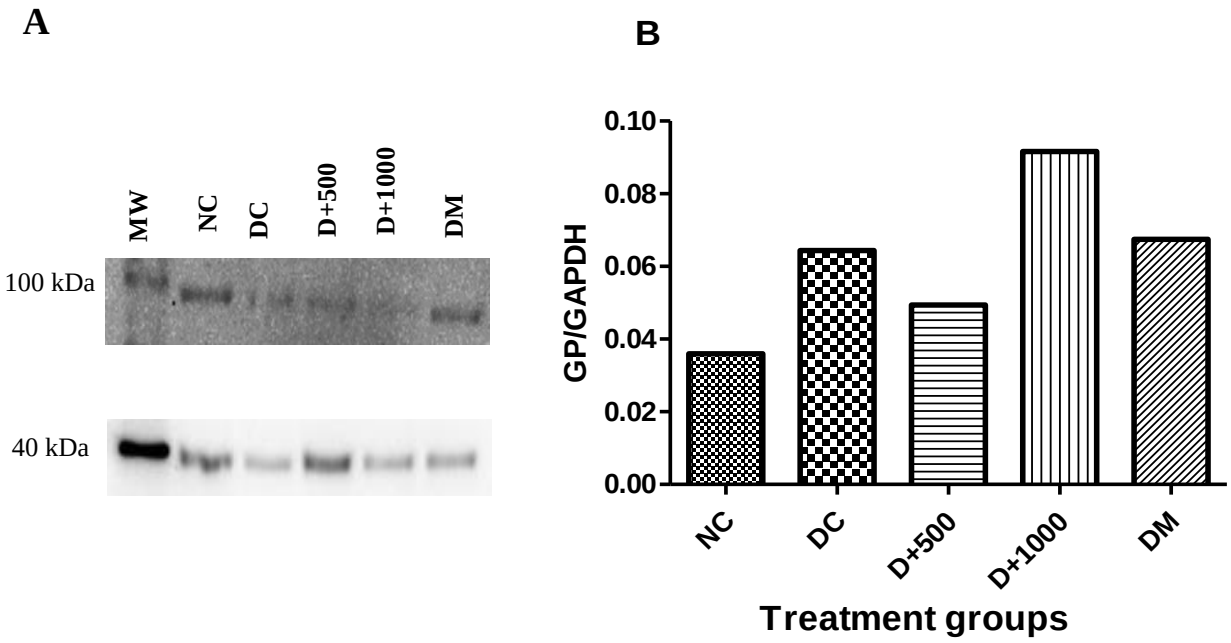


Figure 3.17: (A) Western blots of GP (97 kDa) and GAPDH (37 kDa) in skin following 14 days of treatment with CA. 30 µg and 40 µg were loaded for PFK and GAPDH respectively. (B) PFK was normalized against GAPDH and were expressed as a PFK/GAPDH ratio. MW = Molecular Weight; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The effects of CA on glycogen phosphorylase (GP) expression in skin are shown in Figure 3.17. Diabetic control animals had higher GP expression levels than the non-diabetic control animals. Treatment of diabetic animals with 500 mg/kg b.w. of CA lowered the GP expression compared to the diabetic controls. Diabetic groups treated with 1000 mg/kg b.w. of CA and with metformin showed higher levels of GP expression compared to the diabetic control animals.

Our GS antibody did not cross react with skin as it does with skeletal muscle GS (Mukundwa *et al*., 2016a).

3.5 Histological examinations

The following are morphological and statistical observations of hematoxylin an eosin (H and E) stained longitudinal and cross sectional images of skeletal muscle and skin of non-diabetic and diabetic animals.

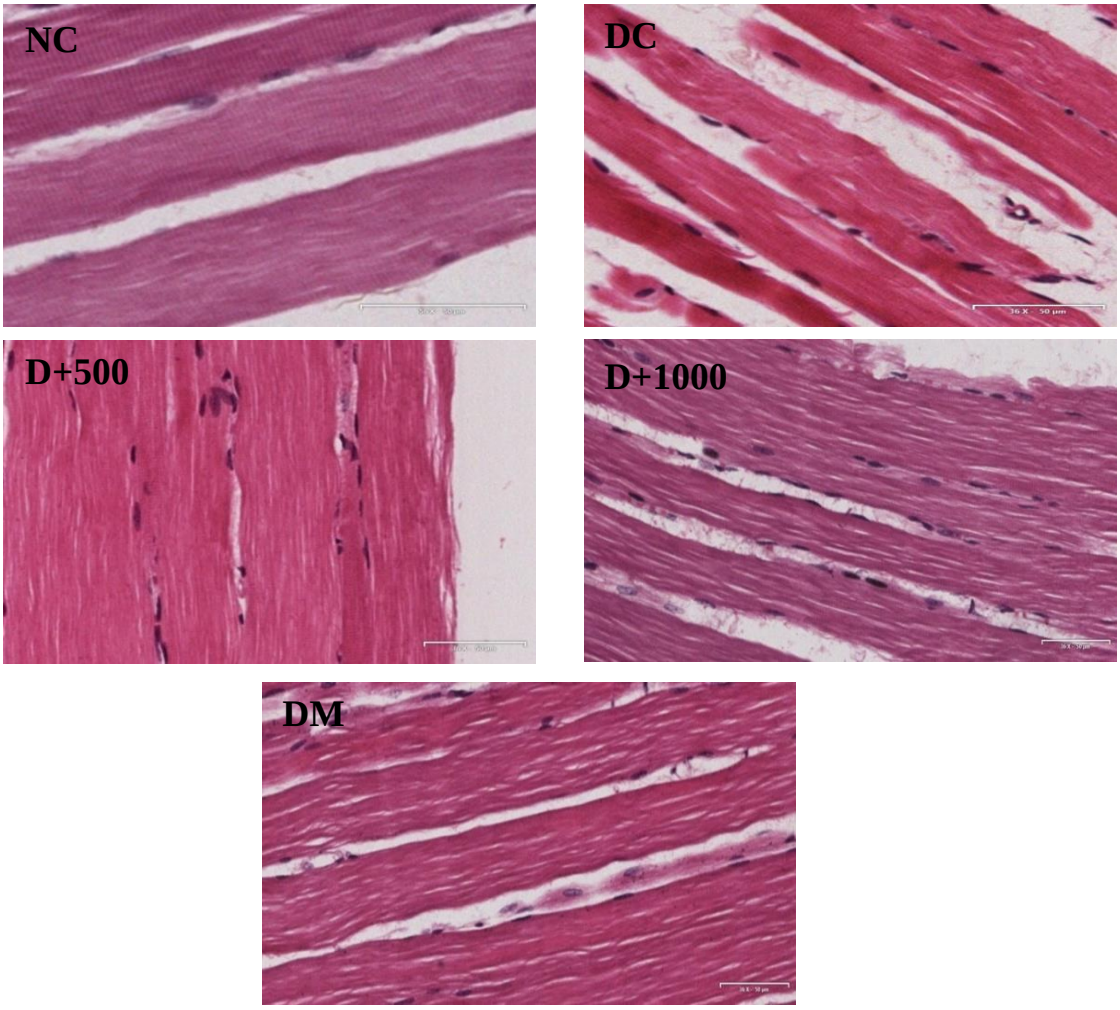


Figure 3.18: H and E stains of longitudinally arranged skeletal muscle fibres with peripherally located multi nuclei. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w) and DM = Diabetic + Metformin. (Magnification = 36 X 50 µm).

Figure 3.18 illustrates H and E stain of longitudinally cut skeletal muscle fibres that run parallel to each other enclosed by an endomysium. In non- diabetic controls, each muscle fibre has striations running perpendicular to the muscle fibre length and oval shaped peripherally located multinuclei. Skeletal muscle fibres of a diabetic control animal with peripherally located multinuclei showed discontinuity between muscle fibres, apparent decrease in fibre thickness and an increase in spaces containing connective tissue compared to the non-diabetic control animals. Diabetic animals treated with 500 mg/kg b.w. of CA show skeletal muscle fibres that appear close with little space between the fibres and damage to fibres appear less compared diabetic control animals. The muscle fibres also appear continuous with less breakage between the fibres. Diabetic animals treated with 1000 mg/kg b.w. of CA appear to have thin but continuous and closely spaced muscle fibres with peripherally located nuclei compared to diabetic control animals. There was also an apparent increase in spaces that encompass connective tissue and within fibres. Muscle fibres of diabetic animals treated with metformin appear continuous, but there was a slight increase in the spaces encompassing connective tissue and within muscle fibres.

Table 3.4: Morphometric data of longitudinal and cross sections of skeletal muscle in normal and diabetic rats.

	Longitudinal sections	**Cross sections**
Treatment groups	**Muscle fibre thickness (µm)**	**Muscle fibre diameter (µm)**
NC	38.85 ± 2.2	53.87 ± 0.52
DC	32.68 ± 0.54	48.33 ± 1.17
D+500	46.98 ± 2.01*****	53.21 ± 0.46
D+1000	40.23 ± 0.88*****	53.42 ± 1.25
DM	33.39 ± 0.92	50.88 ± 1.67

Values are expressed as mean ± SEM, (n = 3). * = p < 0.05 by comparison with DC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The muscle fibre thickness of longitudinal sections and muscle fibre diameter of cross sections of non-diabetic and diabetic animals are shown in table 3.4 above. Muscle fibre thickness in diabetic animals had lower mean muscle fibre thickness than that of non-diabetic animals although the changes were not statistically significant ($p > 0.05$). There was a significant ($p < 0.05$) 44% and 24% increase in muscle fibre thickness of both D+500 and D+1000 respectively compared to the diabetic control animals. There were no significant changes in muscle fibre diameter of other groups compared to the diabetic control animals.

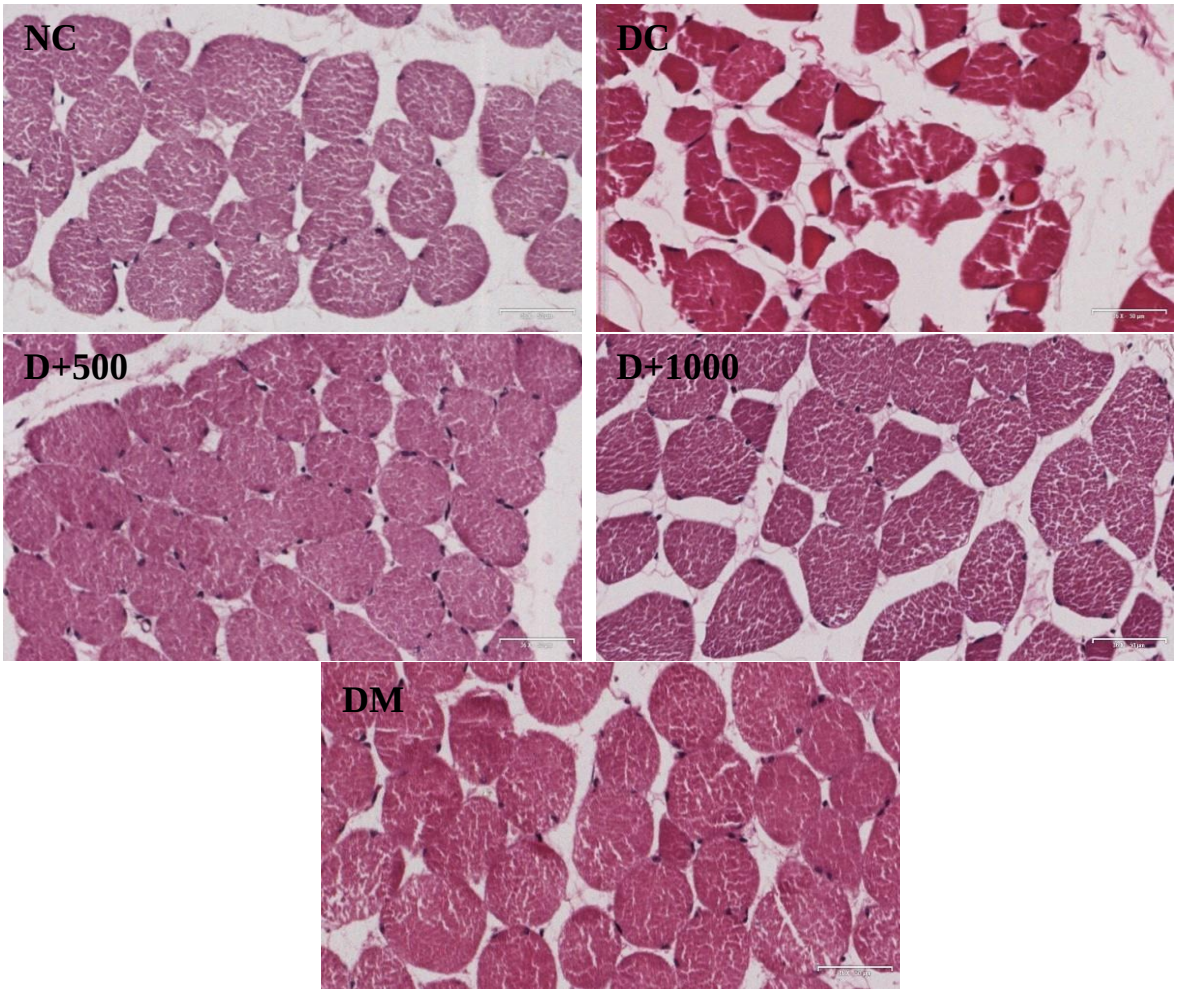


Figure 3.19: H and E stains of cross section skeletal muscle fibres with peripherally located multi nuclei. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin. (Magnification = 36 X 50 µm).

Figure 3.19 illustrates hematoxylin and eosin section of cross-sectionally cut skeletal muscle fibre with peripherally located multinuclei enclosed by an endomysium. The polygonal shaped muscle fibres appear irregularly shaped in diabetic control animals compared to non-diabetic control animals. There also appeared to be more spaces between the muscle fibres in diabetic animals and an increase in connective tissue between the fibres. All the treated diabetic groups (D+500,

D+1000 and DM) appear to have more regularly shaped muscle fibres and less connective tissue between the fibres compared to the diabetic control animals.

Table 3.5: Morphometric data of cross sections of skin epidermal thickness in non-diabetic and diabetic rats.

Treatment groups	Granular thickness (µm)	Spinous and basal thickness (µm)
NC	16.30 ± 087	23.31 ±1.76
DC	12.33 ± 0.86	20.15 ±1.67
D+500	20.24 ±1.99*	24.31 ±1.02
D+1000	17.82 ±1.44	20.48 ±1.08
DM	15.94 ±1.93	22.15 ±3.15

Values are expresses as mean ± SEM (n =3 in each group). * = p < 0.05 by comparison with DC. NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic + Metformin.

The morphometric data of cross-sectionally cut skin epidermis of non-diabetic and diabetic rats are shown in Table 3.5. The keratin layer was not measured due to its free-flowing nature which made it hard to accurately gauge. The Spinous and basal layer were difficult to differentiate between beginning and end without compromising the results; therefore, they were measured together. Both the granular, spinous and basal length were shorter in diabetic control animals compared to the normal control animals. Diabetic animals treated with CA showed an increase granular length with those treated with 500 mg/kg b.w. of CA being 64% higher. Diabetic animals treated with metformin also showed an in increase in granular length.

The spinous and basal layers were thinner in diabetic control animals than in the non-diabetic control animals. The diabetic groups treated with CA (D+500 and D+1000) and metformin had

thicker spinous and basal layers compared to the diabetic control animals. These differences however were not statistically significant.

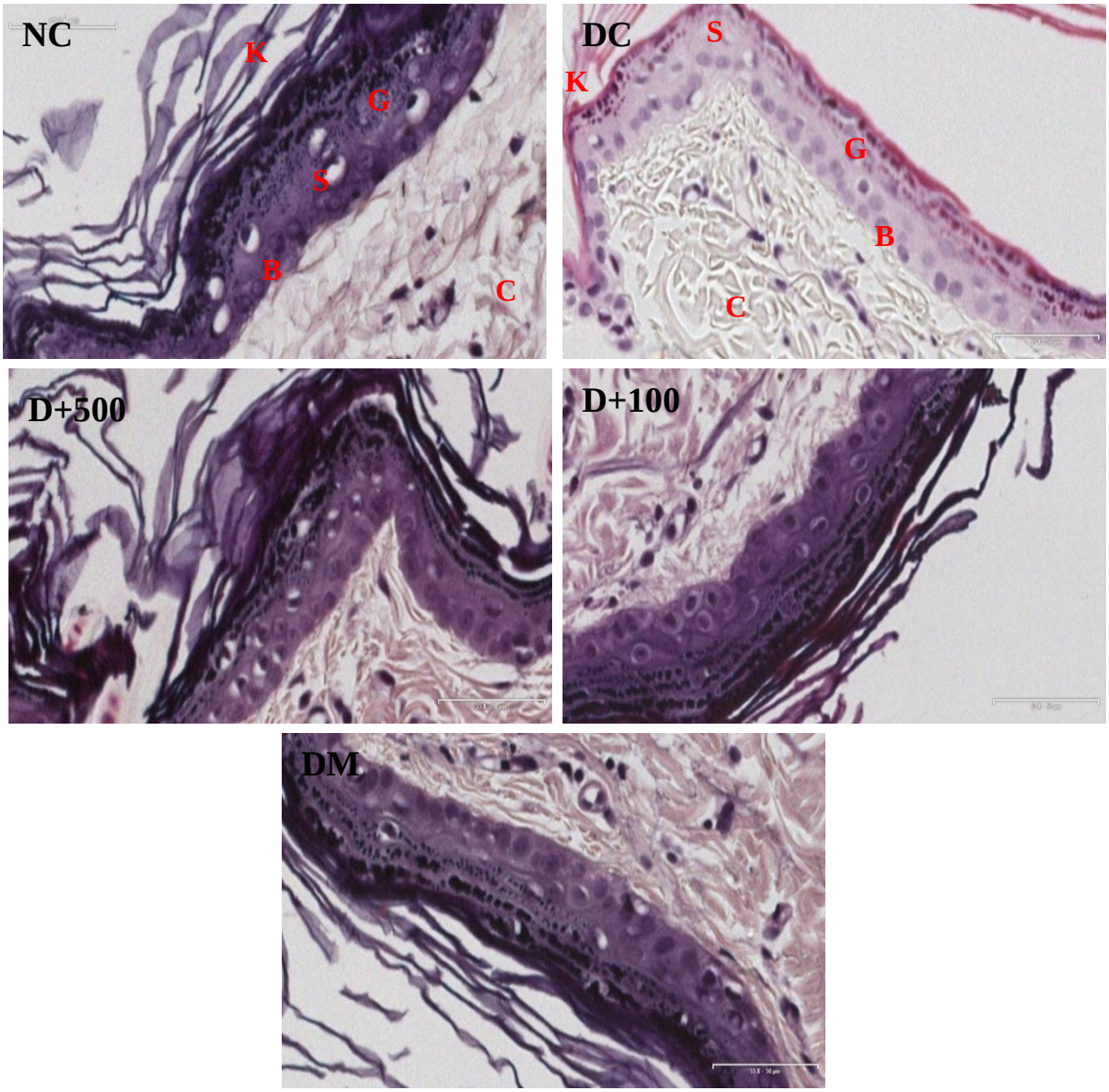


Figure 3.20 H and E stain skin longitudinal- section skin epithelium of non-diabetic and diabetic animals. K = keratin layer; G = granular layer; S= spinous layer; B = basal layer and C = connective tissue; NC = Non-diabetic control; DC = Diabetic control; D+500 = Diabetic + CA (500 mg/kg b.w.); D+1000 = Diabetic + CA (1000 mg/kg b.w.) and DM = Diabetic Metformin (Magnification = 55 X 50 µm).

Longitudinal-sections of skin epithelium and connective tissue of normal and diabetic animals are shown in Figure 3.20. The skin is mainly made up of epidermis and dermis. Structural characteristics of skin include 4 distinct layers in epidermis and connective tissue in dermal layer. The 4 layers include, keratin, granular, spinous and basal layer. The basal layer rests on basement membrane and is formed by round columnar cells while spinous layer is formed of polyhedral cells. The granular layer is formed by basophilic granules that appear deeply stained and keratin layer is made up of flat keratin cells with no nuclei. Diabetic animals appear to have a thinner epidermis and less connective tissue compared to non-diabetic animals. Note the granular layer is reduced and basal cells appear deeply stained. Amongst the diabetic animals, treatment with both concentrations of CA and metformin resulted in visible increase in epithelial thickness, granular cells and increase in the amount of connective tissue. The number of keratinized cells also appear to be increased in treated animals.

CHAPTER 4

Discussion

The aim of this study was to investigate the effects of oleanolic acid and methanol extract of *Centella asiatica* leaves on selected enzymes of glucose metabolism in muscle and skin of streptozotocin-induced diabetic male Sprague-Dawley rats; and to analyze the histological changes occurring in these tissues. The study also investigated changes in serum marker enzymes of tissue damage. This study was conducted to assess how OA and CA achieve their hypoglycaemic potential in a type-II insulin insufficiency diabetic model and insulin insufficiency and insulin resistance Type-II diabetic model respectively. Diabetes was induced using 40 mg/ kg b.w. of streptozotocin (STZ). STZ is a cytotoxic glucose analog that mimics the effects of diabetes when administered to animals. Development of hyperglycaemia in animal models induced with streptozotocin is mainly due to the destruction of pancreatic β-cells via uptake by the GLUT-2 transporter (Szkudelski, 2001). STZ also increases reactive oxygen species formation and nitric oxide production contributing towards DNA damage (Lenzen, 2008). This causes DNA alkylation followed by the activation of poly ADP ribose polymerase (PARP) leading to the reduction of NAD^+ and ATP followed by inhibition insulin production (Szkudelski, 2001; Lenzen, 2008; Ventura-Sobrevilla *et al.*, 2011). Chronic hyperglycaemia can result in glucose-induced insulin resistance, particularly in skeletal muscle. Hyperglycaemia also impairs glucose metabolism, predominantly in rate-limiting enzymes involved in glycolysis, gluconeogenesis, glycogenesis, and glycogenolysis. Theses enzymes include hexokinase, phosphofructokinase, fructose-1,6-bisphosphatase glycogen synthase and glycogen phosphorylase.

Effects of OA treatment in diabetic animals

This study investigated the effects of OA on enzymes of glucose metabolism in both muscle and skin using an insulin insufficiency type-II diabetes model. Metformin was used as a positive control as it is used to manage type-II diabetes. Deployment of OA was to investigate whether OA had any activity that enhances insulin action in a type-II diabetic animal model *in vivo*, as it does *in vitro* (Teodoro *et al.*, 2008). This study could also, in part, be used to compare the effect of STZ

at higher doses that induce type I diabetes to its effect at current lower doses that induce insulin insufficiency type-II diabetes (Mukundwa *et al.*, 2016a; Mukundwa *et al.*, 2016b).

Skeletal muscle is responsible for 75% of insulin stimulated glucose disposal (Saltiel and Kahn, 2001). Glucose uptake and subsequent phosphorylation in skeletal muscle occurs via of GLUT4 transporters and hexokinase, respectively (Dimitriadis *et al.*, 2011). Hexokinase is the first rate limiting enzyme of glycolysis that catalyzes the phosphorylation of glucose to glucose-6-phosphate trapping glucose in the cells. The second rate limiting enzyme in glycolysis, phosphofructokinase catalyzes the phosphorylation of fructose-6-phosphate yielding fructose-1,6-bisphosphate. These are essential in glucose regulation and are linked to complete glycolysis regulation. This study investigated the effects OA on glycolytic enzymes hexokinase and phosphofructokinase in skeletal muscle of diabetic and non-diabetic rats following a 14-day treatment period. The results in Figure 3.1 and 3.2A showed that hexokinase and phosphofructokinase had 19% and 77% decrease in activity, respectively in diabetic animals compared to the non-diabetic animals. A similar decrease in activities in these enzymes in muscle has been observed in previous studies (Da Silva *et al.*, 2010; Rasineni et al., 2010). In this study, treatment of diabetic animals with OA ameliorated this decrease in enzyme activity by increasing hexokinase and phosphofructokinase activity by 82% and 432% respectively. This suggests that OA possibly increases activities of hexokinase and phosphofructokinase through enhancing insulin secretion from β-cells, enhancing the effects of insulin or promoting direct activation of hexokinase and phosphofructokinase leading to subsequent stimulation in rate of glycolysis. It has been previously suggested that OA may enhance insulin secretion *in vitro* (Teodoro *et al.*, 2008). Expression of PFK in skeletal muscle is said to be influenced by genetic variation and insulin sensitivity (Keildson *et al.*, 2014). PFKM encodes for the enzyme PFK in muscle. Changes in gene expression can affect the phenotype of expressed gene therefore affect the regulatory effect of that protein. Mutation of this gene has been shown to cause glycogen storage diseases. Findings of this study indicate that the expression of PFK was slightly increased in diabetic animals compared to non-diabetic animals in skeletal muscle. The same phenomenon of overexpression of PFK phenomenon was seen in a previous study (Keildson *et al.*, 2014). Keildson *et al.* (2014) suggested that the increase in PFK expression might be an attempt to overcome inhibition of PFK activity. Treatment of diabetic rats with OA further increases PFK expression. In rat skin, however (Figure 3.14), PFK expression was also increased in diabetic control animals but reduced in diabetic

animals after treatment with OA. This suggests that the effect of OA on expression may vary depending on the target tissue.

This study also investigated the effects of OA on gluconeogenic enzyme fructose-1,6-bisphosphatase in skeletal muscle following a 14-day treatment regimen. Fructose-1,6-bisphosphatase catalyzes the hydrolysis of fructose-1, 6-phosphate yielding fructose-6-phosphate and an inorganic phosphate. FBPase plays a role in overall regulation of gluconeogenesis and is considered a potential target for diabetes treatment (Erion *et al.*, 2005). FBPase activity is increased in diabetic animals due to insulin insufficiency. The results in Figure 3.2B show that FBPase activity is increased by 25% in diabetic animals compared to the non-diabetic animals. A similar increase in FBPase activity was seen in liver, kidney and skeletal muscle of alloxan induced type-II diabetic model (Alam *et al.*, 2014). Treatment of diabetic rats with OA did not affect FBPase activity compared to the diabetic control animals. This may be because OA requires a threshold amount of FBPase to exert its effects in skeletal muscle. Since skeletal muscle is not a gluconeogenic tissue it produces less amount of FBPase compared to gluconeogenic tissues such as liver and kidney (Mizunuma and Tashima, 1986).

The skin makes up 15% of a person's total body weight and reduced glucose uptake in skin cells could contribute towards higher blood glucose levels in diabetes (Spravchikov *et al.*, 2001). In our previous study, where a type-I diabetic model was used, hexokinase activity was reduced but not significantly in diabetic animals (Mukundwa *et al.*, 2016a). In this study hexokinase activity in skin was only slightly increased by 8% in diabetic animals compared to non-diabetic control animals (Figure 3.3). Thus, diabetes does not seem to cause major change in skin hexokinase activity. This could be attributed towards the fact that during diabetes, glucose breakdown for energy production may be channeled towards major glycolytic tissues such as liver, kidneys and skeletal muscle. This may be the reason for increased dermatologic complications observed in diabetic patients causing poor wound healing properties and reduced collagen formation (Bermudez *et al.*, 2011; Knas *et al.*, 2013). Diabetic animals treated with OA showed increased hexokinase activity compared to the control animals. OA possibly increases hexokinase activity by enhancing insulin secretion from β-cells (Teodoro *et al.*, 2008).

Skin contains rapidly dividing cells that have high rates of proliferation and therefore has a high rate of glycolysis required for high metabolic energy (Keast *et al.*, 1989). The presence of

glycolysis and PFK enzyme activity has been shown to exist in skin samples (Kondo and Adachi, 1971). The results in Figure 3.4A show that diabetic animals had a 74% decrease in PFK activity compared to non-diabetic animals while Figure 3.14 shows that PFK expression is increased by 250% in diabetic control animals compared to the non-diabetic control animals. This increase in PFK expression accompanying a decrease in activity might be as an attempt to overcome inhibition of PFK activity as suggested by Keildson *et al.* (2014) in a study involving muscle. In diabetes, insulin secretion is reduced due to damaged β-cells. Reduced PFK activity of diabetic animals has also been observed in glycolytic tissues such as liver, skeletal muscle and heart (Da Silva *et al.*, 2010; Da Silva *et al.*, 2012). Diminished PFK activity in skin has also been observed in immunocompromised animals (Gupta *et al.*, 2004). The fructose-6-phosphate substrate used for PFK activity in diabetic animals possibly comes from increased glycogenolysis within the cell producing glucose-1-phosphate that is converted by phosphoglucose isomerase to yield fructose-6-phospahte rather than directly from hexokinase phosphorylation of glucose entry through in glycolysis. Treatment of diabetic animals with OA increased PFK activity compared to diabetic control animals. The increased PFK activity may be due to increased fructose-6-phosphate intermediate required for PFK activity. This suggestion is supported by the reduced glycogen phosphorylase activity in animals treated with OA and increased glycogen synthase activity in animals treated with OA and insulin found in type-I diabetic animals treated with OA (Mukundwa *et al.*, 2016a).The increased PFK expression in diabetic animals might be an attempt to overcome PFK activity inhibition (Keildson *et al.*, 2014). The same trend is seen in skeletal muscle of diabetic rats in Figure 3.13. Treatment of diabetic animals with OA reduces PFK expression. This may be because OA alleviated the need for PFK over expression thus no longer required to overcome the inhibition of PFK activity in diabetes.

Figure 3.4B shows a 30% decrease in skin fructose-1,6-bisphospahatase activity of diabetic animals compared to non-diabetic animals. This may be attributed to reduced glucose production capability of skin. The activities of gluconeogenic enzymes FBPase and PEPCK have been shown to be 20 times lower in skin compared to the liver (Peters and White, 1976). However, Peters and White (1976) suggested that FBPase does not necessarily contribute towards glucose metabolism through gluconeogenesis, but also through other pathways. Such a role has been seen in muscle where FBPase participates in glucose homeostasis where its product is isomerized and used as a substrate for glycogen synthesis. Pyruvate in skin may rather contribute more towards the TCA

cycle activity and hence less intermediates products are available for FBPase activity. FBPase may also have an entirely different function in skin not related to gluconeogenesis. This has been observed in muscle FBPase enzyme which is involved in interacting with mitochondrial proteins (Gizak *et al.*, 2012). Treatment of animals with OA lowered FBPase activity in both non-diabetic and diabetic animals. This suggests that OA lowers FBPase activity through other mechanisms that are not involved with metabolic changes caused by diabetes.

In diabetes, there is reduced glycogen formation due to low insulin levels produced by STZ destroyed β-cells (Szkudelski, 2001). However, in this study, muscle glycogen content increased in diabetic control animals compared to non-diabetic animals (Table 3.1). OA treated non-diabetic animals showed a significant ($p < 0.05$) 122% increase in glycogen content compared non-diabetic control animals. Similarly, 41% increase in glycogen content was seen in OA treated diabetic animals compared to diabetic control animals. This agrees with other studies that have also showed that OA can increase glycogen content in both non-diabetic and diabetic animals (Musabayane *et al.*, 2010; Ngubane *et al.*, 2011). These results correlate with the increase in hexokinase activity in OA treated diabetic animals seen in Figure 3.1 above. OA is therefore able to reduce hyperglycaemia by regulating glycogen metabolism possibly by modulating activity of glycogenesis regulatory enzymes. OA may also enhance insulin action of remaining β-cells thus stimulating glycogen formation. This concept is supported by the increase in glycogen synthase activity while reducing glycogen phosphorylase activity in the presence of insulin in OA-treated type-I diabetic animals (Mukundwa *et al.*, 2016b).

There was no difference in glycogen content observed between skin of non-diabetic and diabetic animals (Table 3.1). However, Mukundwa *et al.* (2016a) showed that glycogen content is reduced in a type-I diabetic model. Both OA treated non-diabetic and diabetic animals had reduced glycogen content compared to their respective untreated control groups. The decrease in glycogen in both these groups suggests that OA might induce glycogen break down in skin of diabetic animals. The increase in glycogen breakdown regulated by glycogen phosphorylase may be a way of providing free glucose to be channeled towards energy formation during diabetes. As mentioned by previous studies and suggested in this study, OA increases glycogen content in muscle of diabetic animals (Musabayane *et al.*, 2010; Ngubane *et al.*, 2011). However, this phenomenon is not observed in the skin. The mechanism of action of OA in skin on glycogen synthesis may not

be the same as seen in skeletal muscle. In type-I diabetes, OA and insulin treated animals showed no significant changes in glycogen content in skin compared to their control animals (Mukundwa *et al.*, 2016a). Therefore, OA might have different effects depending on model of diabetes and tissue.

Effects of CA treatment in diabetic animals

A high fat-STZ model is a common model used to induce a non-genetic type-II diabetes (Srinivasan *et al*., 2005). However, although the high fat diet induces insulin resistance, it requires complicated dietary formulations (Srinivasan *et al.*, 2005). Some studies have argued that low doses of just 40 mg/kg b.w. STZ may not be effective in inducing type-II diabetes while higher doses (45-50 mg/kg b.w) can cause the animal to resemble type-I rather than type-II diabetes. STZ alone does not induce insulin resistance rather it selectively destroys pancreatic cells subsequently causing hyperglycemia and subsequently only inducing insulin insufficiency (Srinivasan *et al*., 2005). Studies have shown that feeding animals with 10-15% fructose over a short or long period induces insulin resistance (Dai *et al.*, 1994; Hininger-Favier *et al.*, 2009). Therefore, when fructose feeding is coupled with a low dose of STZ it can induce both hyperglycemia and insulin resistance (Wilson and Islam, 2012). In this study, animals were given 10% fructose *ad libitum* for two weeks before being given a 40 mg/kg b.w. STZ injection to produce an insulin insufficiency and insulin resistance model of type-II diabetes. Insulin resistance results in defects in glucose uptake, phosphorylation, glucose oxidation and reduced glycogen synthesis in skeletal muscle (Petersen and Shulman, 2002).

Figure 3.5 shows that hexokinase activity is reduced by 13.4% in skeletal muscle of diabetic animals compared to non-diabetic animals. This observation is similar to those obtained from other studies in both Type-I and -II diabetic models (Ngubane *et al.*, 2011; Ramachandran and Saravanan, 2013). The lack of insulin sensitivity in skeletal muscle causes an impairment of peripheral glucose uptake and utilization. Glucose uptake by GLUT4 transporter is also impaired where both translocation and activity of transporter are diminished (Williams *et al.*, 2001; Karlsson and Zierath, 2007). Defects in intracellular glucose uptake also play a role in reducing hexokinase activity in skeletal muscle because they lower the amount of glucose substrate available for

hexokinase action. Reduced glucose phosphorylation by hexokinase causes a decrease in glucose metabolism and an increase in blood glucose levels in diabetic animals. Treatment of diabetic animals with 500 mg/kg b.w. of CA increased hexokinase activity by 4.4% while treatment with 1000 mg/kg b.w. of CA and metformin decreased hexokinase activity by 11.1% and 11.7% respectively. Increase in hexokinase activity in diabetic animals treated with 500 mg/kg b.w. of CA implies that CA may facilitate or stimulate glucose entry into the cell by targeting translocation and activation of glucose transporter, GLUT4. CA might also reduce insulin resistance in skeletal muscle promoting signaling of glucose uptake and subsequent phosphorylation by insulin sensitive enzyme, hexokinase. The results indicate that lower concentrations of CA are required for the extract to act on skeletal muscle as D+500 yielded an increase in hexokinase activity while D+1000 decreased activity. This suggests that lower doses of CA may be more effective in affecting against hexokinase activity while higher doses activate hexokinase activity. Asiatic acid, a triterpene of CA has also been shown to reduce hexokinase activity of type-II diabetic animals (Ramachandran and Saravanan, 2013).

The effects of CA on PFK activity and expression in skeletal muscle is shown in Figures 3.6A and 3.15 respectively. PFK is a key control point in glycolysis regulation and is diminished in skeletal muscle of diabetic animals. In this study PFK activity was reduced by 87% in diabetic animals compared to non-diabetic animals. In contrast, PFK expression was increased in diabetic control animals compared to non-diabetic control animals. PFK is not directly affected by insulin resistance, rather the reduced of response of skeletal muscle to insulin affects the insulin signaling pathway thus preventing the phosphorylation of PFK (Vestergaard *et al.*, 1993). It has been shown that PFK is regulated by different protein kinases that phosphorylate its tyrosine and serine residues on PFK in erythrocytes (Real-Hohn *et al.*, 2010). The decrease in hexokinase activity also directly brings about diminished PFK activity as the intermediate product, glucose-6-phosphate is required for isomerization to fructose-6-phosphate before it can act as a substrate for PFK. This decrease in hexokinase activity is also observed in diabetic animals in Figure 3.5 of the OA study which involved an insulin insufficiency model of diabetes. As suggested above, the increase in PFK expression may be a mechanism to compensate for PFK activity inhibition. The same trend of increased PFK expression was seen in insulin insufficient type-II diabetic model of muscle in Figure 3.13. Treatment of diabetic animals with CA (500 and 1000 mg/kg b.w.) and metformin increased PFK activity by 624%, 278% and 518% respectively while the expression of PFK is

reduced compared to diabetic control animals. The increase in PFK activity and reduction of expression seen in CA suggests that CA can reduce or overcome insulin resistance thus increasing PFK activity of diabetic animals. CA may also stimulate re-association of PFK oligomeric structure into stable tetramers restoring balance between inactive dimers and active tetramers thus increasing its catalytic activity (Luther *et al*., 1986; Zancan *et al*., 2008). A lower dose of CA resulted higher PFK activity further showing that CA may be a potent glycolytic activator at low doses.

Since skeletal muscle is not a gluconeogenic tissue, a lower (26%) decrease in FBPase activity was observed in diabetic animals (Figure 3.6B) In a gluconeogenic tissue such as liver, FBPase and PEPCK activities increase in diabetic animals due to increased endogenous glucose production to compensate for lack of glucose entry into cell (Srinivasan *et al.*, 2014). Treatment of diabetic animals with 500 mg/kg b.w. of CA increased FBPase activity by 98% but treatment with 1000 mg/kg b.w. of CA and metformin showed a 4.8% and 12% increase in activities respectively suggesting CA might increase insulin sensitivity the same way metformin does. Reduction of blood glucose levels in diabetic animals treated with metformin has been observed in alloxan induced type-I diabetic animals (Rahman *et al.*, 2012).

Epidermal skin tissue requires insulin for proliferation and differentiation and development of keratinocytes using insulin receptors (Wertheimer *et al.*, 2001).The Skin also requires insulin for migration and differentiation in keratinocytes during wound healing (Liu *et al.*, 2009). Diabetic animals showed a 20% and 57% decrease in hexokinase and phosphofructokinase activity respectively in skin compared to non-diabetic animals (Figures 3.7 and 3.8A). Figure 3.16 shows the expression of PFK is increased in diabetic control animals compared to non-diabetic animals. A similar decrease in hexokinase activity has been observed in skin of type-I diabetic animals (Mukundwa *et al.*, 2016a). This decrease occurs due to reduced response to insulin by the skin. The skins' degree of insulin response is unclear, but insulin receptor substrate proteins have been observed in skin keratinocytes (Shen *et al.*, 2000). The lack of insulin receptor (IR) caused elevated blood glucose levels in IR-null keratinocytes (Spravchikov *et al.*, 2001). In IR-null keratinocytes, the IGF-I receptor also shows reduced phosphorylation and reduced GLUT1 expression consequently resulting in reduced glucose uptake (Spravchikov *et al.*, 2001). The decrease in hexokinase activity reduces intermediate products required for PFK activity thus have a domino

effect on PFK activity of diabetic animals. Impaired hexokinase and phosphofructokinase activity in skin is also seen in immunocompromised and aging animals (Gupta *et al.*, 2004). Treatment of diabetic animals with 500 mg/kg b.w. of CA increased hexokinase and PFK activity by 11% and 35% respectively. In contrast, the expression of PFK in diabetic animals decreased upon treatment with both concentrations of CA. CA may increase hexokinase activity to protect skin cells from hyperglycaemia induced skin damage similar to its hepatoprotective mechanism (Antony *et al.*, 2006). CA has also been shown to be effective in enhancing skin healing properties by increasing collagen formation (Shukla *et al.*, 1999; Somboonwong *et al.*, 2012). CA may ameliorate changes in IGF-I receptor phosphorylation and GLUT-1 expression during diabetes resulting in more glucose being transported into cells for phosphorylation by hexokinase and subsequently phosphofructokinase enzymes. CA may also reduce PFK expression by directly acting on the PFKM gene. A similar reduction in PFK expression is seen in skin of diabetic animals treated with OA in the type-II insulin insufficiency model; and also in muscle of diabetic animals treated with CA in the insulin resistance and insulin insufficiency model in this study.

Figure 3.8B Shows the effects of CA on FBPase activity in skin of non-diabetic and diabetic animals. A 36% decrease in FBPase activity is seen in diabetic animals compared to non-diabetic animals. A similar decrease in activity is observed in the insulin insufficiency type-II diabetic model in Figure 3.4B. This further suggests that FBPase activity may be involved in other mechanisms beyond glucose regulation. Treatment of diabetic rats with 500 mg/kg b.w. of CA and metformin increased FBPase activity by 7% and 61% respectively while treatment with 1000 mg/kg b.w. decreased enzyme activity by 12%. CA may alter FBPase activity though other mechanisms not involved in glucose metabolism.

Excess glucose is stored in liver and muscle as glycogen but glycogen has also been observed to be present in skin epidermis (Jensen *et al.*, 2012). The presence of glycogen observed in epidermis is thought to be play a role in the hair cycle, sweat production and skin keratinization (Montagna *et al.*, 1951; Adachi, 1961). Reduced glycogen content in response to starvation has been observed in tail skin of mice (Harmon and Phizackerley, 1983). Glycogen accumulation in response to injury and abnormal cell proliferation has also been observed in epidermis and keratinocytes respectively (Lobitz and Holyoke, 1954; Wertheimer *et al.*, 2001). A 15% increase in glycogen content of diabetic animals compared to non-diabetic animals was observed in the current study (Table 3.2).

Treatment with CA reduced glycogen levels suggesting that CA may ameliorate changes caused by diabetes in glycogen metabolism.

Glycogen isolated from dog skin suggested the presence of GP and GS activity in this tissue (Adachi, 1961). In this study, no statistical difference in GS activity was observed between non-diabetic and diabetic animals (Figure 3.9). These results are similar to those observed in a type-I diabetic model reported by Mukundwa *et al.* (2016a). Treatment with 500 mg/kg b.w. of CA increased GS activity this may be due to accumulated G-6-P from increased hexokinase activity seen in Figure 3.6A. CA may protect β-cells from STZ induced damage seen in triterpene, asiatic acid, which preserves β-cell mass and reduces hyperglycaemia in STZ-induced type-I diabetic animals (Liu *et al.*, 2010). Asiatic acid also promotes survival of remaining β-cells by activating cell survival signaling. This occurs by activating protein kinase B in islets which plays a role in signaling cell death or survival (Liu *et al.*, 2010).

The skin of diabetic animals showed a reduction in glycogen phosphorylase activity (Figure 3.10) accompanied by an increase in glycogen content levels (Table 3.2). This implies that less glycogen is broken down resulting more of the glucose being stored than used up in skin of diabetic animals. A similar reduction in glycogen phosphorylase and increase in glycogen content has been observed in skin of type-I diabetic animals (Mukundwa *et al.*, 2016a). In diabetes, liver glycogen phosphorylase is enhanced thus increasing the rate of glycogenolysis to contribute towards hepatic glucose production and reducing liver glycogen content (Docsa *et al.*, 2011). Increase in glycogen levels have been observed in a variety of skin diseases and wound healing processes (Steiner, 1955). Glycogen content may not necessarily be for glucose production like in gluconeogenic tissue but may be for energy production within the skin during stressful conditions such as diabetes and wound repair. Treatment of diabetic animals with both concentrations of CA increased GP activity while reducing glycogen content compared to non-diabetic animals. The increase in GP activity may be due to GS levels returning to normal (Figure 3.9) after treatment thus no need to compensate for reduced glycogen synthesis allowing glycogenolysis to return to its normal rate in skin. In epidermis GS and GP occur at equal rates thus maintaining a specific concentration of glycogen (Halprin and Ohkawara, 1966a).

Serum AST and ALT enzymes are considered as biomarkers of tissue damage particularly the liver. Elevated levels of AST and ALT seen in diabetic animals indicates leakage of enzymes from

cytosol of liver into the bloodstream resulting from damaged liver due to defects in glucose utilization (Murali *et al.*, 2013). Table 3.3 shows that ALT and AST levels are elevated by 17.6% and 7.7%, respectively in diabetic animals compared to non-diabetic animals. High levels of serum ALT are associated with decreased insulin sensitivity in the liver and considered as a marker for the potential risk of type-II diabetes (Vozarova *et al.*, 2002). Vozarova *et al.* (2002) suggested that these high ALT levels indicate that the liver may play a potential role in pathogenesis of diabetes. Hyperglycaemia results in increased formation of reactive oxygen species in mitochondria subsequently resulting in oxidative stress causing tissue damage (Fiorentino *et al.*, 2013). Increase in oxidative stress also slows down the pentose phosphate pathway which affects the concentration of NADPH thus causing more diabetic complications (Giacco and Brownlee, 2010). G-6-P, the substrate required for pentose phosphate pathway is provided by hexokinase. Thus, changes in hexokinase activity seen in skeletal muscle and skin may also indirectly contribute towards liver damage. Treatment of diabetic animals with 500 and 1000 mg/kg b.w. of CA lowered AST by 17.3% and 18.7% respectively. ALT was lowered by 41% and 29.2% following treatment of diabetic animals with 500 and 1000 mg/kg b.w. of CA respectively. The ability of CA to lower these enzymes suggests that CA may protect animals from liver damage. These results are in agreement with those reported by Ramachandran and Saravanan (2013) using asiatic acid, a triterpene present in CA.

The reduction of muscle mass and altered physical capacity has been documented in diabetic animals (Andersen *et al.*, 2005). Both morphometric and statistical results show a decrease in muscle fibre thickness and diameter in diabetic animals compared to the non-diabetic control animals (Figure 3.18 and Table 3.4). Morphological studies revealed muscle fibres with distorted striations, reduction in the number of peripheral nuclei and increased spaces between muscle fibres. Statistical analysis showed a reduction in muscle fibre thickness and diameter of 16% and 10% respectively. These results are in agreement with those reported by other studies (Cotter *et al.*, 1993; Aughsteen *et al.*, 2006; Mostafa, 2008). The decrease in muscle fibre thickness is reported to be due to a reduction of protein synthesis and increased protein degradation (Cotter *et al.*, 1989). Apart from distorted muscle fibres, other structural changes observed include damaged myofibrils, distorted sarcomere Z line and swollen irregular mitochondria are as from electron microscopy (Mostafa, 2008). Increased inflammatory cells in response to damaged myofibrils has also been observed (Aughsteen *et al.*, 2006). CA treatment improved structure of muscle fibre,

increased the number of peripheral nuclei and reduced space between connective tissue. Muscle fibre thickness was improved by 44%, 23% and 2% in diabetic animals treated with 500 mg/kg b.w. of CA, 1000 mg/kg b.w. of CA and metformin respectively. Muscle fibre diameter was also improved by 10%, 11% and 5% in diabetic animals treated with CA (500 and 1000 mg/kg b.w.) and metformin respectively. Treatment with CA may have ameliorated morphological changes and muscle fibre thickness by enhancing insulin sensitivity in skeletal muscle as treatment of diabetic rats with insulin is said to improve structural changes and subsequently protein synthesis (Moore *et al.*, 1983). CA may also protect skeletal muscle from hyperglycaemia thus reducing damage caused by diabetes.

At least 30% of diabetic patients experience some form of dermatological complications. The results in Figures 3.19, 3.20 and Table 3.5 indicate the morphological and statistical effects of CA in non-diabetic and diabetic skin. Diabetic animals showed a thinner epidermal layer and reduced connective tissue (Figure 3.20). The thinner epidermis was mainly due to reduced spinous layer rather than all the other layers that make up the epidermis. Similar results have been observed in other studies (Tanga *et al.*, 2003). Other structural changes observed by other studies include deeply stained nuclei and increased pixel intensity of collagen (Omar, 2010; Knas *et al.*, 2013). Immunohistochemical changes observed include reduction of CD34 percentage (Omar, 2010). Treatment of diabetic animals with CA ameliorated changes in skin structure. This further suggests that CA protects skin from structural changes caused by diabetes. CA has also been shown to be effective in enhancing skin healing properties by increasing collagen formation (Shukla *et al.*, 1999; Somboonwong *et al.*, 2012).

CHAPTER 5

Conclusion

OA and CA have hypoglycaemic effects in insulin insufficient and insulin resistant type-II diabetic models respectively. OA increased hexokinase and phosphofructokinase activity in skeletal muscle and skin of diabetic rats. This suggests that OA might modulate its hypoglycaemic activity in skeletal muscle by regulating glycolysis. OA also reduced excessive PFK expression in skin of diabetic animals suggesting OA may have modulating activity and expression of glycolytic enzymes. A 500 mg/kg b.w. dose of CA increased hexokinase, PFK and GS activity while reducing PFK expression in skeletal muscle and skin of diabetic animals and hence may reduce hyperglycaemia by controlling glycolysis and glycogen synthesis. This study also suggests that a lower concentration of 500 mg/kg b.w dose of CA is enough to potentiate its hypoglycaemic activity while a higher dose of 1000 mg/kg b.w. is inhibitory. CA might be able to protect tissues from damage caused by diabetes as it reduced AST and ALT levels in diabetic animals. Histological changes in muscle and skin caused by diabetes were decreased after treatment with diabetes further confirming tissue protective capabilities of CA.

Limitations and further studies

The limitations of this study were that that FBPase was the only gluconeogenic enzyme investigated and Western blots of only PFK and GS were performed. Studies on OA also require more assays on gluconeogenic rate limiting enzyme PEPCK and glycogenic enzymes GS, and GP in both skeletal muscle and skin. Western blot procedures on HK, FBPase and PEPCK expression studies should be explored in both OA and CA studies. Further studies should also involve Gas Chromatography mass spectrometry investigation of the chemical profile of the methanol extract of CA. Chronic studies on OA and CA should be done to investigate the long-term effects of both CA and OA.

CHAPTER 6

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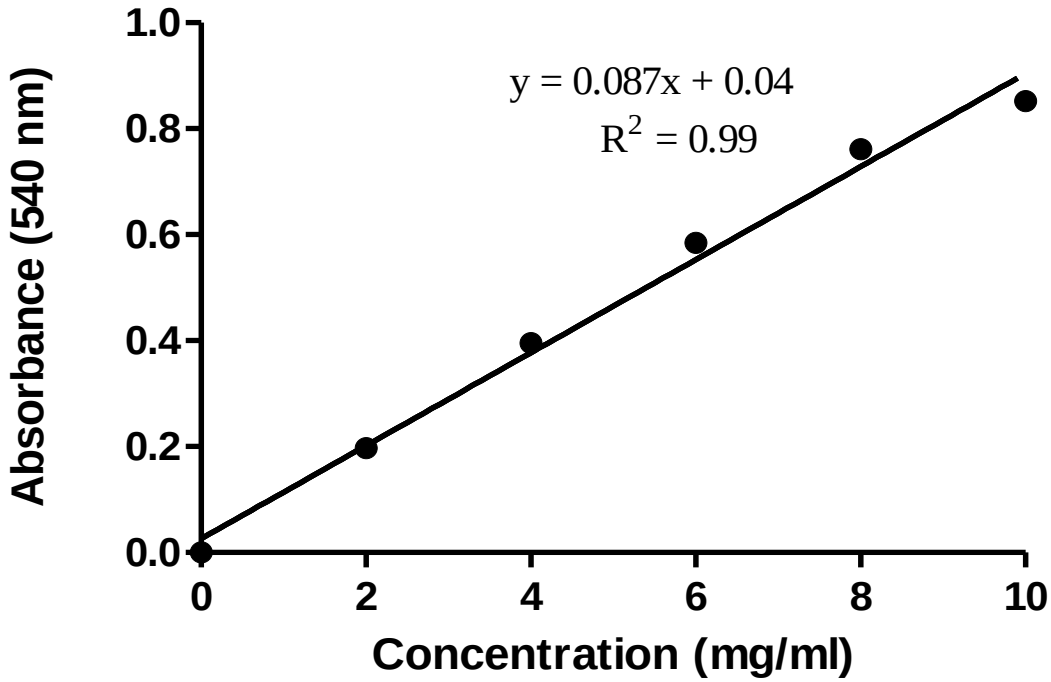
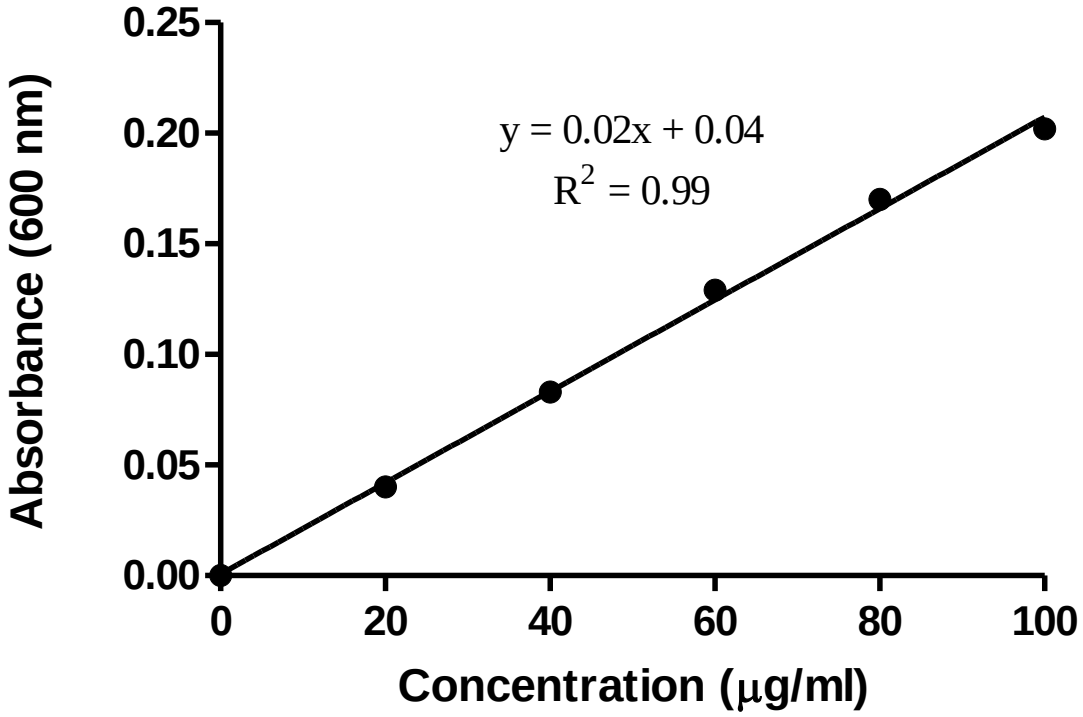
Appendices

Appendix 1: Biuret assay standard curve



Appendix 2: Folin-Lowry standard curve



Appendix 3: Glycogen content standard curve