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RENAL AND METABOLIC EFFECTS OF *TULBAGHIA VIOLACEA* HARV. AND CAPTOPRIL IN FRUCTOSE-FED STREPTOZOTOCIN INDUCED DIABETIC RATS

By

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Renal and metabolic effects of *Tulbaghia violacea* Harv. and captopril in fructose-fed streptozotocin induced diabetic rats

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Human Physiology, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor I Mackraj and co-supervision of Dr K Moodley.

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DECLARATION

I, Ms Kimane Megan Joseph, declare as follows:

1. That the work described in this thesis has not been submitted to the University of KwaZulu-Natal or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows: The project in its entity has been completed by myself under the guidance of my supervisor, Professor I Mackraj and co-supervisor, Dr K Moodley

3. That the contributions of others to the project were as follows: The concept, strategising and execution of the project have been administrated by my supervisor and co-supervisor.

Signe  Date 1 June 2017

DEDICATION

I dedicate this dissertation to my family members who are currently affected by or have passed on due to Diabetes Mellitus.

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LIST OF ACRONYMS

ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end product
AGT	Angiotensinogen
AMP	Adenosine monophosphate
ANGII	Angiotensin II
ARB	Angiotensin receptor blocker
AT1	Angiotensin type 1 receptor
AUC	Area under the curve
BP	Blood pressure
CAGE	Chymostatin-sensitive-AngII-generating enzyme
CAP	Captopril
DC	Diabetic control
DM	Diabetes mellitus
DN	Diabetic nephropathy
DPP-4	Dipeptidyl peptidase 4
ELISA	Enzyme linked immunosorbent assay
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIP	Glucose dependent insulinotropic polypeptide
GLIB	Glibenclamide

GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporters
H&E	Haematoxylin and eosin
HDL	High density lipoproteins
HF	High fat
IDDM	Insulin dependent diabetes mellitus
IR	Insulin resistance
IRS	Insulin receptor substrates
i RAS	Intrarenal renin angiotensin system
IUGR	Intrauterine growth reduction
LDL	Low density lipoproteins
MDA	Malondialdehyde
MET	Metformin
MSG	Monosodium glutamate
NADPH	Nicotinamide adenine dinucleotide phosphate
NDC	Non-diabetic control
NEDD	N-1-napthylethylenediamine dihydrochloride
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitric oxide
NOS	Nitric Oxide Synthase
$ONOO^-$	Peroxynitrite

P13K	Phosphatidylinositol 3 kinase
PCR	Polymerase chain reaction
PKB	Protein kinase B
RAS	Renin angiotensin system
RT-PCR	Real time polymerase chain reaction
RER	Rough endoplasmic reticulum
ROS	Reactive Oxygen Species
SD	Sprague-Dawley
SGLT2	Sodium-glucose co-transporter 2
STZ	Streptozotocin
SULF	Sulphanilamide
SOD	Superoxide dismutase
TZD	Thiazolidinediones
TBARS	Thiobarbituric acid reactive substances
TVL	*Tulbaghia violacea* Harv.
VLDL	Very low density lipoproteins

ABSTRACT

Background: Diabetes mellitus has rapidly emerged as a worldwide epidemic resulting in significant morbidity and mortality. The incidence of type 2 diabetes mellitus, induced by a high carbohydrate diet, a sedentary lifestyle as well as obesity, is increasing rapidly. Despite the availability of current conventional drugs, the complications of diabetes mellitus continue to progress. Therefore the search for alternate therapies that are antidiabetic in nature and elicit minimal side effects is essential. This study investigated the effects of the medicinal plant *Tulbaghia violacea*. Harv and angiotensin converting enzyme inhibitor, Captopril in a fructose-fed streptozotocin induced diabetic rat model.

Methods: Thirty-six, male Sprague-Dawley rats (six-week old) were randomly divided into six groups' namely non-diabetic control, diabetic control, diabetic treated with *Tulbaghia violacea* Harv. (60 mg/kg bw), diabetic treated with captopril (50 mg/kg bw), diabetic treated with metformin (250 mg/kg bw) and diabetic treated with glibenclamide (10 mg/kg bw). Diabetes was induced by fructose feeding for 1 week followed by a single intraperitoneal injection of 40 mg/kg.bw streptozotocin. Animals with a fasting blood glucose concentration >25mmol/L were considered diabetic and included in the study. Thereafter, respective doses of *Tulbaghia violacea* and conventional drugs were daily administered to the diabetic groups by oral gavage for seven weeks. At week six, an oral glucose tolerance test was performed. At the end of week 7, the animals were euthanized by halothane overdose. Blood was collected and organs were harvested for further biochemical analyses.

Results**:** *Tulbaghia violacea* treatment significantly increased plasma insulin and liver glycogen content. *Tulbaghia violacea* treatment also reduced liver thiobarbituric acid reactive substances levels, increased liver superoxide dismutase concentration and increased plasma nitric oxide levels. Furthermore, *Tulbaghia violacea* administration reduced serum triglycerides, total cholesterol, VLDL cholesterol, LDL cholesterol and increased HDL cholesterol. The plant treated group showed increased pancreatic islet counts as well as improved glomerular morphology. The angiotensin converting enzyme inhibitor captopril showed a significant increase in angiotensin converting enzymeactivity as well as increased angiotensin type 1 receptor expression compared to the diabetic controls and *Tulbaghia violacea.*

Conclusion: *Tulbaghia violacea* did not decrease fasting blood glucose levels or improve glucose tolerance. However, in this study, the data obtained demonstrated the ability of *Tulbaghia violacea* to elicit antioxidant and hypolipidemic effects, augment plasma insulin levels, improve pancreatic and glomerular morphology and positively impact β- cell function in a fructose-fed streptozotocin induced diabetic rat model.

CHAPTER 1

1. Introduction

Diabetes Mellitus (DM) is recognized as an emerging epidemic worldwide, accounting for increased morbidity and mortality. According to the World Health Organisation (WHO), 422 million people were affected by DM in 2014. Furthermore the prevalence of DM has significantly increased from 4.7% to 8.5% globally and 3.1% to 7.1% in Africa (World Health Organisation, 2016). Diabetes Mellitus is a chronic metabolic disorder, which is characterized by hyperglycaemia resulting from insulin deficiency, insulin resistance or both (American Diabetes Association, 2015). Hyperglycaemia is known as the primary clinical manifestation of diabetes that is strongly linked to the development of the diabetic complications described as microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications (Ahmed *et al*., 2010; Patel *et al*., 2012; Ullah *et al*., 2015).

This disorder can be classified into Type 1, which is insulin dependent and Type 2, which is non-insulin dependent (Ullah *et al*., 2015).There has been an alarming increase in Type 2 diabetes induced by high dietary carbohydrates such as fructose rich foods as well as a sedentary lifestyle (Dhar *et al*., 2013). Alongside Type 2 diabetes, obesity and dietary carbohydrates are considered to be major health concerns worldwide (Dhar *et al*., 2013; Selvaraju *et al*., 2012). Majority of the patients diagnosed with Type 2 diabetes have been classified with obesity (Scherer and Hill, 2016). Interestingly obesity is characterised by an increase in activation of the Renin angiotensin system (RAS) and multiple lines of evidence suggests that this system is also involved in the development of type 2 diabetes (Goossens. 2012). Studies have shown that the blockade of RAS using either angiotensin (Ang) II type 1 receptor blockers (ARBs) or angiotensin- converting enzyme (ACE) inhibitors is a promising strategy for the management of type 2 diabetes (Putnam *et al*., 2012).

Despite the availability of therapeutic innovations in type 2 diabetes, the existing conventional treatments such as sulphonylureas, Metformin and thiazolidinediones do not fully combat the consequences of insulin resistance (IR) (e.g. hyperglycaemia, diabetic dyslipidemia, and pancreatic β-cell damage) or manage the pathophysiological aspects of this disease (Del Pilar Solano and Golberg *et al*., 2005; Eleazu *et al*., 2013). Furthermore, clinical uses of the current drugs are accompanied by adverse side effects such as severe hypoglycaemia, lactic acidosis, peripheral edema and abdominal discomfort (Lorenzati *et al*., 2010). Therefore, the search for alternate treatments with improved efficacy and minimum side effects is still ongoing.

Medicinal plants have been identified as an alternate remedy recommended for the management and treatment of diabetes. Numerous experimental studies (i.e. animal models and *in vitro*) have documented the antidiabetic activity of various plants (Ghorbani, 2013). In South Africa, indigenous

medicine has become increasingly popular with an estimated 27 million people being dependant on the healing characteristics of plants (Sobiecki, 2014).

Amongst the various alternate remedies involving plant based treatments, *Tulbaghia violacea* (TVL) has shown potential in combating disorders ranging from diabetes mellitus to infectious diseases. This is a herbaceous plant that is indigenous to Southern Africa. In a previous study *TVL* was reported to elicit hypoglycaemic, antioxidant, hepatoprotective and hypolipidemic effects in STZ induced diabetic rats (Moodley *et al*., 2015). Therefore, the aim of the present study is to investigate the renal and metabolic effects of Tulbaghia violacea and captopril in a fructose-fed streptozotocin induced diabetic rat model.

CHAPTER 2

2. Literature Review

2.1 Definition and Classification of Diabetes mellitus

Diabetes mellitus (DM) is a chronic metabolic disorder that is characterized by hyperglycaemia (elevated blood glucose levels) as a consequence of insufficient insulin production, insulin action or both (American Diabetes Association, 2015).There are different types of DM namely, type 1 diabetes or insulin dependent diabetes mellitus (IDDM); type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) and gestational diabetes which only develops during the late stages of pregnancy.

2.1.1 Type 1 Diabetes mellitus

Type 1 DM or insulin-dependent diabetes mellitus (IDDM) usually develops in childhood and progresses with age. It represents about 5-10% of all diabetic cases (Szkudelski *et al*., 2013). Type1 DM is caused by cell-mediated autoimmune destruction of pancreatic β cells resulting in insulin deficiency that predisposes the patient to diabetic ketoacidosis. Genetic mutations and environmental factors are also responsible for the destruction of pancreatic β cells. Patients diagnosed with type 1 DM use prescribed insulin products (e.g. Humilan, Novek) to regulate blood glucose levels (Surya *et al*., 2014; Ullah *et al*., 2015).

2.1.2 Type 2 Diabetes mellitus

Type 2 DM, previously known as non-insulin-dependent diabetes mellitus (NIDDM) or maturity-onset diabetes. The late onset of this condition accounts for 90-95 % of all diabetic cases (Szkudelski *et al*., 2013). It is a heterogeneous disorder with a more complex aetiology and more common than type 1 DM. However, the pathogenesis of type 2 DM is not fully understood. Insulin resistance, inadequate insulin secretion and elevated blood glucose levels are clinical characteristics associated with type 2 DM. It is also characterised by a slowly progressing onset of hyperglycaemia. Some of the primary facets that play a role in the development of this disease include multiple genetic defects and specific environmental factors like obesity (Surya *et al*., 2014; Ullah *et al*., 2015).

2.2 Physiology of insulin release and action

Insulin is a peptide hormone that is produced and secreted by pancreatic β cells found in the islets of Langerhans, which ranges between 100 – 200 µm in size in mammals. The islets of Langerhans play a key role in balancing the blood glucose levels by secreting hormones, like insulin (Jo *et al*., 2007). This hormone plays an essential role in many systems and organs of the body. It has an effect on fat, carbohydrate and protein metabolism, as well as on blood glucose concentration (Cartailler, 2015).

2.2.1 Insulin synthesis and secretion

The mRNA of insulin is translated as an inactive single chain precursor called preproinsulin which has an N-terminal signal peptide that directs its passage across the rough endoplasmic reticulum (RER) membrane into the lumen (Fu *et al*., 2013). During the translocation process, proinsulin is produced when the signal peptide is removed. Proinsulin undergoes folding and the development of three disulphide bonds to create a characteristic three dimensional structure (Rains and Jain, 2011; Fu *et al*., 2013). The folded proinsulin is transported to the Golgi apparatus where the proinsulin enters immature secretory vesicles. Proinsulin is cleaved by specific peptidases to form C-peptides together with the mature and active insulin molecule which is made up of A chains (21 amino acids) and B chains (30 amino acids), linked by two disulphide bonds. Secretory vesicles store the insulin and C-peptide until a vagal stimulation or metabolic signal initiates exocytosis of the mature vesicles, and the release of insulin into circulation (Fu *et al*., 2013).

Insulin is released in response to alterations in blood glucose levels. The entry of glucose into β cells is mediated by type 2 glucose transporters (GLUT 2). A rate-limiting enzyme such as glucokinase catalyses the phosphorylation of glucose, which is further, metabolized to create ATP. Increased ATP levels cause ATP-gated K^+ channels in the cellular membrane to close preventing the movement of K^+ ions out the cell. An increase in the positive charge of the cell results in depolarization. Voltage-gated Ca^{2+} channels become activated, and Ca^{2+}ions are transported into the cells. The net effect on voltage-gated channels leads to the export of insulin containing granules via exocytosis (Figure 2.1). Insulin released from pancreatic β cells is absorbed in the blood stream (Cartailler, 2015).

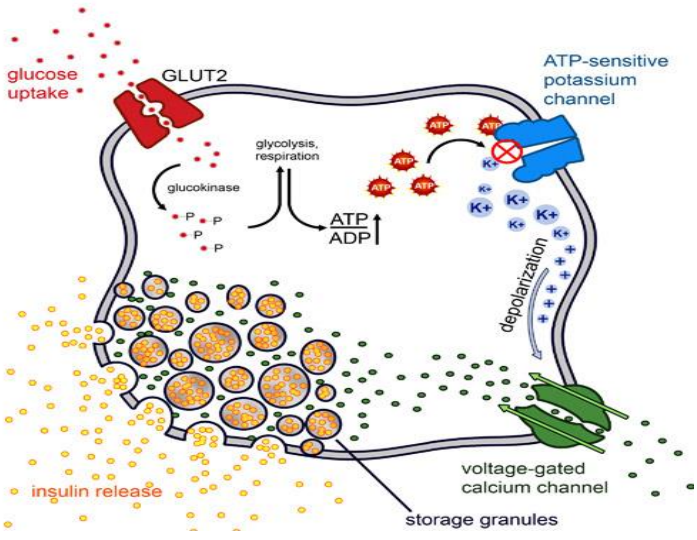

Figure 2.1 An illustration of the steps that lead to the exocytotic release of insulin from their storage granule (Cartailler, 2015)**.**

2.2.2 Insulin receptors and insulin binding

Insulin molecules released by pancreatic β cells remain in the blood stream until they bind to insulin receptors. The up-take of glucose into various tissues that contain type 4 glucose transporters (GLUT 4) is promoted by the insulin receptors. The insulin receptors consist of two extracellular α and two transmembrane β glycoprotein subunits, joined by disulphide bonds. Autophosphorylation of the β subunit occurs when insulin binds to the α-subunit. This enables tyrosine phosphorylation of regulatory proteins known as insulin receptor substrates (IRS-1 to IRS-4). The most important substrates are IRS-1 and IRS-2 (Wilcox, 2005; Rains and Jain, 2011). Effector molecules bind to the tyrosine phosphorylated IRS, which stimulates different signalling pathways such as phosphatidylinositol 3 kinase (P13K), and protein kinase B (PKB; Akt). This leads to translocation of the glucose transporter (GLUT4) to the cell membrane of skeletal muscle and adipose cells, resulting in glucose uptake as shown in figure 2 (Gual *et al*., 2005; Rains and Jain 2011; Styskal *et al*., 2012). The key component of the insulin-signalling cascade is the P13K pathway, which mediates the metabolic effects of insulin on glucose transport and GLUT 4 translocation (Choi and Kim, 2010).

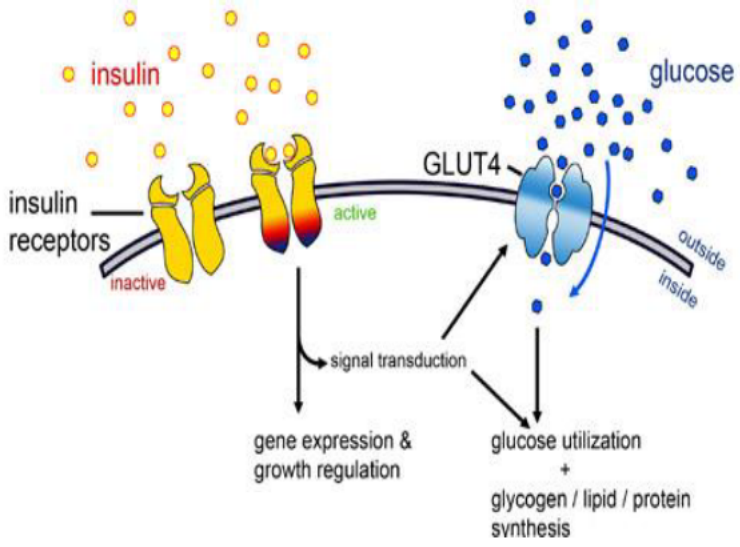

Figure 2.2 An illustration of the steps that lead to insulin-mediated glucose uptake (Cartailler, 2015).

2.2.3 Glucose Transporters

The GLUT 4 protein molecules, which assist in transporting glucose into the cell, regulate insulin activated glucose uptake by the muscle and adipose cells (Wilcox, 2005). The identification of at least five subtypes of transporter proteins with different affinities for glucose and tissue distribution has been documented. GLUT 1 is distributed throughout the body; GLUT 2 is found in the gut, liver, kidney and β cell; GLUT 3 located in neurons, placenta and foetal muscles; GLUT 4 is found in the heart, muscle and fat cells, whereas GLUT 5 is present in the small intestine as well as in the testes (Wilcox, 2005).

2.3 Insulin action

Insulin is an anabolic hormone that enhances the production of carbohydrates, proteins and fats. This hormone has major effects on the metabolism of carbohydrates, lipids and fats, resulting in an increase in the glycogen synthesis rate and a decrease in the glycogen breakdown rate as well as a reduction in lipolysis rate in adipose tissue, which consequently decreases the fatty acid level in the liver and elevating the protein synthesis rate in the muscle, adipose tissue and liver (Dimitriadis *et al*., 2011). The sites of insulin action are specific to the physiological role of the organs and tissues taking into consideration the metabolic processes that require insulin.

2.3.1 Muscle

The muscle depends on insulin to facilitate glucose absorption via GLUT 4 and accounts for 60-70% of the body's insulin mediated uptake. Glycogen synthesis via glycogen synthesis activation in the postprandial state is promoted by insulin in the muscle. However, glycogen synthesis is impaired during insulin resistance, which can be attributed to decreased intracellular glucose translocation (Wilcox, 2005). Protein catabolism is repressed by insulin and enhanced by insulin deficiency, resulting in the release of amino acids for gluconeogenesis (Wilcox, 2005). A balance is created between the synthesis and degradation of protein. Essentially the aim is to impede the release of amino acids from muscle and stimulate its inclusion into protein (Dimitriadis *et al*., 2011).

2.3.2 Adipose tissue

In the postprandial state, intracellular transportation of glucose into adipocytes is insulin dependent via GLUT 4. An estimated 10% of insulin mediated glucose uptake is accounted for by adipose tissue. Insulin stimulates lipogenesis while inhibiting lipolysis and therefore there is a flux of free fatty acid into the blood stream. Adipose tissue plays an important role in maintaining the level and flux of fatty acids in the postprandial state. Diabetic and insulin resistant patients have adipocytes with decreased GLUT 4 translocation and altered intracellular signalling (Wilcox, 2005).

2.3.3 Liver

In the liver glucose uptake is independent of insulin and contributes to about 30 % of the body's insulin mediated glucose disposal (Wilcox, 2005). In the liver metabolic processes like inhibition of glucose 6-phosphate, which promotes glycogen accumulation and stimulates glycogen synthesis; amino acid uptake and protein synthesis; lipid metabolism regulation; the production of ketone bodies and the suppression of gluconeogenesis, are facilitated by insulin. (Wilcox, 2005; Rains and Jain, 2011).

2.4 Insulin Resistance

A major contributing factor in the pathogenesis of type 2 DM is resistance to the biological functions of insulin (Roberts *et al*., 2013). Insulin resistance is a condition in which the hormone fails to carry out its functions such as increasing glycogen synthesis and decreasing the production of glucose molecules (gluconeogenesis). The pancreas compensates in the early stages of insulin resistance by elevating the

release of insulin into the bloodstream. This occurs as an attempt to recover from defects in peripheral insulin action. The pancreatic β cells hypertrophy in response to the insulin demand (Roberts *et al*., 2013).

In addition to insulin resistance, progressive pancreatic β cell dysfunction significantly contributes to the pathogenesis of type 2 DM. The determination of insulin resistance and pancreatic β cell function is facilitated by the homeostasis model assessment (HOMA). This tool is formulated from a mathematical assessment, which requires the fasting levels of glucose and insulin to assess the balance between hepatic glucose output and insulin secretion (Wallace *et al*., 2004). It has reported that the measure of insulin resistance using the HOMA of insulin resistance (HOMA-IR) index is simple and inexpensive. The HOMA of pancreatic β cell function (HOMA- B) index has been regarded as a good quality measure of pancreatic β cell function (Wallace *et al*., 2004; Song *et al*., 2007).

In vivo studies using fructose-fed rats indicated that the ability of insulin to limit hepatic glucose is impaired. The glucose outflow in fructose-fed rats was also enhanced in comparison to the control rats. This is due to the suppression by insulin in fructose-fed rats being lower than the insulin suppression in the control rats (Tobey *et al*., 1982; Tran *et al*., 2009). Impairments in the body's insulin sensitivity are also similar in fructose-fed rats. An assessment of reductions in the insulin sensitivity index by an oral glucose tolerance test showed that fructose-fed rats were insulin resistant (Song *et al*., 2007). An additional characteristic of insulin sensitivity is an increased constant state of plasma glucose levels, which has been observed in fructose-fed rats (Chen *et al*., 1996; Tran *et al*., 2009). Evidently, the pathogenesis of hypertension, obesity and type 2 DM has been linked to insulin resistance and an overactive renin angiotensin system (Zhou *et al*., 2016).

2.5 Renin Angiotensin System – classical and alternative RAS

The renin-angiotensin system (RAS) is one of the oldest studied hormone system and a complex regulatory system with many distinct actions. Nevertheless, it may principally be viewed as a powerful regulatory system for the conversion of salt and blood volume, and the preservation of an adequate blood pressure (BP). This system slowly over the years was elucidated. In response to specific stimuli a proteolytic enzyme synthesised by the kidney is released into the circulation and acts on the angiotensinogen (AGT), a circulating protein (α_2-globulin) produced by the liver.

Reduction in blood volume and renal perfusion stimulates the secretion of renin by the kidney. An inactive decapeptide angiotensin I (Ang I) is formed by renin cleaving to angiotensinogen. Angiotensin-converting enzyme (ACE) and non-ACE pathways convert Ang I to active octapeptide angiotensin II (Ang II). This octapeptide is a strong vasoconstrictor and results in the release of catechcholamines by the adrenal medulla and prejunctional nerve endings. Ang II results in an increase in blood pressure by stimulating the aldosterone secretion and sodium reabsorption.

Ang II provides a negative feedback to the system by inhibiting the release of renin. This cycle is regarded as the classical RAS (Hsueh and Wyne, 2011).

The long-standing classical theory implicates that the RAS is seen as an endocrine system with the circulating active metabolite, Ang II (Ribeiro-Oliveira Jr *et al*., 2008). However, recent emphasis has been placed on the tissue-specific paracrine and/or autocrine functions of Ang II, classed as the alternate RAS (Kobori *et al*., 2007; Zhuo and Li, 2011). It has been suggested by Ribeiro-Oliveira Jr *et al*., (2008) that the vital components of the RAS are present in all organs and tissues of the body. In addition, these researchers state that the tissue/ local RAS are regulated with the circulatory RAS, even though they can work together (Ribeiro-Oliveira Jr *et al*., 2008; Carey, 2013). Several non-ACE pathways have been identified and involved in the generation of AngII in tissue.

Angiotensinogen can directly generate local Ang II via the activity of the enzymes cathepsin G and chymostatin-sensitive-Ang II- generating (CAGE)-enzyme. In addition, human chymase efficiently generates AngII. Chymase has been reported to be the major catalyst for tissue Ang II formation in the human heart (>80%) and in the arteries (>60%) (Probstfield and O'Brian, 2010; Carey, 2013). The ACE2 enzyme has been linked to the alternate RAS pathway and is found in the heart, kidney, testes and the gastrointestinal tract. A homologue of ACE is ACE 2 which acts as a catalyst in the conversion of Ang I to inactive angiotensin 1-9 [Ang (1-9)] and Ang II to angiotensin 1-7 [Ang (1-7)] (Macia-Heras *et al*., 2012). Through the action of endopeptidases, Ang I can also be converted to Ang (1-7) (Probstfield and O'Brien, 2010). The conversion of Ang (1-9) to the vasodilator and anti-proliferative Ang 1-7 is catalysed by the 'classic' ACE (Macia-Heras *et al*., 2012).

It has been proposed that over activation of the RAS with subsequent increases in Ang II is a contributing factor to the progression of hypertension following insulin resistance. Ang II may mediate the development of insulin resistance through its vaso-constrictor actions, thus decreasing blood flow and glucose uptake into insulin-sensitive tissues. This hypothesis is supported by the findings, that Ang II infusion induced endothelial dysfunction, hepatic insulin resistance and elevations in blood pressure (Tran *et al*., 2009).

Studies have suggested that hyperglycaemia and insulin both stimulate the RAS by elevating the expression of angiotensinogen, Ang II and AT1 receptor. On several levels, there is cross talk between the RAS and insulin signalling. The action of insulin may be inhibited by the activation of RAS via the PI3 signalling pathway (Thaman and Arora, 2013).

A decrease in the incidence of Type 2 diabetes has been reported in hypertension trials using RAS blockade. This sparked interest in the possibility of the RAS blockade being a preventative measure against diabetes. The study confirmed the demonstration of decrease in Type 2 diabetes in non-diabetic individual administered an ACE inhibitor or ARB (Scheen, 2004a; Scheen, 2004b). The over-activity

of RAS has been shown to be a contributing factor towards altered glycaemia (Luther and Brown., 2011).Similarly, to increase glucose levels, Ang II induces the production of reactive oxygen species (ROS) as well as impedes antioxidant mechanisms, leading to the development of oxidative stress (Motawi *et al*., 2013).

2.6 Oxidative Stress

As a result of normal cellular metabolism, living organisms produce chemically reactive molecules known as reactive oxygen species (ROS). These molecules play a role in physiological cell processes, in low to moderate concentrations. However, at high concentrations, ROS induces adverse alterations to cell components like lipids, proteins and DNA (Valko *et al*., 2006; Stadtman, 2004). Oxidative stress is defined as a shift in the oxidant /antioxidant balance in favour of oxidants. In numerous pathological conditions, oxidative stress is a contributing factor. These conditions include cancer, neurological disorders (Jenner, 2003), atherosclerosis, hypertension, ischemia/perfusion (Kasparava *et al*., 2005), diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Fitzpatrick *et al*., 2009).

Oxidative stress is essentially an imbalance between the generation of reactive oxygen species and their eradication by protective mechanisms. Free radicals are commonly known as Reactive Oxygen Species (ROS) and are formed when oxygen interacts with certain molecules and are therefore also known as products of oxygen metabolism. They can be defined as reactive chemical species having a single unpaired electron in an outer orbit (Riley, 1994). A balance between the production and elimination of free radicals is maintained by the body's endogenous antioxidant systems and the ingestion of exogenous antioxidants (Rahman, 2007). A substance that can delay the oxidation process of a substrate is regarded as an antioxidant. A variety of species or molecules, both endogenous and exogenous, contributes significantly to antioxidant defence mechanisms and is used as biomarkers of oxidative stress (Ullah *et al*., 2015).

2.7 Oxidative stress and diabetes mellitus

Chronic hyperglycaemia induces a number of biochemical abnormalities (Moniruzzaman *et al*., 2012). Complications induced by diabetes are mainly mediated through increased production of ROS or altered antioxidant defence systems, commonly known as oxidative stress (Patel *et al*., 2012). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, uncoupled nitric oxide synthase (NOS) , xanthine oxidase, cyclooxygenase (COX) as well as mitochondrial electron transport chain, are the main sources of ROS as illustrated in the figure 2.3 (Paravicini and Touyz, 2008; Fostermann, 2010).

Studies have shown that free radical induced damage facilitates the development of insulin resistance, pancreatic β islet cell dysfunction, impaired glucose tolerance and type 2 DM (Wright *et al*., 2006). Hyperglycaemia, through various mechanisms such as glucose autoxidation, formation of advanced

glycation end products (AGEs) and activation of the polyol pathway, induces oxidative stress (Jay *et al*., 2006). It has been noted that there is a significant increase in protein glycation (AGE) in diabetics. In addition, the incidence of microvascular lesions increases due the accumulation of AGEs. Microvascular lesions are found in patients with diabetic retinopathy and also contributes to cardiovascular problems (Jay *et al*., 2006). The main cause of irriversible blindness is primary open angle glaucoma (POGA) which is a result of damage induced by ROS (Izzotti *et al*., 2006).

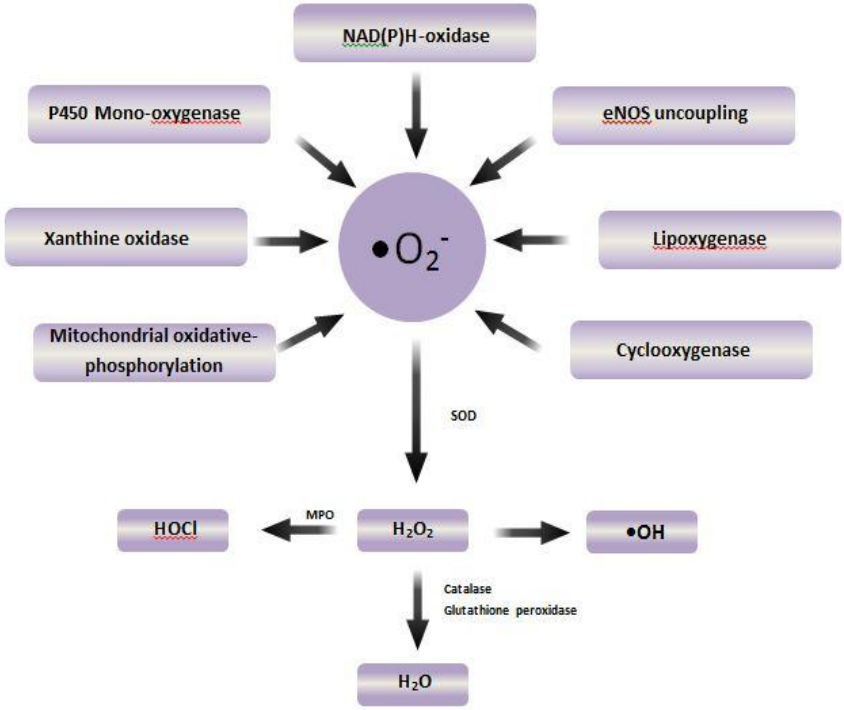

Figure 2.3 An overview of enzymatic systems producing ROS (Sachse and Wolf, 2007)

A link between oxidative stress and diabetes mellitus which includes the measurement of biomarkers like DNA damage markers and lipid peroxidation products has been highlighted by various studies. The onset and development of late diabetic complications is influenced by free radicals that play a pivotal role in damaging lipids, proteins and DNA (Ayepola, 2014; Ullah *et al*., 2015).

2.8 Lipid peroxidation

The cells of the body become more susceptible to lipid peroxidation due to disturbances in the lipid profile caused by diabetes mellitus (Ullah *et al*., 2015). Polyunsaturated fatty acids in the cell membrane are the primary sites of lipid peroxidation (Adly, 2010). The toxic effects of hydroperoxides on cells are mediated both directly and through degradation to highly toxic hydroxyl radicals. Stable aldehydes may form when hydroperoxides react with transition metals such as iron or copper. An example of a stable aldehyde is Malondialdehyde (MDA) (Tiwari *et al*., 2013), which is an important biomarker of

free radical mediated lipid damage and oxidative stress (Tiwari *et al*., 2013). Following a reaction between MDA and thiobarbituric acid (TBARS ASSAY), the levels of lipid peroxides can be determined (Adly, 2010).

2.9 Oxidative DNA damage

DNA is regarded as a key target for ROS. Structural changes in DNA may be induced by free radicals leading to base pair mutations, deletions, insertions and sequence amplifications (Adly, 2010). It has been reported that mitochondrial DNA is more prone to oxidative induced damage and may be involved in the development of various pathologies (Lobo *et al*., 2010).

2.10 Oxidative protein damage

Proteins are essential biomolecules of the cell that play a role in numerous physiological functions like cell signalling and transport across the cells. Another latent target of ROS is proteins, where their structure and function can be altered by oxidative modification (Adly, 2010; Tiwari *et al*., 2013). Some amino acids react with ROS, producing modified, denatured or non-functioning proteins, which can further contribute to oxidative stress. Diabetic glycaemia causes protein glycation and oxidative degeneration through the process of free radical production. Glycated haemoglobin and fructosamine levels are biomarkers that can be used to estimate the degree of protein glycation (Ullah *et al*., 2015).

Hyperglycaemia contributes significantly to the development of oxidative stress by increasing the formation of ROS as well as decreasing the action of the intrinsic antioxidant system (Fatehi-Hassanabad *et al*., 2010; Schaffer *et al*., 2012). Currently oxidative stress is the combining factor in the progression of diabetes complications. Alterations in oxidative stress, due to of either glucose-mediated elevated free-radical generation and/or decrease in endogenous antioxidants, are possible factors of the title 'root cause' of diabetes related complications (Bagul *et al*., 2012).

The role of NADPH oxidase and mitochondrial-derived O_2^- together with the NOS-derived NO and $ONOO^-$ has been the focus of studies on diabetes and its complications (Lazo-de-la-Vega-Monroy and Fernandez- Meija, 2013). O_2^- acts in tandem with NO to form the toxic $ONOO^-$ leading to the generation of several harmful effects including: i) stimulation of lipid peroxidation and protein nitration, ii) degradation of single stranded DNA; iii) impairment of anti-proliferative and vaso-relaxation effects of NO as a result of a decrease in NO bioavailability, and iv) oxidation of the co-factor of NOS, BH_4, resulting in the production of O_2^- and the uncoupling of NOS (Adly, 2010). Hyperglycaemia impedes endothelial nitric oxide synthase (e NOS) in endothelial and vascular smooth muscle cells, resulting in decreased nitric oxide production and elevated ROS production (Mohamadin *et al*., 2007).

It has been proven that the elevation of extra and intra-cellular glucose concentrations result in oxidative stress (West, 2000). Increased blood glucose levels up regulates oxidative stress through several mechanisms as mentioned above. Free fatty acids and leptin are other circulating factors that are

augmented in diabetes, contributing to the increase in ROS (Jay *et al*., 2006). An increased production and accumulation of AGEs also contributes to the development of diabetic complications such as retinopathy, neuropathy and renal dysfunction through pathological changes (Patel *et al*., 2012).

2.11 Diabetic complications

Diabetic patients become susceptible to complications associated with the disease, which contributes significantly to the morbidity and mortality rate (Patel *et al*., 2012). These complications can be regarded as macrovascular (coronary heart disease, peripheral vascular disease and stroke), microvascular (neuropathy, retinopathy and nephropathy) or both micro and macrovascular (diabetic foot). Furthermore, these complications categorized into two groups: metabolic acute complications (short term) and systemic late complications (long term) (Patel *et al*., 2012; Ullah *et al*., 2015).

2.12 Dyslipidemia in diabetes

Evidence has suggested that insulin resistance plays an important role in the pathogenesis of diabetic dyslipidemia (Mooradian, 2009). Three lipoprotein abnormalities are characteristic of diabetes-related dyslipidemia, namely increased plasma triglyceride levels, decreased high density lipoproteins cholesterol (HDL-C) and elevated small dense LDL particles (Del Pilar Solano and Goldberg, 2005). Triglycerides form as a result of circulating free fatty acids (FFA) in the incidence of insulin resistance and hyperinsulinemia (Thaman and Arora, 2013).

Generally, an increase in free fatty acid (FFA) flux to the liver leads to increased production of very low density lipoproteins (VLDL). Physiologically the secretion of VLDL into the systemic circulation is inhibited by insulin. In the case of insulin resistance, hepatic triglyceride synthesis is increased because of increased FFA flux to the liver. Dyslipidemia is characterised by increased triglycerides and decreased levels of high density lipoproteins (HDL) cholesterol (Thaman and Arora, 2013).

During a state of hyperglyceridaemia there is a decrease in the content of cholesterol in HDL due to decreases in the cholesteryl ester content of lipoprotein core, with inconsistent increases in triglyceride. In a similar way, the composition of LDL is also modified. LDL composition change is attributable to diminution of unesterified and esterified cholesterol and phospholipids, with either no increase or change in LDL triglyceride. In most cases, this amendment in LDL composition is related to the associated changes in other lipoproteins and other risk factors (Mooradian, 2009; Del Pilar Solano and Goldberg, 2005).

2.13 Diabetic Nephropathy

One of the most common microvascular complications in DM is diabetic nephropathy (DN). At the outset, DN manifests with microalbuminuria and progressively leads to end stage renal failure (Stanton and King, 2011; Zheng *et al.*, 2016). Alterations in specific renal functions and morphology lead to diabetic nephropathy (Surya *et al*., 2014). DN is a renal disease that is progressive, irreversible and

characterised by the build-up of extra cellular matrix in glomerular masangium and kidney interstitial tissues (Tavafi, 2013). An index of renal dysfunction is an increase in serum creatinine and urea, which is associated with DN (Kadian *et al*., 2013). Other early manifestations of functional disorders in nephropathy include glomerular hyper filtration, renal hypertrophy, tubulointerstitial and mesangial growth, and glomerular endothelial dysfunction (Balakumar *et al*., 2009).

The result of chronic hyperglycaemia is the development and accumulation of advanced glycated end products (AGEs) in the kidney of diabetics. One of the key contributing factors to the development and progression of diabetic nephropathy is the glycation of proteins (Alhaider *et al*., 2011).

It has been implicated that a principle player in the pathogenesis of DN is the activation of the intrarenal renin-angiotensin system. A critical regulator of the i RAS and a major contributor to renal damage is angiotensin-converting enzyme (ACE) (Motawi *et al*., 2013). The upregulation in intrarenal ACE expression has shown in previous studies. Numerous diabetes-associated stimuli could possibly lead to the upregulation of intrarenal ACE and may contribute towards the ferocious circle of renal damage (Konoshita *et al*., 2006; Motawi *et al*., 2013). Studies based on diabetes mellitus require all complications to be taken into consideration in an experimental model in order to mimic the true characteristics of the disease.

2.14 Experimental models of diabetes mellitus

Diabetes research has placed significant focus on the use of animal models, more specifically, the rodent model. Small body size, omnivorous nature, serene behaviour and decreased dietary and housing costs, makes rodents the ideal choice for experimental studies. Broadly, the two major categories of rodent models are genetic or spontaneously induced models, and non-genetic, non-spontaneously or experimentally induced models (Islam and Loots, 2009). Experimentally diabetes-induced rodent models involve the use of animals that are not naturally or genetically diabetic (Islam and Loots, 2009). Not only are these models widely available globally but also cost effective in comparison to genetically modified models of diabetes. The development of induced models requires the use of chemicals or dietary changes or the combination of both. The popularity of these models in recent years can be attributed to the resemblance achieved of the pathogenesis of diabetes in humans (Islam and Wilson, 2012).

The development of the full pathogenesis of Type 2 diabetes is not achievable. There are models, however, which induce one or both of the two key pathogenic features of Type 2 diabetes. These features are insulin resistance and pancreatic beta cell dysfunction which are induced by a variety of methods, namely incomplete pancreatectomy, neonatal streptozotocin (STZ) or alloxan injection, a once off dose of STZ or alloxan, a high fat (HF) diet or fructose feeding over long period of time, HF diet and STZ injection combined, intrauterine growth reduction (IUGR), monosodium glutamate (MSG) injection and fructose feeding and STZ injection combined (Islam and Wilson, 2012).

2.14.1 Fructose-fed diabetic model

The widely used model of insulin resistance is the high fructose diet fed to Sprague Dawley (SD) rats (Hwang *et al*., 1987). The first researchers to demonstrate fructose-fed induced symptoms of hypertriglyceridemia, hyperinsulinemia and insulin resistance in rats were Zavaroni *et a*l (1980) and Tobey *et al* (1982). The development of these symptoms require the intake of diets consisting of 35% and 72% fructose or 10-15% fructose solutions in drinking water *ad libitum* for 2-12 weeks (Islam and Wilson, 2012). Insulin resistance and partial pancreatic beta cell dysfunction are two major pathogeneses that should be replicated in order to achieve an ideal model of Type 2 diabetes. It is not feasible to induce insulin resistance or Type 2 diabetes using only fructose feeding, as this would require an extended experimental period. Therefore, Wilson and Islam (2012) have hypothesized and reported that a shorter period of fructose feeding combined with a low dose of Streptozotocin (STZ) injection, would mimic the major pathogenic pathways of Type 2 diabetes in rat models.

2.14.2 Streptozotocin-induced diabetes

Experimental diabetes in rats is commonly induced using diabetogenic chemicals, which destroy the pancreatic β cells partially or completely, depending on the dose of the chemical. The most widely used chemicals are Alloxan and Streptozotocin (STZ), both of which are capable of adequately inducing diabetes in rat models. The method of β cell destruction by these compounds is identical. Their cytotoxicity, however, is achieved by means of different pathways (Lenzen, 2008). Alloxan and STZ enter the pancreatic β cells by way of the GLUT2 glucose transporter, which is the factor that influences the selectivity of cellular destruction. The mode of inducing cellular toxicity, nonetheless, is different. In the case of STZ, DNA alkylation is the main mode of action, while Alloxan is the production of reactive oxygen species (Lenzen, 2008). Alloxan is more suitable in instances where there is an interest in β cell toxicity; induced by reactive oxygen species (ROS) mediated mechanisms. Hence, due to its stable chemical properties, Streptozotocin is the toxin of choice in terms of inducing experimental diabetes (Lenzen, 2008).

2.15 Conventional Treatment of diabetes

Insulin therapy and oral hypoglycaemic agents are used as the first line of treatment in DM. Despite their vital role in managing or treating DM, they elicit side effects and are not completely successful in changing the course of diabetic complications (Patel *et al.,* 2012). The pathways targeted classify the various available drugs used to manage DM.

2.15.1 Sulphonylureas

Since 1942, sulphonylureas (Glibenclamide) have been used to treat Type 2 diabetes and requires pancreatic cells to be functional for their hypoglycaemic effect (Lebovitz, 2004). These drugs are easily absorbed from the intestine (Patel *et al*., 2012) and promote the release of insulin by binding to beta cell K channels. This causes a blockage in the ATP dependent K^+ channel. Sulphonyl urea becomes bound

to a sulphonyl urea receptor, resulting in a closure of potassium channels, depolarisation of membranes, opening of calcium channels and finally the release of insulin (Amod *et al*., 2012).Hypoglycaemia, weight gain, headache, hypersensitivity and abdominal irregularities are some of the side effects associated with sulphonylureas (Talreja and Kaur, 2014).

2.15.2 Biguinides

Another class of oral anti-diabetic drugs, used to treat all types of diabetes, is biguinides (Metformin) (Patel *et al*., 2012). These drugs induce peripheral use of glucose, enhanced sensitivity of muscle to insulin action, decrease in intestinal absorption and reduction in hepatic glucose production (Surya *et al*., 2014). These drugs act on adenosine monophosphate (AMP) kinase leading to a decrease in the production of hepatic glucose through multiple intracellular pathways. In the 1950's, Metformin and other biguinides were developed. The only biguinide currently prescribed, however, is Metformin (Amod *et al*., 2012). This hypoglycaemic agent has an insulin sensitizing effect, reduces insulin levels and controls hyperglycaemia (Alhaider *et al*., 2011). Gastrointestinal disturbances like diarrhoea, cramping, bloating and flatulence are the most common side effects of Metformin (Amod *et al*., 2012).It is an insulin sparing drug and does not induce weight gain or incite hyperglycaemia (Surya *et al*., 2014).

2.15.3 Thiazolidinediones (TZDs)

Thiazolidinediones (TZDs) include rosiglitazone and pioglitazone, which act by binding to perisome proliferator activator receptor-gamma. Their action results in the increase in peripheral glucose disposal, decreased hepatic glucose output and elevated peripheral sensitivity to insulin .TZDs elicit side effects like peripheral edema and are not administered to those with potential heart and liver failure risks (Surya *et al*., 2014).

2.15.4 Alpha-glucosidase inhibitors

Acarbase and miglitol are examples of alpha- glucosidase inhibitors, which induce carbohydrate and alpha- amylase inhibition. Nevertheless, these drugs are less frequently used due to negative gastrointestinal symptoms, and they are not recommended to patients experiencing inflammatory bowel disease and renal disorders (Surya *et al*., 2014).

2.15.5 Incretin-based therapies

Glucose dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1) and exenatide are incretin-based therapies that act by enhancing glucose dependent insulin secretion. The major side effects reported include nausea, vomiting and hypoglycaemia (Surya *et al*., 2014).

2.15.6 Dipeptidyl peptidase 4 (DPP-4) inhibitors

DPP-4 inhibitors are oral drugs used to treat diabetes by inhibiting the enzyme DPP-4. Sitagliptin and saxagliptin are DPP-4 inhibitors that belong to a class of anti-hyperglycaemic drugs used in the improvement of glycaemic control in patients with type 2 diabetes (Sujatha and Vijaya, 2015). Varieties

of bioactive peptides become deactivated by DPP-4, including glucose–dependant insulinotropic polypeptide (GIP) and GLP-1. Adverse effects most commonly experienced by 5% or more of patients who have received DPP-4 inhibitors include upper respiratory tract infection, nasopharyngitis and headache with Sitagliptin and upper respiratory tract infection, urinary tract infection and headache with saxagliptin (Sujatha and Vijaya, 2015).

2.15.7 Sodium-glucose co-transporter 2 (SGLT2) inhibitors

A sodium-glucose co-transporter 2 (SGLT 2) inhibitor is a new type of diabetic medication used only for the treatment of Type 2 diabetes. SGLT2 inhibitors can improve glycaemic control when combined with exercise and a healthy diet. The re-absorption of glucose, back into the plasma, is facilitated by SGLT's. The inhibition of this process stimulates glucosuria, which subsequently decreases blood glucose levels (Chao, 2014).

2.16 Alternate Remedies

Over the years, the general populace to prevent, treat and manage various ailments has used herbal remedies extensively. Plants remain as one of the most commonly exploited natural resources in the world, from ancient times. Information and knowledge of the medicinal properties of plants have been passed on from generation to generation amongst tribal folks. The medicinal uses of plants are rising in popularity, in both developed and developing countries (Yirga *et al*., 2011). For a plant to be considered medicinal, it must have compounds that can be utilized in drug development as precursors for drug development or for therapeutic reasons (Sofowora *et al*., 2013). A major advantage of medicinal plants is their availability and minimal perceived side effects (Arumugam *et al*., 2013).

More than 1200 plants to date have been shown to possess antidiabetic abilities (Baharvand-Ahmadi *et al*., 2015). Plant sources boasting about 200 pure compounds have been reported to elicit blood glucose lowering effects. These compounds include alkaloids, carbohydrates, glycosides, flavonoids, steroids, terpenoids, peptides and amino acids, lipids, phenolics, glycopeptides as well asiridoids (Singh, 2011).

The World Health Organisation Expert Committee on DM encouraged further investigation into the effectiveness and therapeutic potential of medicinal plants in 1980. Despite the availability and efficacy of conventional treatment for DM, the demand for herbal remedies, with reduced adverse side effects, is growing (Patel *et al*., 2012). Another factor, which favours the use of herbal remedies, may be that concoctions containing multiple compounds could have a wide array of effects targeting multiple pathways. Since many of the lifestyle disorders are multi-factorial, such an approach would improve the efficacy of these compounds and be favoured by the public.

2.16.1 Tulbaghia violacea Harv.

2.16.1.1 Distribution

Recent years have seen a surge of interest in the purported medicinal properties of *Tulbaghia violacea* Harv. (TVL). This plant belongs to the *Alliaceae* family, which is known to have 600 species in 30 genera (Benham, 1993). This genus is found growing in Asia, Europe, North and South America and Southern Africa, and is mostly confined to the Southern Hemisphere. *Tulbaghia violacea* is an herbaceous plant that is indigenous to Southern African countries like Tanzania, Lesotho, Malawi, Botswana and South Africa (Vosa, 2007; Lyantagaye, 2011). In South Africa, this plant is distributed in the Eastern Cape, Southern KwaZulu-Natal and the Transvaal where it is found growing in rocky grasslands (Kubec *et al*., 2002). *Tulbaghia violacea* has a 'garlic-like odour' released by the cysteine-derived sulphur compound when the flesh is bruised or decomposed. Hence, it is commonly known as "society garlic", "sweet garlic", or "wild garlic" (Hutchings *et al*., 1996; Van Wyk *et al*., 1997; Kubec *et al*., 2013).

2.16.1.2 Phytochemistry

The *Tulbaghia* species are believed to have similar biological activities and secondary metabolites as garlic (Van Wyk *et al*., 1997; Bungu *et al*., 2008). The secondary metabolites present in this family (*Alliaceae*) are steroidal saponins (Hutchings *et al*., 1996; Aremu and Van Staden., 2013). *Tulbaghia violacea* does indeed have steroidal saponins, as well as quercetin, kaempherol, sugars and sulphur containing compounds like marasmin (Van Huyssteen *et al*., 2011). The precursor of the typical odor in TVL is a cysteine-derived amino acid, S- (methylthiomethyl) cysteine -4-oxide (Marasmin) that is primarily found in the cytoplasm (Lyantagaye, 2011). Marasmin reacts with an enzyme (C-S lyase) to form thiosulfinatemarasmicin (2, 4, 5, 7-tetrathiaoctan-4-oxide, 2), when the plant is damaged. Studies have reported that marasmicin is subject to chemical breakdown forming various sulphur-containing degradation products. Anticancer, antifungal and antimicrobial properties of some of these compounds have been reported (Jager and Stratford, 2012).

2.16.1.3 Medicinal uses

Tulbaghia violacea has been used in African folkloric medicine to manage, control and treat numerous human disorders, including fever and colds, tuberculosis, asthma, gastro-intestinal disorders, hypertension, oesophageal cancer, inflammation, bacterial and other infective diseases. It is used to treat colic and restlessness in babies and the leaves can treat sinus headaches when rubbed on the head (Hutchings *et al.,* 1996; Kubec *et al*., 2002). This plant has a history of medicinal use and has been extensively studied. It has proven to have a variety of biological activity against many diseases and ailments, making it a plant of interest.

In previous studies, TVL exhibited antihypertensive effects in experimental rats over a long period (Moodley *et al*., 2013), as well as resulting in hypoglycaemic, antioxidant and hypolipidemic effects

in diabetic rats (Moodley *et al*., 2015). Studies have also indicated that this plant may be involved in various biochemical pathways. It may be effective in decreasing plasma lipid levels (TG) and it could be useful in preventing oxidative stress (Olorunnisola *et al*., 2012). It has been suggested that TVL decreased blood pressure and heart rate by inhibiting angiotensin converting enzyme (ACE) and β 1 andrenoceptors (Raji *et al*., 2012). A hypertension study by Raji *et al* in 2013, reported that the antihypertensive effects of TVL in male rats could possibly be involved in the decrease in plasma aldosterone levels. Olorunnisola *et al* (2012) proved that TVL has properties that affect the antioxidant enzyme levels and the lipid profile in normal rats. The results showed a decrease in malondialdehyde (MDA) in the treated groups. MDA is a product of lipid peroxidation, which is measured as TBARS. This indicates that TVL possesses properties or compounds that combat oxidative stress by increasing the antioxidant levels in the cells. Tannins, flavonoids, vitamin C and E are natural antioxidants found in plants that protect beta cell function and avoid diabetes induced ROS production (Patel *et al*., 2012). Although there are many reports on the chemical, physiological and pharmacological qualities of the various plant species belonging to the Alliaceae family, specifically *Allium sativum Linn* (garlic) and *Allium cepa Linn* (onion), there are only a few reports on the *Tulbaghia violacea* species (Benham, 1993; Bungu *et al*., 2008; Aremu and Van Staden., 2013).

With the growing interest in TVL, researchers have studied its properties, which have proven the efficacy of the plant in respect of possessing properties of being antimicrobial (43%), assisting in the alleviation of cardiovascular-related disease (19%) and antioxidant (15%) (Aremu and Van Staden, 2013).

2.17 Project aim

The aim of this study is to investigate the *in vivo* renal and metabolic effects of methanolic extracts of *Tulbaghia violacea Harv.* and captopril in a fructose-fed streptozotocin induced diabetic rat model.

Study objectives

To determine the effect of TVL and conventional drug treatments on:

i. Plasma insulin levels
ii. Liver glycogen
iii. Liver SOD
iv. Kidney and liver lipid peroxidation
v. Plasma nitric oxide levels
vi. Serum lipid levels
vii. Histology of pancreatic and renal tissue
viii. Gene expression of AT1 and NADPH in kidney tissue

CHAPTER 3

3. Materials and Methods

3.1 Plant identification and harvesting

The medicinal plant *Tulbaghia violacea* Harv. Botanist, Professor H Baijnath (University of KwaZulu-Natal), identified (TVL). Thereafter, a voucher specimen was deposited in the Ward herbarium at the University of KwaZulu-Natal, Durban, South Africa.

3.2 Preparation of plant extract

Tulbaghia violacea rhizomes were freshly harvested from Durban, KwaZulu-Natal thoroughly washed and air-dried for 48hours at room temperature. Once dried, the rhizomes were weighed, crushed using Waring Blender before soaking in methanol (Merck Chemicals (Germany) and placing on a shaker for 48hours. The crude extract mixture was then filtered using Whatman filter paper. The filtrate was concentrated in a rotary evaporator (Heldolph, Schwabach, Germany) after which the extract was. Freeze-dried and stored in a desiccator at 4°C for further analysis. The yield of the methanolic extract was calculated as 2.36%.

3.3 Animal Studies

3.3.1 Ethical clearance and experimental design

Ethical clearance was obtained from the Experimental Animal Research Ethics Committee based at the University of KwaZulu-Natal, South Africa (Ethical approval number: 044/15/Animal).Six-week-old male Sprague-Dawley (SD) rats were obtained from the University of KwaZulu-Natal, South Africa (Westville campus), with an initial mean body weight (bw)185±1.56g. All animals were housed in polycarbonate cages (*n*=2-3/cage) and were maintained under standard laboratory conditions (24 ± 1ºC, relative humidity 40-60%) on a 12-hour light/dark cycle, fed a standard rat pellet diet and given access to water *ad-libitum*.

The animals were randomly divided into six groups namely: NDC: Non diabetic control (*n*=5), DC: Diabetic control (*n*=5), TVL: Diabetic+TVL extract (60mg/kg bw, *n*=7), CAP: Diabetic+Captopril (50mg/kg bw, *n*=7), MET: Diabetic+Metformin (250mg/kg bw, *n*=6),GLIB: Diabetic+Glibenclamide (10mg/kg bw, *n*=6). The animals were allowed to acclimatize for one week before starting the experiment.

The protocol to induce DM using fructose and Streptozotocin to was adapted from Wilson and Islam, (2012). The NDC group was given drinking water whilst the remaining groups received 10% fructose solution. After two weeks, a low dose of STZ (40mg/kg bw) dissolved in citrate buffer (pH: 4.5) was intraperitoneally (i.p) injected into the fructose fed animals for partial pancreatic β-cell dysfunction. The NDC group received citrate buffer (pH 4.5) via intraperitoneal (i.p.) injection.

After a period of one week following the STZ injection, the fasting blood glucose (FBG) of all groups was determined using a portable glucometer (One Touch Select, Life scan, Inc., CA. USA.). Animals with a fasting blood glucose concentration of>25mmol/L were classified as diabetic and included in the study. The treated diabetic rats then received a respective dose of the extract/drugs dissolved in distilled water and orally administered once in every morning for 7 weeks (Moodley *et al*., 2015) using a gastric gavage. The animals in NDC and DC groups were treated with similar volume of the vehicle only. Throughout the experimental period, food and water intake were measured daily whilst body weights and FBG levels were measured weekly for the duration of the treatment period.

3.3.3 Oral glucose tolerance test (OGTT)

In week six of the experimental period, an oral glucose tolerance test was performed. Following an overnight fast the animals were administered an oral dose of glucose solution (1.2 g/kg bw). Blood glucose concentrations were determined as follows: 0 (prior to glucose dosage), 15, 30, 45, 60, 90, 120 and 180 minutes following the glucose load.

3.3.4 Collection of blood and organs

At the end of the 7th week, the animals were euthanized by halothane overdose using Isofor^{TM} for blood collection which was later centrifuged for serum and plasma. Heart, left liver lobe and left kidney were harvested, weighed and snap frozen in liquid nitrogen for storage at -80°C. The pancreas, right kidney and right liver lobe were fixed in buffered neutral formalin for histological preparations.

3.4 Determination of plasma insulin levels

Plasma insulin levels were determined by an Ultrasensitive Rat Insulin (10-1251-01) quantitative sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Mercodia) according to the manufacturer's instructions.

3.5 Histopathology of the pancreas

Segments of the pancreas were removed and preserved in 4% buffered neutral formalin for 24 hours or more. The tissues were dehydrated using alcohol and cleared in xylene. Thereafter the tissues were embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin (H&E). Histology slides were examined using a Leica SCN400 scanner for evaluation of morphology and morphometric measurements.

3.5.1 Quantitative analysis of islets

According to a modified method of Masjedi *et al*., (2013), the quantitative analysis of islets was performed as follows: The average number of islets for each group was calculated by counting the number of islets in 10 microscopic fields of 10 x 100 µm. The Leica SCN software was used to assess the pancreatic islet size by measuring the area (µm^2) of each counted islet and calculation of the mean area per group.

3.6 Homeostatic model of assessment for insulin resistance (HOMA-IR) and β cell function (HOMA-β)

At the end of the experimental period, the HOMA-IR and HOMA-β scores were calculated using plasma insulin and fasting blood glucose concentrations according to the following formula:

HOMA-IR = [Insulin (U/l) x Blood glucose (m mol/l)]/ 22.5

HOMA-β = [20 x Insulin (U/l)] / [Blood glucose (m mol/l) – 3.5]

Conversion factor: Insulin (1U/l = 7.174 p mol/l) and blood glucose (1 mol/l = 18 mg/dl)

3.7 Determination of liver glycogen content

Hepatic and muscle glycogen concentrations were determined as described by Ong and Khoo (2000). Briefly, the tissue samples of 1-1.5 g were homogenized in 2 ml of 30% potassium hydroxide (300g/l) and heated at 100^{o}C for 30 min, and then cooled on ice. The glycogen was precipitated with ethanol, pelleted and re-dissolved in distilled water. Absorbance was read at 620 nm using a Novaspec II spectrophotometer and the glycogen concentration of the samples was determined from a standard curve of (10-2000 mg glycogen/l).

3.8 Determination of serum lipids

Serum samples were sent for analysis to Global Clinical and Viral Laboratory (Amanzimtoti, South Africa).

3.9 Determination of lipid peroxidation in liver and kidney

Lipid peroxidation was assessed using the modified method of the thiobarbituric acid reactive substances (TBARS) assay (Masjedi *et al*., 2013). Liver and kidney tissues (50 mg) were homogenized in 0.2% H_3PO_4 (450 µl) and centrifuged at 10000 rpm for 15 min at 4^{o}C. The supernatant was decanted into glass tubes, into which 500 µl of 2%H_3PO_4 was added and vortexed. This was followed by the addition of 200 µl of 7% H_3PO_4, TBA/BHT (400 µl) and1M HCl (200 µl). The tubes were heated at 100^{0}C for 15 min and then allowed to cool at room temperature. Butanol (1500 µl) was then added to each tube and vortexed. The top phase of each solution was transferred into a 96-well plate in triplicate and the absorbance was measured at 532 nm and 600 nm on a plate reader Spectrostarnano (BMG LABTECH, Ortenberg, Germany). Concentration was calculated using the following formula:

Concentration= [(Absorbance at 532 nm –Absorbance at 600 nm)/156] X1000

3.10 Determination of plasma SOD levels

The activity of superoxide dismutase (SOD) (K335-100) in liver samples was determined using Biovision (California, USA) assay kit according to the manufacturer's instructions.

3.11 Determination of plasma nitric oxide levels

Plasma nitric oxide (NO) production was determined through the measurement of NO_2 and NO_3 under acidic conditions. NO levels were measured as an indirect indicator of reactive nitrogen species (RNS) in plasma. To perform this assay, 25 µl of plasma was added in triplicate to the wells of a 96-well microtitre plate. Sodium nitrite (0 µM-200 µM) was used as a standard in this assay and 50µl of each concentration was added in triplicate to the wells. This was followed by the rapid addition of 50 µl vanadium chloride (VCl_3), 25 µl 2% sulphanilamide (SULF) and 50 µl 0.1% *N*-1-napthylethylenediamine dihydrochloride (NEDD) into each well. The plate was then incubated (37ºC) for 45 minutes under dark conditions and the optical density was read at 540/690 nm using a spectrophotometer (Spectrostarnano, BMG LABTECH, Ortenberg Germany). A standard curve was constructed using the results obtained from the sodium nitrite standard and the resultant NO concentration for each sample was determined by extrapolation.

3.12 Histopathology of the kidney

Right kidneys were excised and preserved in 4% buffered neutral formalin for 24 hours or more. The tissues were dehydrated using alcohol and cleared in xylene. Thereafter the tissues were embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin (H&E). Histology slides were examined using a Leica SCN400 scanner for evaluation of morphology and morphometric measurements.

3.12.1 Semi-quantitative analysis of glomeruli

According to the method adapted from Benigni *et al*., (1998), a semi-quantitative analysis of renopathology was performed. For the determination of glomerular injury score, 100 glomeruli were analysed for each slide and the results were shown as the percentage of glomeruli illustrating normal, segmental and global sclerosis.

3.13 Determination of serum ACE activity

Serum samples were sent for analysis to Global Clinical and Viral Laboratory (Amanzimtoti, South Africa).

3.14 Expression of renal AT1 and NADPH using real-time polymerase chain reaction (PCR)

RNA was isolated from kidney tissues using the Quick RNA MicroPrep Kit (Zymo Research, USA). The tissues were weighed and 40mg of tissue was homogenised in 400 µl RNA lysis buffer using a sonicator. An equal volume of 100 % ethanol was added to lysate. The lysate was transferred into a spin column and collection tube and centrifuged for 1 min at 12, 000 x g with the flow through being discarded. Then 400 µl of RNA pre-wash buffer was added to the column and centrifuged at 12, 000 x g for 1 min, again the flow through was discarded. 700 µl of RNA wash buffer was added to the column and centrifuged at 12, 000 x g for 30 sec, the flow through was discarded and followed by a second wash using 400 µl of the wash buffer. The flow through was discarded, and to ensure complete removal

of the wash buffer the column was centrifuged in the empty collection tube for 2 min at 12,000 x g. The spin column was then placed in an empty RNAse-Free tube, 25 µl of RNAse-Free water was added directly to the spin column matrix and left at room temperature for 1 min. The tube was then spun at 13, 500 x g for 30 sec to completely elute the RNA.

3.14.1 Determination of RNA purity and concentration

The RNA concentration was determined using the NanoDrop 1000 (Thermo Scientific, USA) spectrophotometer. The absorbance was measured at 260 nm and 280 nm, the ratio of the absorbance ($\frac{A\,260}{A\,280}$) was used to determine the purity of RNA. A value between 1.7 and 2.1 was accepted, while higher values showed signs of possible DNA contamination. The concentration was calculated by the Nanodrop software and expressed as ng/µl. The RNA was then stored at -80°C.

3.14.2 cDNA synthesis

Reverse transcriptase PCR (RT-PCR) was used to synthesise cDNA from the RNA templates made in 3.4.2. This was performed using the iScript Reverse Transcriptase cDNA synthesis kit (Bio-Rad, USA).

The reaction mix contained 1 µl of the reverse transcriptase (RT) enzyme, 4 µl of the dNTP containing reaction mix, RNA template (volume of RNA template was determined using the concentration of RNA) and made up to 20 µl using nuclease free water.

The reaction mixture was added to a thin-walled PCR tube and incubated in the GeneAmp 9700 Thermal Cycler (Applied Biosystems, USA). The reaction mixture was incubated for 5 minutes at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes, followed by a final hold at 4°C. The cDNA was then stored in a freezer at 4°C until used for Real-time PCR.

3.14.3 Real time PCR detection of AT1 and NADPH

The presence of genes AT1 and NADPH were measured using the Lightcycler 96 (Roche Diagnostics). The concentrations of the two genes were expressed relative to the concentration of GAPDH (housekeeping gene). The reaction mixture for each gene contained cDNA, SYBR green I master mix (Roche Diagnostics, Germany) forward primer (10 mM) and reverse primer (10 mM) as well as PCR grade water.

Table 3.1: Oligonucleotide sequences of PCR primers

Primer	Sequence	
AT1	**F**	5'-CAC CTA TGT AAG ATC GCT TC -3'
	R	5'-GCA CAA TCG CCA TAA TTA TCC-3'
NADPH	**F**	5'-GCA GGG ATT GGC GTC ACT -3'
	R	5'- ACG CTG GAA TTT GTA CCA GAT AGA-3'

AT1 primer sequence (Miguel-Carrasco et al., 2010); NADPH primer sequence (Savchenko, 2013)

Reaction mixtures underwent real-time PCR using Lightcycler 96 (Roche Diagnostics). Conditions for AT1 were as follows: Pre-incubation: One cycle consisting of (95°C for 10 minutes) followed by PCR: 45 cycles of 95°C for 15 seconds; 55°C for 5 seconds; 72°C for 16 seconds; 82°C for 1 second. Melting curve analysis: 95°C for 10 seconds, 65°C for 60 seconds with a single fluorescent measurement done at 97°C; with a ramp rate of 4.4°C per second and a continuous fluorescent measurement. This was followed by a final cooling step of 37°C for 30 seconds.

Conditions for NADPH were as follows: Pre-incubation: One cycle consisting of (95°C for 10 minutes) followed by PCR: 45 cycles of 95°C for 10 seconds; 65°C for 10 seconds; 72°C for 10 seconds. Melting curve analysis: 95°C for 10 seconds, 65°C for 60 seconds with a single fluorescent measurement done at 97°C; with a ramp rate of 4.4°C per second and a continuous fluorescent measurement. This was followed by a final cooling step of 37°C for 30 seconds.

3.15 Statistical analysis

The data was tested for normality using Kolmogorov-Smirnov test. Parametric data was then subjected to one-way ANOVA with Bonferroni post test. Nonparametric data for two or more groups was done using Kruskal-Wallis test. All values were expressed as mean ± SEM. A probability of $p<0.05$ was considered statistically significant. Graphical representation and analysis of the data was plotted using the GraphPad Prism (V 5.00) software.

CHAPTER 4

4. Results

4.1 The effect of TVL and CAP on body and organ weight and food and water intake

The effect of TVL on pre-treated and post-treated body weights, relative organ weights and food and water intake in rats during the experimental period is shown in table 4.1. The pre-treated body weights showed no significant differences between the various diabetic experimental groups. There was a significant 24% increase in the post-treated body weights (g) of the non-diabetic control compared to the pre-treated body weights ($p< 0.05$). MET showed a significant increase (26.71%) in post-treated body weights (g) in comparison to the pre-treated body weights ($p< 0.05$). TVL and GLIB showed increases while CAP showed a decrease in post-treated body weights (g) in comparison to pre-treated body weights (g); however these changes were not significant. The post-treated body weights of the diabetic groups were significantly reduced compared to the non-diabetic ($p< 0.05$; $p<0.001$). However, the post-treated weights showed significant decreases in the diabetic control (33.01%), TVL (27.67%), CAP (30.32%), MET (14.44%) and GLIB (20.69%) in comparison to the non-diabetic control. In terms of organ weights, there was a significant increase in the % left kidney weight in the diabetic control (0.41 ± 0.01), TVL (0.40 ± 0.01), CAP (0.40 ± 0.01), MET (0.41 ± 0.02) and GLIB (0.40 ± 0.01) groups in comparison to the non-diabetic control (0.30 ± 0.02). However, there were no significant changes between the diabetic control and diabetic treated groups. The percentage (%) of left lobe of liver weight showed significant increases in the TVL (1.70 ± 0.10), CAP (1.60 ± 0.10) and MET (1.70 ± 0.10) in comparison to the non-diabetic control (1.20 ± 0.11) group. Further, significantly higher mean food (gram) and water consumption was observed in the diabetic control (18.56 ± 0.716; 79.3 ± 9.912) than the non-diabetic control (10.13 ± 0.684, 14.30 ± 0.898) while the TVL (16.71 ± 1.07; 75.8 ± 6.012), CAP (16.69 ± 1.231; 74.35 ± 8.374), MET (16.97 ± 0.698; 76.79 ± 6.510) and GLIB (16.47 ± 1.014; 77.01 ± 6.178) groups had lower, though insignificant food and water intake compared to the diabetic control group.

Table 4.1: Average body weights, left kidney weights %, left liver weights % and heart weights % of the non-diabetic and diabetic groups.

Parameter	NDC	DC	TVL	CAP	MET	GLIB
Body Weight Pre-treated(gram)	268.40 ± 12.2	234.40 ± 13.6	235.29 ± 7.9	241 ± 8.5	231.50 ± 7.5	248.50 ± 7.4
Body weight Post-treated(gram)	335 ± 19.9	224.40 ± 22.3***	242.29 ± 8.5***	233.43 ± 13.4 ***	293.33 ± 5.7***	265.67 ± 8.3*
Heart(gram)	0.31 ± 0.02	0.34 ± 0.01	0.34 ± 0.01	0.32 ± 0.01	0.32 ± 0.1	0.33 ± 0.02
Left Kidney (gram)	0.30 ±0.02	0.41 ± 0.01***	0.40 ± 0.01***	0.40 ± 0.01**	0.41 ± 0.02***	0.40 ± 0.01***
Left Liver (gram)	1.20 ± 0.1	1.61 ± 0.0	1.70 ± 0.1**	1.60 ± 0.1**	1.70 ± 0.1*	1.40 ± 0.20
Food gram/rat/day	10.13 ± 0.6	18.56 ± 0.7***	16.71 ± 1.07***	16.69 ± 1.2***	16.97 ± 0.6***	16.47 ± 1.01***
Water ml/rat/day	14.30 ± 0.8	79.3 ± 9.9***	75.8 ± 6.01***	74.35 ± 8.4***	76.79 ± 6.5***	77.01 ± 6.1***

Non-diabetic control (NDC), diabetic control (DC); *Tulbaghia violacea* 60mg/kg.bw (TVL), Captopril 10mg/kg.bw. (CAP), Metformin 250mg/kg.bw (MET) and Glibenclamide10mg/kg.bw (GLIB). All data are expressed as mean ± SEM

***Significantly different from non-diabetic control $p < 0.001$

** Significantly different from non-diabetic control $p < 0.01$

*Significantly different from non-diabetic control $p < 0.05$

4.2 The effect of TVL and CAP on fasting blood glucose levels

The profiles of weekly mean fasting blood glucose (FBG) levels indicated that all diabetic groups had elevated blood glucose levels in comparison to the non-diabetic control, $p<0.0001$ at the first week after induction (Fig.4.2.1). The TVL treatment decreased the T2D-induced elevation in FBG in the first week after the treatment period and the same pattern was observed for the entire experimental period with no significance.

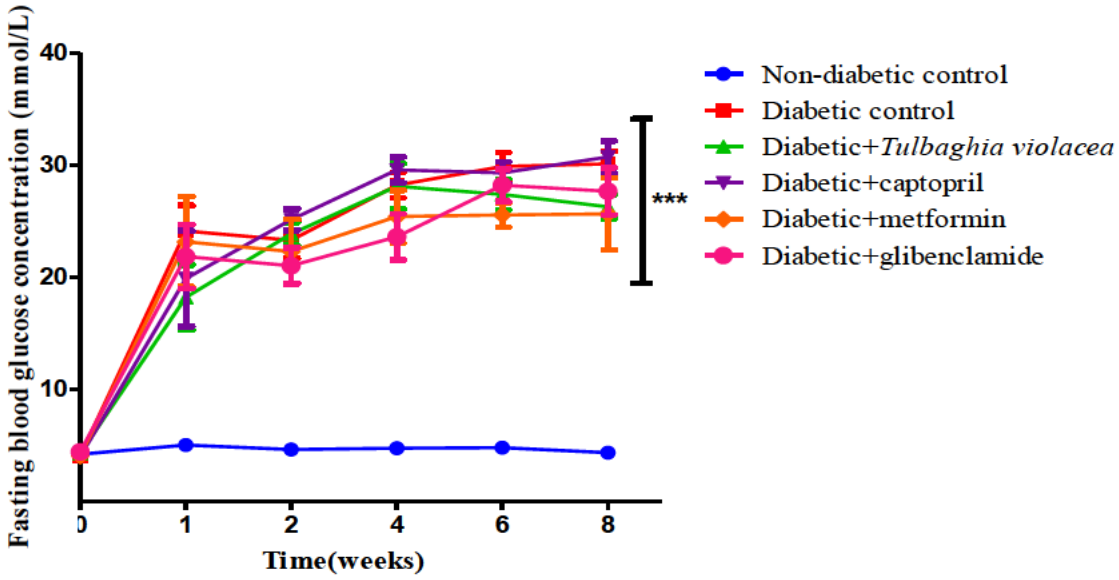

Figure 4.2.1: The effect of TVL and CAP on weekly fasting blood glucose levels in non-diabetic and diabetic rats. All data is expressed as mean ± SEM. ***Significantly different to non-diabetic control; $p< 0.0001$.

4.3 The effect of TVL and CAP on OGTT

The effect of TVL on OGTT is shown in Fig. 4.3.1. The glucose levels in the diabetic control, TVL, CAP, MET and GLIB groups were significantly increased, compared to the non-diabetic control group at 0, 15, 30, 45, 60, 90, 120 and 180 minutes, $p < 0.001$.

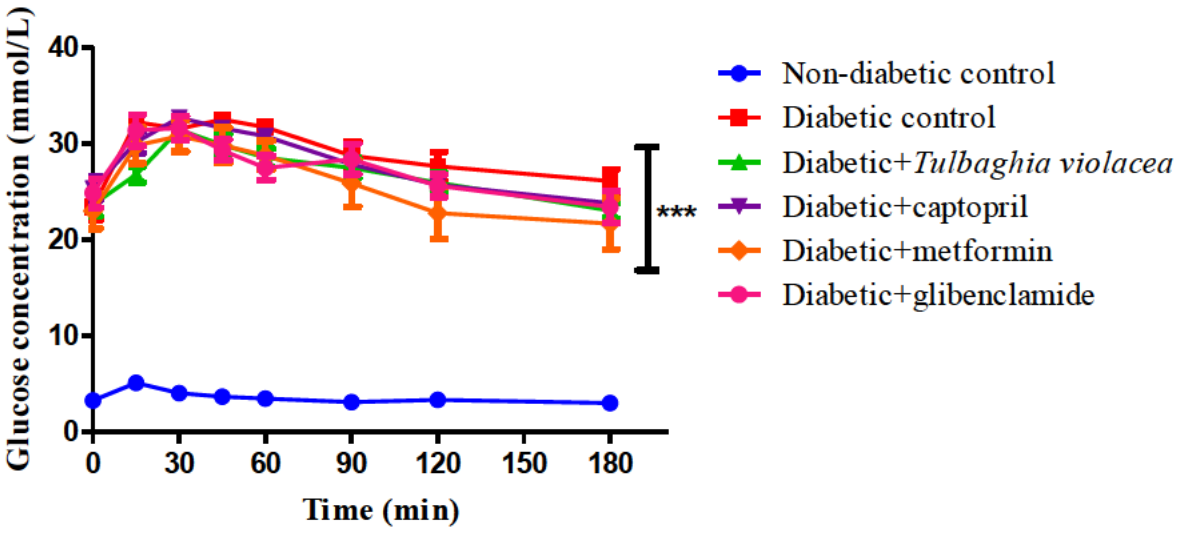

Figure 4.3.1: The effect of TVL and CAP on oral glucose tolerance test (OGTT) in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. ***Significantly different to non-diabetic control; $F_{(5)} = 497$, $p < 0.0001$

4.3.1 Area under Curve (AUC)

The figure below represents the AUC determined from the OGTT graph. The $AUC_{glucose}$ values were significantly increased in the untreated as well as the treated diabetic groups in comparison to the non-diabetic control group, $p < 0.001$.

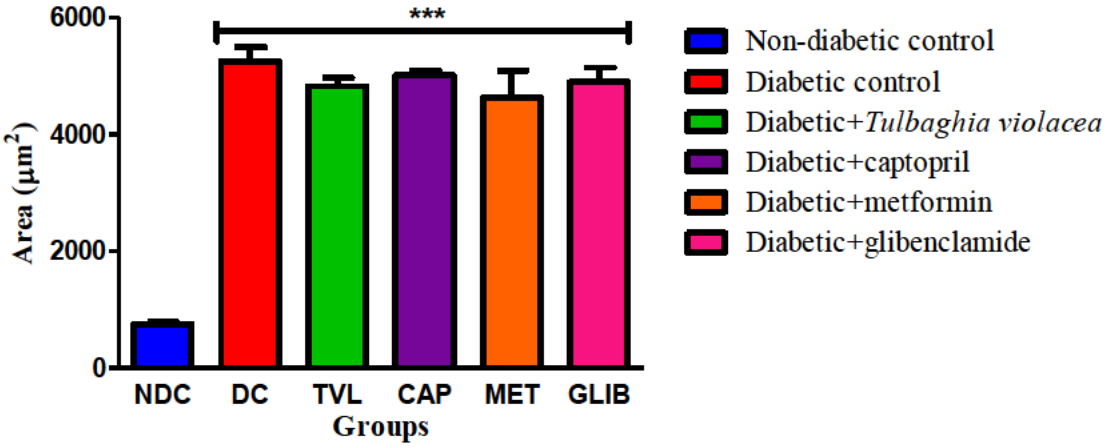

Figure 4.3.2: Area under the curve determined from OGTT graph of non-diabetic, diabetic and diabetic treated groups. All data expressed as mean ± SEM. ***Significantly different to non-diabetic control, $p < 0.001$

4.4 The effect of TVL and CAP on plasma insulin concentration

The plasma insulin levels in the various groups are shown in Fig. 4.4.1. The plasma insulin level in the diabetic group (0.045 ± 0.003) was significantly lower compared to non-diabetic group (0.318 ± 0.0549), but TVL (0.066 ± 0.005), CAP (0.0702 ± 0.007), MET (0.079 ± 0.026) and GLIB (0.077 ± 0.006) groups recorded significantly ($p<0.05$) higher insulin levels than the diabetic control group.

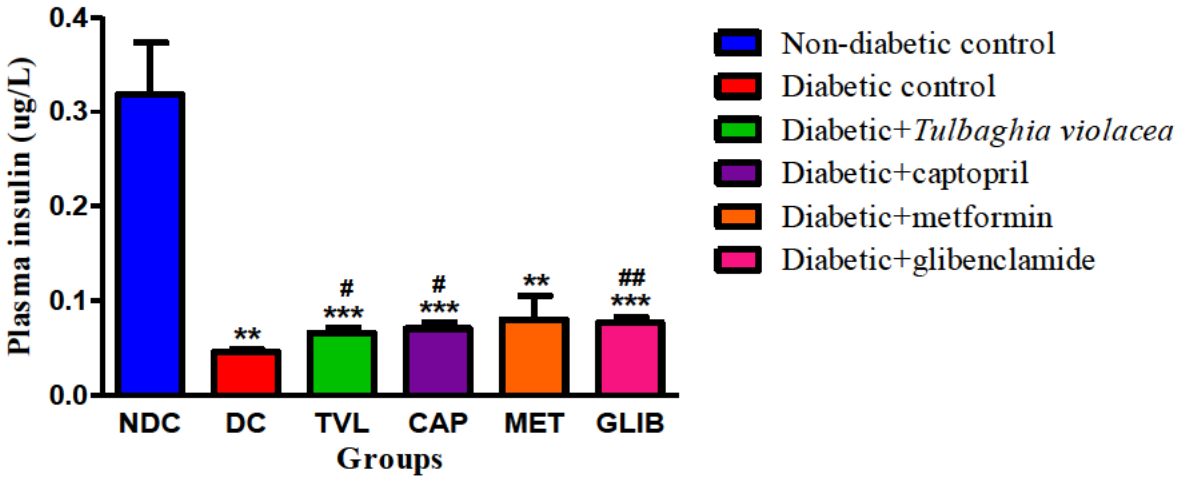

Figure 4.4.1: Effect of TVL and CAP on plasma insulin concentration in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. ***Significantly different to non- diabetic control $p < 0.001$. **Significantly different to non-diabetic control $p< 0.01$. ##Significantly different to diabetic control $p< 0.01$. #Significantly different to diabetic control $p<0.05$.

4.5 Histological and semi-quantitative analysis of the pancreas

4.5.1 Light Microscopy

Histopathological analysis of the pancreatic islets showed extensive damage to the islets and reduction in size of islets in diabetic group (Figure 4.5.1 B). However, the diabetic groups treated with TVL (Figure 4.5.1 C) and MET (Figure 4.5.1 E) showed improved morphology with visibly intact islets and minimal reduction in size of islets.

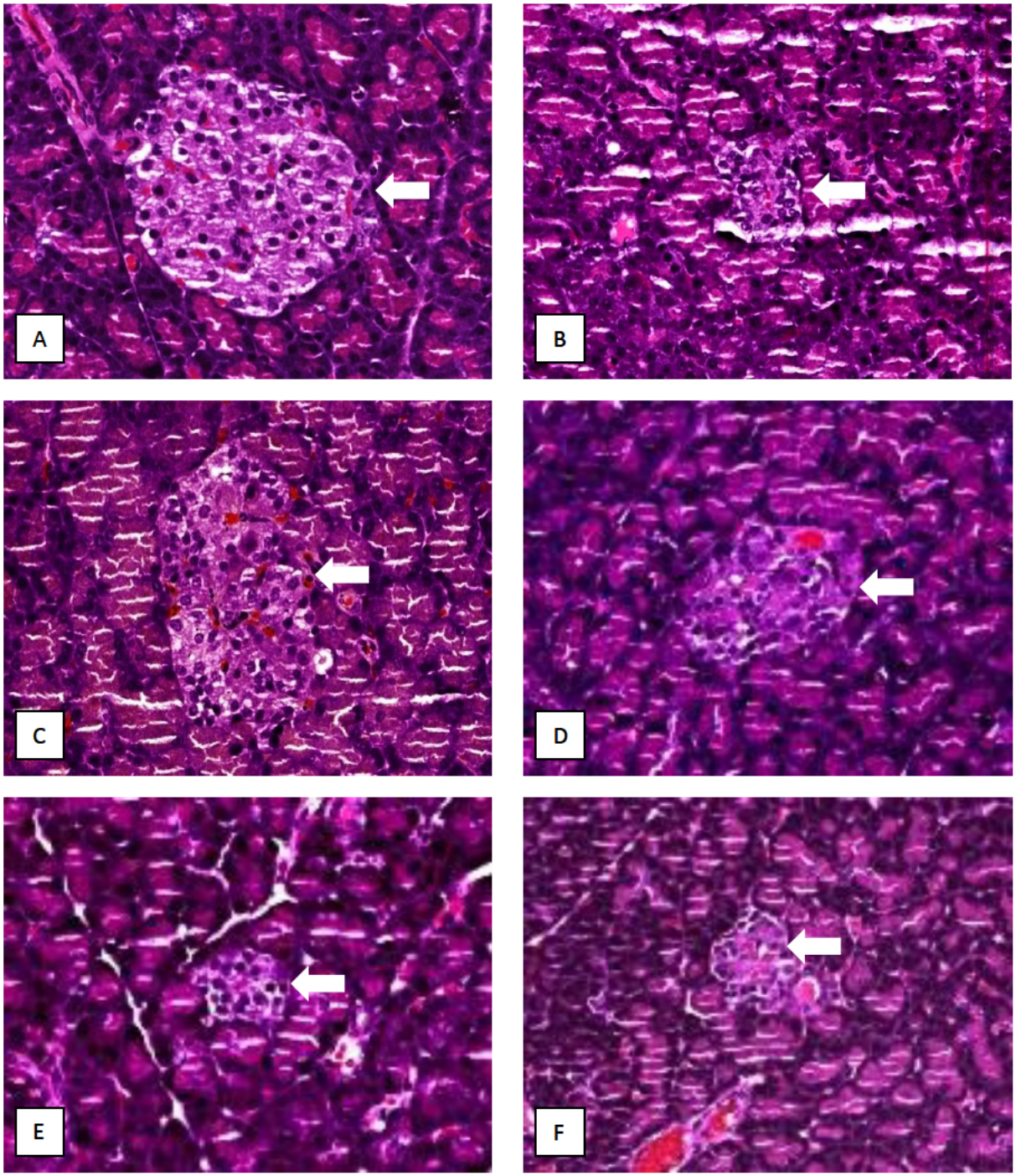

Figure 4.5.1: Photomicrographs of the pancreatic islets of non-diabetic and diabetic rats. (**A**)Non-diabetic control; (**B**) Diabetic control; (**C**) TVL; (**D**) CAP; (**E**) MET; (**F**) GLIB. Magnification 30X.

4.5.2 Pancreatic Islet Count

A quantitative analysis of islets is depicted in Fig. 4.5.2. As expected a significant reduction in the number of islets was seen in the diabetic control (51.81%), CAP (31.75%), MET (31.75%) and GLIB (34.76%) groups in comparison to the non-diabetic control. A significant increase in the number of islets is observed in the diabetic treated groups, TVL (45.47%), CAP (29.39%), MET (29.39%) and GLIB (26.13%) in comparison to the diabetic control. Within the diabetic treated groups, a significant decrease (26.18%) in the pancreatic islet count in the GLIB group in comparison to the TVL is shown.

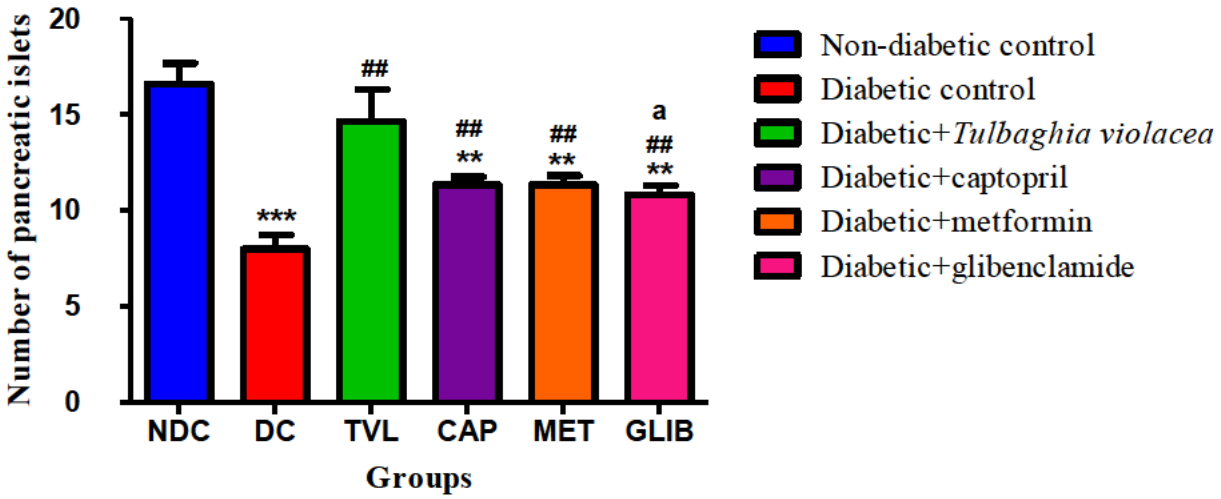

Figure 4.5.2: Effect of TVL and CAP on pancreatic islet numbers in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. *** Significantly different from non-diabetic control, $p< 0.001$. **Significantly different from non-diabetic control, $p<0.01$. ##Significantly different from diabetic control, $p< 0.01$. ªSignificantly different from TVL, $P< 0.05$.

4.5.3 Area of pancreatic Islets

The evaluation of the islet size is shown in the Fig.4.5.3. The diabetic untreated and treated groups showed significant decreases in comparison to the non-diabetic control; diabetic control (11.68%), TVL (27.10%), CAP (18%), MET (19.74%) and GLIB (18.60%). Significant increases are shown in the TVL (43.10%), CAP (64.89%) and GLIB (62.91%) groups in comparison to the diabetic control. The CAP group showed a significant decrease (66.44%) in comparison to TVL (43.10%), $p< 0.05$.

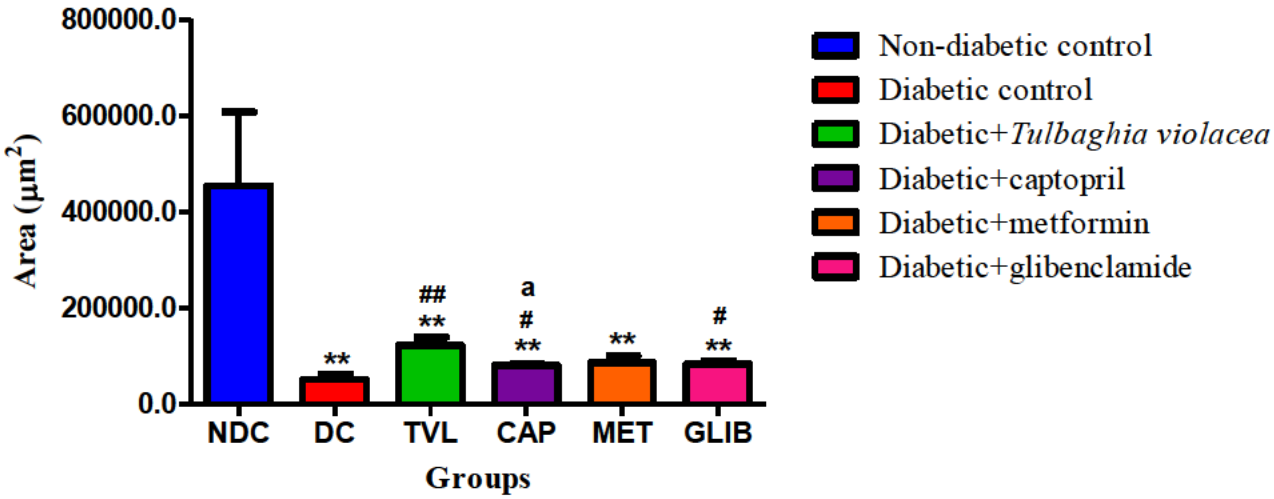

Figure 4.5.3: Effect of TVL and CAP on area (µm^2) of pancreatic islet in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. ** Significantly different from non-diabetic control, p< 0.01. ##Significantly different from diabetic control, p< 0.01. #Significantly different from diabetic control, p<0.05. aSignificantly different from TVL, P<0.05.

4.6 The effect of TVL and CAP onHOMA IR and HOMA β scores

The HOMA IR and HOMA β scores are presented in table 4.2. There were no significant differences between the non-diabetic and diabetic untreated and treated groups. The HOMA β score showed significant decreases in the diabetic control (0.899 ± 0.090; p<0.001), TVL (1.177 ± 0.030; p<0.001), CAP (1.207 ± 0.064; p<0.001), MET (1.027 ± 0.138; p<0.001) and GLIB (2.019 ± 0.138; p<0.001) groups in comparison to the non-diabetic control (142.2 ± 3.678). Significant increases were also shown in the TVL (1.177 ± 0.030; p<0.05), CAP (1.207 ± 0.064; p<0.05) and GLIB (2.019 ± 0.138, p<0.001) in comparison to the diabetic control (0.899± 0.090).

Table 4.2: Effect of TVL and CAP on the HOMA IR and HOMA β scores in non-diabetic and diabetic rats

Parameter	NDC	DC	TVL	CAP	MET	GLIB
HOMA IR	1.65 ± 0.07	1.93 ± 0.2	2.03 ± 0.2	2.25 ± 0.3	1.68± 0.06	1.8 ± 0.2
HOMA β	142.2 ±3.6	0.89± 0.1***	1.18 ± 0.03***#	1.207 ± 0.1***#	1.027±0.14 ***	2.019 ± 0.14***###

Non-diabetic control (NDC), diabetic control (DC); *Tulbaghia violacea* 60mg/kg.bw (TVL), Captopril 10mg/kg.bw. (CAP), Metformin 250mg/kg.bw (MET) and Glibenclamide10mg/kg.bw (GLIB). All data are expressed as mean ± SEM

*** Significantly different from non-diabetic control, $p < 0.001$

*Significantly different from non-diabetic control, $p < 0.05$

###Significantly different from diabetic control, $p < 0.001$

#Significantly different from diabetic control, $p < 0.05$

4.7 The effect of TVL and CAP on liver glycogen levels

The liver glycogen levels in the TVL group showed a significant increase (81%) in comparison to the diabetic control group, $p < 0.05$. The diabetic control group showed a decrease in liver glycogen levels when compared to the non-diabetic control however; it was of no significance (Fig. 4.6.1). There were no significant differences in the liver glycogen levels between the conventionally treated groups in comparison to both the non-diabetic and diabetic control groups; however, they did show a trend of increase in comparison to the diabetic control.

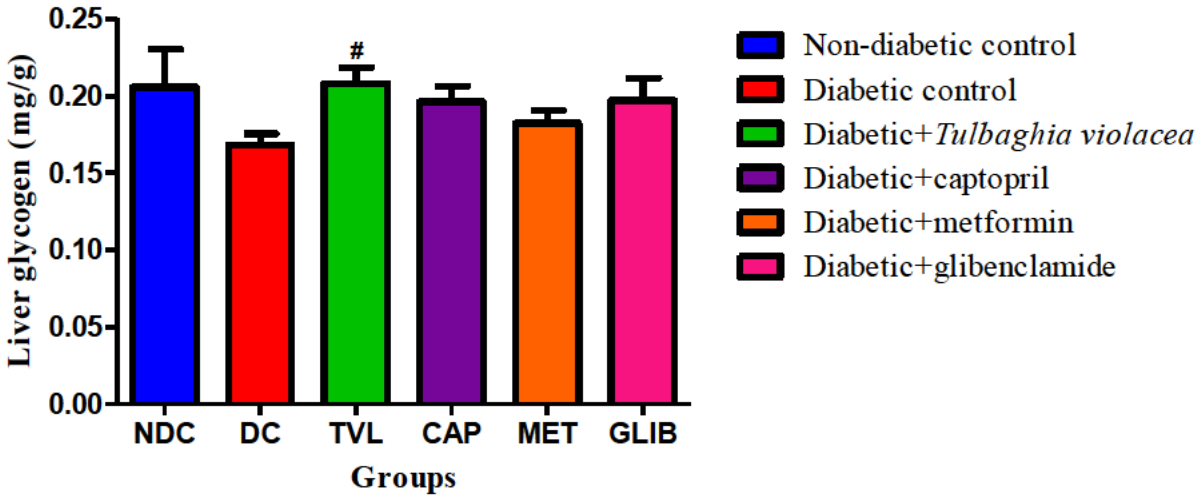

Figure 4.6.1: Effect of TVL and CAP on liver glycogen levels in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. #Significantly different to diabetic control, $p < 0.05$.

4.8 The effect of TVL and CAP on serum lipids

The serum lipid profile is represented in table 4.3. The level of triglycerides showed a significant increase in the diabetic control (2.34 ± 0.350; $p < 0.05$) in comparison to the non-diabetic control (0.844 ± 0.290). The GLIB (1.25 ± 0.135; $p < 0.05$) also showed a significant reduction in comparison to the diabetic control (2.34 ± 0.350). A significant increase is shown in the TVL (2.031 ± 0.278; $p < 0.05$) and CAP (2.304 ± 0.381; $p < 0.05$) in comparison to the GLIB (1.25 ± 0.135).

The total cholesterol level of the MET (1.633 ± 0.108) showed significant increase in comparison to the non-diabetic control. The CAP (1.343 ± 0.057; $p <0.05$) and GLIB (1.350 ± 0.050; $p <0.05$) groups were significantly decreased in comparison to MET (1.633 ± 0.108).

The HDL Cholesterol level of the diabetic control (0.527 ± 0.16; $p<0.01$) dropped significantly in comparison to the non-diabetic control (0.854 ± 0.05). The TVL (1.03 ± 0.056; $p < 0.01$) and MET (1.095 ± 0.080; $p < 0.01$) showed significant increases in comparison to the non-diabetic control. Within the diabetic treated groups, the MET (1.095 ± 0.080; $p<0.05$) showed a significant increase in comparison to CAP (0.861 ± 0.051).

The GLIB (0.638 ± 0.053; $p<0.05$) significantly decreased LDL cholesterol levels in comparison to the diabetic control (0.702 ± 0.125). Within the diabetic treated groups, the TVL (0.822± 0.061; $p< 0.05$) and MET (1.022 ± 0.15; $p< 0.05$) increased significantly in comparison to GLIB (0.638 ± 0.053).

The VLDL levels showed significant increases in the diabetic control (0.468 ± 0.069; $p< 0.05$), TVL (0.406 ± 0.056; $p< 0.05$) and CAP (0.407 ± 0.076; $p<0.05$) in comparison to the non-diabetic control (0.169 ± 0.057). The TVL (0.406 ± 0.056; $p< 0.05$) and CAP (0.407 ± 0.076; $p<0.05$) increased significantly in comparison to GLIB (0.250 ± 0.027).

Table 4.3: Effect of TVL and CAP on serum lipids in non-diabetic and diabetic rats

Serum Lipids and indices	NDC	DC	TVL	CAP	MET	GLIB
TG (mg/dl)	0.844 ± 0.3	2.34 ± 0.4*	2.031 ± 0.4*^{d}	2.304 ± 0.4*^{d}	2.42 ± 0.6	1.25±0.135$^{\#}$
TC (mg/dl)	1.260 ± 0.1	1.580± 0.1	1.443± 0.04	1.343± 0.05^{c}	1.633± 0.1*	1.350±0.050^{c}
HDL-C(mg/dl)	0.726±0.03	1.096± 0.04**	1.027± 0.06**	0.861± 0.05$^{\#}$	1.095±0.08^{**b}	0.962± 0.045
LDL-C (mg/dl)	0.702±0.13	0.952± 0.1	0.822± 0.06^{d}	0.942± 0.1	1.022± 0.2^{d}	0.638±0.053$^{\#}$
VLDL (mg/dl)	0.169 ± 0.05	0.468± 0.07*	0.406± 0.06^{*d}	0.407± 0.07^{*d}	0.484± 0.12	0.250±0.027

Non-diabetic control (NDC) and diabetic control (DC); *Tulbaghia violacea* 60mg/kg.bw (TVL), Captopril 10mg/kg.bw. (CAP), Metformin 250mg/kg.bw (MET) and Glibenclamide10mg/kg.bw (GLIB). All data are expressed as mean ± SEM

*Significantly different from non-diabetic control p <0.05,

**Significantly different from non-diabetic control p <0.01,

***Significantly different from non-diabetic control p <0.001,

$^{\#}$Significantly different from diabetic control p <0.05,

$^{\#\#}$Significantly different from diabetic control p <0.01,

$^{\#\#\#}$Significantly different from diabetic control p <0.001,

^{b}Significantly different from Captopril p <0.05,

^{c}Significantly different from Metformin p < 0.05,

^{d}Significantly different from Glibenclamide p < 0.05

4.9 The effect of TVL and CAP on TBARS

4.9.1 TBARS in liver

The lipid peroxidation level in liver is represented in Fig. 4.7.1. The diabetic control group (0.24 ± 0.05) showed a significant increase in liver TBARS levels in comparison to the non-diabetic control (0.072 ± 0.01; $p<0.001$). The liver TBARS levels in the TVL (0.119 ± 0.002; $p<0.01$), CAP (0.0119 ± 0.02; $p<0.01$) and GLIB (0.098 ± 0.019; $p < 0.01$) groups significantly decreased in comparison to the diabetic control group. There were no significant differences between the non-diabetic control and the treated groups.

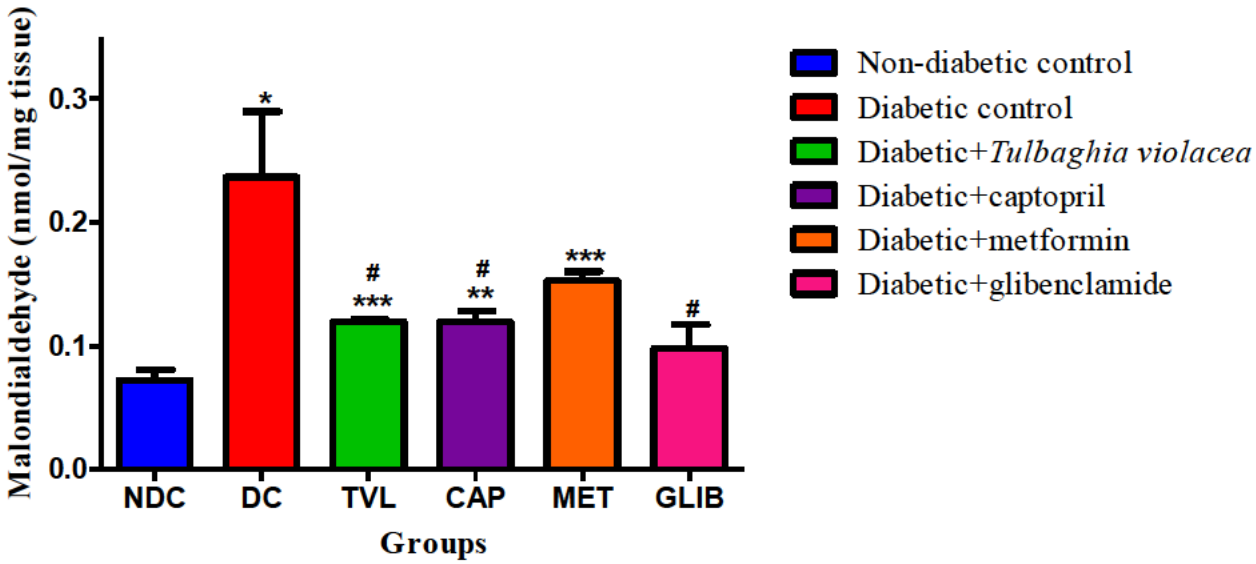

Figure 4.7.1: Effect of TVL and CAP on liver TBARS in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. ***Significantly different to non- diabetic control, $p < 0.001$. **Significantly different to non-diabetic control, $p < 0.01$. *Significantly different to non-diabetic control, $p < 0.05$. #Significantly different to diabetic control $p<0.05$.

4.9.2 TBARS in kidney

The lipid peroxidation level in kidney is represented in Fig. 4.7.2. The conventionally treated groups, CAP (1.04 ± 0.15; $p < 0.05$), MET (0.81 ± 0.14; $p< 0.05$) and GLIB (0.85 ± 0.13; $p<0.01$), showed a significant decrease in comparison to diabetic control group (1.23 ± 0.20). The TVL group showed a decrease in comparison to the diabetic control, however, it is not significantly different. There were no significant differences between the treated and non-diabetic control groups.

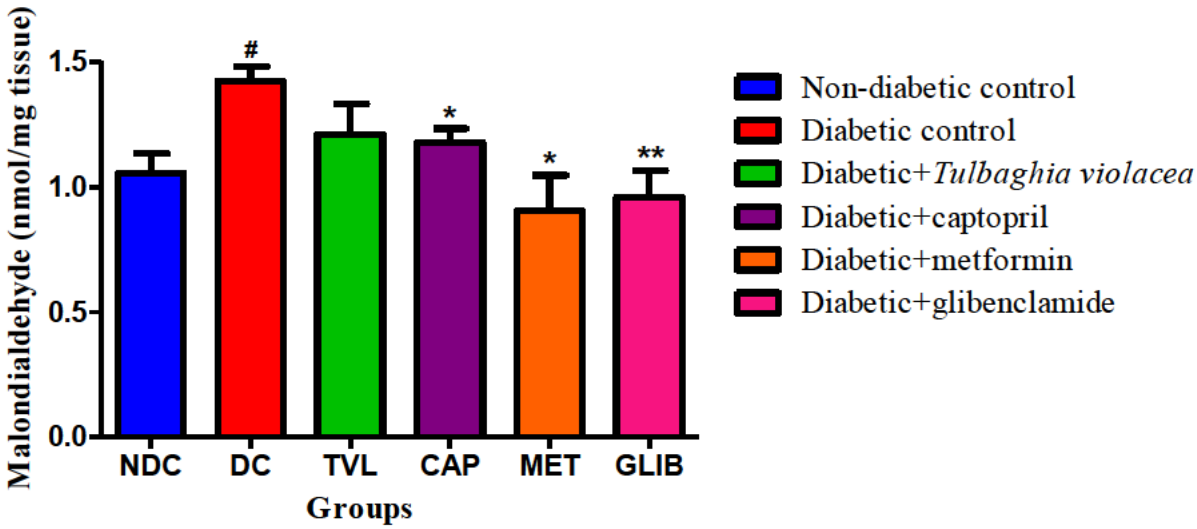

Figure 4.7.2: Effect of TVL and CAP on kidney TBARS in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. #Significantly different to non-diabetic control, $p<0.05$. *Significantly different to diabetic control, $p < 0.05$. **Significantly different to diabetic control, $p <0.01$.

4.10 The effect of TVL and CAP on the percentage inhibition of SOD

The percentage inhibition of superoxide dismutase is represented in Fig. 4.8.1. The diabetic control (0.72 ± 0.053; $p<0.05$) and GLIB (0.078 ± 0.022; $p<0.05$) groups showed a significant decrease in comparison to non-diabetic control (0.923 ± 0.033). The TVL (0.904 ± 0.033; $p<0.05$), MET (0.966 ± 0.010; $p<0.05$) and CAP (0.94 ± 0.023; $p<0.05$) groups showed a significant increase in comparison to the diabetic control (0.72 ± 0.053). The conventionally treated groups, CAP (0.94 ± 0.023; $p<0.05$) and MET (0.966 ± 0.01; $p<0.05$) showed a significant increase when compared to the GLIB (0.780 ± 0.022) group.

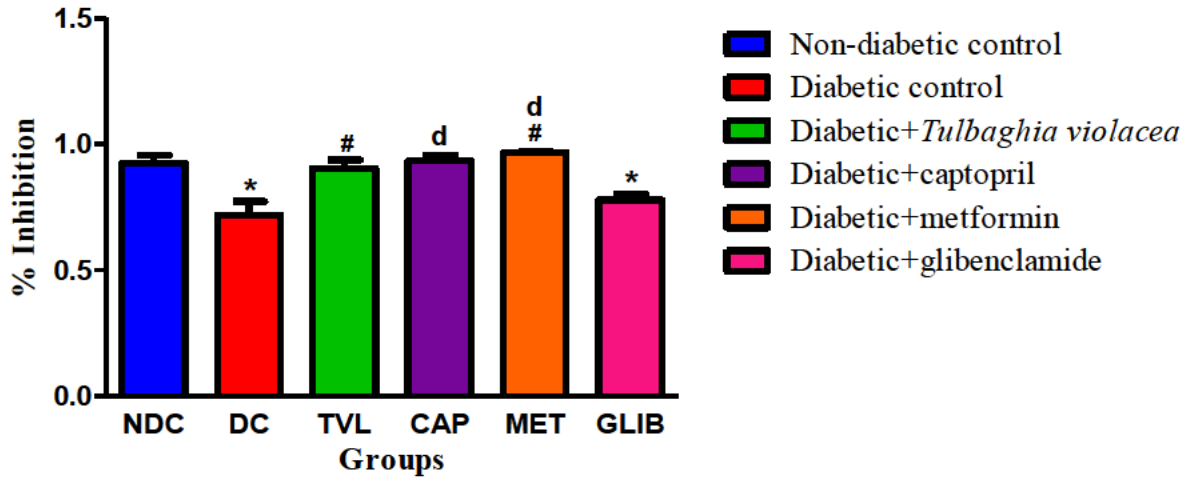

Figure 4.8.1: Effect of TVL and CAP on superoxide dismutase in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. *Significantly different to non-diabetic control, $p < 0.05$. #Significantly different from diabetic control, $p<0.05$. dSignificantly different from Glibenclamide, $p< 0.05$.

4.11 The effect of TVL and CAP on plasma nitric oxide levels

The nitric oxide levels in plasma are represented in Fig. 4.9.1. The diabetic control group (551.7 ± 48.36) showed a significant decrease in comparison to the non-diabetic control (1293 ± 188; $p < 0.01$). The TVL (1161 ± 112.4; $p < 0.01$), CAP (1169 ± 195.1; $p < 0.05$), MET (1126 ± 180.7$p < 0.05$) and GLIB (932.7 ± 112.5; $p < 0.05$) were significantly higher in comparison to the diabetic control (551.7 ± 48.36).

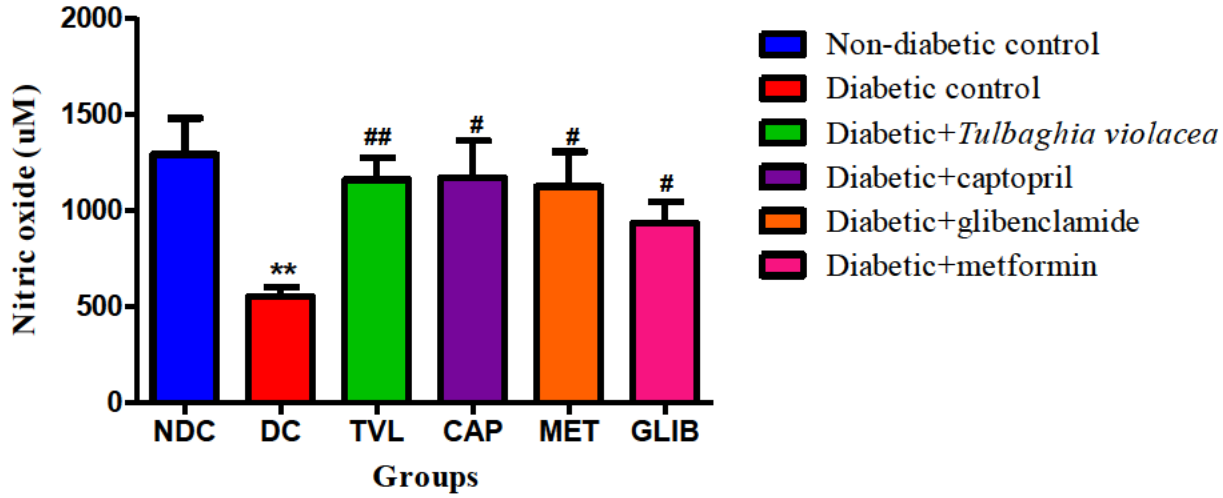

Figure 4.9.1: Effect of TVL and CAP on nitric oxide concentration in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. **Significantly different to non- diabetic control, $p < 0.01$. ##Significantly different to diabetic control, $p <0.01$. #Significantly different to diabetic control, $p<0.05$.

4.12 The effect of TVL and CAP on histopathology of renal tissue

4.12.1 Light Microscopy

Histopathological analysis of the kidney showed damaged glomeruli in the diabetic group (Figure 4.12.1 B). However, the diabetic groups treated with TVL (Figure 4.5.1 C) and CAP (Figure 4.12.1 D), MET (Figure 4.12.1 E) and GLIB (Figure 4.12.1 F) showed decreased glomerulosclerosis.

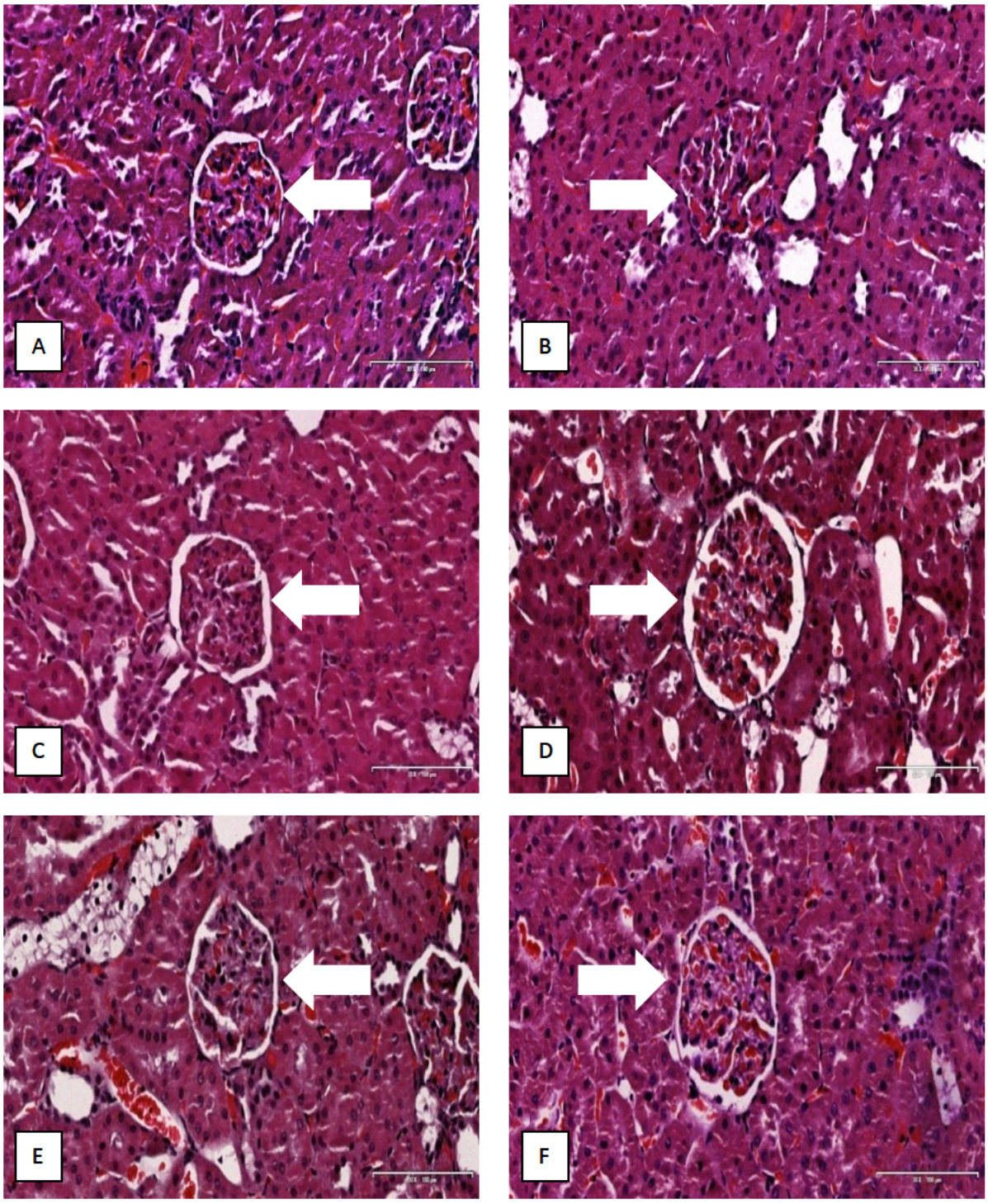

Figure 4.10.1: Photomicrographs of the kidney of normal and diabetic rats. (**A**) Non-diabetic control; (**B**) Diabetic control; (**C**) TVL; (**D**) CAP; (**E**) MET; (**F**) GLIB. Magnification 30X.

4.12.2 Glomerular Injury Score

The injury score of the kidney is represented in Fig. 4.10.2 and is characterized into normal, segmental and global sclerosis. The normal glomeruli count showed a significant difference between the non-diabetic and diabetic groups. The diabetic control (40.69%), TVL (17.94%), CAP (28.75%), MET (31.55%) and GLIB (25.77%) groups showed significant decreases in the normal glomeruli count in comparison to the non-diabetic control. The TVL (27.73%) significantly increased in the normal count in comparison to the diabetic control. Within the treated diabetic group, the CAP (13.17%) and MET (16.58%) decreased significantly in the normal count in comparison to the TVL group. There were no significant differences between the segmental non-diabetic, diabetic control and diabetic treated groups.

The global sclerosis count showed a significant increase in the diabetic control and diabetic treated groups in comparison to the non-diabetic control. The diabetic control (75.49%), TVL (55.12%), CAP (69.70%), MET (71.83%) and GLIB (64.70%) groups showed a significant increase in comparison to the non-diabetic control. A significant decrease in the global sclerosis count is shown in the TVL (45.39%), CAP (19.12%) and GLIB (30.56%) in comparison to the diabetic control group. Within the treated diabetic groups there were significant decreases in the global sclerosis count in the CAP (32.48%) and the MET (37.24%) in comparison to the TVL.

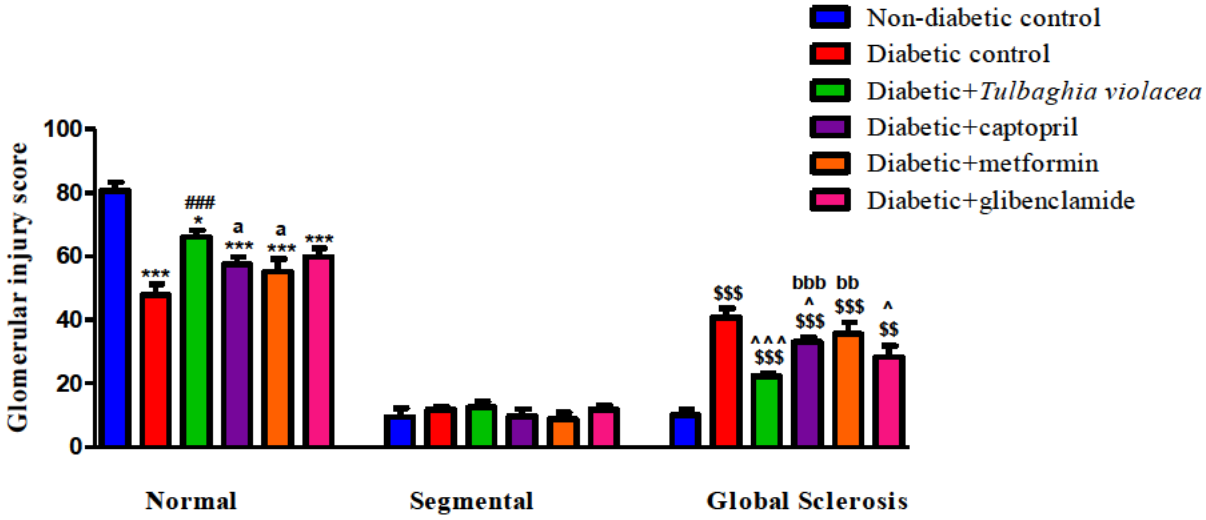

Figure 4.10.2: The effect of TVL and CAP on the injury score of the kidney in non-diabetic and diabetic rats. All data is expressed as mean ± SEM.

Normal - *** Significantly different from non-diabetic control, $p < 0.001$. * Significantly different from non-diabetic control, $p< 0.05$. ### Significantly different from diabetic control, $p< 0.001$. a Significantly different from TVL, $P< 0.05$. Global Sclerosis- $$$ Significantly different to non-diabetic control, $p<0.001$. $$ Significantly different from non-diabetic control, $p<0.01$. ^^^ Significantly different from diabetic control, $p<0.001$. ^ Significantly different from diabetic control, $p <0.05$. bbb Significantly different from TVL, $p<0.001$. bb Significantly different from TVL, $p <0.01$

4.13 The effect of TVL and CAP on serum ACE activity

Serum ACE activity levels are represented in Fig. 4.11.1. The CAP group (550.6 ± 35.47) showed a significant increase in ACE activity in comparison to the non-diabetic control group (167.1±6.68; $p<0.001$). The untreated and treated diabetic groups, diabetic control (207.4±4.49), TVL (229.1 ± 7.41), MET (224.8 ± 7.05) and GLIB (205.5 ± 4.78), were significantly reduced in comparison to the CAP group (550.6 ± 35.47; $p<0.001$). There were no significant differences between the non-diabetic control and the diabetic control groups.

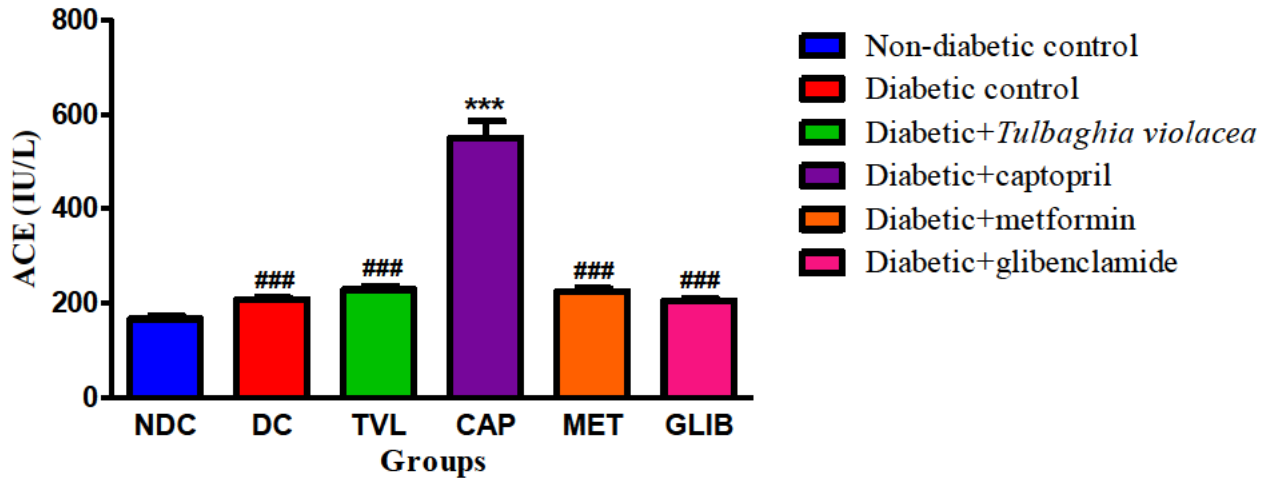

Figure 4.11.1: Effect of TVL and CAP on serum ACE activity in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. ***Significantly different to non-diabetic control, $p < 0.001$. ###Significantly different to Captopril $p < 0.001$

4.14 The effect of TVL and CAP on the expression of renal AT1 and NADPH

4.14.1 The effect of TVL and CAP onAT1 gene expression

The gene expression of AT1 in the left kidney is represented in Fig 4.12.1. The diabetic control (0.029 ± 0.011) and the CAP group (0.064 ± 0.003) showed a significant increase in AT1 gene expression in comparison to the non-diabetic control group (0.005 ± 0.001; $p < 0.001$). The treated diabetic groups, TVL (0.0196 ± 0.007), MET (0.022 ± 0.006) and GLIB (0.007 ± 0.006), showed significantly reduced AT1 expression in comparison to the CAP group (0.064 ± 0.003; $p<0.001$).

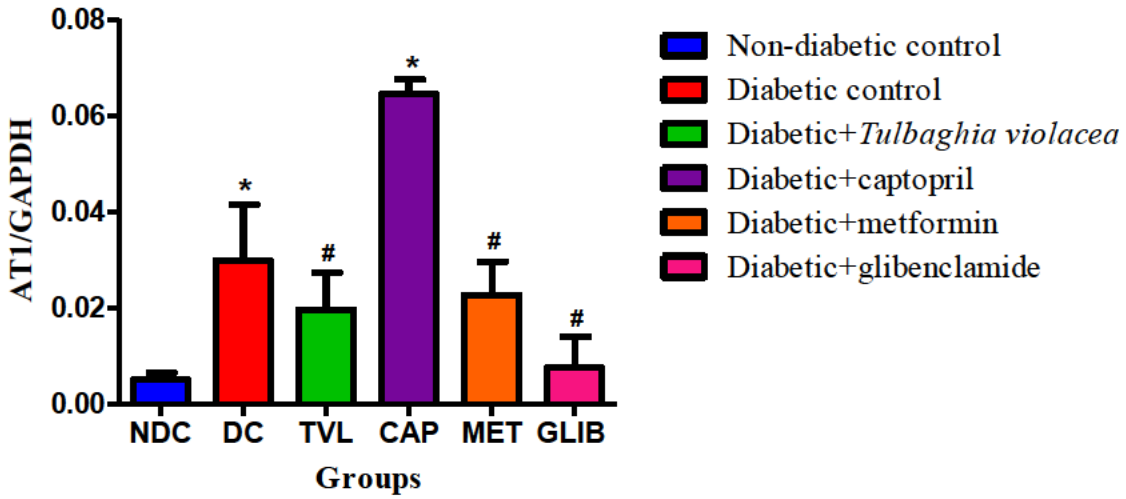

Figure 4.12.1: Effect of TVL and CAP on AT1 gene expression in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. *Significantly different to non-diabetic control, $p < 0.001$. #Significantly different to CAP $p < 0.001$

4.14.2 The effect of TVL and CAP onNADPH gene expression

The gene expression of NADPH in the left kidney is represented in Fig 4.12.2. The diabetic control (2.554 ± 0.450) showed a significant increase in NADPH gene expression in comparison to the non-diabetic control group (1.402 ± 0.106; $p< 0.001$)

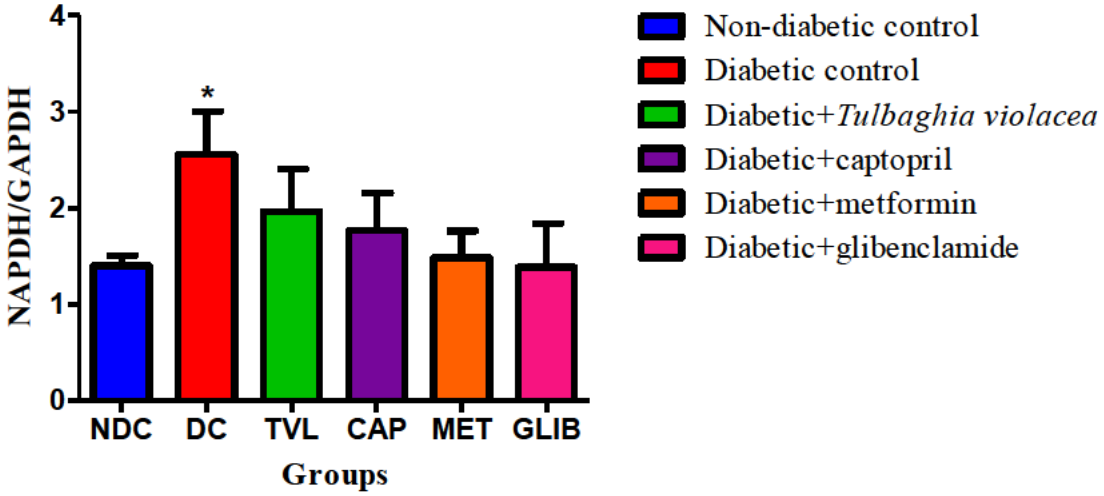

Figure 4.12.2: Effect of TVL and CAP on NADPH gene expression in non-diabetic and diabetic rats. All data are expressed as mean ± SEM. *Significantly different to non-diabetic control, $p < 0.001$

CHAPTER 5

5. Discussion

This study investigated the renal and metabolic effects of *Tulbaghia violacea* Harv. (TVL) and captopril in a fructose-fed streptozotocin (STZ) induced diabetic rat model. The results of this study demonstrated the potential of TVL to elicit antioxidant and hypolipidemic effects, increase plasma insulin levels, improve pancreatic and glomerular morphology as well as improve β cell function, however TVL did not reduce fasting blood glucose levels or improve glucose tolerance.

There are many methods of induction of DM representative of various models, however, not all are successful in mimicking all the symptoms of diabetes mellitus. The model chosen for this study required a two-week period of fructose feeding combined with a 40mg/kg.b.w. dose of STZ injection, which potentially mimics all the major pathogeneses of Type 2 diabetes in rats (Wilson and Islam, 2012). In this study the efficacy of TVL was investigated, a medicinal plant, which has been previously reported to be effective in treating hypertension in Dahl-salt sensitive rats (Moodley *et al*., 2014) and STZ-induced diabetes in Wistar rats (Moodley *et al*., 2015) but not in an STZ-fructose fed model. In addition, the effects of an ACE inhibitor in the amelioration of the symptoms or complications of diabetes mellitus were investigated. ACE inhibitors can impede the progression of renal pathology related to diabetes mellitus or diabetic nephropathy. It has been reported that the further development of diabetic nephropathy can be delayed through both glycaemic control and inhibition of RAAS, in particular using ACE inhibitors (Ahmad, 2015). Hence, the use of CAP in the study was not particularly for its blood pressure lowering effects but rather for its reported ability to reduce further development of end organ damage.

In the present study, increased blood glucose levels, significantly decreased body weights, polydipsia and polyphagia as well as polyuria in the diabetic untreated and treated groups in comparison to the non-diabetic control were observed indicating the successful induction of diabetes mellitus in this model. Administration of TVL to diabetic rats showed a tendency to reduce the fasting blood glucose levels and improve glucose tolerance; which did not reach levels of significance. Diabetic rats treated with TVL did not show significant differences in body weights, food consumption and water intake in comparison to the diabetic control; however, there were tendencies of improvement in post-treated body weights and reduction in both food consumption and water intake in comparison to the non-diabetic control. In this study, reduced body weight was found which is in keeping with others, as body weight reduction and decreased growth rate in diabetic rats, regardless of increased food intake, is characteristic of elevated catabolism of protein due to insulin insufficiency causing structural protein degradation and muscle wasting (Eleazu *et al*., 2013). The slight improvement in the body weight of the diabetic group treated with TVL suggests that the plant could be protective against the breakdown of structural proteins.

Eleazu *et al*., (2013) has reported that several organs showed hypertrophic effects in the model. Elevated liver and kidney to body weight ratios in the untreated and treated diabetic rats in comparison to non-diabetic control were also shown in this study. Hypertrophy of the liver could be due to hyper-insulinaemia-induced influx of fatty acids and reduced secretion of lipoprotein from the liver, resulting in accumulation of triglycerides in the model. The kidney related findings are unusual as increased kidney weights in diabetes have been linked to elevated protein synthesis and lipogenesis. Renal hypertrophy is regarded as an early symptom of glomerular pathology related to diabetes (Eleazu *et al*. 2013).

It is common knowledge that resistance to the biological effects of insulin is a major contributing factor to the pathogenesis of Type 2 diabetes, which ultimately results in increased fasting, and postprandial blood glucose levels (Shen *et al*., 2008; Roberts *et al*., 2013).Hence therapeutic intervention would seek to improve insulin action through increased sensitivity of the organs to insulin. In a previous study, we reported a significant decrease in fasting blood glucose levels as well as improved glucose tolerance following TVL 60mg/kg administration to STZ induced DM (Moodley *et al*., 2015). In this study, TVL did not significantly reduce fasting blood glucose levels or improve glucose tolerance. These results are contrary to the findings of Moodley *et al*., 2015, where TVL demonstrated its hypoglycaemic effects in STZ induced diabetic rats. For the current study, a different model of diabetes was used to induce an insulin resistant state whereas in the previous study a state of hyperglycaemia was induced. Therefore, it can be speculated that TVL is not successful or efficient in reducing blood glucose levels or improving glucose tolerance effectively in an insulin resistant model created by fructose feeding coupled with an STZ injection. However, in the present study the TVL did show a tendency to reduce the fasting blood glucose levels and improve glucose tolerance which did not reach levels of significance. This difference in response can be attributed to the mode of diabetes induction through fructose feeding. It can be inferred that the presence of fructose loading (analogous to glucose overload), may be too great a stimulus to benefit from a multi-targeted approach using extracts.

Significantly increased plasma insulin levels were found in the TVL, CAP and GLIB treated groups. The significant increase observed in the TVL treated group suggests that the plant could possibly elicit insulinogenic activity and is able to influence the secretion of insulin from pancreatic β cells. These results are in keeping with a previous study (Moodley *et al*., 2015) and other studies reporting that plant extracts exert hypoglycaemic action through stimulatory effects on insulin release (Patel *et al*., 2012; Ghandi *et al*., 2012).

It is characteristic of insulin to enhance glycogen synthesis and impede glycogenolysis in the liver, which is the principle site for endogenous glucose production (Rao *et al*., 2013). Hence, insufficient insulin leads to the slowing down of glycogen synthesis and the enhancement of glycogenolysis, which consequently decreases liver glycogen levels in diabetic rats (Subash-Babu *et al*., 2008; Kondeti *et al*.,

2010). The administration of TVL in the present study showed a significant increase in the glycogen levels in comparison to the diabetic control rats. The significant increases in both plasma insulin and liver glycogen levels may imply that the use of TVL promoted the secretion of insulin from intact pancreatic β-cells, which subsequently improved the reduced capacity of the liver to synthesize glycogen.

In conjunction to plasma insulin levels, HOMA β scores are regularly used to authenticate pancreatic β-cell function (Song *et al*., 2007; Wilson and Islam 2012). The two major pathogeneses of type 2 diabetes include partial pancreatic β cell dysfunction and insulin resistance (Islam and Wilson, 2012). While the model of diabetes used in the present study induced insulin resistance, as reflected by the increased HOMA IR, the increase did not reach levels of significance. Regarding therapeutic efficacy; in the present study, we found an increase in the HOMA β scores of the TVL, CAP and GLIB groups illustrating the ability of the treatments to improve β cell function.

The pancreas secretes insulin from the beta cells and is implicated in the pathogenesis of DM. In this study, histopathological analysis of pancreatic tissue showed severe damage to the islet cells as well as a decrease in islet number and size in the diabetic control group. This finding further affirms the successful induction of DM using fructose feeding in conjunction with an STZ injection. In this study, we found that both pancreatic islet number and size significantly increased in the treated group. This may represent a protective response against the deleterious action of fructose feeding and STZ induced islet damage, which may also have led to the observed improved insulin secretion as indicated by the elevated plasma insulin levels.

Despite the increase in plasma insulin levels, the blood glucose levels did not decrease, which could be attributed to fructose feeding. The consumption of high fructose diets contribute significantly to the excessive formation of ROS leading to oxidative stress and more specifically insulin resistance. In addition, increased cellular ROS directly triggers the activation of serine/threonine kinase cascades that in turn phosphorylates multiple targets including the insulin receptor and insulin receptor substrate (IRS). Increased serine phosphorylation of IRS reduces its capability of undergoing tyrosine phosphorylation and speeds up the breakdown of IRS-1, resulting in impaired glucose uptake in tissues, because of fructose feeding the tissues are not effectively stimulated by insulin to dispose of glucose (Sivaraman *et al*., 2013).The pathogenesis of DM is associated with alterations in key markers of oxidative stress viz. increased ROS, NADPH oxidase and decreased NO levels as well as antioxidant enzyme activity (Henriksen *et al*., 2011; Miguel-Carrasco *et al*., 2010). An excessive accumulation of ROS induces oxidative stress, which contributes to the development of DM. The development of diabetes induced oxidative stress is due to increased production of free radicals or impaired antioxidant defence mechanisms (Ramachandran *et al*., 2011; Olorunnisola *et al*., 2012). Fructose feeding or fructose rich diets are linked to the development of oxidative stress. This occurs due to an increase in

the concentration of free radicals and the decrease of antioxidant levels under the influence or consequent to the presence of fructose in excessive quantities (Sivaraman *et al*., 2013).

In the present study the role of TVL in diabetes, induced oxidative stress was analysed. Evidence provided in literature implies that free radicals in excessive amounts play a vital role in diabetes; specifically reactive oxygen species produced by hyperglycaemia-induced glucose autoxidation and protein glycosylation (Masjedi *et al*., 2013). Furthermore, one of the principle targets of ROS is lipids (Tiwari *et al*., 2013). The lipid profile of the body undergoes alterations or disturbances induced by DM which results in susceptibility to lipid peroxidation (Patricia, 2009). In the present study a decrease in SOD concentration and an increase in TBARS levels were observed, which is obtained from a reaction between malondialdehyde and thiobarbituric acid, in the liver and kidney of untreated diabetic rats, providing evidence of diabetes induced oxidative stress through damage caused by fructose feeding and STZ injection. The administration of TVL, CAP and GLIB decreased the levels of TBARS in the liver and kidney, however, only the liver samples reached levels of significance. In addition to the decreased TBARS level, TVL showed a significant increase in SOD concentration. Similar results were observed in a previous study using a different rat model (Moodley *et al*., 2015).

The ability of TVL to reduce the generation of TBARS and elevate the levels of SOD can most likely be attributed to the organosulphur compounds present in the plant (Capasso, 2013). Garlic organosulphur compounds have been extensively studied and shown to have numerous biological effects, including antioxidant and anti-inflammatory properties (Vazquez-Prieto and Miatello, 2010). Additionally, the significant decrease in the TBARS levels and significant increase in concentration of SOD in the TVL treated diabetic rats may be due to polyphenolic compounds present in the plant (Olorunnisola *et al*., 2011) and/or the elevation of antioxidant defence mechanisms. It is possible that the plant extract is capable of reducing the generation of free radicals or increasing free radical scavenging mechanisms. Many previously published studies have reported decreases in oxidative stress due to the potential antioxidant properties of TVL (Moodley *et al*., 2015; Olorunnisola *et al*., 2012; Zheng and Wang 2001).

Hyperglycaemia is one of the characteristic features of diabetes that contributes to the inhibition of endothelial nitric oxide synthase (eNOS). This inhibition results in decreased nitric oxide production and ultimately the elevation of ROS production (Juarez-Rajop *et al*., 2012; Mohamadin *et al*., 2007). The administration of TVL in the present study significantly increased the plasma NO levels as seen in the previous study (Moodley *et al*., 2015). This increase can be attributed to NO-generating compounds, which have been reported to improve hyperglycaemia and oxidative stress in diabetic rats (Mohamadin *et al*., 2007).

Diabetic nephropathy (DN) is a severe and common complication that contributes significantly to the mortality rate of people afflicted with chronic DM (Duran-Salgado and Rubio-Guerra, 2014; Tsutsui *et*

al., 2015). In addition to the analysis of electrolyte concentrations, glomerulosclerosis and tubular changes, which are structurally associated with DN, need to be considered in treatment (Varatharajan *et al*., 2015).The evaluation of renal histopathology showed that TVL, CAP, MET and GLIB are associated with decreased glomerulosclerosis as evidenced by the glomerular injury scores. Glomerulosclerosis is one of the morphological hallmarks of renal injury and ultimately, end-stage renal failure (Wolf, 2006).

The Renin Angiotensin Aldosterone system plays a key role in the maintenance of oxidative balance and therefore our strategy included drugs or plant extracts which would inhibit the RAS pathway. It is well known that the over activity of the RAS is known to induce oxidative stress and renal dysfunction via the AT1 receptor (Kinoshita *et al*., 2011) and therefore our strategy included drugs or plant extracts which would inhibit the RAS pathway. The G protein coupled receptor AT1, is present in multiple tissues of the body and especially on the kidney. It is the cognate receptor for the octapeptide angiotensin II, which is the key role player on the RAS cascade (Motawi *et al*., 2013). Hence, one of the main objectives of this study was to focus on the gene expression of AT1. We anticipated a decrease in AngII production or down-regulation of AT1, which would then decrease the production of reactive oxygen species derived from NADPH oxidase and ultimately decrease oxidative stress and renal dysfunction. Therefore, the use of CAP, an ACE inhibitor, could potentially result in reduced renal damage by down regulating the activity of renal NADPH oxidase via the AngII blockade. In this study, the CAP treated group showed a significant increase in the expression of AT1 and TVL as well as the conventional diabetic treatments showed a significant decrease. Regarding the serum ACE activity, TVL had no significant effect and CAP showed significantly elevated levels. In previous studies, TVL exhibited ACE inhibitor activity (Duncan *et al*., 1999; Ramesar *et al*., 2008), however, in this study the results differed from that which was expected. Interestingly other studies have revealed results for CAP (Ninahuaman *et al*., 2007; Moodley *et al*., 2013) that are in keeping with the trend observed in this study. It is possible that the increase in ACE serum activity seen in the CAP group could be attributed to a phenomenon known as 'aldosterone escape', a limiting factor on the effect of RAS inhibition (Lu *et al*., 2010). Clinically this occurrence has been observed over an extended period of time in which ACE inhibitor treatment was administered, resulting in an increase in aldosterone levels in the case of DN (Sato *et al*., 2003).

Insulin resistance in a diabetic state has been established by various reports as a pre-disposing factor for dyslipidemia. Characterization of dyslipidemia includes increased total cholesterol, triglycerides (TG), low-density lipoprotein cholesterol (LDL) and very low density lipoprotein cholesterol (VLDL) levels as well as decreased high-density lipoprotein cholesterol (HDL) (Kondeti *et al*., 2010; De Silva and Frayling, 2010). Dyslipidemia occurs due to increased movement of free fatty acids from fat depots, enhancing the activity of HMG-CoA and diminishing lipoprotein lipase (LPL) activity (De Silva and Frayling, 2010). Triglycerides are hydrolyzed by the enzyme lipoprotein lipase, which is controlled by

insulin under normal conditions. However, lipoprotein lipase becomes deactivated due to insulin deficiency in a diabetic state, leading to elevated hepatic synthesis of triglycerides and disturbances in the release and rate of clearance of VLDL –cholesterol lipoprotein lipase (Juarez-Rajop *et al*., 2012).

In the present study, we found increased triglycerides, total cholesterol, VLDL cholesterol and LDL cholesterol levels in the diabetic model. The TVL treatment reduced the levels of triglycerides, total cholesterol, VLDL cholesterol and LDL cholesterol; however, levels of significance were not reached. These results are in keeping with our previous study and validate the ability of TVL to exert hypolipidemic effects (Moodley *et al*., 2015). The HDL cholesterol levels of the untreated diabetic group in the present study showed a significant increase, which is not usually characteristic of dyslipidemia; however, this result is not isolated. Divi *et al*., 2012 reported similar changes and attributed the increase in HDL- cholesterol levels to high fructose feeding in various animals like Sprague-Dawley rats.

CHAPTER 6

6. Conclusion

This study aimed to investigate the renal and metabolic effects of TVL and captopril in a fructose-fed STZ induced diabetic rat model. Metabolically, the administration of TVL and captopril exhibited antioxidant effects, improved plasma insulin levels, significantly reduced pancreatic islet damage and positively impacted the β –cell function. However, only TVL significantly increased the liver glycogen content. These findings support the investigation of the metabolic effects of TVL and captopril in this model of diabetes. Furthermore, the potential of TVL and captopril to exert positive renal effects is illustrated in the significantly improved glomerular morphology, as evidenced by the increased normal glomerular count coupled with a decrease in the global sclerosis count. Despite the metabolic and renal effects of the plant and captopril, these treatments did not reduce fasting blood glucose levels or improve glucose tolerance. Hence, this study showed that TVL might not be as effective in a model with a high glycaemic index. On the contrary, TVL potentially demonstrated the ability to impede complications associated with DM. Albeit, the metabolic effects of TVL it is well documented, the novelty of this study focused on the model of induction and the use of an ACE inhibitor (an established antihypertensive - Captopril) in the treatment of DM. This well-known ACE inhibitor was not particularly used for its blood pressure lowering effects (haemodynamic effects) but for its ability to decrease the progression of end organ damage (non-haemodynamic effects) and therefore Captopril was not expected to exert glycaemic control in this study. Further studies are warranted to isolate and identify the bioactive compounds present in TVL as well as to investigate its beneficial biological mechanisms to combat DM and various other conditions. The rising problem of DM requires a rigorous and multifaceted therapeutic intervention involving lifestyle changes coupled with effective herbal regimes that elicits minimal side effects.

Future Recommendations

1. Isolation and identification of bioactive compounds of TVL as well as further testing of each isolated bioactive compound for specific biologically activity (i.e. antidiabetic, anticancer, antifungal, antimicrobial etc).
2. Thereafter, nanoencapsulation of the isolated and identified bioactive compounds that show therapeutic effects against diseases.
3. Natural products may improve the mechanism of action through coupling with conventional drugs in a lower dose. This could elicit a multifaceted approach to treating DM due to the multiple characteristics of plant extracts and the target specific ability of conventional drugs.

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APPENDICES

Appendix A: Animal ethical clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

28 January 2015

Reference: 044/15/Animal

Miss KM Joseph
Dept of Physiology
School of Laboratory Medicine and
Medical Sciences
WESTVILLE CAMPUS

Dear Miss Joseph

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2015** on the following project:

"The effect of ACE inhibition in Streptozotocin induced diabetic rats."

[redacted]

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar
Research Office – Dr N Singh
Supervisor – Prof. I Mackraj
HOS – Prof. W Daniels
BRU – Dr S Singh

Appendix B: TBARS Reagents

Preparation of Reagents

1. BHT: 20mM → 0.449g in 100µl of ethanol
2. NaOH: 50mM → 0.2g into 100ml of H_2O

Took 5ml of 20mM BHT, added to and mixed with 100ml of NaOH solution, then added 1g of TBA. Now you have BHT/TBA solution.

3. 0.2% phosphoric acid → 170µl of 85% phosphoric acid into ± 99.83ml of H_2O.
4. 2% phosphoric acid → 1.7ml of 85% phosphoric acid into ±98.3ml H_2O.
5. 7% phosphoric acid → 5.95ml of 85% phosphoric acid into ±94.05ml H_2O.

1M HCl→ 9.5ml HCl into 90.5ml H_2O

Appendix C: Nitric Oxide Materials and Reagents

Materials:

1. N-1-Naphthyl ethylenediaminedihydrochloride (NEDD)
2. Sulphanilamide (SULF)
3. Vanadium (III) Chloride
4. Sodium Nitrate
5. HCl

Reagents:

1. 1M HCl: Added 9.83ml of 32% HCl to 90.17ml dH_2O.
2. 5% HCl: Added 30ml HCl to 150ml dH_2O.
3. VCl_3: Dissolved 100mg VCl_3 in 12.5ml 1M HCl, Filter sterilize. Wrap in foil, stored at 4°C.
4. NEDD: Dissolved 10mg NEDD in 10ml dH_2O, stored at 4°C
5. SULF: Dissolved 200mg in 10ml of 5% HCl. Stirred on hot plate, stored at 4°C wrapped in foil.
6. Standards: Dissolved 6.06mg Sodium Nitrate in dH_2O. Prepare 8 serial dilutions (0-200 µM).

Appendix D:

Table 8.1: Fasting blood glucose levels of the non-diabetic and diabetic groups.

Week	NDC	DC	TVL	CAP	MET	GLIB
0	4.18 ± 0.3	4.04 ± 0.24	4.27 ± 0.1	3.99 ± 0.11	4.06 ± 0.06	4.4 ± 0.34
1	5.02 ± 0.26	24.09± 2.29	18.19±2.87	19.84± 4.27	23.14± 4.01	21.81± 2.85
2	4.62 ± 0.27	23.28± 1.62	23.84± 1.03	25.11± 0.95	22.27± 2.88	21± 1.6
4	4.72±0.12	28.18± 1.16	28.09± 2.02	29.54 ± 1.15	25.38± 2.37	23.58±2.06
6	4.78± 0.27	29.84± 1.26	27.36± 1.36	29.29± 0.97	25.53± 1.11	28.18± 1.46
7	4.32±0.2	30.08±1.17***	26.26±1.07***	30.7±1.42***[a]	25.62±3.22***[a]	27.63±2.11***[a]

Non-diabetic control (NDC), diabetic control (DC); *Tulbaghia violacea* 60mg/kg.bw (TVL), Captopril 10mg/kg.bw. (CAP), Metformin 250mg/kg.bw (MET) and Glibenclamide10mg/kg.bw (GLIB). All data are expressed as mean ± SEM

***Significantly different from non-diabetic control $p < 0.0001$

[a] Significantly different from *Tulbaghia violacea* 60mg/ kg.bw $p < 0.05$

Appendix E:

Table 8.2: Oral glucose tolerance test in the non-diabetic and diabetic groups.

Minutes	NDC	DC	TVL	CAP	MET	GLIB
0	3.27 ± 0.13	23.42 ± 1.40	23.66 ± 1.19	25.36 ± 1.22	23.00 ± 1.86	24.87 ± 1.52
15	5.11 ± 0.39	32.26 ± 0.62	26.80 ± 0.83	30.16 ± 1.14	29.82 ± 1.86	31.38 ± 1.62
30	4.05 ± 0.22	31.54 ± 0.94	31.44 ± 0.62	32.71 ± 0.39	30.80 ± 1.62	31.62± 1.28
45	3.67 ± 0.15	32.50 ± 0.62	29.88 ± 1.10	31.61 ± 0.51	29.85 ± 1.82	29.33 ± 1.08
60	3.50 ± 0.19	31.7 ± 0.73	28.54 ± 0.99	30.79 ± 0.43	28.80 ± 1.48	27.48 ± 1.28
90	3.13 ± 0.27	28.75 ± 1.52	27.48 ±1.07	27.86 ± 0.51	25.87 ± 2.42	28.40 ±1.58
120	3.35 ± 0.23	27.65 ± 1.51	25.94 ± 1.08	25.70 ± 0.49	22.78 ± 2.70	25.63 ± 1.18
180	3.02 ± 0.23	26.13 ± 1.22***	23.04 ± 0.81***	23.83 ± 0.37***	21.67 ± 2.65***	23.43± 1.67***

Non-diabetic control (NDC), diabetic control (DC); *Tulbaghia violacea* 60mg/kg.bw (TVL), Captopril 10mg/kg.bw. (CAP), Metformin 250mg/kg.bw (MET) and Glibenclamide10mg/kg.bw (GLIB). All data are expressed as mean ± SEM

***Significantly different from non-diabetic control p <0.0001