Cape Peninsula University of Technology

Effects of kolaviron–a *Garcinia kola* biflavonoid on biochemical and histological parameters in streptozotocin - induced diabetes and diabetic complications (nephrotoxicity and hepatotoxicity) in male Wistar rats

By

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DECLARATION

I, Omolola Rebecca Ayepola declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

…….. **……**

Signed **Date**

PREFACE

The thesis has been written in an article format based on the guidelines of the journal where it has been published or submitted for review and consists of seven chapters. Chapter one is a brief introduction on the meaning of diabetes and the worldwide endemic nature of the disease. It also summarizes the association between diabetes, oxidative stress and inflammation. Due to the limitation of the current anti-diabetic drugs, the need to search for medicinal plants that can target oxidative stress-inflammatory cytokines as alternative and/or complementary therapy to this disease and the associated complications was highlighted. The aims of the study were included at the end of the first chapter. Chapter two encompasses a detailed literature review on the involvement of oxidative stress and inflammation in diabetes and its associated complications. Kolaviron, a *Garcinia* biflavonoid complex with antioxidative and anti-inflammatory properties was proposed as a potential therapeutic candidate in the management of diabetes mellitus and its complications. Chapter three is the research article entitled "Kolaviron improved resistance to oxidative stress and inflammation in the blood (erythrocyte, serum and plasma) of streptozotocin-induced diabetic rats'' which has been published in the Scientific World Journal (Omolola R. Ayepola, Nicole L. Brooks and Oluwafemi O. Oguntibeju. *The Scientific World Journal*, 2014:1-8.). Chapter four is the research article entitled "Kolaviron**,** a *Garcinia* biflavonoid complex ameliorates hyperglycemia-mediated hepatic injury in rats via suppression of inflammatory responses" which has been published in the BMC Complementary and alternative medicine. (Ayepola *et al*., *BMC Complementary and Alternative Medicine*, 2013: 13;363). Chapter five is the research article entitled "Kolaviron modulates apoptosis by suppressing oxidative stress and inflammation in diabetes-induced nephrotoxic rats'' which is under review in *Phyomedicine* (International Journal of Phytotherapy and Phytopharmacology). Chapter six is the research article entitled "Effects of kolaviron on liver oxidative stress and beta-cell damage in streptozotocin-induced diabetic rats". Included in the last chapter of the thesis (Chapter seven) is a general discussion and conclusion of the entire study. The published articles are included in the appendix of the thesis

ABSTRACT

Diabetes mellitus (DM) results in severe metabolic imbalances and pathological changes in many tissues. Chronic inflammation and oxidative stress have been implicated in the pathophysiology of diabetes mellitus. *Garcinia kola (*Family: *Guttiferae)* is a plant well known for its ample medicinal values. The seed of the plant also known as 'bitter kola' due to its bitter taste is used as a masticatory agent in traditional hospitality, cultural and social ceremonies in Africa. Kolaviron (KV) is a defatted ethanol extract from the seeds of *Garcinia kola* (GK). Kolaviron has been shown in experimental models of diseases to have numerous beneficial effects due to the presence of flavonoids (mainly *Garcinia* biflavonoid (GB)-1, GB-2 and kolaflavanone). However, there is paucity of information regarding the possible effect of kolaviron on inflammatory mediators and oxidative stress in diabetes mellitus. Therefore, this study was carried out to investigate the potential beneficial effects of kolaviron on antioxidant status, inflammatory mediators and apoptosis. Other biochemical and histological alterations in the blood, liver and kidney of streptozotocin-induced diabetic rats were also evaluated.

A single intraperitoneal injection of freshly prepared solution of streptozotocin (50 mg/kg.b.wt.) in citrate buffer (0.1M, pH 4.5) was administered to overnight fasted rats for diabetes induction. Diabetes was confirmed by stable hyperglycemia (>18 mmol/l) in the tail blood glucose after 5 days of streptozotocin injection. Kolaviron (100 mg/kg b.wt.) was administered to diabetic rats (by gastric gavage) on the 6^{th} day after the induction of diabetes and treatment continued for 6 weeks (5 times weekly). The effects on blood glucose, body weight, organ (liver and kidney) weight, serum biochemical parameters, oxidative status, inflammatory mediators and histology of the liver, kidney and pancreas were assessed.

Kolaviron (KV) treatment lowered blood glucose in diabetic and normoglycemic rats and reduced glycated haemoglobin [HbA_{1C} (%)]. Plasma insulin level was raised in diabetic rats treated with KV. Histomorphometric analysis of the pancreas revealed increased β-cell area of pancreatic islets of kolaviron-treated diabetic group**.** The indices of organ (liver and kidney) damage were increased in diabetic rats. However, KV treatment protected against liver and kidney damage. The characteristic features of diabetic dyslipidemia such as elevated serum

triglyceride and cholesterol concentration which are major risk factors for cardiovascular disease were also significantly reduced in KV-treated diabetic rats.

Alteration in antioxidant enzymes status was observed in the liver, kidney and blood (erythrocyte, plasma and serum) of diabetic rats. Lowered catalase (CAT) activity was observed in the liver and kidney of diabetic rats while KV treatment significantly ($p < 0.05$) elevated catalase activity in the liver and kidney. There was no significant change ($p > 0.05$) in erythrocyte catalase activity among all treatment groups. Erythrocyte of diabetic rats showed a marked reduction in the activity of superoxide dismutase (SOD) with no significant changes in liver and kidney SOD activity of diabetic rats compared to control whereas KV administration to rats markedly increased SOD activity. Glutathione peroxidase (GPX) activity was elevated in the erythrocyte and kidney of STZ-induced diabetic rats with no significant effect on liver GPX activity. KV treatment reversed the alteration in GPX activity in the kidney and erythrocyte.

Level of reduced glutathione (GSH), a non-enzymatic antioxidant was decreased in the both liver and kidney of diabetic rats and treatment of diabetic rats with KV elevated GSH concentration in both tissues. Also, malondialdehyde (MDA), a marker of lipid peroxidation was elevated in the liver, kidney and plasma of diabetic rats and significantly ($p < 0.05$) lowered following KV treatment. Diabetes induction reduced the capacity of liver and kidney to absorb oxygen radicals as demonstrated by lowered oxygen radical absorbance capacity (ORAC) values. KV administration to normal and diabetic rats significantly increased ORAC values.

Increased rate of apoptosis, a major cellular response to high glucose induced stress was observed in the renal and hepatic tissues of diabetic control rats. Kolaviron treatment of diabetic rats protected the liver and kidney against hyperglycemia-induced apoptosis and decreased the number of TUNEL positive cells

A significant ($p < 0.05$) elevation of pro-inflammatory cytokines; monocyte chemoattractant protein (MCP-1), Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor (TNF)-**α** was observed in the liver of diabetes rats. KV treatment lowered these inflammatory biomarkers. On the other hand, the kidney of diabetic rats showed elevated concentration of pro-inflammatory IL-1β with no significant effect on kidney TNF-**α**. An increase in the serum concentration of MCP-1 and

IL-1β was observed in the untreated diabetic rats while kolaviron treatment normalized the alteration in serum concentration of MCP-1, IL-1β and vascular endothelial growth factor (VEGF).

In conclusion, persistent and chronic hyperglycemia promotes the generation of free radicals and inflammatory molecules which contributes to progressive development of micro- and macro vascular complications and multi-organ damage. Kolaviron demonstrated beneficial effects on markers of oxidative stress and inflammation in the diabetic rats and also promoted the survival and functional integrity of the liver and kidney.

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DEDICATION

This thesis is dedicated to God almighty.

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GLOSSARY

Abbreviations	Definition / Explanation
8- OHdG	8-hydroxy-2'-deoxyguanosine
$O_2^{-\cdot}$	Superoxide radical
$ONOO^-$	Peroxynitrite
AGE's	Advanced-glycation end products
ALT	Alanine amino transferase
ANOVA	Analysis of variance
AST	Aspartate transaminase
CAT	Catalase
CVD	Cardiovascular disease
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ET-1	Endothelin-1
GB	*Garcinia* biflavonoid
GH	Glycogenic hepatopathy
GK	*Garcinia kola*
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase

GLUT	Glucose transporter
HPLC	High performance liquid chromatography
IDDM	Insulin-dependent diabetes mellitus
IL-1 β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
KV	Kolaviron
LDL	Low density lipoprotein
MDA	Malondialdehyde
MCP-1	Monocyte chemo-attractant protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NF-ĸB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDM	Non- Insulin-dependent diabetes mellitus
NOS	Nitric oxide synthase
PARP	Poly (ADP-ribose) polymerase
PKC	Protein kinase C
PLA_2	Phospholipase A_2
RAGE	Receptor for Advanced Glycation End products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SOD	Superoxide dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid-reactive substances
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor- alpha
TUNEL	Terminal deoxynucleotidyl transferased UTP nick end labeling
VEGF	Vascular endothelial growth factor

CHAPTER ONE

Introduction

The condition of diabetes has been known since around the first century B.C.E., when a Greek physician, Aretus the Cappadocian, named it *diabainein*, meaning "a siphon," or "to pass through," a reference to increased urination (polyuria), a common symptom of the disease. The word *diabetes* was first recorded in 1425. In 1675, the Latin word *mellitus*, meaning "like honey," was added to the name, to reflect the sweet taste of the patient's urine due to the presence of glucose (glycosuria). Diabetes mellitus is sometimes referred to as sugar diabetes but usually it is simply called diabetes. There is also a rare disease called diabetes insipidus (water diabetes) in which the kidneys release too much water. Like diabetes mellitus, it has excessive urination as a symptom, but these two endocrine disorders are otherwise unrelated (Diabetes Control and Complication Trial (DCCT), 1993). Diabetes mellitus (DM) is a serious metabolic disorder resulting from a defect in insulin secretion, insulin action, or both (WHO, 1999). Insulin is a hormone produced in the pancreas. Insulin deficiency leads to chronic hyperglycemia (high blood glucose) with disturbances of carbohydrate, fat and protein metabolism. The effects of DM include long term damage, dysfunction and failure of various organs, especially the pancreas, liver, kidney, eyes (retinopathy), kidneys (nephropathy), nerves (neuropathy), livers, hearts, and blood vessels (Bennett & Knowler, 2005).

The United Nations (UN) has accorded diabetes a right of place as a global jeopardy and chronic killer (Diabetes Care and Research in Europe, 1990; WHO, 1994; Alberti *et al*., 2007). The UN defines DM as a chronic, debilitating, and costly disease associated with severe complications which pose severe risks for families, member states, and the entire world; and serious challenges to the achievement of the internationally agreed developmental goals, including the millennium development goals (MDGs) (Oputa & Chinenye, 2012). The rapidly increasing prevalence of DM worldwide is one of the most serious and challenging health problems in the 21st century. According to the 2012 estimate of the International Diabetes Federation (IDF) as shown in figure 1, 371 million adults, aged 20–79 years, of the world's population have diabetes, giving a prevalence of 8.3% and that the number of diabetic patients would increase to 552 million in

2030. IDF also estimated that 4.6 million and 4.8 million people died in 2011 and 2012 respectively due to diabetes complications.

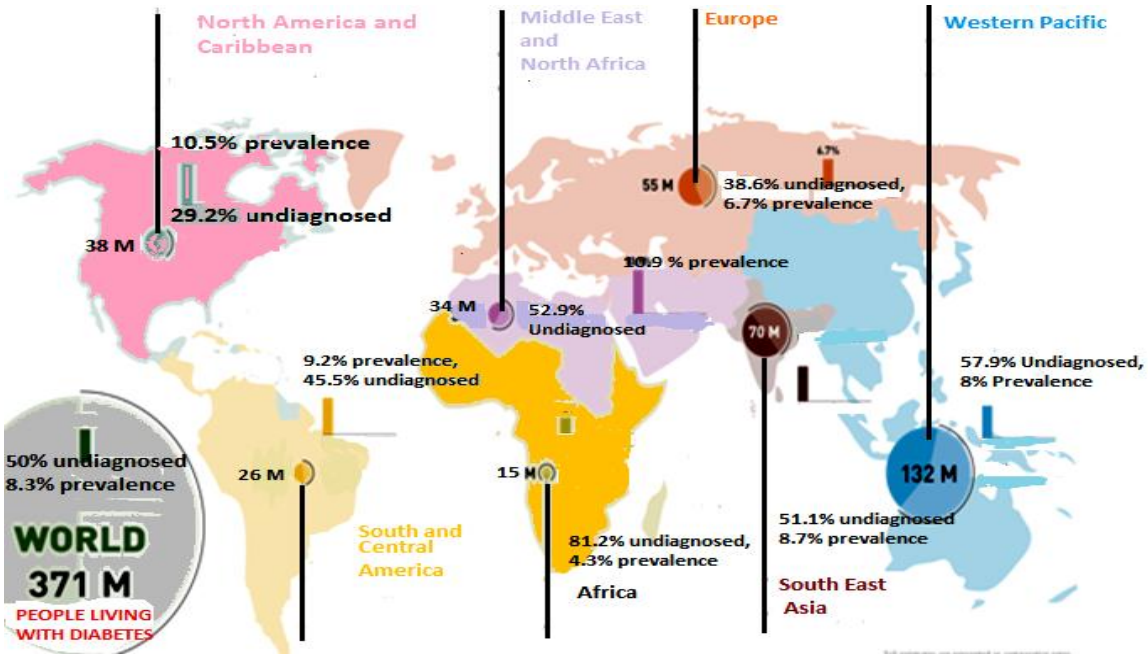

Figure 1: Regional estimates of diabetes by the International Diabetes Federation in 2012

Source: IDF, 2012

- *North America and Caribbean*; More dollars were spent on diabetes here than any other region. About 1 in 10 adults has diabetes.
- In the *Middle East and North Africa*, 1 in 9 adults have diabetes.
- *Europe*: 21.2 million People in this region have diabetes and are not aware of it.
- *Western Pacific*; One in three adults with diabetes lives in this region.
- *South and Central America*; One in eleven adults in this region have diabetes
- *Africa*; This region have the highest mortality rate due to diabetes. Also, over the next 20 years, the number of people with diabetes in the region will almost double
- *South East Asia*; One in five of all undiagnosed cases of diabetes is in this region. One in four deaths due to diabetes occurred in this region.

Chronic hyperglycemia is known to damage almost all cell types in the body. A relationship has been established between hyperglycemia, oxidative stress and numerous pathways which can lead to organ and tissue damage. Oxidative stress occurs as a result of excessive formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) collectively describing free radicals and an imbalance between the rate of free radical scavenging by the antioxidant system. Some of the mechanisms by which hyperglycemia causes oxidative stress includes, increased production of the superoxide anion in the mitochondria (Kiritoshi *et al.,* 2003), non enzymatic glycation of proteins (Baynes, 2003), glucose auto-oxidation (Wolff, 1993) and increased levels of inflammatory mediators (Navarro *et al.,* 2011).

An increase in inflammatory cytokines IL-6 (Interleukin-6), IL-18 (Interleukin -18), IL-1 (Interleukin -1) and TNF-α (Tumour necrosis factor alpha) has been reported in the blood of patients with diabetes mellitus (Esposito *et al*. 2002; Foss *et al*. 2007). Hyperglycemia can also activate NF-KB (nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor leading to an increase in the expression of inducible nitric oxide synthase (iNOS) which is accompanied by an increased generation of nitric oxide (Spitaler & Graier, 2002). Nitric oxide can react with superoxide to produce the strong oxidant peroxynitrite, which in turn can increase lipid peroxidation, protein nitration, and low density lipoprotein (LDL) oxidation, affecting many signal transduction pathways (Griendling & FitzGerald, 2003). Hyperglycemia-induced oxidative stress has been demonstrated to result in beta cell dysfunction and death (Donath *et al*., 1999; Robertson *et al*., 2000), as well as in fibrosis of pancreatic islets (Hayden, 2007; Hong *et al*., 2007).

Diabetes is also associated with the production of pro-apoptotic factors and induction of apoptosis in diabetic liver and renal tubular cells (Stitt-Cavanagh *et al.,* 2009; Kim *et al.,* 2011). The initial cellular response to a high glucose challenge is the generation of ROS, which rapidly induces apoptotic cell death (Park *et al.,* 2001). As the disease progresses, tissue or vascular damage develops, leading to severe diabetic complications such as nephropathy (Huang *et al.,* 2002), chronic liver injury (Kim *et al.,* 2009), retinopathy (Bearse *et al.,* 2004; Hove *et al.,* 2004), neuropathy (Seki *et al.,* 2004), cardiovascular complications (Saely *et al.,* 2004; Svensson *et al.,* 2004) and ulceration (Wallace *et al.,* 2000).

Many drugs such as alpha-glucosidase inhibitors, sulfonylurea, metformin, thiazolidinediones and meglitinides have been used in the treatment of diabetes in addition to insulin. However, their use is limited due to their side effects such as; abdominal bloating, hypoglycemia, gastrointestinal (GI) disturbances, liver injury, and hypersensitivity reactions. The World Health Organization (WHO) has since recommended the evaluation of the effectiveness of plants in diabetic conditions especially where safe modern drugs are lacking or not readily accessible (World Health Organization, 1980) therefore the search for natural, safe anti-diabetic plant products is therefore on-going.

The administration of antioxidant-rich compounds could be a promising therapeutic approach in the management of diabetes. Many plants containing flavonoids, glycosides, alkaloids, terpenoids and carotenoids have been shown to exhibit anti-diabetic effects (Loew & Kaszkin, 2002). Flavonoids are phenolic phytochemicals that are thought to promote health, partly via their antioxidant and antiinflammatory effects in protecting cellular components against reactive oxygen species (ROS). *Garcinia kola* seeds (Family: Guttiferae) are eaten in West and Central Africa and are known to contain a high amount of biflavonoids. The biflavonoids of *Garcinia kola* have been shown to be pharmacologically active with several pharmacokinetic advantages over simple monomeric flavonoids as they survived first-pass metabolism (Iwu, 1986).

Kolaviron is a fraction of the defatted ethanol extract of *Garcinia kola,* containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone as its major components (Iwu, 1985). Results of some studies have revealed the protective effects of kolaviron against hepatotoxicity and oxidative stress induced by carbontetrachloride (Farombi, 2000), 2-acetylaminofluorene (Farombi *et al*, 2000) aflatoxin B 1 (Farombi *et al*., 2005) and potassium bromate-induced nephrotoxicity (Farombi *et al*., 2002) via scavenging of reactive metabolites and induction of increases in carcinogen detoxifying enzymes (Farombi *et al.*, 2002; Farombi, 2003). In addition, the ability of kolaviron to inhibit hydroxyl and superoxide anion radicals, which are known to play an important role in the process of lipid peroxidation, has also been demonstrated (Farombi & Nwaokeafor, 2005). Considerable experimental evidence has been adduced in support of the antihepatotoxic efficacy of kolaviron in animals against such hepatotoxicants as paracetamol),

carbon tetrachloride, thioacetamide, galactosamine, phallodine, and ethanol (Iwu *et al*., 1987; Akintonwa & Essien, 1990; Farombi *et al*., 2000; 2005).

1.1 Aim

Although the hypoglycemic effect of kolaviron has been demonstrated in diabetic rats, no study has explored the effect of kolaviron on oxidative stress, inflammation and apoptosis which are characteristic features of diabetes mellitus and which has been implicated to play a major role in the progression to the complications that accompanies the disease. This study investigated the effects of kolaviron (KV) administration in a streptozotocin-induced diabetic rat model examining its effects (i.e KV) on diabetic-induced liver injury and nephrotoxicity in rats. Imunohistochemical staining and morphometric analysis of the pancreas was also carried out to determine the possible regenerative effects of kolaviron on the pancreas. Specific alterations in the blood (serum, plasma and erythrocyte) and organs of diabetic rats and the effect(s) of kolaviron were investigated through the assessment of the following biochemical parameters:

1. Antioxidant status
2. Inflammatory proteins
3. Apoptotic index
4. Biomarkers of liver and kidney function
5. Glycaemic and lipidaemic parameters
6. Histological alteration in the liver and kidney

CHAPTER TWO

Literature Review

Uptake of blood glucose from the bloodstream into the cells, liver, skeletal muscle, and adipose tissue is promoted by insulin (Prabhakar & Doble, 2008). All tissues have energy requirement that is usually met by metabolizing glucose. The maintenance of glucose homeostasis by insulin depends on the secretion of appropriate insulin and the efficacy of the secreted insulin to stimulate uptake of glucose into peripheral tissues. Diabetes mellitus is a syndrome characterized by chronic hyperglycemia and disturbances of carbohydrate, fat and protein metabolism associated with absolute or relative deficiency in insulin secretion or insulin action (Jayakar & Suresh, 2003).

The human pancreas is composed basically of 2 types of secretory cells- exocrine (98%) and endocrine (2%) cells. The small endocrine secretory cells are of 4 main types namely; alpha, beta, delta and pancreatic polypeptide cells distributed throughout the pancreas in areas known as islets (Kloppel & In'tVeld, 1997). The beta cells synthesize the 51 amino acid peptide insulin which maintains glucose homeostasis. Insulin is first synthesized as preproinsulin by the ribosomes of the rough endoplasmic reticulum (Figure 1). It is then cleaved into proinsulin and transported into the golgi apparatus for packaging into secretory granules. The granules undergo further cleavage to give insulin and C-peptide and stored until it is released by an appropriate stimulus (Fu *et al.,* 2013). Insulin biosynthesis by the pancreatic beta cells is regulated transcriptionally and translationally. Glucose is the main regulator of insulin secretion although, it can also be regulated by the availability of other nutrients (such as amino acids, fatty acids), growth factors and hormones (such as melatonin, leptin, estrogen) (Fu *et al.,* 2013). Structural deterioration of the pancreatic islets is a key factor in both type 1 and type 2 forms of DM (Ryu *et al.*, 2001; Meier *et al*., 2005; Hayden, 2007). Severe beta cell loss is found in type 1 DM while islets without severe loss of beta cells are usually seen in type 2 DM (Kloppel & In'tVeld, 1997).

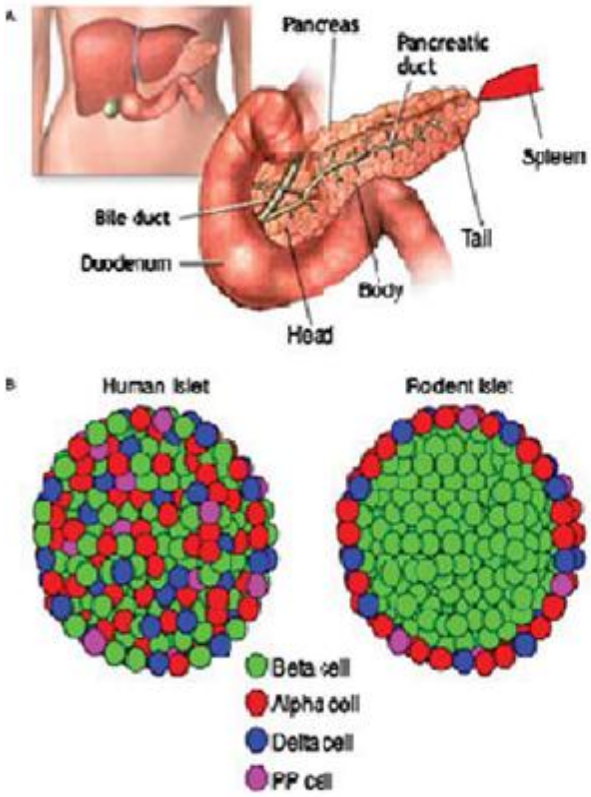

Figure 1a:Architecture of the pancreas and cell distribution within the islets of Langerhans

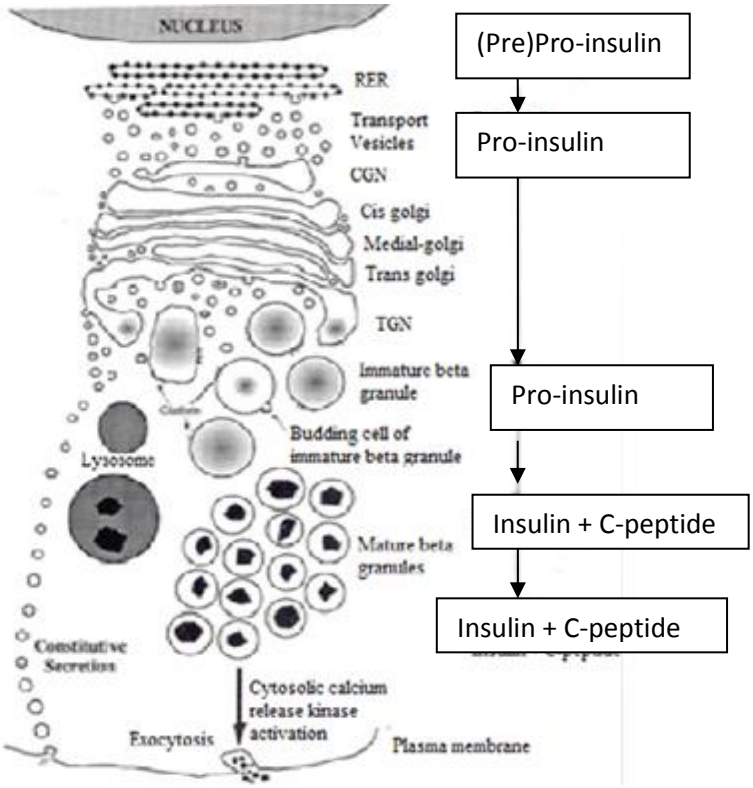

Figure 1b: Insulin Secretion Pathway in the β-cells

Source: Martinic & Herrath, 2008

Once insulin has entered the circulation, it is bound by specialized receptors found on the membrane of most cells. This binding of insulin to its receptor is highly specific and triggers a lot of biological responses especially in the liver, muscle and adipose tissues. Insulin promotes the storage of glucose and fat within these target cells and influences cell growth and the metabolism of a wide variety of tissues (Nolte & Karam, 2001). In the liver, insulin acts to increase the storage of glucose as glycogen and resets the liver to the fed state by inhibiting processes such as glycogenolysis, gluconeogenesis and ketogenesis. This is achieved by insulin-induced phosphorylations, which activates glucokinase, phosphofructokinase and pyruvate kinase, while inactivating glucose-6-phosphatase, fructose bisphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase (Nolte & Karam, 2001). Due to the pivotal role of the liver in glucose and lipid homeostasis, it is severely affected during diabetes with major

biochemical and functional abnormalities, including alterations in carbohydrate, lipid, and protein metabolism, and changes in the antioxidant status (McLennan *et al.,* 1991, Saxena *et al.,* 1993, Harrison *et al.,* 2006). Hence, the prevalence of hepatobiliary diseases is increased in patients with either Type 1 or Type 2 diabetes (Saxena *et al.,* 1993; Bell & Allbright, 2007). The kidney also contributes to glucose homeostasis by; filtering and reabsorbing or excreting glucose, protecting the body against wide variations in glucose demand and glucose supply and accounting for one-third to one-half of the metabolic clearance of insulin and glucagon (Gerich, 2010). Due to the kidney's involvement in glucose homeostasis, a progressive decline in kidney function is also observed in the diabetic state.

2.1 Mechanisms of action of Streptozotocin (STZ)

The selective destruction of the pancreatic beta cells by chemical agents has been used to establish diabetic animal models. Streptozotocin (STZ) is a diabetogenic agent that is selectively toxic to insulin-secreting beta-cells of the pancreatic islets which induces impairment of islet glucose oxidation and of glucose-induced insulin secretion. STZ, a methylnitrosourea with a 2-substituted glucose, causes beta-cell necrosis and insulin-dependent diabetes mellitus in many species (Merzouk *et al.,* 2000; Hayden & Tyagi, 2002). The mechanism of action of STZ on rodent beta cells is well known. The glucose moiety allows preferential uptake of STZ into beta cells, probably via the glucose transporter (GLUT)-2. Intracellular metabolism of STZ yields nitric oxide that precipitates additional DNA strand breaks. Within 48hr, severe insulin dependent diabetes ensues, characterized histologically by massive β cell necrosis and macrophage infiltration (Hua & Wright, 2002; Hayden & Tyagi, 2002). Streptozotocin induces diabetes in rats that mimic the human hyperglycaemic diabetes mellitus (Weir *et al.,* 1981) hence justifying its selection as a diabetogenic agent in experimental studies. The molecular structure of STZ was first described by Herr *et al.,* (1967). The structure of STZ (figure 2) is composed of nitrosourea moiety with a methyl group attached at one end and a glucose molecule at the other end. STZ has a molecular weight of 265g/mol.

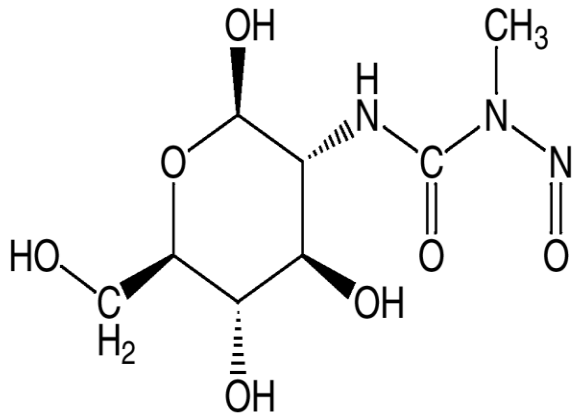

Figure 2: Structure of Streptozotocin (STZ).

2.2 Classification of diabetes

The two major types of Diabetes mellitus (DM) are insulin dependent (IDDM) - Type 1 and non-insulin dependent (NIDDM) - Type 2. Type 1 is present in patients who have little or no endogenous insulin secretory capacity and require insulin therapy for survival while Type 2 is characterized by a combination of insulin resistance coupled with insufficient production of insulin to overcome the insulin resistance (DeFronzo *et al*., 1997; Zimmet *et al*., 2004).

2.2.1 Type 1 diabetes mellitus (DM)

Type 1 DM is characterized by a specific destruction of the pancreatic β cells, commonly associated with immune-mediated damage (Zhao, 2011). Type 1 diabetes represents around 10% of all cases of diabetes. Although type 1 diabetes affects all age groups, the majority of individuals are diagnosed either at around the age of 4–5 years, or in their teens and early adulthood (Bloom *et al*, 1975). The disease recognizes two major subtypes: 1A (autoimmune) and 1B (idiopathic) (Vlad & Timar, 2012).

Autoimmune Diabetes Mellitus

Autoimmune diabetes mellitus is a genetically determined chronic immune-mediated disorder that leads to selective loss of pancreatic insulin-secreting β-cells and is associated with several

immunologic abnormalities. The rate of autoimmune mediated destruction of the beta cells of the pancreas is rapid in some individuals and slow in others (Zimmet *et al*., 1994). The rapidly progressive form is commonly observed in children, but also may occur in adults (Humphrey *et al.*, 1998). The slowly progressive form generally occurs in adults and is sometimes referred to as latent autoimmune diabetes in adults (LADA).

Although the genetic susceptibility to type 1diabetes is inherited, only 12–15% of this subtype of type 1diabetes occurs in families and the discordance of type 1 diabetes in monozygotic twins shows that genetic factors alone will not explain the aetiology of the disease. Asides genetic factors, other environmental triggers, including viral infections, nutritional factors, parental age and low birth weight, have been implicated in the development of type 1 diabetes (Akerblom *et al*., 2002).

Idiopathic Diabetes Mellitus

This is a form of Type 1 diabetes with no known aetiology. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity (McLarty *et al*., 1990).

2.2.2 Type 2 diabetes mellitus (T2DM)

Type 2 diabetes mellitus is the most common form of diabetes, accounting for 85 to 95%. Unlike Type 1, Type 2 diabetes is not caused by autoimmune destruction of the pancreatic β cells.Type 2 diabetes is a heterogeneous disorder characterized by insulin resistance coupled with impaired insulin secretion by β-cells in the pancreas (Kahn, 2008*)*. The three key defects in the onset of hyperglycemiain T2DM are;

(1) increased hepatic glucose production
(2) Reduced insulin secretion and
(3) Impaired insulin action/insulin resistance (DeFronzo *et al*. 1992, Stumvoll e*t al*. 2005).

Insulin resistance refers to suppressed or delayed responses to insulin. Insulin resistance is generally ‘post-receptor’, which refers to a problem with the cells that respond to insulin rather than a problem with insulin production.

There is strong evidence that Type 2 diabetes is inherited and has a genetic origin. Also, very high concordance rates of Type 2 diabetes have been reported in monozygotic twins and about 25% of those with the disease have a family history of DM (Rother, 2007). There is also strong evidence for a significant environmental causal involvement in Type 2 diabetes. A number of lifestyle factors are known to be important to the development of type 2 DM. These are physical inactivity, sedentary lifestyle, cigarette smoking and generous consumption of alcohol. Obesity has been found to contribute to approximately 55% of cases of type 2 DM (Olokoba *et al*., 2012).

2.2.3 Other types

Other specific types of diabetes are due to other causes, e.g., genetic defects in β cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (these include drugs that are directly toxic to the β cell, such as cyclosporine and pentamidine, or drugs that worsen insulin resistance, such as glucocorticoids and high-dose thiazide diuretics) (Holt, 2004; ADA, 2013). Gestational diabetes mellitus (GDM) is also another type of diabetes diagnosed during pregnancy that is not clearly overt diabetes.

2.3 The diagnostic criteria for diabetes

- Glycated haemoglobin (A_{1C}) > 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT (Diabetes Control and Complications Trial) assay.
- Fasting blood glucose (FBG) >126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 hr.*
- 2-h plasma glucose > 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT). The test should be performed as described by the World Health Organization (WHO), using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water.*
- In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose > 200 mg/dL (11.1 mmol/L).

* In the absence of unequivocal hyperglycemia, result should be confirmed by repeat testing (ADA, 2013).

2.4 Symptoms of diabetes

- Polydipsia - Excessive thirst
- Polyuria- Excessive urination and dehydration
- Polyphagia- Excessive hunger or appetite
- Unexplained weight loss
- Blurred vision, nearsightedness or other vision problems
- Frequent infections, including skin infections, thrush, gingivitis, urinary tract infections and yeast infections
- Slow healing of sores
- Skin problems, such as itchiness or acanthosis nigricans
- Fatigue, lethargy or drowsiness
- Shakiness or trembling
- Dizziness or fainting
- Numbness, tingling or pain in the feet, legs or hands (Riaz, 2009).

2.5.1 The complications of Diabetes Mellitus (DM)

Chronic hyperglycaemia is known to damage almost all cell types in the body. The injurious effects of hyperglycemia are separated into microvascular (involving small vessels, such as capillaries) and macrovascular complications (involving large vessels, such as arteries and veins). Microvascular complications include diabetic nephropathy, neuropathy, and retinopathy, hepatopathy while macrovascular complications include coronary artery disease, peripheral arterial disease, and stroke (Fowler, 2008).

2.5.1 Diabetic Nephropathy (DN)

Diabetic nephropathy is a major cause of end-stage renal disease worldwide. It is a progressive decline in the glomerular filtration rate, characterized by glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix proteins (ECM) (Jain, 2012). Alteration of the permeability characteristics of the glomerular capillary wall manifests clinically as abnormal albuminuria (Lewis & Xu, 2008).

Microalbuminuria progresses to end-stage renal disease through a number of stages including normoalbuminuria, microalbuminuria and macroalbuminuria (O'Connor & Schelling, 2005).

2.5.2 Diabetic Retinopathy (DR)

DR results from the damage of the small vasculature of the retina, multi cellular and the light sensitive tissue at the back of the eye. It is a major cause of visual impairment worldwide (Marshall & Flyvbjerg, 2006; Santos *et al*., 2011). The retina capillaries are lined with endothelial cells responsible for maintaining the blood retinal barrier, and are surrounded by smooth muscle cells, pericytes, which provide tone to the vessels (Santos *et al*., 2011). The vascular lesions that are identified at the early stage of diabetic retinopathy include pericytes disappearance from capillaries resulting in pericyte ghosts, obliteration of capillaries and small arterioles, gradual thickening of vascular basement membrane, increased permeability of endothelial cells, and formation of microaneurysms (i.e. weakening of vessel walls that results in the projection of a balloonlike sac), vessel leakage, exudate, and hemorrhage (Engerman, 1989; Hammes, 2005).

2.5.3 Diabetic Neuropathy

Neuropathies are characterized by a progressive loss of nerve fiber function. A widely accepted definition of diabetic neuropathy is "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with mellitus after exclusion of other causes" (ADA, 2007). In the peripheral nervous system, diabetes causes a progressive deterioration of sensory nerves and damage to motor nerves (Pazdro & Burgess, 2010). Diabetic neuropathy is ultimately the leading cause of lower extremity amputation (Obrosova, 2009). Peripheral neuropathy is thought to develop because of cellular damage to endothelial cells, affecting nerve blood flow and also damage to the neurons affecting conductivity of impulses (Obrosova, 2009). Signs and symptoms of diabetic neuropathy include decrease or no sweating, numbness, or tingling, and some sort of burning sensation, weakness and loss of reflexes (Soumya & Srilatha 2011).

2.5.4 Diabetic Cardiomyopathy

Both Type 1 and 2 diabetes are powerful and independent risk factors for coronary artery disease (CAD), stroke, and peripheral arterial disease (Schwartz *et al*., 1992; ADA, 1993; Orchard*et al*.,

2006). Diabetics have a 2- to 4-fold higher risk for cardiovascular events (Ding & Triggle, 2005) and nearly 80% of diabetes-associated deaths are caused by cardiovascular disease (CVD) (Winer & Sowers, 2004). Atherosclerosis, (excessive accumulation of lipids, cholesterol, inflammatory cells, and connective tissue in the vessel wall) accounts for more than 80% of the CVD-associated death and disability (Epstein & Ross, 1999; Libby *et al*., 2011). Formation of atherosclerotic plaques can result in occlusion of vessel lumen and a rapid cessation in blood flow to target tissue (Funk *et al*., 2012). Hyperglycemia, increased free fatty acids, and insulin resistance induce a large number of alterations at the cellular level that contribute to vascular dysfunction and accelerate the atherosclerotic process. These include increased oxidative stress, decreased bioavailability of nitric oxide (NO), disturbances of intracellular signal transduction and increased production of several prothrombotic factors (Creager & Lüscher, 2003; Funk *et al*., 2012).

2.5.5 Diabetic Hepatopathy

The effects of diabetes on the kidneys, eyes, heart and nerves are well known, but less emphasized on the liver. Diabetic hepatopathy is a type of advanced liver disease which is characterized by liver cirrhosis, liver failure or the need for a liver transplant. Diabetes, by most estimates, is now the most common cause of liver disease in the U.S.A and wide spectrum of liver disease is seen in patients with type 2 diabetes (Tolman *et al*., 2007). This includes abnormal liver enzymes, nonalcoholic fatty liver disease (NAFLD), cirrhosis, hepatocellular carcinoma, and acute liver failure (Abolfathi *et al*, 2011). Cryptogenic cirrhosis, of which diabetes is, by far, the most common cause, has become the third leading indication for liver transplantation in the U.S. (Caldwell *et al.,* 1999) accounting for 12.5% of deaths in patients with diabetes (Balkau *et al*., 1991). The standardized mortality ratio (SMR) for cirrhosis was 2.52 compared with 1.34 for cardiovascular disease (CVD). Glycogenic hepatopathy (GH) is characterized by abnormal glycogen accumulation in hepatocytes, a major cause of hepatomegaly in type 1 diabetes mellitusalthough it has been recently reported in type 2 (Saadi, 2012; Jardim *et al*., 2013). GH presents with abdominal pain, nauseas, vomiting, hepatomegaly and elevated liver enzymes (Sweetser & Kraicheyl, 2010; Saxena *et al*., 2010).

2.6 Involvement of oxidative stress in diabetes mellitus

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Biological free radicals are highly unstable molecules which are products of normal cellular metabolism. They have electrons available to react with various organic substrates such as lipids, proteins and deoxyribonucleic acid (DNA). Free radicals are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko *et al.,* 2007). At low or moderate levels free radicals (ROS and RNS) exerts beneficial effects such as defense against infectious agents, induction of a mitogenic response and the maturation process of cellular structures (Droge, 2002; Pacher *et al*., 2007; Sachdev & Davies, 2008). ROS include superoxide anion ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), hydrogenperoxide (H_2O_2) and hypochlorous acid (HOCl) while RNS include nitric oxide ($^{\cdot}NO$), nitrogen dioxide ($NO_2^{\cdot}$) and peroxynitrite ($OONO^{-}$) (Sies, 1991; Halliwell & Gutteridge, 2007). High concentrations of free radicals on the other hand result in deleterious processes that can damage cell structures due to oxidative stress (Bahorun *et al*., 2006; Halliwell, 2007).

Numerous experimental evidences have highlighted a direct link between oxidative stress and diabetes through the measurement of oxidative stress biomarkers in both diabetic patient and rodents. A hyperglycemic state can lead to an increase in the levels of oxidative DNA damage markers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG); lipid-peroxidation products measured as thiobarbituric acid-reactive substances (TBARS); protein oxidation products such as nitrotyrosine and carbonyl levels and also lower the activity of antioxidant enzymes. Cell culture studies using pancreatic beta cells, aortic smooth muscle cells and endothelial cells have also provided evidence for an increase in ROS production in diabetes (Inoguchi *et al*., 2000; Lee *et al*., 2010).

Exposure of β-cell line and isolated pancreatic islet cells to oxidative stress has been shown to inhibit the promoter activity and mRNA expression of the insulin gene therefore, decreasing insulin gene expression (Hanazaki, 2009). Oxidative stress is also strongly suspected to be involved in chronic hyperglycemia-induced insulin resistance (Eriksson, 2007).

2.7 Pathways of free radical generation in diabetes mellitus and its associated complications

In diabetes, ROS is thought to be generated through increased polyol pathway (Chung *et al*., 2003), increased formation of advanced-glycation end products (AGEs) (Baynes & Thorpe, 1999) and protein kinase C (PKC) activation (Inoguchi *et al*., 2003) resulting in damage to macromolecules and organ malfunction as shown in figure 3.

2.7.1 Aldose reductase pathway

Aldose reductase is the rate limiting enzyme of the polyol pathway. This nicotinamide adenine dinucleotide phosphate (NAD(P)H)-requiring aldose reductase, catalyses the reduction of glucose to sorbitol followed by the oxidation of sorbitol to fructose by NAD^+ dependent sorbitol dehydrogenase. At normal blood glucose concentration (5.5 mM), aldose reductase catalyzed reaction represents less than 3% of total glucose utilization (Morrison *et al*., 1970). However, hyperglycemia results in saturation of hexokinase and more than 30% of glucose is directed into the polyol pathway (Gonzalez *et al*., 1984). In a diabetic state, polyol pathway increases in tissues that do not require insulin for cellular glucose uptake, such as retina, kidney, peripheral nerves and blood vessels (Stephen *et al*., 2003).

The overall reaction of the polyol pathway leads to a shortage of intracellular NAD(P)H and a surplus of NADH, i.e, a reductive imbalance. Increased NADH generation during conversion of sorbitol to fructose provides substrate for NADH oxidase to generate ROS (Morre, 2000). NADH serves as a source of electrons in complex 1 of the electron transport chain resulting in increased mitochondrial generation of superoxide radical. In diabetic cells, oxidative phosphorylation in mitochondria is enhanced due to increase flux of electron donors into the electron transport chain. This drives the inner mitochondrial membrane potential upward causing blockage of electron transfer inside complex III (Yang and Trumpower, 1990). Electrons back up to coenzyme Q results and electrons are transferred one at a time to molecular oxygen, generating superoxide. DNA damage by superoxide and peroxynitrite results in the activation of poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme. PARP reduces the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (an enzyme of the glycolytic pathway which catalyses the conversion of glyceraldehyde-3-phosphate to 1, 3-biphosphoglycerate) by

ADP- ribosylation (Soriano *et al*., 2001; Du *et al*., 2003). A consequence of GAPDH inhibition by PARP is an increase in triose phosphate pool, upstream of GAPDH and increase flux of intermediates into the damaging pathways of diabetic complications.

The polyol pathway also results in reduction in the bioavailability of NAD(P)H. The reduced bioavailability of NAD(P)H negatively affects the antioxidant defence system by depleting glutathione (GSH) a very important antioxidant. This is because the activity of GSH reductase, an antioxidant enzyme that generates GSH from its oxidized form (GSSH) depends onNAD(P)H. Depletion of NAD(P)H also decreases the synthesis of nitric oxide ($^{\cdot}NO$), a vaculoprotective agent. NAD(P)H serves as a cofactor for nitric oxide synthase (NOS) which synthesizes $^{\cdot}NO$ from L-arginine. If endothelial nitric oxide synthase (eNOS) lack its substrate, L-arginine or one of its co-factor, it may produce superoxide radical ($^{\cdot}O_2^-$) instead of $^{\cdot}NO$ and this is referred to as ‘‘uncoupled state of nitric oxide’’(Vasquez-Vivar *et al*., 1998). Nitric oxide performs several physiological roles such as inhibition of platelet activation, vascular relaxation (Mehta *et al*., 2006) and acts as an anti-inflammatory agent by reducing platelet aggregation and adhesion (Sharma *et al*., 2007). These properties inhibit atherogenesis and protect the blood vessel. Reduced bioavailability of $^{\cdot}NO$ level will therefore increase inflammation, enhance thrombosis and disrupt the integrity of endothelial cells. Reduction in $^{\cdot}NO$ has been documented in diabetes subjects with nephropathy (Tessari *et al.*, 2010). Superoxide anion directly quenches $^{\cdot}NO$ by forming highly reactive peroxynitrite ($ONOO^-$) which initiates lipid peroxidation, oxidizes sulfhydryl group in protein and nitrates amino acids such as tyrosine, thereby affecting many signal transduction pathways. The polyol pathway serves as a main source of ROS generation in the retina (Mara & Oates, 2008). In addition, sorbitol accumulation has been implicated in osmotic swelling of the eye lens and cataractogenesis (Patel *et al.*, 2011).

2.7.2 Advanced Glycation Endproducts (AGEs) formation pathway

Glucose can react spontaneously with free amino groups of protein to form Schiff bases. These Schiff bases through complex reactions such as amadori rearrangement, dehydration and condensation forms cross-linked heterogeneous fluorescent derivatives called advanced glycation end products (AGEs). Advanced glycation end products constitute a heterogeneous group of molecules formed by non-enzymatic reactions of reducing sugars, ascorbate and other

carbohydrates with amino acids, lipids and nucleic acids (Vlassara & Palace., 2002; Peppa *et al*., 2004). Glycation end product's adducts such as pyraline, pentosidine and N- Carboxy- methyl lysine (CML) are found to be elevated in diabetic tissues (Stitt, 2001; Wautier & Gullausseau, 2001; Ahmed, 2005).

Once formed, AGEs can cause tissue damage by two main pathways which are: (1) formation of cross links that alter protein structure and function and, (2) interaction of AGE with AGE-cell surface receptors on the surfaces of various cells such as endothelial cells, macrophages, neurons, and smooth-muscle cells resulting in activation of cell signaling and gene expression that induces oxidative stress and inflammation (Nishikawa *et al*., 2000; Vlassara &Palace, 2002; Peppa *et al*., 2004; Ahmed, 2005; Sourris & Forbes, 2009; Giacco & Brownlee, 2010). Oxidative stress can accelerate AGE formation while AGE formation can also amplify the production of more ROS resulting in a vicious cycle of AGE formation and oxidative stress.

AGE's mediate some of their effect via interaction with some receptors that have been shown to bind to these chemical moieties. Among these receptors, Receptor for Advanced Glycation End products (RAGE) is the most extensively studied (Ramasamy *et al.,* 2005). Evidence from numerous studies suggests that AGEs are involved in a vicious cycle of inflammation, generation of ROS and increased production of AGEs. Ligand RAGE interaction results in activation of pathways such as p21ras, erk1/2 (p44/p42), MAP kinases, p38 and SAPK/JNK MAP kinases (Yan *et al*., 1994; Lander *et al*., 1997; Goldin *et al*., 2006). A consequence of the activation of these pathways is the nuclear translocation of transcription factor, Nuclear Factor Kappa B (NF-KB). Translocation of NF-KB to the nucleus increases the transcription of a number of proteins such as, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, 1L-18 and tumour necrosis factor (TNF)-**α** which are centrally involved in the endothelial recruitment of neutrophil and subsequent development or progression of atherosclerotic plaque (Cybulski & Gimbrone, 1991; Rubanyi, 1993; Schiekofer *et al*., 2003; Goldin *et al*., 2006).

The gene regions of NF-KB are located at the promoter region of RAGE. Moreover, the binding of NF-KB to the promoter region of RAGE results in up-regulation of RAGE itself (Barbosa *et al*., 2008). Interaction of AGE with RAGE generates more oxidative stress and this further potentiates the formation of AGE's (Park *et al*., 1998; Goldin *et al*., 2006). Generation of ROS by ligand stimulated RAGE activation is mediated at least in part via activation of NADPH oxidase (Wautier *et al*., 2001). Other mechanisms by which AGE's may be linked to increased generation of ROS is by reducing the activities of enzymatic antioxidant such as SOD and CAT, lowering of glutathione stores, and activation of PKC (Yan & Harding, 1997; Obrosova, 2002; Jiang *et al*., 2004).

Increased renal AGE in diabetic animals and patients have been linked to structural abnormality observed in diabetic nephropathy such as mesangial expansion, glomerular basement membrane thickening glomerulosclerosis and tubulointerstitial fibrosis (Yamagishi & Matsui, 2010). Advanced Glycation End Products level is increased with decreased renal function in type 1 diabetic patients (Miura *et al*., 2003). Evidence from clinical studies indicates a correlation between progression of diabetic retinopathy and the level of AGE in serum and retinal blood vessels of diabetic patients (Ono *et al*., 1998; Stitt, 2001). In diabetes, increased AGEs are observed within retinal capillary cells and causes pericyte loss in diabetic retinopathy (Stitt, 2003). AGE's induce toxic effects on retinal pericytes by causing oxidative stress and subsequent apoptosis (Chen *et al*., 2006).

High levels of serum AGEs have been documented in patients with type 2 diabetes mellitus and coronary heart disease (Kilhovd *et al*., 1999). Glycation increases susceptibility of low density lipoprotein (LDL) to oxidative modification which is considered a critical step in its atherogenicity (Bowie *et al*., 1993). Glycation end product's can also enhance atherosclerosis by trapping LDL in the subendothelium and decrease the recognition of AGE-modified LDL by LDL receptor (Bucala *et al*., 1994). Modification of LDL and its increased localization in vessels increases foam cell production and accelerates atherosclerosis development (Sobenin *et al*., 1993). Oxidative stress induces AGEs formation on collagen leading to cross-linking which is considered to play a role in diabetic cardiomyopathy (Goh & Cooper, 2008). The intermolecular collagen cross-linking caused by AGE increases vascular stiffness and interferes with arterial

blood flow (Eble *et al*., 1983; Cooper *et al*., 2001) and this partly explains the diastolic dysfunction and systolic hypertension seen in diabetic subjects**.**

2.7.3 Protein Kinase C (PKC) activation pathway

PKC activation is related to vasoconstriction, proliferation and overgrowth of smooth muscle cells as well as accelerated synthesis of extracellular matrix proteins, and thus plays significant roles in the onset and progression of vascular cell dysfunction in diabetes mellitus (Koya & King, 1998; Meier & King, 2000; Way *et al*., 2001). Two major pathways have been implicated in the activation of PKC in hyperglycemia. Persistent and excessive activation of several PKC isoforms result primarily from enhanced de novo synthesis of diacylglycerol (DAG) from glucose via increase in triose phosphate availability (Inoguchi *et al*., 1992; Shiba *et al*., 1993; Du *et al*., 2003; Giacco & Brownlee, 2010). There is also evidence that the interaction between AGEs and their cell-surface receptors can result in enhanced activity of PKC isoforms (Derubertis and Craven, 1994; Thallas-Bonke *et al*., 2008).

PKC likely regulates diabetic complications on multiple levels such as activation of endothelial nitric oxide synthase (eNOS), NAD(P)H oxidase, phospholipase A_2 (PLA_2), endothelin-1 (ET-1), Vascular endothelial growth factor (VEGF), Transforming growth factor-β (TGF-β), and by activating NF-KB. Diacylglycerol activated PKC alters the gene expression of key proteins leading to decrease blood flow, capillary occlusion, inflammation, free radicals generation and damage to cellular macromolecule (Klann *et al*., 1998; Way *et al*., 2001; Meier & King, 2000; Noh& King, 2007).

High glucose levels can stimulate ROS production via a PKC-dependent activation of NAD(P)H oxidase in cultured aortic endothelial cells, smooth muscle cells, and renal mesangial cells (Inoguchi *et al*., 2003). Nicotinamide adenine dinucleotide phosphate oxidase, which is primarily found in phagocytic cells, is the main source of ROS in non-phagocytic cells such as endothelial cells (Jones *et al*., 1996), fibroblasts (Pagano *et al*., 1997), podocytes (Greiber *et al*., 1998) and smooth muscle cells (Patterson *et al*., 1999). The expression of NAD(P)H oxidase components is up-regulated in vascular tissues from animal models of diabetes and in patients with diabetes and coronary artery disease (Hink *et al*., 2001; Guzik *et al*., 2002; Kim *et al*., 2002). Experimental

evidence indicates that NAD(P)H oxidase-dependent production of ROS may cause DNA damage in diabetic renal tissues leading to the development of nephropathy (Etoh *et al*., 2003). Increased activity of the NAD(P)H oxidase has also been reported in the retina of diabetic rats suggesting its involvement in the development of diabetic retinopathy (Ellis *et al*., 1998).

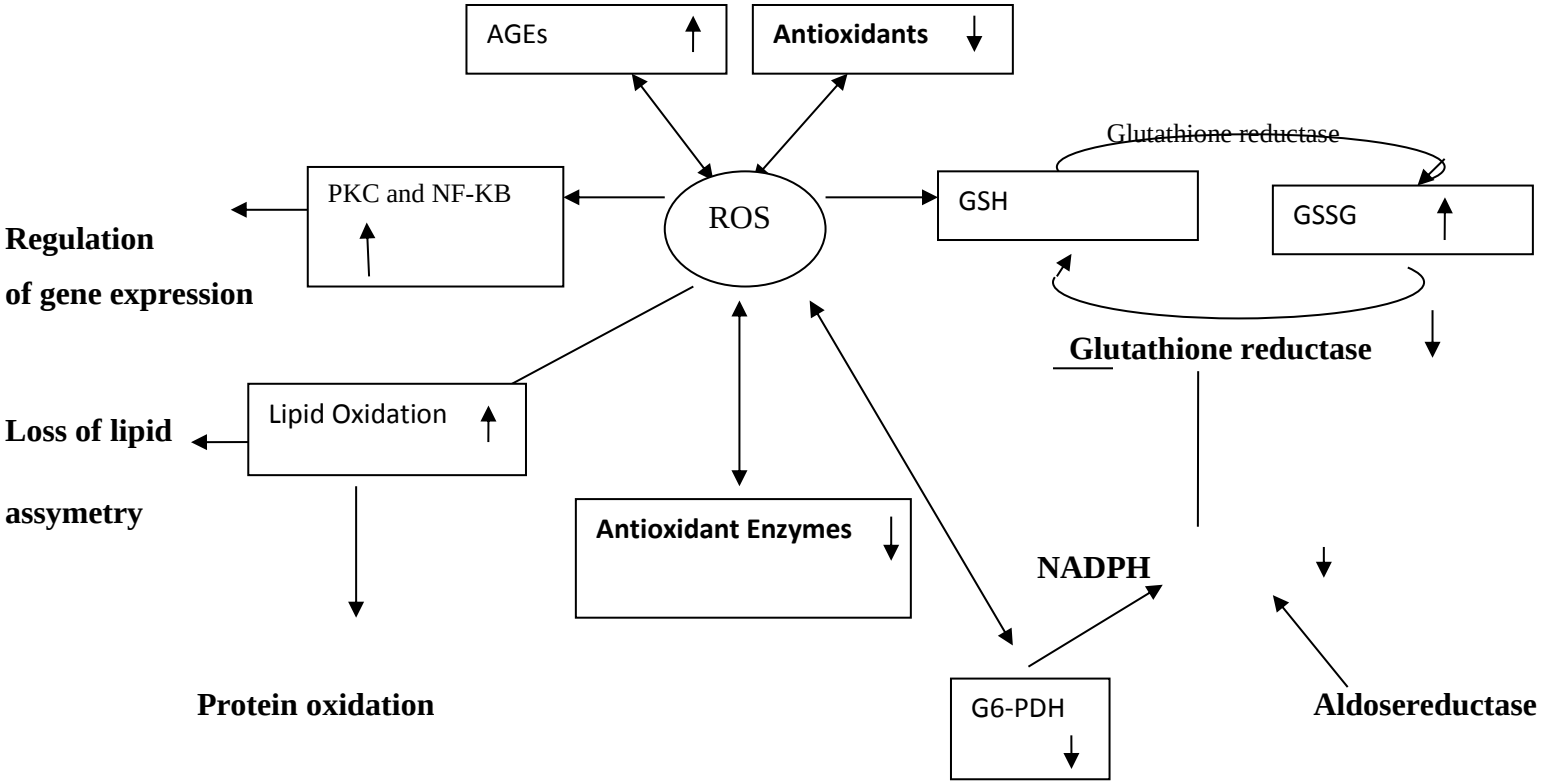

Figure 3: Oxidative stress-induced macromolecular damage and organ malfunction (Niedowicz & Daleke, 2005)

2.8 Antioxidants as potential therapeutic agents in the management of diabetes mellitus

Despite efforts to control blood glucose, tissue and organ damage are cumulative over many years in most diabetic patients. Varying degrees of hyperglycemia are virtually unavoidable in subjects with diabetes mellitus and glycemic memory has been used to describe the development of diabetes-related complications in diabetic patients even after normoglycemia has been restored and initial glycemic environment is remembered in the target organs (Ceriello *et al*., 2009; Giacco & Brownlee, 2010).

It is noteworthy that ROS has been implicated as a major cause of the metabolic memory after glucose normalization due to the chains of reactions leading to cell damage and loss of cellular function. The ability of ROS to directly oxidize and damage DNA, proteins, and lipids has implicated oxidative stress as a major 'player' in the onset and progression of late-diabetic complications (Rosen *et al*., 2001). In the absence of an appropriate condensation by antioxidant defense network, increased oxidative stress leads to activation of stress-sensitive intracellular signaling pathways and the formation of gene products that cause cellular damage and contribute to late diabetic complications (Nourooz-Zadeh *et al*., 1997; Brownlee, 2001; Evans *et al*., 2002; Shih *et al*., 2002). Due to the implication of hyperglycemia-induced oxidative stress in diabetes, these patients should in theory benefit from antioxidant supplementation.

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Free radicals produced under physiological conditions are maintained at steady state levels by endogenous or exogenous antioxidants (externally supplied through foods or supplements) which act as free radical scavengers. However, oxidative stress occurs when the production of free radicals overwhelms the detoxification capacity of cellular antioxidant system causing biological damage (Abdollahi *et al*., 2004; Ridnour *et al*., 2004; Halliwell, 2011).The endogenous antioxidants (Table 1) comprise of the enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and non-enzymatic antioxidants including glutathione (GSH), α lipoic acid, vitamins C and E (Halliwell & Gutteridge, 2007; Fukai & Ushio-Fukai, 2011; Lubos *et al.*, 2011). On the other hand, the exogenous antioxidants include micronutrients and other exogenously administered compounds such as vitamin E, vitamin C, trace metals (selenium,

manganese, and zinc), carotenoids and flavonoids (Halliwell & Gutteridge, 2007; Pham-Huy *et al.*, 2008; Halliwell, 2011).

The beneficial effect of antioxidants has been reported in animal models of diabetes and in diabetic patients (Maritim *et al.*, 2003; Liu *et al*., 2006). Quercetin for example is a well-known flavanoid and a strong antioxidant which has been shown to reduce oxidative stress and apoptosis in diabetes (Bakhshaeshi*et al*., 2012). Curcumin, an extract from the roots of the *Curcuma longa* plant (Aggarwal *et al*., 2007) is also a potent antioxidant and antiinflammatory agent (Khan *et al*., 2008) which has also shown beneficial effect in the management of diabetes and its associated complications (Sharma *et al*., 2006; 2007).

The inherent antioxidative properties of some common antidiabetic drugs such as aminoguanidine, statins, thiazolidinediones, glibenclamide and repaglinide also provides additional support to the involvement of oxidative stress in diabetes and therefore suggest that the use of antioxidants as therapeutic agents in diabetes is a promising approach (Fukui *et al*., 2000; Dobsak *et al*., 2001; Elmali *et al*., 2004; Haendeler *et al*., 2004; Gumieniczek *et al*., 2005).

Table 1: Role of antioxidants in the protection against free radical damage

Antioxidants	Cellular location	Role	Reference
Enzymatic Antioxidants			
(A) Catalase	Peroxisomes	Decomposition of H_2O_2 to water and oxygen	Winterbourn, 1993
(B) Glutathione peroxidase	Cytoplasm, mitochondria, and nucleus	Detoxifies H_2O_2 and lipid peroxides with simultaneous oxidation of GSH and generation of GSSG	Jurkovič *et al*., 2008
(C) Glutathione reductase	Cytoplasm, mitochondria, and nucleus	Recycles Glutathione disulfide back to glutathione using the cofactor NADPH	Maritim *et al.*, 2003
(D) Superoxide dismutase	Cytoplasm, nucleus lysosomes, Mitochondria	Conversion of superoxide radical to H_2O_2	Zelko *et al*., 2002
Antioxidants	**Cellular location**	**Role**	**Reference**
Non enzymatic antioxidants			
(A) GSH	Cytoplasm, mitochondria and nucleus	Acts as a cofactor for antioxidant enzymes (GPx, GST), regenerates other antioxidants such as Vitamins C and E to their active forms	Livingstone & Davis, 2007
(B) Vitamin-E	Membrane	Directly scavenge singlet oxygen, peroxyl and superoxide radicals , protects against peroxidation of membrane lipids	Valko, *et al*., 2007
(C) Vitamin-C	Cytosol	Acts synergistically with vitamin E to terminate radical induced lipid peroxidation	May *et al*., 1996; Valko, *et al*., 2007
(D) **α**-Lipoic acid	Cell membrane and cytoplasm	Increases glutathione and vitamin C levels	Smith *et al*., 2004

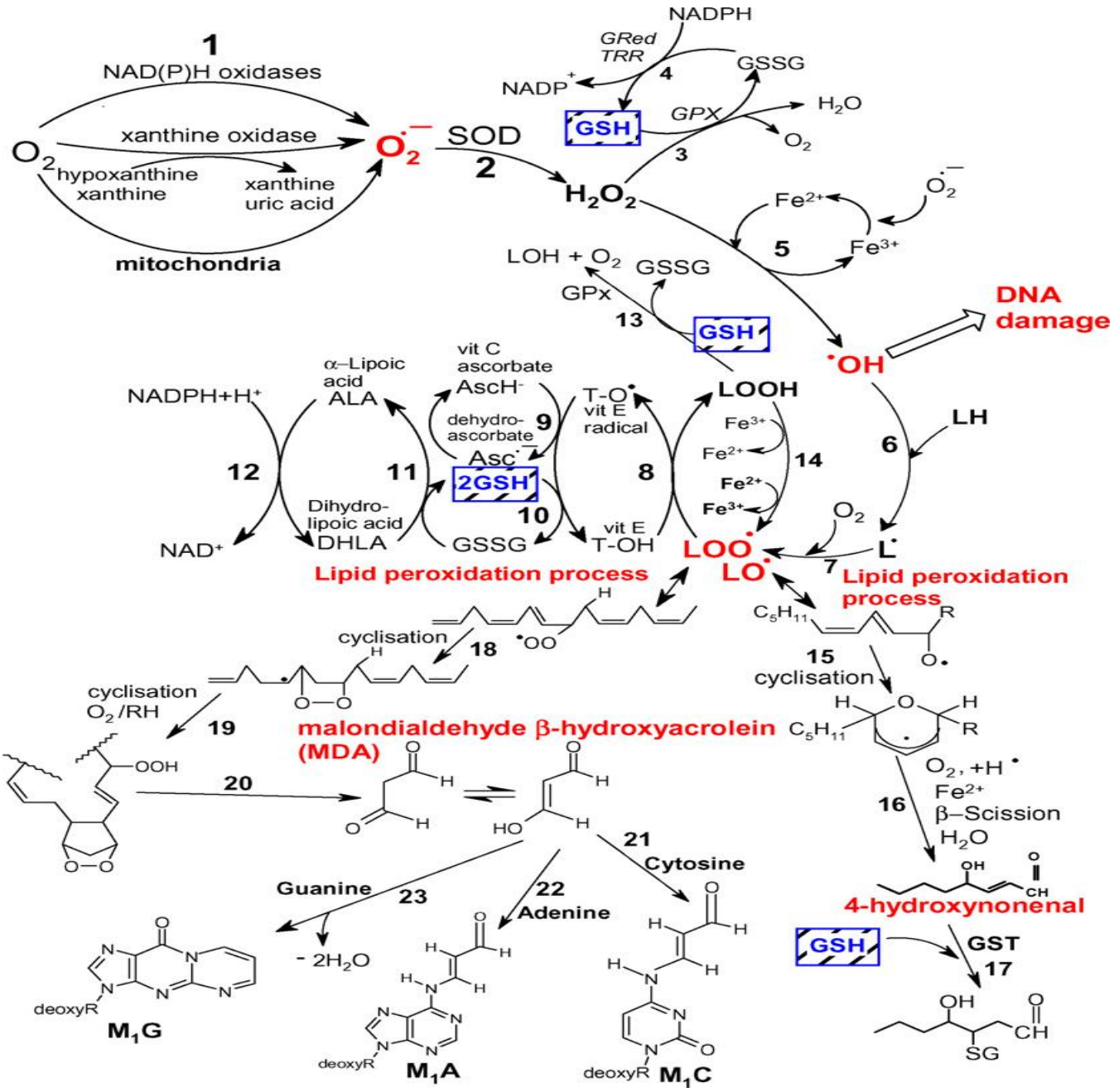

Figure 4: Pathways of ROS formation, and the role antioxidants in the management of oxidative stress (Valko, 2007).

The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPX) which requires GSH as the electron donor. The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GR) which uses NADPH as the electron donor.

Some transition metals (e.g. $Fe^{2+,}$ Cu^{+} and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•). The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•). The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, AscH−) leaving behind the ascorbyl radical (Asc•−). The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH. The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbate monoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to _-lipoic acid (ALA). Reaction 12: The regeneration of DHLA from ALA using NADPH. Lipid hydroperoxides are reduced to alcohols and dioxygen by GPX using GSH as the electron donor. Lipid hydroperoxides can react fast with Fe^{2+} to form lipid alkoxyl radicals (LO•), or much slower with Fe3+ to form lipid peroxyl radicals (LOO•).

Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. Six-membered ring hydroperoxide udergoes further reactions (involving-scission) to from 4-hydroxy-nonenal. 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione *S*-transferase). A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce cyclic peroxide adjacent to a carbon-centred radical. This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. The compound formed is an intermediate product for the production of malondialdehyde. Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively.

2.9 Interplay between oxidative stress and inflammation

Inflammation is an essential physiological response of the body to diverse pathological events like pathogen invasion, tissue injury and other irritants. This response involves infiltration and subsequent activation of the cells of the innate and adaptive immune system to the site of injury

and the production of inflammatory mediators such as chemokines, cytokines, cell adhesion molecules, prostaglandins etc. It is hypothesized that the release of these mediators is induced by high glucose concentration and mediated by oxidative stress (Gumieniczek *et al*., 2005). Chronic inflammation and oxidative stress have been implicated in the pathophysiology of diabetes mellitus. Inflammation and oxidative stress are inextricably connected in physiologic as well as disease states; they have even been termed ‘‘essential partners’’ in certain diseases (Ambade & Mandrekar, 2012). Complex interactions between the oxidative and inflammatory pathways contain mechanisms for both mutual amplification (positive feedback or a ‘‘vicious cycle’’)**.** Inflammation is the primary immune system reaction to eliminate pathogens or other stimuli in order to restore the cells to normal state or replace destroyed tissue with scar (Emmendoerffe *et al*., 2000). After activation, innate immune system cells secrete pro-inflammatory cytokines and chemokines that induce ROS/RNS production (Costa & Garlid, 2008).

Pro-inflammatory cytokines can indirectly provoke oxidative stress by activating macrophages which play a pivotal role in eliminating the pathogen through the generation of reactive oxygen species including superoxide, nitric oxide, hydrogen peroxide, hydroxyl radical, peroxynitrite and hydrochlorous acid (Hocl) (Fialkow *et al*., 2007). However, chronic inflammation is a prolonged pathological condition characterized by tissue destruction and fibrosis resulting in cell damage following ROS overproduction from inflammatory cells. Therefore, chronic inflammation exerts its cellular side effects mainly through continuous and excessive production of free radicals and depletion of antioxidants (Hold & El-Omar, 2008).

On the other hand, ROS also increases inflammation by activating certain stress-activated kinases such as ERK, JNK, and p38. Also, ROS can stimulate transcription factors, e.g. NF-kB and activator protein-1 (AP-1), to stimulate pro-inflammatory cytokine expression. Thus, targeting oxidative stress-inflammatory cytokine signaling seems an attractive strategy for the treatment of diabetes. Under normal physiological conditions, oxidative stress and activation of the immune system are generally short-lived due to intrinsic negative feedback mechanisms, such as increased production of anti-oxidant compounds or of anti-inflammatory cytokines. In certain chronic disease states, like diabetes however, both of these systems remain activated and

may, indeed, form a positive self-sustaining feedback loop, or a ‘‘co-activation’’ state worsening the disease condition (Jesmin *et al*., 2010).

2.10 Potential roles of medicinal plants in the management of diabetes mellitus

The limitations and side effect of conventional anti-diabetic drugs necessitate the search for alternative or complementary medicine that can enrich the therapeutic arsenal of diabetes mellitus. The enormous contributions of plants derived medicines to the human health are inspiring and cannot be overlooked. Plants are reputed in the indigenous systems of medicine for the treatment of various diseases. Various medicinal plants have been established for their antidiabetic effects. These include; *Garcinia kola, Allium sativa, Eugenia jambolana, Panax ginseng, Gymnema sylvestre, Momrodica charantia, Ocimum sanctum, Phyllanthus amarus, Pterocarpus marsupium, Trigonella foenum graecum* and *Tinospora cordifolia* (Khan *et al*., 2012).

Most of these plants have been found to contain phytochemicals like glycosides, alkaloids, terpenoids, and flavonoids etc. that are frequently implicated as having antidiabetic effects. Different mechanisms of action have been identified for the beneficial effects of these plants in diabetes (Table 2). These include rejuvenation of beta cells and stimulation of insulin secretion and insulin release from beta cells of islets, reduction in insulin resistance, blood glucose lowering effect, anti-oxidant activity and antiinflamatory action. The table below (Table 2) shows different medicinal plants that have been studied and their antidiabetic effects.

Table 2: Selected plants with antidiabetic properties

Botanical name	Family	Active constituents	Antidiabetic and other beneficial effects	References
Eugenia jambolana	Myrtaceae	Kaempferol, anthocyanins glucoside, isoquercetin, ellagic acid, tannins, myricetin	Hypoglycemic, anti-hyperlipidemic antioxidant and anti-inflammatory effects	Rizvi & Mishra, 2013
Ocimum sanctum L.	Lamiaceae	Apigenin, tannins, cirsimaritin, triterpenoids, saponins, rosmarinic acid, orientin, isothymonin	Stimulates insulin secretion, anti-hyperglycemic, anti-hyperlipidemic antioxidant and anti-inflammatory properties,	Pattanayak *et al*., 2010; Rizvi & Mishra, 2013
Nigella sativa	Ranunculaceae	Alkaloids, saponin, thymoquinone	Anti-hyperglycemic, decreases oxidative stress and preserves pancreatic beta-cell integrity.	Mathur *et al*., 2011
Allium sativum	Amaryllidaceae	Allicin, allixin, ajoene	Improvement in insulin sensitivity,anti-hyperglycemic, anti-hyperlipidemic and antioxidant effect.	Kavishankar *et al*., 2011, Rizvi & Mishra, 2013

Botanical name	**Family**	**Active constituents**	**Antidiabetic and other beneficial effects**	
Gymnema sylvestre	Asclepiadaceae	Gymnemic acid, tartaric acid, flavones, gurmarin, calcium oxalate, glucose, stigmasterol, betaine and choline	Anti-hyperglycemic, antiinflammatory and hypolipidemic effeects	Kanetkar *et al*., 2007.
Tinospora cordifolia	Menispermaceae	Alkaloids, tannins, cardiac glycosides, flavonoids, saponins and steroids	Antioxidant activity, promotes insulin secretion, inhibition of gluconeogenesis and glycogenolysis, immunomodulatory action	Saha & Ghosh, 2012.
Trigonella foenum graecum	Fabaceae	Saponins, flavonoids, coumarin, trigonelline	Isletcells regeneration, glucose lowering effect, antioxidant activity	Premanath *et al*., 2012; Neelakantan *et al*., 2014

2.10.1 *Garcinia kola*

Garcinia kola Heckel (family-*Guttifera*) is a dicotyledonous plant found in moist forest, riverine and swampy areas. It occurs naturally in Sierra Leone, Nigeria and Angola. The plant grows to a medium sized tree of about 12-14 m high and produces reddish, yellowish or orange coloured fruit (Okwu, 2005; Adesanya *et al*., 2007). The flowering of the plant occurs between December and January while the fruits mature between June and August. Each fruit contains 2 to 4 yellow seeds and a sour tasting pulp. The seeds have a bitter astringent taste; hence, it is called bitter kola in Nigeria. *Garcinia kola* seed is also referred to as orogbo (Yoruba-Western Nigeria), cida goro (Hausa-Northern Nigeria), Aku ilu or Ugugolu (Igbo-Eastern Nigeria), Efiari (Efik), and Igoligo in Idoma-Middle Belt (Yakubu & Quadri, 2012). The plant has been referred to as a "wonder plant" because every part of it has medicinal importance (Dalziel, 1937).

The plant has been cultivated for various medicinal uses. Split stems and twigs of the plant are used as chewing sticks in many parts of Africa, and have been commercialized in the major cities for years, offering natural dental care. In fact, it is probably the most important source of chewing sticks in West Africa (Agyili *et al.,* 2006). Also, its applicability as an adaptogen (rejuvenating, stimulating and/or anti-stress agent) has been reported (Iwu, 1999; Esimone *et al.,* 2007). In this respect, the seed, which is edible, has been shown to help organisms to adapt to stress by influencing multiple regulatory systems responsible for stimulus-response coupling such as the immune system (Iwu, 1999; Meserole, 1999; Esimone *et al.,* 2007). It is used to treat throat infections, cough and stomach upset in African traditional medicine (Hussain *et al.,* 1982; Iwu & Igboko, 1986; Igoko, 1986; Middleton & Kandaswani, 1991; Iwu, 1999; Meserole, 1999).

The bitter kola nut is also called''malekola''because of its aphrodisiac and fertility enhancing activities (Iwu, 1993; Okoko, 2009; Ralebona *et al*., 2012) which has been suggested to be related to its vasodilator effects on the genitalia smooth muscles (Adegbehingbe *et al*., 2008). Extracts of the various parts of the *Garcinia kola* plants have been used in folk lore medicine for the treatment of ailments such as liver disorders, urinary tract infections, hepatitis, diarrhoea, laryngitis and bronchitis (Akintowa & Essien, 1990; Iwu, 1993; Orie & Ekon, 1993; Adesina *et al*., 1995). Other known uses include anti-atherogenic and antilipoperoxative effects (Adaramoye *et al.,* 2005).

Phytochemistry of GK have shown its content to include benxophenones, kolaviron (containing a complex of *Garcinia* biflavonoids (GB-1, GB-2) , kolaflavonone and kolaflavone), xanthones, oleoresin alkaloids, phenols, tannins and saponins (Cotterih *et al.*, 1978; Hussain *et al*., 1982; Ebana *et al*., 1991;Terashima *et al*., 1995, 1999; Onayade *et al*., 1998; Farombi *et al*., 2002; Okunyi *et al*., 2002; Okwu, 2005). The chromanols, garcioic and garcinal, together with Gamma-tocotrienol have also been isolated from *G. kola* (Terashima *et al*., 2002). These constituents have several effects including antiviral, anti-inflammatory, anti-diabetic, bronchodilator and anti-hepatotoxic properties.

2.10.2 Kolaviron

Kolaviron is a fraction of the deffated ethanol extract of *Garcinia Kola seeds*, containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone and kolaflavone as its major components (Iwu, 1985). Kolaviron has shown ample beneficial health effects (as summarized in figure 5) in animal models of diseases and also in the prevention of hepatotoxicity induced by several toxins such as thioacetamide, paracetamol, carbontetrachloride and amanitatoxins.

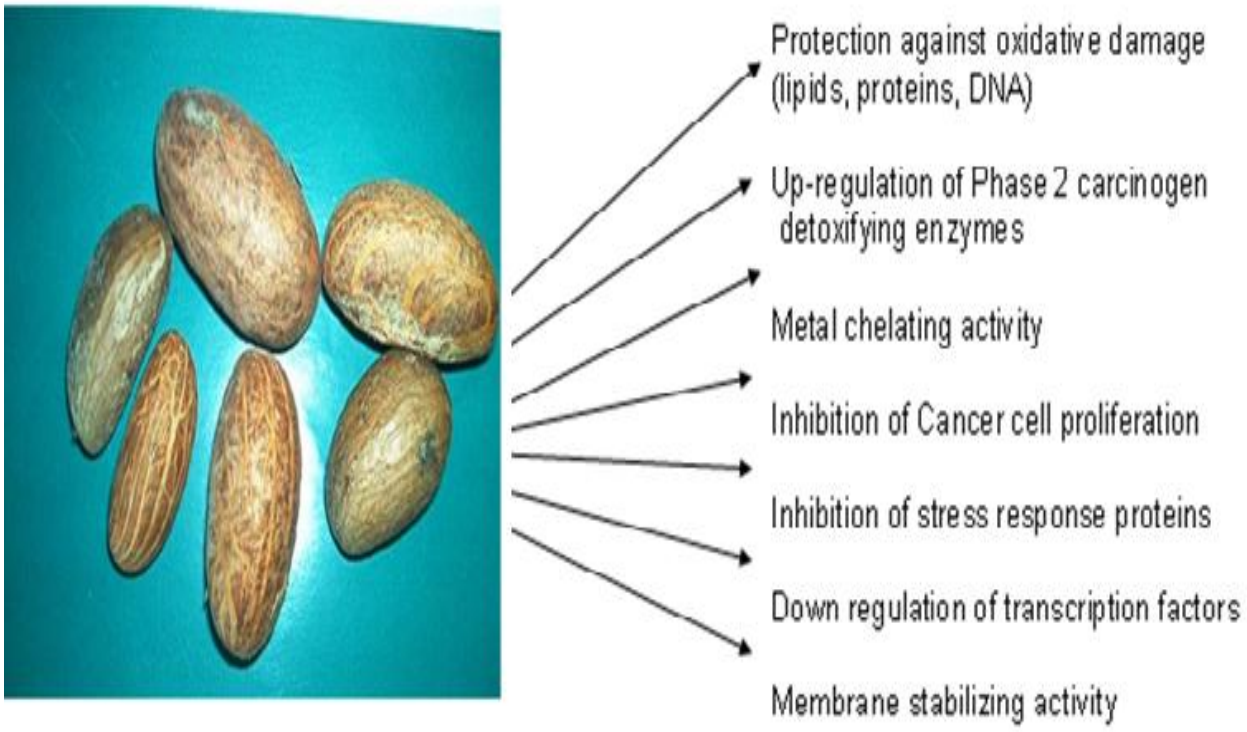

Figure 5: Biochemical mechanisms underlying the beneficial properties of *Garcinia kola* derived-bioflavonoids (Farombi & Owoeye, 2011).

The protective effects of kolaviron against insults from various xenobiotics have been attributed to its antioxidant properties. Kolaviron reduced damage to lipids and proteins induced by Fe^{3+}/EDTA/ascorbate mixtures *ex vivo* (Farombi *et al*., 2004). In the plasma and liver, kolaviron lowered biomarker of protein oxidation (2-aminoadipic semialdehyde) and also lipid oxidation marker (malondialdehyde) in the liver. In addition, the role of kolaviron in the chemoprevention of chemically-induced genotoxicity is demonstrated by its inhibitory effect on H_2O_2-induced ROS production in HepG2 cells (Eddy *et al*., 1987; Nwankwo *et al*., 2000). Kolaviron also improved antioxidant status by enhancing antioxidant gene expressions and scavenging ROS in atrazine-induced cytotoxicity of rat leydig cells. In addition, kolaviron exhibited antiulcerogenic and gastroprotective role which may be related to its intrinsic anti-oxidant properties. Kolaviron showed protective effects against ethylene glycol monoethyl ether (EGEE)-mediated haematotoxicity and renal apoptosis in rats (Adedara & Farombi, 2013) and oxidative damage in boar spermatozoa (Adedara & Farombi, 2013).

The inhibitory actions of kolaviron on inflammatory mediators such as, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) in rats treated with dimethylnitrosamine is another indication of its protective effect on drug-induced hepatotoxicity (Farombi *et al*., 2009). Previous studies have suggested the use of kolaviron as a prophylactic agent in the protection against atherosclerosis due to its antioxidative effects on serum lipoprotein oxidation both *in vitro and ex vivo.* The possible mechanisms of protection were suggested to involve metal chelation, anti-oxidant and scavenging of radical species (Farombi & Nwaokeafor, 2005). The hypoglycemic effect of kolaviron and its anti-atherogenic effects in rats fed on high cholesterol diet have also been documented (Adaramoye *et al*., 2005; Adaramoye & Adeyemi, 2006). Kolaviron treatment also protected against carbon tetrachloride (CCl_4)-induced alterations in erythrocyte membrane structure and function. The vasorelaxant effects of kolaviron in isolated superior mesenteric arteries from normotensive rats has also been demonstrated via mechanisms involving the blockade of extracellular calcium ions (Ca^{2+}) influx and inhibition of Ca^{2+} release from intracellular stores (Adaramoye & Medeiros, 2009).

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CHAPTER THREE

Kolaviron Improved Resistance to Oxidative Stress and Inflammation in the Blood (Erythrocyte, Serum and Plasma) of Streptozotocin-Induced Diabetic Rats

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Abstract

Aims

Bitter kola seed (*Garcinia kola,* Family: *Guttiferae)* has been used as a social masticatory agent in Africa for several years and is believed to possess many useful medicinal properties.The present study evaluates the anti-oxidative, anti-inflammatory and anti-lipidemic effects of kolaviron (an extract from the *Garcinia kola* seeds) in the blood of streptozotocin (STZ) - induced diabetic rats.

Methods

Diabetic rats were treated with kolaviron (100 mg/kg b.wt.) orally five times a week for a period of 6 weeks. Serum glucose and HbA_{1c} concentration were estimated in diabetic rats. The activities of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) (in erythrocytes) as well as plasma concentration of malondialdehyde (MDA), a product of lipid peroxidation, oxygen radical absorbing capacity (ORAC) and ferric-reducing antioxidant power (FRAP) were investigated. Serum levels of pro-inflammatory cytokine, chemokine and growth factor; interleukin (IL)-1, monocyte chemotactic protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) respectively were also analyzed.

Results

Kolaviron treatment markedly improved antioxidant status and abated inflammatory response evidenced by reduction in the levels of pro-inflammatory cytokines and growth factor, lipid peroxidation product and the restoration of the altered activities of erythrocyte antioxidant enzymes in the blood of diabetic rats.

Conclusion

Kolaviron improved antioxidant status, reduced inflammation and protected against hyperglycemia-induced oxidative damage in the blood of diabetic rats.

Keywords: Diabetes; Antioxidant; Blood; Oxidative stress

3.1 Introduction

Diabetes mellitus (DM) reduces life expectancy and adversely affects the quality of life amongst those affected. According to the international diabetes federation (IDF), diabetic patients would increase from 371 million (2012) to 552 million in 2030. The limitations of the currently used antidiabetic drugs suggest an urgent need to discover new compounds that can serve as alternative and/ or complementary therapy against this disease [1].

Hyperglycemia has been associated with an increased state of oxidative stress which is believed to play a crucial role in the onset and progression of late-diabetic complications through activation of stress-sensitive intracellular signaling pathways and the formation of gene products that causes cellular damage [2; 3; 4]. Biological free radicals are products of normal cellular metabolism and are maintained at steady state levels by antioxidants which act as free radical scavengers. At high concentrations, the production of free radicals overwhelms the detoxification capacity of cellular antioxidant system, resulting in oxidative stress and damage to cell structures [5; 6]. Altered antioxidant enzyme activities have been reported in the blood of diabetic patients and in animal diabetic models. Red blood cells (RBC's) are the first cells in the body to be exposed to stressful stimuli and hence prone to oxidative stress [7]. Damage to red blood cells by reactive oxygen species (ROS) results in abnormalities in the function, morphology and metabolism of erythrocytes [8; 9]. Hyperglycemia and oxidation of membrane proteins are strongly associated with an increase in RBC's haemolysis and many pathological consequences [10]. Some of the mechanisms by which hyperglycemia causes oxidative stress include, increased production of superoxide anion in the mitochondria [11], non enzymatic glycation of proteins [12] and glucose autoxidation [13]. In addition, metabolic stress can result in changes in energy metabolism, reduced antioxidant defense and increased levels of inflammatory mediators [14; 15].

Hyperlipidemia and altered antioxidant defenses are companions of oxidative stress. Diabetes-induced hyperlipidemia has been reported as one of the major risk factors for micro and macrovascular complications [16]. Maintaining a balance between reactive oxygen species (ROS) and antioxidants is a major mechanism in preventing damage by oxidative stress

therefore; dietary supplementation of antioxidants could be a promising approach in the treatment of diabetes.

There is considerable interest In the potential beneficial effects of flavonoids on human health due to their biological activities which includeantioxidant, antiviral, antiinflammatory and anti-tumor activities [17]. Kolaviron (KV) is an extract from the bitter *kola* seeds (*Garcinia kola,* Family: *Guttiferae)* containing a complex of *Garcinia* biflavonoids. KV has been proven to be beneficial in various pathological conditions of animal models through its antioxidative, antigenotoxic, analgesic and antiinflammatory properties [18; 19; 20; 21; 22] hence the need to explore its potentials in diabetic conditions. The present study investigated the beneficial effects of kolaviron on oxidative stress and inflammatory biomarkers in the blood of diabetic rats.

3.2 Materials and methods

3.2.1 Chemicals

Streptozotocin (STZ),6-hydroxydopamine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and 2-thiobarbituric acid (TBA) and β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) were obtained from Sigma-Aldrich (Johannesburg, South Africa). Malondialdehyde bis (diethyl acetal) (MDA), hexane and methanol were purchased from Merck (Johannesburg, South Africa). All other chemicals and reagents used were of the highest commercially available purity.

3.2.2 Animals

The study protocol was approved by the Faculty of Health and Wellness Sciences Research Ethics Committee of the Cape Peninsula University of Technology (Ethics Certificate no: CPUT/HW-REC 2012/AO4). All the animals received humane care in accordance to the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Science (NAS) and published by the National Institute of Health (Publication no. 80-23, revised 1978). Male Wistar rats (270 ± 25g), were used for the study. Treatments were

performed at the animal facility of the Medical Research Council (MRC), South Africa and all standard operating procedures (SOPs) were strictly adhered to. All animals were housed individually at room temperature (22 ± 2 °C) with 55 ± 5% humidity and an automatically controlled cycle of 12 hr light and 12 hr dark. Standard laboratory animal feed and water were provided *ad libitum* and animals were acclimatized to the experimental conditions for a period of one week before the commencement of the experiment.

3.2.3 Collection of plant material and extract preparation

*Garcinia kola*seeds were peeled sliced and air-dried (25–28°C). Kolaviron was isolated according to the method of Iwu *et al.* [23]. Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet for 24 hr. The defatted dried product was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded kolaviron, a golden yellow solid.

3.2.4 Experimental design

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ; 50mg kg^{-1} body weight) in citrate buffer (0.1 M, pH 4.5). Five days post STZ injection, diabetes was confirmed by a stable hyperglycemia (>18 mmol/l) in the tail blood glucose with a glucometer (Accu-Chek, Roche, Germany). The animals were divided into 4 groups (n=10 per group): Normal control (NC group), kolaviron treated normal control (KV), diabetic control (DM group) and kolaviron- treated diabetic group (DM + KV group). Kolaviron was dissolved in vehicle [dimethylsulphoxide (DMSO)] and administered orally at a dose of 100mg kg^{-1} five times a week for six weeks. Normal control (NC) rats also received vehicle throughout the study period.

For biochemical estimations in the blood, rats were sacrificed under sodium pentobarbital anesthesia (60mg/kg). Random blood glucose was determined in rats after collection of blood specimen from the abdominal aorta into glucose tubes. Blood samples were collected into tubes with or without EDTA to obtain plasma or serum, respectively and centrifuged at 3500 xg for

10 min at 4 °C. Blood was also collected into another set of tubes used for HbA_{1C} estimation. Erythrocytes were obtained from EDTA-treated blood after plasma separation. Buffy-coat layers were discarded and erythrocytes were washed three times with cold saline and centrifuged at 3000 rpm for 10 min. Samples were haemolyzed by the addition of a three-fold volume of ice-cold double distilled water (ddH_20) and the haemolysate was obtained after removing the cell debris by centrifugation at 3000 rpm for another 10 min. The supernatant was collected and stored at -80^oC prior to the estimation of enzymatic activity.

3.2.5 Analysis of glucose, glycated haemoglobin and lipid profile

Plasma glucose, glycated haemoglobin (HbA_{1C}) and serum lipid profile (total cholesterol, triglycerides) were analyzed with diagnostic kits in an automated clinical chemistry analyzer (Medical Cooperation, Bedford, MA, USA).

3.2.6 Plasma antioxidant capacity assays

The antioxidant capacity of plasma samples was determined by the ferric-reducing antioxidant power (FRAP) assayof Benzie and Strain [24] with slight modifications in a Multiskan Spectrum plate reader (Thermo Fischer Scientific, Waltham, MA USA). Oxygen radical absorbance capacity (ORAC) assay was conducted to kinetically measure the peroxyl radical scavenging activity in plasma samples with Trolox™ as the antioxidant standard according to the method of Ou *et al.* [25]. The total plasma polyphenol was performed using the Folin Ciocalteu's phenol reagent according to the method of Singleton *et al*. [26].

3.2.7 Erythrocyte antioxidant enzyme activity assays

Activities of antioxidant enzymes in the erythrocytes were estimated in aclear 96-well plate using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Catalase (CAT) activity was determined by the method of Aebi [27]. Superoxide dismutase was determined by the method of Crosti *et al*., [28], based on the inhibitory effect of SOD on the spontaneous autoxidation of 6- hydroxydopamine. Glutathione peroxidase (GPX) activity was determined according to the method of Ellerby and Bredesen [29] based on the oxidation of NADPH to

$NADP^+$ in the presence of H_2O_2. The protein concentrations of the erythrocyte were determined by the bicinchoninic acid (BCA) kit (Pierce, Illinois, USA).

3.2.8 Lipid peroxidation (LPO)

Plasma malondialdehyde (MDA), an end product of lipid peroxidation was determined by High-Performance Liquid Chromatography (HPLC) using a method adapted from Khoschsorur *et al.*, [30]. Briefly, 100 µL of plasma samples and standard (MDA) were mixed with 750 µL orthophosphoric acid (0.44 M), 250 µL of aqueous thiobarbituric acid (42 mM) and 450µL distilled water. The mixture was heated in a boiling water bath for 60 min. After cooling on ice, alkaline methanol (50 mL methanol + 4.5 mL 1 M NaOH) was added (1: 1). The samples were centrifuged at 3500 xg for 3 min at 4°C. 1 mL of supernatant was added to 500 µL of n-hexane and the mixture centrifuged at 14000 xg for 40 sec. 50 µL of the supernatant was then chromatographed on an Agilent 1200 series HPLC. A 5 µm YMC-PackPro C18 (1 5 0 mm × 4. 6 m m i.d.) column was used for separation with 60: 40 (v/v) 50 mM phosphate buffer (pH 6.8), and methanol respectively as mobile phase. The flow rate was 1 mL min^{-1}. Fluorometric detection was performed with excitation at 532 nm and emission at 552 nm. The peak of the MDA-TBA adduct was calibrated with the MDA standard.

3.2.9 Assay of IL-1, MCP-1 and VEGF

The serum levels of inflammatory markers including monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF) and interleukin (IL)-1 were measured in the serum using Bio-plex Pro™ magnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-plex® platform (Bio-Rad). Following previous optimization, samples were evaluated undiluted in a blinded manner. Samples were reacted with a mixture of fluorescent polystyrene beads bound with specific anti-cytokine primary antibodies, resulting in binding of the cytokines to the beads with the corresponding antibody. The biotinylated anti-cytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. All analytes levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all the analytes ranged from 3-12000 pg/ml. Bio-Plex Manager™ software, version 6.0 was used for bead acquisition and analysis.

3.2.10 Statistical analysis

Values were expressed as mean ± SD. Data were tested for normality and equality of variance using the Levene's Test. Differences between groups mean were estimated using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for all pairwise comparisons. The Kruskal-Wallis test, a non-parametric analogue to the one-way ANOVA was used to test for group differences when data was not normally distributed. Results were considered statistically significant at $P < 0.05$, or marginally significant at $P < 0.1$. All the statistics were performed using MedCalc version 12.2.1 software (MedCalc software bvba, Mariakerke, Belgium).

3.3 Results

3.3.1 Kolaviron treatment lowered blood glucose, glycated haemoglobin (HbA_{1c}) and levels of lipid profiles

Kolaviron treatment reduced total cholesterol and triglyceride concentrations in the serum of normal and diabetic rats as shown in Figure 1. Serum glucose concentration of the diabetic group was 2.84 fold higher than in the non-diabetic group, indicating a sustained hyperglycemic state in the STZ-induced diabetic rats. Glycated hemoglobin (HbA_{1c}) was significantly elevated in diabetic rats and the administration of kolaviron significantly lowered blood glucose and HbA_{1c} levels in diabetic rats.

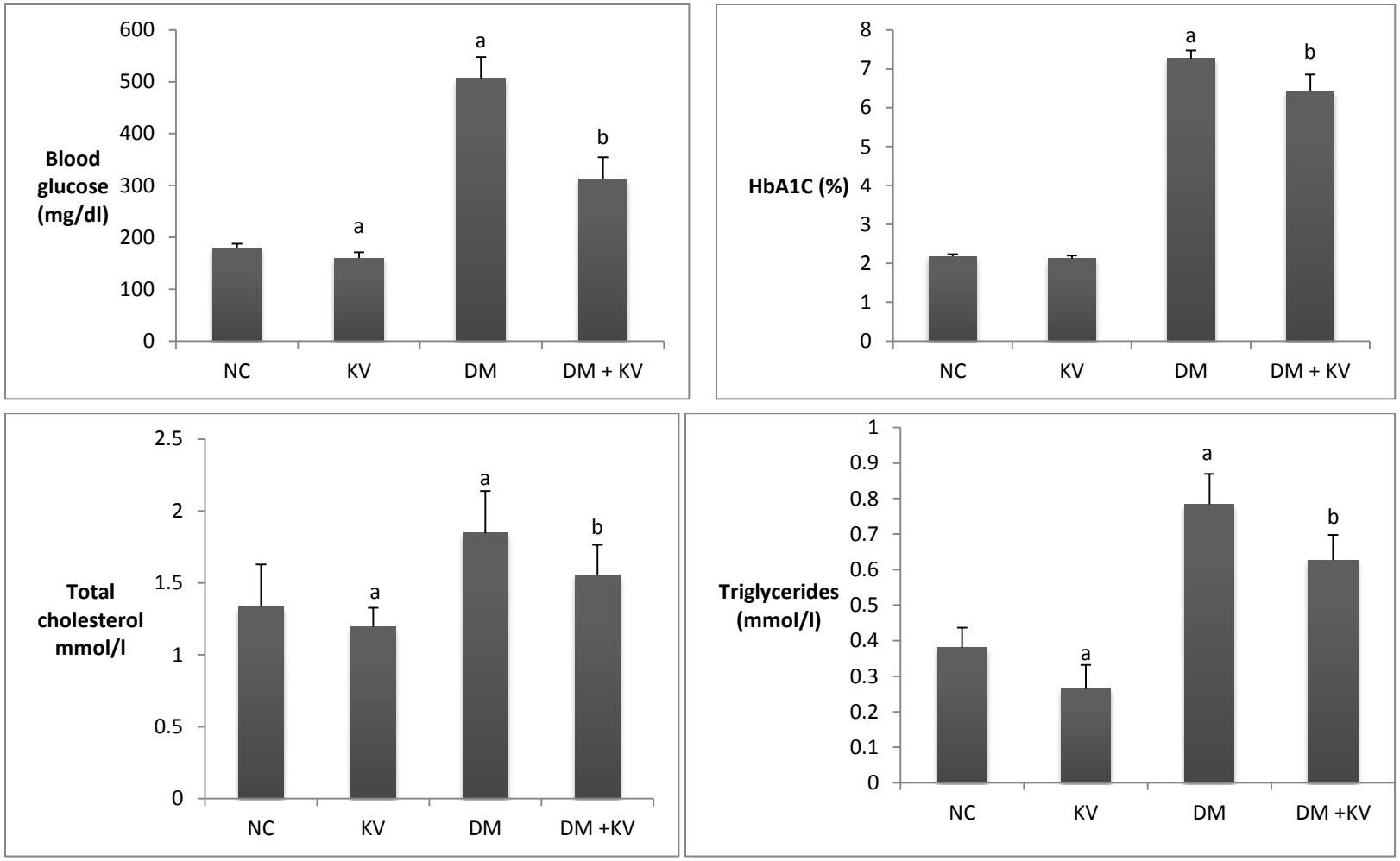

Figure 1: Effects of kolaviron on blood glucose, glycated haemoglobin and levels of lipid profiles (total cholesterol and triglyceride concentrations). [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). NC; Normal control, KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron.

3.3.2 Kolaviron alleviates oxidative stress in the erythrocyte of diabetic rats

A significant increase in GPX and SOD activity was observed in the erythrocytes of diabetic rats compared to control group (Table 1). This alteration was reversed after kolaviron administration to STZ-induced diabetic rats for 6 weeks. No significant change in CAT activity was observed. Diabetic rats showed increased level of the lipid peroxidation product, MDA. Kolaviron significantly reduced the formation of plasma MDA in STZ-diabetic model.

Table 1: Effects of kolaviron on erythrocyte enzymatic activity and plasma lipid peroxidation in diabetic and normoglycemic rats

	CAT	SOD	GPX	MDA
NC	0.34 ± 0.06	0.029 ± 0.008	32.13 ± 4.60	1.60 ± 0.20
KV	0.28 ± 0.12	0.026± 0.008	35.29 ± 4.14	1.5 ± 0.21
DM	0.37± 0.07	0.018 ± 0.004 [a]	48.30 ± 8.21 [a]	2.01 ± 0.3[a]
DM +KV	0.36 ± 0.11	0.022 ± 0.006	30.60 ±8.43 [b]	1.61 ± 0.31[b]

Table 1 illustrates the effect of kolaviron on erythrocyte enzymatic activity and plasma lipid peroxidation in diabetic and normoglycemic rats. CAT: catalase, μmol H_2O_2 consumed/min/mg protein, SOD: superoxide dismutase, U/μg protein, GPx: glutathione peroxidase, µmol NADPH oxidized/min/μg protein, MDA: malondialdehyde, (μmol MDA/L). Data are presented as mean ± S.D. [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). NC; Normal control, KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron.

3.3.3 Effect of kolaviron treatment on plasma antioxidant capacity

Although an increasing trend was observed in plasma antioxidant status assessed as FRAP, ORAC and total polyphenols in diabetic rats, following kolaviron treatment for 6 weeks (Table 2), no statistically significant difference was observed in the estimated parameters in all treatment groups.

Table 2: Effects of kolaviron supplementation on plasma antioxidant status in diabetic rats

	ORAC (μmol TE/L)	FRAP (μmol AAE/L)	Total polyphenol (mg GAE/L)
NC	11.72 ± 1.27	85.68 ± 9.45	48.39 ± 4.5
KV	13.57 ± 1.54	82.87 ± 9.26	47.84 ± 3.3
DM	11.20 ± 1.0	80.60 ± 8.56	43.73 ± 5
DM +KV	11.03 ± 1.71	92.98 ± 15.95	46.45 ± 6.15

Data as shown in Table 2 are presented as mean ± S.D. [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). NC; Normal control, KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron. AAE: ascorbic acid equivalent, TE: trolox equivalent, FRAP: ferric reducing ability of the plasma, ORAC: oxygen radical absorbance capacity.

3.3.4 Kolaviron abates inflammation in the serum of diabetic rats

Diabetic rats had significantly elevated serum levels of MCP-1, VEGF and IL-1β compared to control rats (Table 3) and kolaviron normalized serum levels of these inflammatory markers in diabetic rats. No significant difference was observed in the serum levels of MCP-1, VEGF and IL-1β in normal rats treated with kolaviron compared to untreated control group.

Table 3: Effects of kolaviron treatment on levels of inflammatory proteins and growth factor

	IL-1β (pg/ml)	MCP-1(pg/ml)	VEGF (pg/ml)
NC	28.37 ± 10.54	226.62 ± 75.71	10.45 ± 1.4
KV	20.48± 5.13	231.26 ± 78.31	9.49 ± 2.5
DM	43.32 ± 8.65 [a]	542.77 ± 67.27 [a]	15.41 ± 1.96 [a]
DM +KV	16.43 ± 7.74 [b]	266.61 ± 81.36 [b]	12.44 ± 1.73 [b]

Table 3 shows effects of kolaviron on interleukin (IL)-1β, monocyte chemotactic protein (MCP-1) and vascular endothelial growth factor (VEGF). Data are presented as mean ± S.D. [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). NC; Normal control, KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron.

3.4 Discussion

The glucose lowering effect of kolaviron was observed in our study [31]. Glycated hemoglobin (HbA_{1c}) expressed as a percentage of total blood hemoglobin concentration, is an effective index for the screening of glycemic control over time. Higher level of HbA_{1c} is observed in diabetes due to reaction of excess blood glucose with hemoglobin. An evidence of glycemic regulation by kolaviron is the significant reduction of blood glucose and glycated hemoglobin levels in kolaviron supplemented-diabetic rats.

Increase glucose concentrations results in oxidative stress. Erythrocytes are vulnerable to oxidative stress due to high concentration of polyunsaturated fatty acids, ferrous ions and molecular oxygen [32]. Persistent hyperglycemia and increased oxidative stress are major players in the development of secondary diabetic complications such as nephrotoxicity [33] and hepatic injury [34; 35]. Cells maintain a variety of defenses against reactive oxygen species toxicity and oxidative stress. Among these are an array of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Superoxide dismutase (SOD) scavenges superoxide radical by accelerating its conversion to hydrogen peroxide (H_2O_2) while glutathione peroxidase (GPX) detoxifies H_2O_2 and lipid peroxides [36; 37]. CAT acts in the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Hyperglycemia can interfere with the antioxidant defense network and the alteration in the activity of antioxidant enzymes is a common occurrence in diabetes. However, divergent results have been reported regarding the activities of antioxidants enzymes in diabetics.

Alteration in antioxidant defense in the diabetic rats was evidenced by a significant reduction in SOD activity in the erythrocyte of diabetic rats. The decrease in SOD activity in the hyperglycemic rats could be due to oxidative stress-induced inactivation. Increased H_2O_2 concentration for example is known to inactivate SOD [38]. Glycosylation of SOD and/or loss of Cu^{2+}, a cofactor required for SOD activity can also reduce SOD activity [39]. We observed that supplementation of kolaviron to diabetic rat increased SOD activity to near normal level.

The observed increase in erythrocyte GPX activity in the un-supplemented diabetic rats is an indication of increased H_2O_2 concentration. Increased GPX activity might be due to an increase

generation of H_2O_2 and a compensatory response to erythrocyte membrane oxidative damage. The reduction of oxidative stress in diabetic rats by kolaviron is evidenced by the suppression of GPX activity. Some studies have reported an increase in erythrocyte catalase activity in diabetic rats [40] while others have shown a decrease [41] in erythrocyte CAT activity. However, similar results to ours were found by Bandeira and colleagues [42] who observed no significant difference in erythrocytes CAT activity.The erythrocyte protective effects of kolaviron against free radical attack may be due to its direct free radical scavenging capacity and improvement in antioxidant defense.

The release of free radicals during oxidative stress causes serious damage to the biological system by abstracting electrons from macromolecules causing instability and disintegration. Antioxidants, in general, are compounds and reactions which dispose, scavenge and suppress the formation of ROS [43]. The ability of kolaviron to protect against free radical-induced damage and lipid peroxidation process is also evidenced by the significant decrease in the levels of malondialdehyde (MDA) -a product of lipid peroxidation in the plasma of diabetic rats following treatment with kolaviron. Kolaviron contains *Garcinia* biflavonoids. Flavonoids (bioflavonoids) are a diverse group of polyphenols (phenyl benzopyrans) which function as phytochemicals and are well-known for their multi-directional biological activities [44]. Structure- activity relationships have been shown to play a major role in the antioxidant effects of flavonoids. Structural features which confer on kolaviron its antioxidant and free radical scavenging activity includes its multiple aromatic hydroxyl groups [45; 46].

The ORAC and FRAP assays are two antioxidant capacity assays commonly used to assess the total antioxidant capacity of biological samples [24; 25]. The ORAC assay relies on free radical damage to a fluorescent probe, most commonly fluorescein, caused by an oxidizing reagent resulting in a loss of fluorescent intensity over time. The inhibition of oxidative damage to the fluorescent probe can be correlated with the antioxidant capacity of the compound. Also, the FRAP assay measures the ability of a sample to reduce Fe^{3+} to $Fe^{2+}$and reflects the plasma levels of ascorbic acid, uric acid and **α**-tocopherol [24] although it does not measure the SH-group-containing antioxidants. The antioxidant activity of a compound against a free radical does not

necessarily match its reducing ability. No significant difference was observed in plasma antioxidant capacity assessed by FRAP and ORAC assay between all the groups in our study.

Lipids are main sources of peroxidation products and elevation in lipid levels in diabetes mellitus plays an important role in the development of atherosclerosis and represents an increased risk factor for coronary heart diseases [47]. The hyperlipidemia observed in the untreated diabetic rats in the present study could indicate an increase in the mobilization of free fatty acids from the peripheral fat depots. This could result from the uninhibited actions of lipolytic enzyme lipase caused by insulin deficiency characteristic of the diabetic state. We observed in the present study that kolaviron significantly lowered total cholesterol and triglyceride levels in the serum of normal and diabetic rats.

Hyperglycemia can result in elevated levels of circulating inflammatory mediators [48]. Increase in oxidative stress can increase the production of inflammatory proteins and vice-versa. Interactions between oxidative stress and inflammatory signals play a major role in disease progression and tissue damage in diabetes [49; 50]. Elevated levels of VEGF, IL-1 and MCP-1 have been implicated in diabetes related complications [51; 52; 53]. VEGF is an angiogenic factor with potent vascular permeability and angiogenic effects and plays a central role in vasculogenesis and neoangiogenesis by promoting the survival, migration, and proliferation of endothelial cells, and regulates glomerular permeability [54]. Our findings agree with a previous report of elevated serum levels of VEGF in diabetes [55]. Despite its protective role in non-diabetic renal disease, over-expression of VEGF is implicated in diabetic renal disease by increasing the permeability of vascular endothelium, endothelial cell proliferation and migration, and activation of matrix-degrading and plays a major pathophysiological role in diabetic nephropathy. Factors that modulate expression of VEGF and its receptors are high glucose, AGEs, endothelin 1, angiotensin II, and TGF-β [56]. In the present study, kolaviron normalized VEGF concentration in the serum of diabetic rats. Role of IL-1β has been well reported in diabetes. IL-1β increases the expression of chemotactic factors and adhesion molecules, enhances vascular endothelial permeability, and stimulates the proliferation of mesangial cells and matrix synthesis [49]. Kolaviron significantly reduced IL-1β mediated inflammation in the serum of diabetic rats in comparison to untreated diabetic control rats.

Increased production of monocyte chemoattractant protein (MCP-1) can occur after induction of oxidative stress or growth factors in a number of cells such as monocytes, smooth muscle cells and endothelial cells [57]. MCP-1 mediated macrophage accumulation plays a role in the formation of vascular inflammation, atherosclerotic lesion and kidney damage [58; 59; 60]. The protective effect of kolaviron in our study is evidenced by the restoration of MCP-1 to near normal levels in diabetic rats.

Overall, our study showed the protective effects of kolaviron in the blood of diabetic rats through improvement in glucose level, endogenous antioxidant defense and inflammation. Increased activation of major pathways such as advanced glycation end products (AGEs), protein kinase C (PKC) and aldose reductase due to the hyperglycemic state is thought to amplify the production of free radicals and inflammatory biomarkers in diabetes hence mediating the damaging effect. The ability of kolaviron to interfere with one or more of these pathways in the present study could possibly explain the observed beneficial effects of kolaviron in the blood of diabetic rats.

3.5 Conclusion

The result of this study indicates that the mechanism of antidiabetic effects of kolaviron may be related to its intrinsic anti-oxidative and anti-inflammatory properties and suggests that kolaviron may be beneficial in reducing the risk of vascular complications in diabetes.

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CHAPTER FOUR

Kolaviron, a *Garcinia* Biflavonoid Complex Ameliorates Hyperglycemia-Mediated Hepatic Injury in Rats via Suppression of Inflammatory Responses

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Abstract

Background

Chronic inflammation plays a crucial role in hyperglycemia-induced liver injury. Kolaviron (KV), a natural biflavonoid from *Garcinia kola* seeds have been shown to possess anti-inflammatory properties which has not been explored in diabetes. To our knowledge, this is the first study to investigate the effect of KV on pro-inflammatory proteins in the liver of diabetic rats.

Methods

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (50mg/kg) in male Wistar rats. Kolaviron (100 mg/kg) was administered orally five times a week for six weeks. The concentrations of cytokines and chemokines were measured using Bio-plex Pro™ magnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA). Plasma glucose and serum biomarkers of liver dysfunction were analyzed with diagnostic kits in an automated clinical chemistry analyzer. Insulin concentration was estimated by radioimmunoassay (RIA).

Result

Kolaviron (100 mg/kg) treatment significantly ameliorated hyperglycemia and liver dysfunction. Serum levels of hepatic marker enzymes were significantly reduced in kolaviron treated diabetic rats. Kolaviron prevented diabetes induced increase in the hepatic levels of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF-**α**) and monocyte chemotactic protein (MCP-1).

Conclusion

The results of this study demonstrate that the hepatoprotective effects of kolaviron in diabetic rats may be partly associated with its modulating effect on inflammatory responses.

Keywords: Diabetes; hepatic injury; kolaviron; proinflammatory cytokine; chemotactic protein.

4.1 Introduction

Type 1 diabetes mellitus (DM) is an autoimmune disorder involving immune mediated recognition of pancreatic β cells by auto-reactive T cells with subsequent release of pro-inflammatory cytokines that worsen the disease state [1]. DM characterized by prolonged hyperglycemia in the postprandial and/or fasting state [2] results from impaired insulin mediated glucose metabolism. Uncontrolled hyperglycemia leads to progressive development of microvascular and macrovascular complications, causing morbidity and mortality in diabetic patients [3-5]. Diabetes is associated with an increased risk of hepatic injury [6, 7]. It has been reported that the standardized mortality rate from end-stage liver disease (i.e. cirrhosis) in diabetic patients is higher than those with cardiovascular disease [8].

To a large extent, the effect of hyperglycemia is mediated by an elevation in the levels of pro-inflammatory proteins. Over-production of several inflammatory mediators such as growth factors, pro-inflammatory cytokines and chemokines has been documented in DM [9-11].
Type 1 DM is considered as an inflammatory process in which a significant increase of cytokines was found in the blood of patients with this disease. The response of hepatocytes to pro-inflammatory cytokines promotes the expression of genes that mediate the inflammatory process [12]. Furthermore, increased oxidative stress and chronic inflammation affects insulin secretion and sensitivity [13]. Targeting inflammatory mediators signaling through the use of anti-inflammatory agents could therefore improve therapeutic options for diabetic liver disease, a diabetic complication that is gradually gaining recognition.

Bitter kola (*Garcinia kola*) belongs to the family of plants called Guttiferae and the genus *Garcinia*. *Garcinia kola* seeds have been shown to contain a complex mixture of polyphenolic compounds, biflavonoids, prenylated benzophenones and xanthones which account for the majority of its effects [14, 15]. Kolaviron (KV) is an extract from the seeds of *Garcinia kola*, containing a complex mixture of biflavonoids and polyphenols [16]. A number of studies have confirmed the antioxidative and anti-inflammatory effects of kolaviron in chemically-induced toxicity, animal models of diseases and in cell culture [17-20]. Although the glucose lowering effect of kolaviron has been reported in animal models of diabetes mellitus [21, 22], no study has addressed the effect of KV on inflammatory biomarkers in diabetes. In the present study, we

investigated the effects of kolaviron in modulating inflammatory responses in the liver of streptozotocin-induced diabetic rats.

4.2 Materials and methods

4.2.1 Plant materials

Fresh seeds of *Garcinia kola* were purchased from Bodija market in Ibadan, Oyo State, Nigeria and authenticated by Professor E. A. Ayodele at the Department of Botany, University of Ibadan. A voucher specimen is available at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan.

4.2.2 Extraction of kolaviron

Garcinia kola seeds were peeled sliced and air-dried (25–28°C). Kolaviron was isolated according to the method of Iwu *et al.*, [20]. Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet for 24 hr. The defatted dried product was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded kolaviron, a golden yellow solid [16].

4.2.3 Ethics statement

The study protocol was approved by the Faculty of Health and Wellness Sciences Research Ethics Committee of Cape Peninsula University of Technology (Ethics Certificate no: CPUT/HW-REC 2012/AO4). All the animals received humane care in accordance to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science (NAS) and published by the National Institute of Health (Publication no. 80-23, revised 1978).

4.2.4 Animals

Adult male Wistar rats, weighing about 240–290g were housed in individual plastic cages at the animal facility of the Medical Research Council, South Africa. They were supplied with water and standard rat feed *ad libitum*. The animals were maintained under standard laboratory conditionsat 22 ± 2^{o}C with 12 hr light/dark cycles and humidity at 55 ± 5%.

4.2.5 Induction of diabetes

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ, Sigma, USA) in citrate buffer (0.1 M, pH 4.5) at a dosage of 50 mg/kg body weight (b.wt.). Diabetes was confirmed by stable hyperglycemia (>18 mmol/l) in the tail blood glucose after five days of STZ injection using a portable glucometer (Accu-Chek, Roche, Germany).

4.2.6 Study design

The dose of kolaviron (100mg/kg) was chosen based on our preliminary investigation. One hundred (100) mg/kg kolaviron was a more effective dose among the doses (100 and 200 mg/kg) investigated in our preliminary study. The animals were divided into 4 groups (n=10 per group): Normal control (NC group), Kolaviron treated normal control (NC +KV), diabetic control (DM group), and kolaviron treated-diabetic group (KV + DM group). Kolaviron (100 mg/kg b.wt.), dissolved in dimethylsulphoxide (DMSO) was administered by gastric gavage 5 times a week. Control rats also received DMSO as a vehicle. At the end of the treatment period, the rats were weighed and then anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Blood glucose was measured in 4 hours-fasted animals (usually between 10 am and 2 pm). Blood samples were collected from the abdominal aorta into glucose tubes (containing sodium fluoride/potassium oxalate), EDTA-containing tubes and serum clot activator tubes. Blood samples were centrifuged at 4000g for 10 min at 4^{o}C. Aliquot of the supernatant was stored at -80^{o}C for plasma glucose determination while other biochemical analysis was carried out on the serum. The liver was dissected, rinsed in cold phosphate buffered saline (10 mM pH 7.2), blotted on filter paper and weighed. Liver homogenate was prepared in phosphate buffered saline (10 mM pH 7.2), centrifuged at 15000 rpm for 10 min at 4^{o}C.

4.2.7 Liquid chromatography-mass spectrometry (LC-MS) analysis of Garcinia kola seed extract

LC-MS was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Brucker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). Kolaviron was separated by reversed phase chromatography on a Thermo Fischer Scientific C18 column 5 μm; 4.6 × 150 mm (Bellefonte,

USA) using gradient elution with 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B) as solvent at a flow rate of 1.0 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30°C. MS spectra were acquired in negative mode using the full scan and auto MS/MS (collision energy 25 eV) scan modes with dual spray for reference mass solution. Electrospray voltage was set to +3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 300 °C and nebulizer gas pressure was set to 35 psi.

4.2.8 Analysis of glucose and liver dysfunction biomarkers

Plasma glucose, levels of aspartate transaminase (AST) and alanine transaminase (ALT) in the serum were analyzed with diagnostic kits in an automated clinical chemistry analyzer (Medical Cooperation, Bedford, MA, USA).

4.2.9 Insulin estimation

Plasma Insulin was estimated by radioimmunoassay (RIA) according to the protocol supplied by Merck Millipore (Millipore, cooperation, MA, USA). Separate tubes containing 100 μL and 200 μL of assay buffer, 100 μL of plasma samples or standards were mixed with100 μL ^{125}I insulin tracer and 100 μL of primary antibodies. The mixture was incubated overnight at 4^{o}C. This was followed by the addition of 1 mL of precipitating agent and incubation for 20 minutes at 4^{o}C. Again samples were centrifuged at 4000g for 30 min at 4^{o}C and the supernatant was aspirated. The tubes were subjected to radioactive counting using a ^{125}I gamma counter.

4.2.10 Analysis of inflammatory biomarkers (interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and monocyte chemotactic protein (MCP-1)) in the liver

The levels of 4 inflammatory markers including interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α and monocyte chemotactic protein (MCP-1) were measured in the tissue lysates from all the rats. This was done using Bio-plex Pro™ magnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-plex® platform (Bio-Rad), according to the manufacturer's instructions. Following previous optimization, samples were evaluated undiluted,

in a blinded manner. Bio-Plex Manager^{TM} software, version 6.0 was used for bead acquisition and analysis.

4.2.11 Statistical analysis

Data were analyzed using one-way analysis of variance and expressed as mean ± standard deviation. Statistical analyses were performed using Graph pad Prism version 6.02, for windows (Graph Pad software, San Diego, CA). Differences were considered significant at $P<0.05$.

4.3 Results

4.3.1 Kolaviron treatment lowers blood glucose, prevented loss of body weight and liver hypertrophy in diabetic rats

Effect of kolaviron administration on blood glucose, liver and body weight in STZ-induced diabetic and normoglycemic rats is shown in Table 1. Six weeks after diabetes confirmation, the random blood glucose concentration (mmol/l) in the diabetic and control group was 28.19 ± 2.25 and 9.93 ± 0.51 respectively. The blood glucose concentration for the normal control rats plus KV was 8.91 ± 0.6 and the diabetes mellitus plus kolaviron group was 17.35 ± 2.36. In addition to elevated blood glucose, diabetic rats had decreased mean body weight compared to normal control while treatment with kolaviron for 6 weeks significantly lowered blood glucose and ameliorated the body weight loss when compared to the untreated diabetic group. Although body weight was still significantly lower in comparison with normal control. Diabetes caused an increase in relative liver weight (expressed as % body weight) in rats while treatment of diabetic rats with kolaviron significantly restored liver weight to near normal. STZ diabetic rats exhibited impaired insulin release while KV treatment of diabetic rats significantly increased plasma insulin levels compared with untreated rats.

Table 1: Effect of kolaviron administration on plasma glucose, insulin, liver weight and body weight in STZ-induced diabetic and normoglycemic rats.

Parameters	NC	NC +KV	DM	DM +KV
Glucose (mmol/l)	9.93 ± 0.51	8.91 ± 0.6 [a,b]	28.19 ± 2.25 [a]	17.35 ±2.36 [a,b]
Insulin (ng/ml)	7.99 ± 1.93	7.69 ± 1.94	0.30 ± 0.13 [a,b]	0.79 ± 0.28 [a,b]
Body weight change (g)	+99.59	+101.12	-9.38 [a]	+45.38 [a,b]
Relative liver weight (g)	3.22 ± 0.15	3.38 ± 0.07	4.53 ± 0.16 [a]	3.99 ± 0.15 [a,b]

Data are presented as mean ± S.D Values. [a] $p < 0.05$ vs. normal control. [b] $p < 0.05$ vs. untreated diabetes. NC; Normal control, NC +KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron.

4.3.2 Kolaviron lowers serum levels of hepatic enzymes in STZ-induced diabetic rats

Figure 1 indicates results of kolaviron administration on serum levels of hepatic enzymes in STZ-induced diabetic rats. In the diabetic group, serum levels of liver damage biomarkers; ALT (77.96 ± 11.9) and AST (107 ± 5.43) were elevated compared with normal controls viz: 60.37 ±7.20 and 57.12 ± 6.63 respectively. Kolaviron administration to diabetic rats significantly reduced serum levels of ALT (67.9 ± 6.94) and AST (53.38 ± 4.93) when compared to the diabetic control.

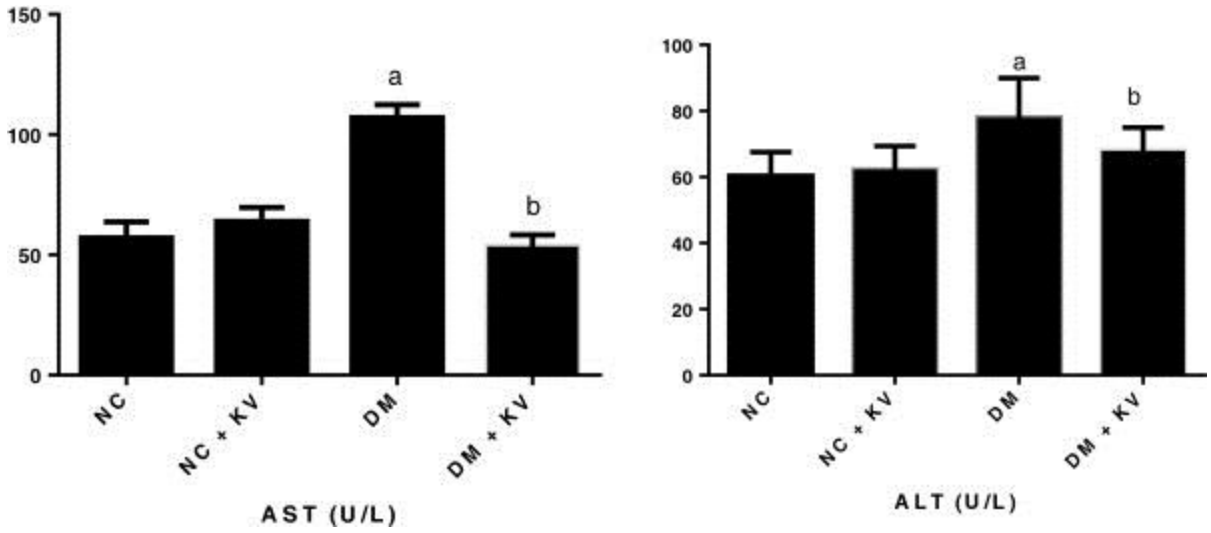


Figure 1: Effect of kolaviron administration on serum levels of hepatic enzymes in diabetic and normoglycemic rats. Data are presented as mean ± S.D Values. [a] $p < 0.05$ vs. normal control. [b] $p < 0.05$ vs. untreated diabetes. NC; Normal control, NC +KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated with kolaviron.

4.3.3 Kolaviron ameliorated hyperglycemia-mediated increase in the levels of proinflammatory proteins in the liver of diabetic rats

The effect of kolaviron on interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF-**α**) and monocyte chemotactic protein (MCP-1) is illustrated in Figure 2. The concentration of proinflammatory cytokines were significantly increased in the liver of diabetic rats when compared with the control rats. Lowered levels of MCP-1 and IL-1β were detected in the liver of kolaviron treated diabetic rats compared to the untreated diabetic group. Administration of kolaviron to diabetic rats 5 times a week for 6 weeks also significantly reduced IL-6 and TNF-**α** when compared with both normal control and diabetic rats. Kolaviron also lowered serum levels of TNF-**α** and IL-6 in normal rats.

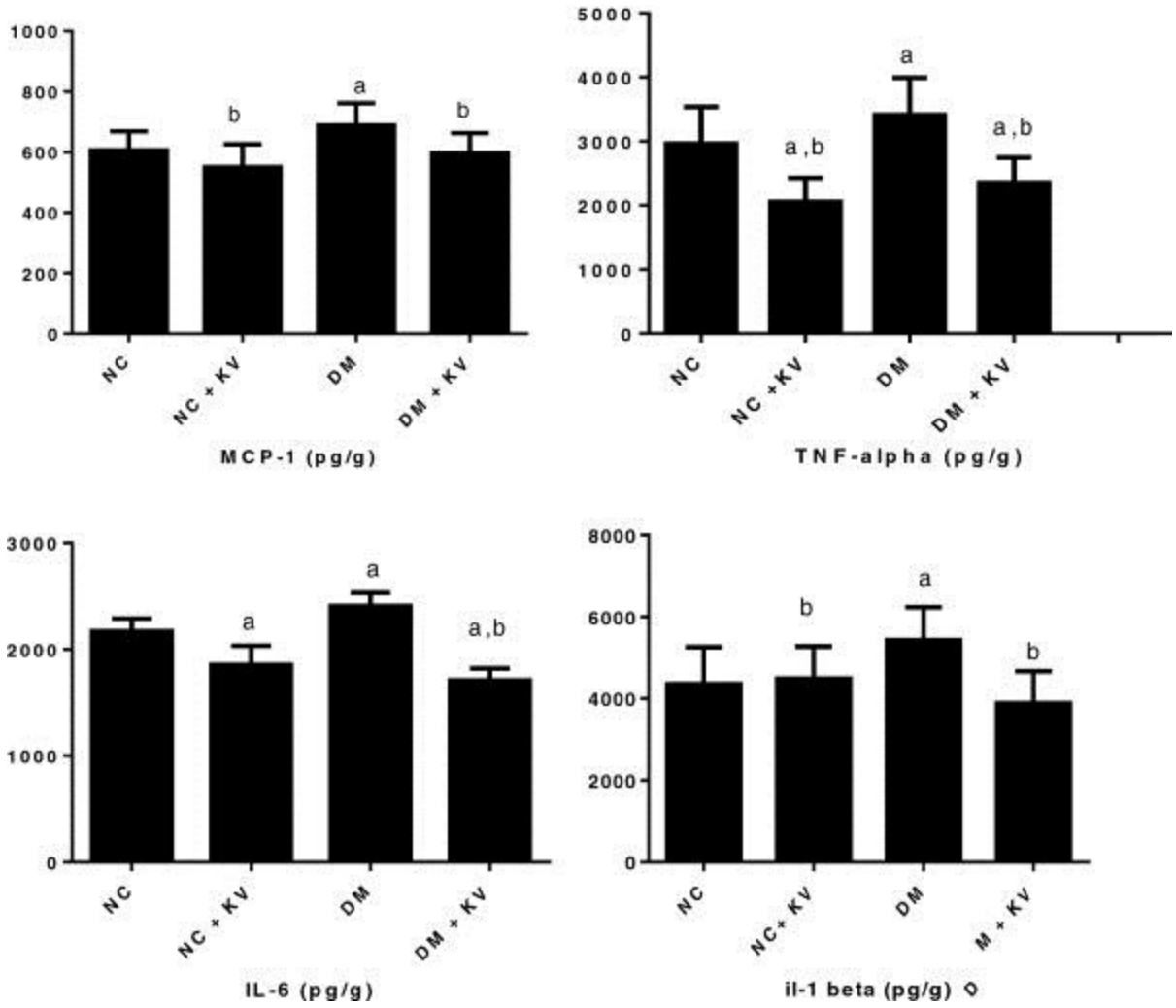
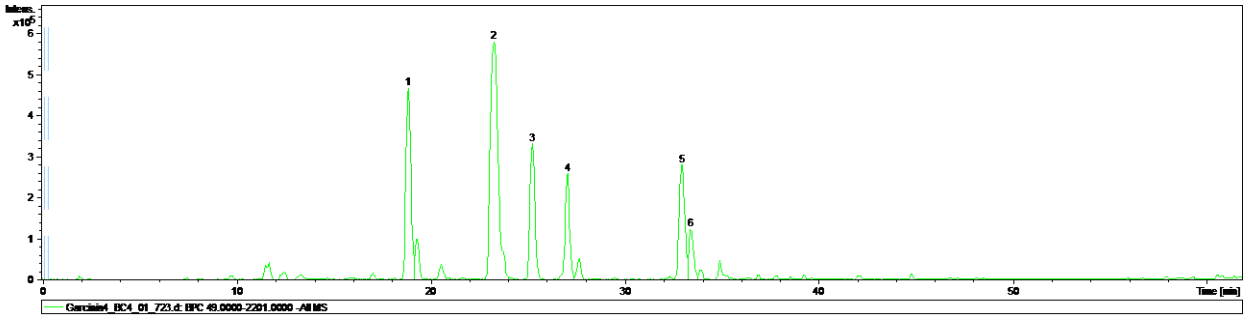


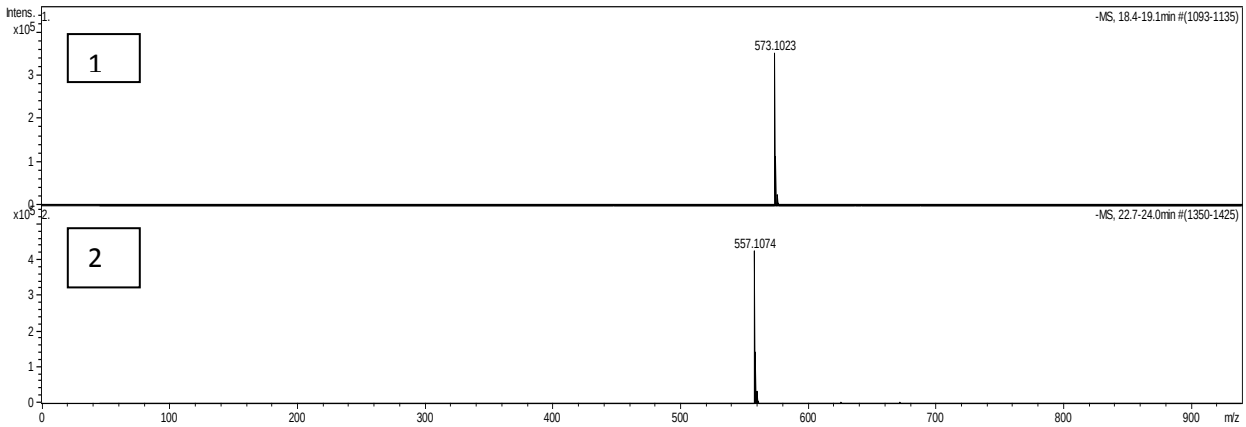
Figure 2: Effects of kolaviron on levels of MCP-1, IL-1β, TNF- α and IL-6 in the liver of normal and diabetic rats. Data are presented as mean pg/g wet tissue ± S.D. [a]p< 0.05 vs. normal control. [b] p < 0.05 vs. untreated diabetes. NC; Normal control, NC +KV; Normal control treated with kolaviron, DM; untreated diabetic rats, DM + KV; Diabetic rats treated withkolaviron.

4.3.4 Liquid chromatography-mass spectrometry (LC-MS) analysis of Garcinia kola seed extract

In the negative-ion, the ESI-MS analysis of kolaviron shows molecular ion peaks $[M-H]^-$ at (1) *m/z* 573.1023, (2) *m/z* 557.1074, (3) *m/z* 587.1178, (4) *m/z* 557.1074(5) *m/z* 541.1123 and (6) *m/z* 555.0909 (Figure 3). Based on the calculated molecular formula, the presence of *Garcinia* biflavonoid 2 (*m/z* 573.1023 = $C_{30}H_{22}O_{12}$), *Garcinia* biflavonoid 1(*m/z* 557.1074, 557.1072=

$C_{30}H_{22}O_{11}$), kolaflavanone (*m/z* 587.1178=$C_{31}H_{24}O_{12}$) and kolaflavones (*m/z* 555.0909=$C_{30}H_{21}O_{11}$), previously reported in literature as components of kolaviron [16] were confirmed (Figure 4a) The mass spectrum of peak 5 shows the ion peak $[M-H]^-$ at *m/z* 541.1123 with the formula $C_{30}H_{22}O_{10}$. On the basis of this data and database searching, the structure of this compound (peak 5) was deduced to be a binaringenin (Figure 4b), a biflavanone commonly found in *Garcinia* species [23.] To our knowledge, this is the first report of a new biflavonoid in kolaviron (a *Garcinia* biflavonoid complex).





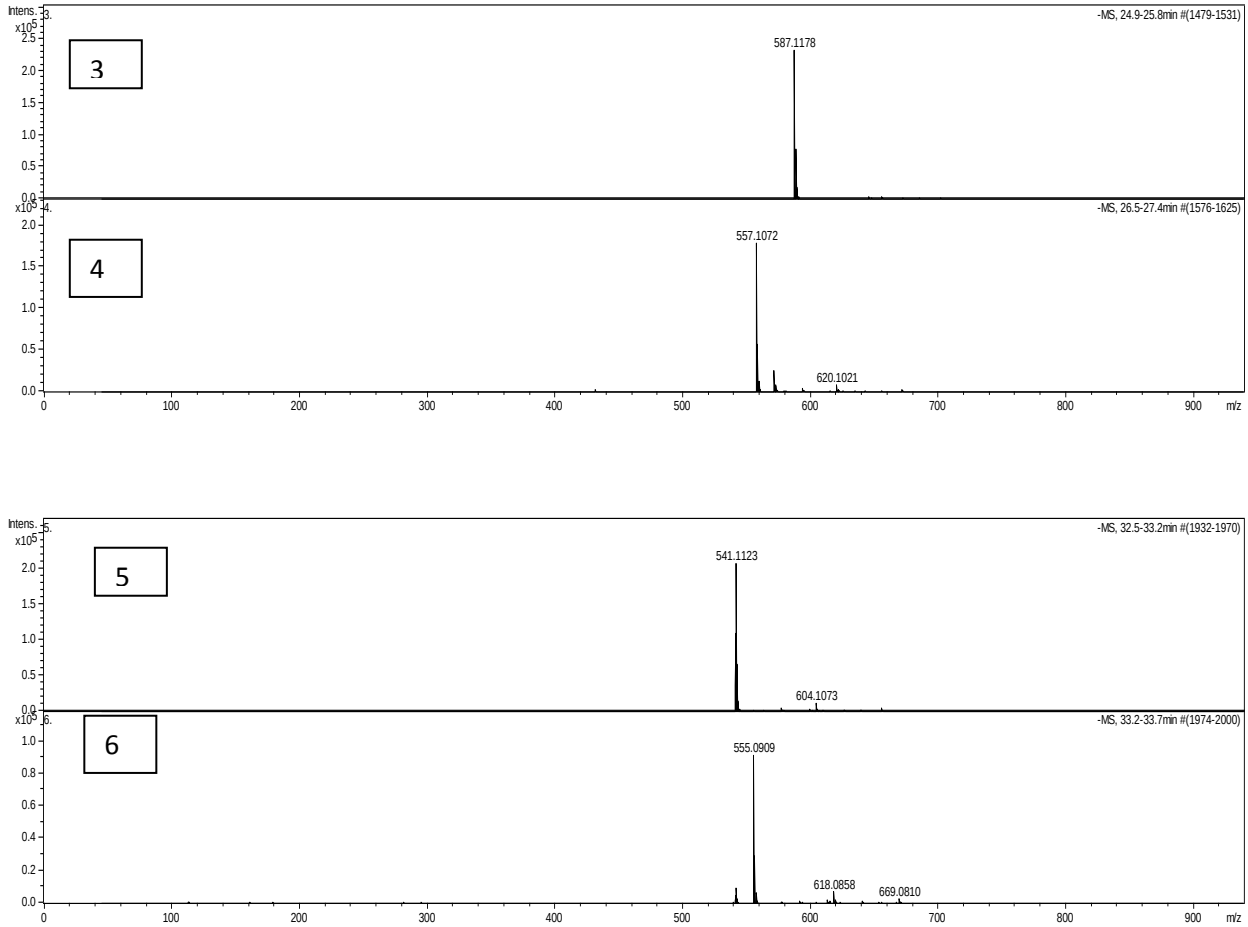
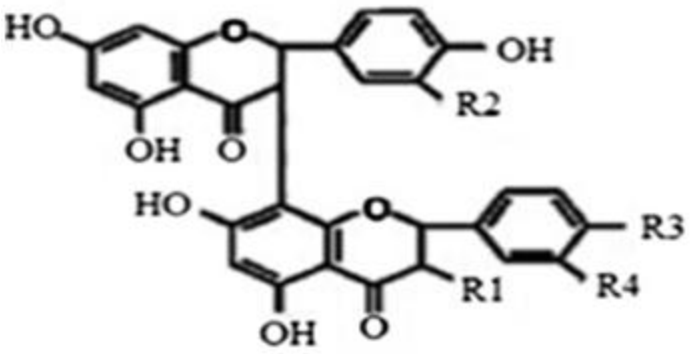
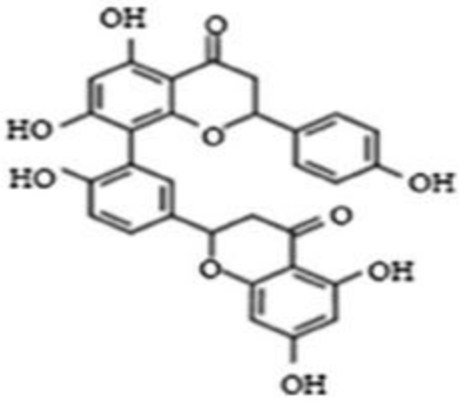


Figure 3: Mass Spectra of kolaviron; (1) Garcinia Biflavonoid 2 ($C_{30}H_{22}O_{12,}$ *m/z* 573.1023,); (2) Garcinia Biflavonoid 1 ($C_{30}H_{22}O_{11,}$ *m/z* 557.1074,);(3)Kolaflavanone ($C_{31}H_{24}O_{12,}$ *m/z* 587.1178), (4) Garcinia Biflavonoid 1 ($C_{30}H_{22}O_{11,}$ *m/z* 557.1074); (5) X($C_{30}H_{22}O_{10,}$ 541.1123), deduced to be binaringenin); (6) Kolaflavone ($C_{30}H_{21}O_{11,}$ *m/z* 555.0909).

4a



4b



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavone	OH	H	OH	----
Kolaflavanone	OH	H	OCH_3	OH

Figures 4a and 4b: Chemical structure of *Garcinia* biflavonoid complex (Kolaviron) containing Garcinia biflavonoid GB-1(3",4',4"',5,5",7,7"-heptahydroxy-3,8" biflavanone), GB-2 (3",4',4"',5,5",5"',7,7"-octa-hydroxy-3,8"- biflavanone), and kolaflavanone (3",4',4"',5,5",5"',7,7" octahydroxy-4"'-methoxy-3,8"-biflavanone) is confirmed (Figure 4a) while binaringenin (Figure 4b) is presumed to be an additional compound in kolaviron based on ESI-MS/MS result.

4.4 Discussion

Limitations of the currently used drugs on glycemic regulation have raised the need for the development of new drugs which can act as alternative and/or complementary therapy. Interest in natural plant products as anti-diabetic agents has increased over the years due to their low side effects and multidimensional mode of action [24]. Kolaviron a natural compound from the bitter kola seed, containing a complex mixture of *Garcinia* biflavonoid, GBI, GB2 and kolaflavones has been demonstrated for its, hypoglycemic, antioxidative, antiinflammatory and antigenotoxic effects [19, 25].

The concept that chronic low grade inflammation is important in the development and progression of diabetes and its associated complication is not new. Prolonged hyperglycemia can generate an inflammatory state leading to an increment in cytokine production and pancreatic beta cell destruction [26]. Antiinflammatory agents have been documented to be beneficial in diabetes. Among these are curcumin and its derivative [27, 28], resveratrol [29] and Cannabidiol [30]. The damaging effect of inflammatory molecules in diabetes can be mediated through its interaction with receptors and activation of signaling pathways which exacerbate the disease state [31]. Due to the existing association between chronic inflammation and diabetic complications including liver injury, identification of therapeutic targets that is able to specifically downregulate proinflammatory responses and mediators could be a promising strategy in the management of diabetes mellitus. It is noteworthy that our study is the first to investigate the effect of kolaviron on inflammatory mediators in diabetes.

Regulation of blood glucose either in the fasting state and/or postprandially is an important factor in diabetic therapy. Sustained glycemic control decreases the risk of developing microvascular and macrovascular complications [32, 33]. The marked reduction of blood glucose in this study following KV treatment is in line with previous studies demonstrating its hypoglycemic effects [34]. Although kolaviron significantly increased the plasma insulin levels in diabetic rats, the magnitude of increase is lower compared to the corresponding effect on blood glucose. The mechanisms of hypoglycemic effect of kolaviron might be due to the combination of its stimulating action on the pancreatic β cells to release insulin and also an insulin independent

effect and extrapancreatic action which involves glucose utilization in extrahepatic tissues [35, 36]. Furthermore, the hypoglycemic effect of flavonoids can be mediated through an increase in hepatic glucose storage by stimulating the action of glycolytic and glycogenic enzymes or by inhibiting glucose-6-phosphatase. This consequently results in the uptake of glucose into cells and the reduction in the blood glucose level through the upregulation of glycogen formation, downregulation of the rate of glycogen breakdown, and glucose synthesis [37-39].

It has been shown that absolute or relative insulin deficiency coupled with decreased ATP production accounts for low protein synthesis [40]. The decreased mean body weight in diabetic rats could be an indication of excessive breakdown of structural proteins in an attempt to compensate for low availability of carbohydrate as an energy source [41]. The ability of kolaviron to protect against weight loss might be due to its glucose lowering effect. Liver hypertrophy (increased liver weight) observed in diabetic rats may be due to hypoinsulinemia-induced increased triglycerides accumulation in the liver as alternative glucose precursors since liver glycogen is usually depleted in STZ-induced diabetic rats [42]. Liver weight (expressed as a percentage of body weight) was significantly lower in kolaviron treated rats. The ability of kolaviron to restore liver glycogen levels may partly explain its beneficial effect on liver hypertrophy in diabetic rats. It was reported in a previous that kolaviron inhibited microsomal glucose-6-phosphatase in STZ diabetic rats [34]. The inhibition of glucose-6 phosphatase by kolaviron can increase hepatic glucose-6 phosphate which serves as substrate for glycogen synthesis thereby resulting in upregulation of hepatic glycogen levels.

Amino transferases, aspartate transaminase (AST) and alanine transaminase (ALT) catalyse amino transfer reactions and are used as markers of hepatic injury [43]. Deleterious effect of hyperglycemia in the liver of diabetic rats observed in the present study is evidenced by serum elevation of liver damage biomarkers. The hepatoprotective effect of kolaviron is demonstrated by the significant reduction of serum levels of ALT and AST in the diabetic treated rats.

Inflammation has been reported to cause direct organ damage in diabetic rats [44, 45] and humans [46]. Increased levels of pro-inflammatory cytokines MCP-1, IL-1β, IL-6, IL-18 and

TNF-**α** have been reported in diabetes [47, 48]. In this study, upregulated levels of these pro-inflamatory proteins were observed in the liver of STZ-induced diabetes rats.

MCP-1 is a chemo-attractant which promotes monocyte and macrophage migration and activation at the site of injury. Over expression of MCP-1 exerts various damaging effects via increased production of superoxide radicals from macrophages, release of lysosomal enzymes, cytokines, growth factors and cellular adhesion molecules [49, 50]. Animal and human studies have also shown a correlation between blood and hepatic levels of MCP-1 and the extent of inflammation [51, 52]. Considering the role of macrophages in perpetuating hepatic inflammation, reduction of MCP-1 levels may be another mechanism by which kolaviron mediates its protective effect in the liver of diabetic rats.

Hepatic infiltrating macrophages and Kupffer cells are sources of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 in the liver [53]. Our study shows upregulated levels of these inflammatory proteins in a diabetic state while kolaviron treatment notably reduced hepatic levels of IL-1β, IL-6 and TNF-α in diabetic rat. The suppressing effect of kolaviron on serum levels of IL-1β has been demonstrated in chemically-induced inflammation of the colon [19]. IL-1β induces the expression of various genes encoding oxidants, cytokines, chemokines, growth factors and adhesion molecules whose promoter region are monitored through interactions with transcription factor, NFκB [54-56]. IL-1β inhibits β- cell function and promotes Fas-triggered apoptosis in part by NF-κB [57]. In a GK rat model of type 2 diabetes, treatment with IL-1 receptor antagonist (IL-1Ra) reduced islet mRNA expression of a number of inflammatory factors which includes: IL-1β, IL-6, TNF-**α**, MCP-1 and MIP-1**α** [58]. Possible mechanisms by which kolaviron elicit its liver protective and anti-inflammatory effect in diabetic rats could be by a direct reduction of macrophage infiltration and/or by repressing NF-κB activation.

TNF-α is one of the major cytokines upregulated in diabetic liver which can promote the activation of NF-κB through interaction with the TNF-**α** receptor resulting in liver inflammation and apoptosis [55]. The involvement of TNF in alcoholic hepatitis, viral hepatitis and ischemia/reperfusion liver injury has also been documented [59]. Our study revealed that Kolaviron treatment abrogated hyperglycemia induced increase in the hepatic concentration of

TNF-α. There is a report that kolaviron (KV) shows inhibitory action on prostaglandin E_2 and TNF-α production in macrophage-like cell line [60]. Kolaviron also downregulates iNOS and COX-2 expression in the liver of dimethyl nitrosamine (DMN)-treated rat via the inhibition of DNA binding activity of NF-κB [61]. The antiinflammatory and hepatoprotective effect observed in our study might be mediated via inhibition of transcription factor NF-κB, a key regulator of inflammatory process.

Although the results of our study demonstrated the beneficial effects of kolaviron on inflammatory response and hepatic injury in the liver of diabetic rats, future studies can address other possible mechanisms of action of kolaviron and the underlying molecular targets of *Garcinia* biflavonoid complex in diabetes. Further investigation may also be necessary for complete elucidation of the structure of kolaviron.

4.5 Conclusion

In summary, our study revealed that kolaviron treatment ameliorated hyperglycemia-induced increase in the levels of proinflammatory cytokines and chemokine in rat's liver and may therefore act as a useful agent in retarding the progression of diabetic liver complications. Another important outcome of this study is the discovery of a new compound from kolaviron. This new compound along with previously identified compounds of kolaviron may partly explain its beneficial effects in diabetes.

Competing Interests

The authors declare that they have no competing interests.

Author's contributions

ORA was responsible for the conception and design, carried out all experiment, performed data analysis and drafted the manuscript. NNC collaborated in the antiinflammatory studies and made

contribution to the revision of the manuscript. NLB and OOO made contribution to the conception and revised the manuscript critically for intellectual content

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CHAPTER FIVE

Kolaviron modulates apoptosis by suppressing oxidative stress and inflammation in diabetes-induced nephrotoxic rats

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Abstract

Aims

Diabetic nephropathy is a complex disease that involves increased production of free radicals which is a strong stimulus for the release of pro-inflammatory factors. We evaluated the renal protective effect of kolaviron (KV) in diabetes-induced nephrotoxic rats.

Methods

Male Wistar rats were divided into 4 groups; Untreated controls (C); Normal rats treated with kolaviron; (C+KV); Untreated diabetic rats (D); Kolaviron treated diabetic rats; (D+KV). A

single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg) was used for the induction of diabetes. Renal function parameters were estimated in a clinical chemistry analyzer. Markers of oxidative stress in the kidney homogenate were analysed in a Multiskan Spectrum plate reader and Bio-plex Promagnetic bead-based assays was used for the analysis of inflammatory markers. The effect of kolaviron on diabetes-induced apoptosis was assessed by TUNEL assay.

Result

In the diabetic rats, alterations in antioxidant defenses such as an increase in lipid peroxidation, glutathione peroxidase (GPX) activity and a decrease in catalase (CAT) activity, glutathione (GSH) levels and Oxygen Radical Absorbance Capacity (ORAC) were observed. There was no difference in superoxide dismutase (SOD) activity. Diabetes induction increased apoptotic cell death and the levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-**α** with no effect on IL-10. Kolaviron treatment of diabetic rats restored the activities of antioxidant enzymes, reduced lipid peroxidation and increased ORAC and GSH concentration in renal tissues. Kolaviron treatment of diabetic rats also suppressed renal IL-1β.

Conclusion

The beneficial effects of kolaviron on diabetes-induced kidney injury may be due to its inhibitory action on oxidative stress, IL-1β production and apoptosis.

Keywords: nephrotoxicity, cell death, antioxidant, cytokine.

5.1 Introduction

Diabetes mellitus has dramatically increased globally. Diabetes can result in a progressive decline in glomerular filtration rate characterized by glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins (Jain, 2012). The progression of renal injury often leads to end-stage renal disease affecting 20-40% of diabetic patients (Hakim and Pflueger, 2010). Oxidative stress induced by the hyperglycemic milieu drives the development of complications including diabetic nephropathy (Kawahito *et al*, 2009). The initial cellular response to high glucose challenge also rapidly induces apoptosis (Park *et al.*, 2001). Furthermore, the recruitment of inflammatory cells from the circulation into renal tissue is a critical feature of diabetic nephropathy (Navarro-González *et al*., 2011). STZ is selectively toxic to insulin-secreting beta cells of pancreatic islets inducing beta cell necrosis and impairs glucose oxidation and insulin release (Hayden and Tyagi, 2002; Merzouk *et al.,* 2000) and, in rats, mimics human diabetes (Weir *et al.,* 1981) therefore it is a good experimental model for diabetic renal injury.

Garcinia kola seeds (Family: Guttiferae) are consumed in west and central Africa and contain high biflavonoid levels which have several pharmacokinetic advantages over simple monomeric flavonoids as they survive first-pass metabolism (Iwu and Igboko, 1986). Kolaviron (KV) is a biflavonoid complex extracted from the seeds of *Garcinia kola* which has numerous therapeutic effects. Although, kolaviron has been reported to produce a hypoglycaemic and hypocholesterolaemic effects in diabetic animals (Iwu *et al.,* 1990; Adaramoye and Adeyemi, 2006) and to protect against oxidative stress induced by toxins in experimental animal models (Akintonwa and Essien, 1990; Farombi, 2000), its efficacy in modulating the complex responses associated with oxidative stress and inflammation in diabetic kidney damage remains to be elucidated. In this study, we hypothesized that supplementation of kolaviron protects against diabetic kidney damage resulting from hyperglycemia-induced oxidative stress, inflammation and apoptosis in rats.

5.2 Materials and methods

5.2 .1 Animals

The study protocol was approved by the Faculty of Health and Wellness Sciences Research Ethics Committee of the Cape Peninsula University of Technology (Ethics Certificate no: CPUT/HW-REC 2012/AO4). All the animals received humane care in accordance with the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science (NAS) and published by the National Institute of Health (Publication no. 80-23, revised 1978). Male Wistar rats (11-12 weeks, 270 ± 25g) were used for the study. Experiments were performed at the animal facility of the Medical Research Council (MRC) and strictly adhered to the standard operating procedures (SOPs). All animals were housed individually at room temperature (22 ± 2°C) with 55 ± 5% humidity and an automatically controlled cycle of 12 h light and 12 h dark. A standard laboratory diet and water were provided *ad libitum* and rats were habituated to the experimental conditions one week prior to experimentation.

5.2.2 Chemicals and reagents

Streptozotocin (STZ), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH),5,5'-dithiobis-2-nitrobenzoic acid (DTNB), fluorescein sodiumsalt, FolinCiocalteu's phenol reagent,L-ascorbic acid, reduced glutathione (GSH), glutathione reductase (GR), 6-hydroxydopamine, β-nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt (NADPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and 2-thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (Johannesburg, South Africa). Diethylenetriaminepentaacetic acid (DETAPAC) and malondialdehydebis (diethyl acetal) (MDA), hexane, methanol (MeOH) and hydrogen peroxide (H_20_2) were purchased from Merck (Johannesburg, South Africa). All chemicals and reagents used were of the highest commercially available purity.

5.2.3 Induction of diabetes

To induce diabetes in rats, a single intraperitoneal injection of freshly prepared solution of 50 mg/kg of STZ (Sigma-Aldrich, SA) in citrate buffer (0.1M, pH 4.5) was administered to overnight fasted rats. Diabetes was confirmed after a tail prick by stable hyperglycemia (>18 mmol/l) five days post-STZ injection using a portable glucometer (Accu-Chek, Roche, Germany).

5.2.4 Plant materials

Fresh seeds of *Garcinia kola* were purchased from Bodija market in Ibadan, Oyo State, Nigeria and authenticated by Professor E. A Ayodele at the Department of Botany, University of Ibadan. A voucher specimen (FHI-109777) is available at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan.

5.2.*5 Extraction of kolaviron*

Garcinia kola seeds were peeled, sliced and air-dried (25-28°C). Kolaviron was isolated according to the method of Iwu *et al.* (1990). Briefly, the powdered seeds were extracted with light petroleum ether (bp 40-60°C) in a soxhlet for 24 h. The defatted dried product was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded kolaviron.

5.2.6 *Study design*

The rats were randomly assigned into four groups (n = 10 per group): control (C), KV-treated control (C+KV), diabetic (D) and KV-treated diabetic (D+KV). Treatment commenced on the sixth day post STZ injection and continued for 6 weeks. The 100 mg/kg dose of Kolaviron was selected based on our preliminary investigation. This 100 mg/kg kolaviron dose was a more effective dose among the doses (100 and 200 mg/kg) investigated in our preliminary study. Kolaviron (100 mg/kg body weight), dissolved in dimethylsulphoxide (DMSO) was administered by gastric gavage five times weekly. The dosage of kolaviron was adjusted weekly according to changes in body weight to standardize the dosages over the duration of the study.

5.2.7 Blood and tissue collection

After treatment, the rats were weighed and anaesthetised with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Blood was collected in tubes without anticoagulant for serum separation. Blood samples were centrifuged at 4,000g for 10 min at 4^{o}C. Aliquots of the supernatant were stored at -80^{o}C for biochemical analysis. The left kidney was rapidly excised, washed in ice-cold phosphate buffered saline, blotted, frozen in liquid nitrogen and stored at -80°C until use whereas the right kidney was excised and fixed in 10% (v:v) neutral buffered formalin.

5.2.8 Determination of relative kidney weight

Hypertrophy of the kidney was estimated by comparing the kidney weight to the body weight (relative kidney weight).

$$\text{Relative kidney weight (mg/100g body weight)} = \frac{\text{Kidney weight (g)}}{\text{Total body weight (g)}} \times 100$$

5.2.9 Analysis of renal function parameters

Serum uric acid, blood urea nitrogen (BUN) and albumin concentrations were analysed with diagnostic kits in an automated clinical chemistry analyzer (Medical Cooperation, Bedford, MA, USA).

5.2.10 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay kinetically measured the peroxyl radical scavenging activity in kidneys with trolox as the antioxidant standard according to the method of Ou *et al.*, 2001. The fluorescence of the reaction mixture was monitored and recorded every minute (excitation = 485 nm and emission = 535 nm) for 2 h with a Fluoroscan Ascent plate reader (Thermo Fischer Scientific, Waltham, MA, USA). Results were determined with a regression equation relating trolox concentrations and the net area under the kinetic fluorescein decay curve ($y = ax2 + bx + c$). The ORAC value was expressed in micromoles of trolox equivalents per gram of tissue (μmol TE/g).

5.2.11 Measurement of antioxidant enzyme activities

Activities of antioxidant enzymes were determined in a clear 96-well plate using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Catalase (CAT) activity was determined by the method of Aebi (1984). Superoxide Dismutase (SOD) was determined by the method of Crosti *et al*., (1987). Glutathione Peroxidase (GPX) activity was determined according to the method of Ellerby and Bredesen (2000). The protein level in the kidney was estimated with a bicinchoninic acid (BCA) kit (Pierce, Illinois, USA).

5.2.12 Determination of glutathione (GSH) concentrations

The concentration of reduced glutathione (GSH) in the kidney was estimated by the method of Jollow *et al.,* (1974).

5.2.13 Measurement of lipid peroxidation

Kidney malondialdehyde (MDA) was determined by high performance liquid chromatography (HPLC) using a method adapted from Khoschsorur *et al.* (2000). Fluorometric detection was performed with excitation at 532 nm and emission at 552 nm. The peak of the MDA-TBA adduct was calibrated with the MDA standard.

5.2.14 Determination of renal interleukin (IL)-1β, IL-10 and TNF-α concentrations

The levels of IL-1β, IL-10 and TNF-α were determined in the rat kidney homogenate using Bio-plex Promagnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-plex platform (Bio-Rad). Following previous optimisation, undiluted samples were blindly evaluated. Samples were reacted with a mixture of fluorescent polystyrene beads bound with specific anti-cytokine primary antibodies, resulting in binding of the cytokines to the beads with the corresponding antibody. The biotinylated anti-cytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. Bio-Plex Manager software version 6.0 was used for bead acquisition and analysis.

5.2.15 Assessment of apoptosis

The right kidney was excised, fixed in 10% (v: v) neutral buffered formalin and dehydrated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin. Paraffin-embedded kidney tissue sections (5 μm) were layered onto glass slides then dewaxed in xylene and rehydrated in graded dilutions of ethanol. Kidney apoptotic indices were quantified by terminal deoxynucleotidyltransferase mediated dUTP-biotin nick end labeling (TUNEL) assay according to the manufacturer's instruction using a Apo Tag fluorescein *in situ* apoptosis detection kit (Millipore, Temecula, CA). Sections were counter stained with propidium iodide (PI), mounted and viewed on a Carl Zeiss LSM 780 confocal microscope using a Plan-Neofluar 40X objective. Samples were excited with an argon mutline laser with excitation at 488 nm (for green) and 561 nm (for red). Images were acquired with a GaAsP detector set up for standard FITC and PI detection and optimised with the Carl Zeiss ZEN 2011 (Germany) software package. Total cell population and apoptotic cells were counted using the ImageJ image analysis software. TUNEL positive cells were expressed as percentage of total cells.

5.2.16 Histology

Kidney tissue was fixed in 10% (v: v) neutral buffered formalin and embedded in paraffin wax. Fixed kidney tissues were cut into 5μm slices. After being deparaffinized using xylene and ethanol dilutions and rehydration, tissue sections were stained with hematoxylin and eosin (H&E). Mounted slides were examined in a masked fashion under a light microscope and photographed using a digital camera.

5.2.17 Statistical analysis

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p<0.05$.

5.3 Results

5.3.1 Kolaviron's effect on weight parameters and renal function in normal and STZ-induced diabetic rats

There was an increase in the relative kidney weight of the diabetic rats compared to control rats (Table 1). Elevated serum levels of blood urea nitrogen and uric acid and a decrease in albumin levels were also observed in the diabetic rats compared to control rats. Treatment of diabetic rats with kolaviron reduced the relative kidney weight and the serum levels of kidney function biomarkers, viz., uric acid, blood urea nitrogen and increased albumin concentration. Administration of kolaviron to non-diabetic control rats had no adverse effect on the kidney.

Table 1: Effect of KV on weight parameters and serum markers of kidney damage

	C	C + KV	D	D + KV
Change in body weight (%)	+33.34%	+33.84%	-3.52%[a]	+17.30%[a,b]
Kidney weight (g)	1.3 ± 0.09	1.4 ± 0.07	1.3 ± 0.09	1.4 ± 0.09
Relative kidney weight (g/100g)	0.33 ± 0.02	0.35 ± 0.04	0.5 ± 0.03 [a]	0.43 ± 0.04[a,b]
Uric acid (unit)	48.8 ± 9.9	49.3 ± 7.06	82.8 + 11.35 [a]	55.3 ± 12.34 [b]
BUN (unit)	6.5 ± 0.77	6.2 ± 0.5	18.4 ± 2.90 [a]	8.3 ± 1.21[a,b]
Albumin (unit)	30.8 ± 0.91	30.2 ± 0.62	26.2 ± 0.72 [a]	27.4 ± 0.98[a,b]

Data are presented as means ± S.D. [a]Values differ from control rats ($p < 0.05$). [b]Values differ from diabetic rats ($p < 0.05$). C; Control, C + KV; KV-treated control rats, D; untreated diabetic rats, D + KV; KV-treated diabetic rats. BUN, blood urea nitrogen

5.3.2 Effect of kolaviron on kidney antioxidant enzymes (CAT, SOD, GPX) and non-enzyme (GSH) in normal and STZ-induced diabetic rats

Table 2 illustrates the effect of kolaviron on glutathione concentration and activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the control and diabetic rat kidney. The induction of diabetes with STZ reduced kidney glutathione (GSH) concentration and CAT activity in the diabetic rats. GPX activity was elevated in diabetic rats compared to control rats with no differences in SOD activities amongst the groups. Kolaviron

treated diabetic rats demonstrated increased GSH concentration and CAT activity compared to diabetic controls. GPX activitiy was also normalized after kolaviron administration to diabetic rats. Kolaviron also increased GSH concentration in renal tissues of non-diabetic rats.

Table 2: Effect of kolaviron on renal antioxidant status

	C	C + KV	D	D +KV
CAT	1.32 ± 0.26	1.31 ± 0.24	0.95 ± 0.24 [a]	1.16 ± 0.19
SOD	401.70 ± 40.32	386.85 ± 43.53	423.68 ± 28.56	391.87 ± 39.17
GPX	4.76 ± 0.37	3.93 ± 0.61	6.09 ± 0.89 [a]	4.70 ± 0.90 [b]
GSH	1.19 ± 0.07	1.33 ± 0.10[a]	0.96 ± 0.14 [a]	1.10 ± 0.13[b]

Data are presented as mean ± S.D. [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). C; Normal control, C+ KV; kolaviron-treated control rats, D; untreated diabetic rats, D + KV; kolaviron-treated diabetic rats.CAT; catalase, μmol H_2O_2 consumed/min/mg protein, SOD; superoxide dismutase, U/mg protein, GPx; glutathione peroxidase, µmol NADPH oxidized/min/μg protein, GSH; reduced glutathione, µmol/g tissue.

5.3.3 Oxygen radical absorbance capacity of kolaviron and its effect on lipid peroxidation in the kidney of normal and STZ-induced diabetic rats

As shown in Table 3, malondialdehyde (MDA; lipid peroxidation marker) levels were elevated in the kidneys of diabetic control rats whereas oxygen radical absorbance capacity (ORAC) values were reduced compared to non-diabetic control. Kolaviron treatment reduced MDA levels and increased ORAC values in diabetic rats compared to diabetic control rats. Kolaviron- treated control rats also had increased ORAC values compared to untreated control rats.

Table 3: Effect of kolaviron on oxygen radical and lipid peroxidation in the kidney of diabetic and non-diabetic rats.

	MDA (µmol/g tissue)	ORAC (µmol TE/g tissue)
C	2.09 ± 0.11	4.21 ±0.86
C + KV	2.06 ± 0.11	7.05±0.78 [a]
D	2.26 ± 0.12 [a]	3.62 ±0.76[a]
D + KV	1.96 ± 0.14[b]	7.13 ±0.81[a,b]

Data are presented as mean ± S.D. [a]Values differ significantly from normal control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). C; Normal control, C + KV; kolaviron-treated control rats, D; untreated diabetic rats, D + KV; kolaviron-treated diabetic rats. ORAC: oxygen radical absorbance capacity, TE: trolox equivalent, MDA: malondialdehyde.

5.3.4 Effect of kolaviron on pro-inflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF-α)] and anti-inflammatory cytokine (IL-10) in normal and STZ-induced diabetic rats

The induction of diabetes increased renal TNF-α and IL-1β concentrations (Figure 2). The increase in IL-1β was inhibited after the administration of kolaviron although no statistically significant effect was observed in the renal concentration of TNF-α in the diabetic rats after kolaviron treatment. No significant difference was observed in the levels of IL-10 amongst the groups.

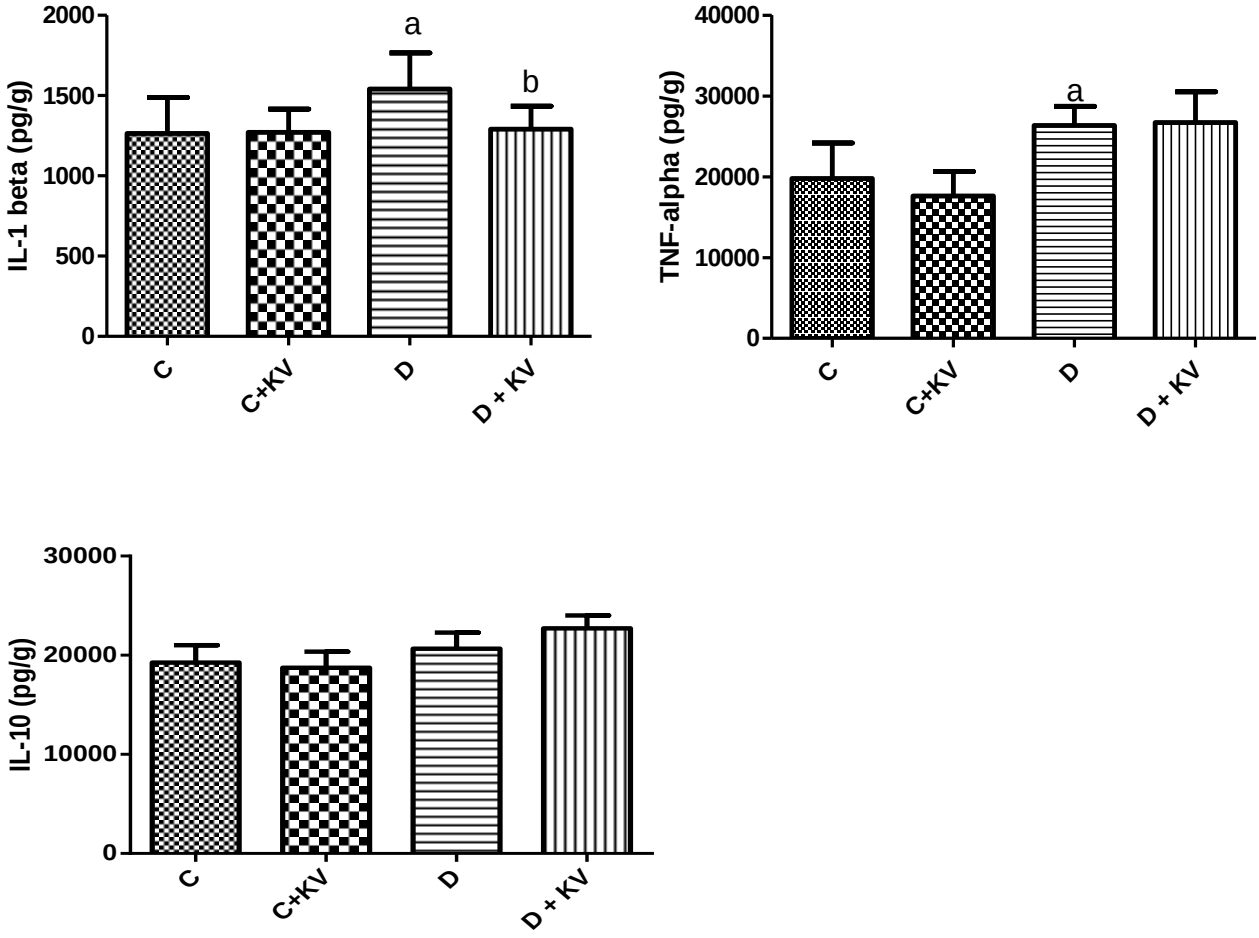
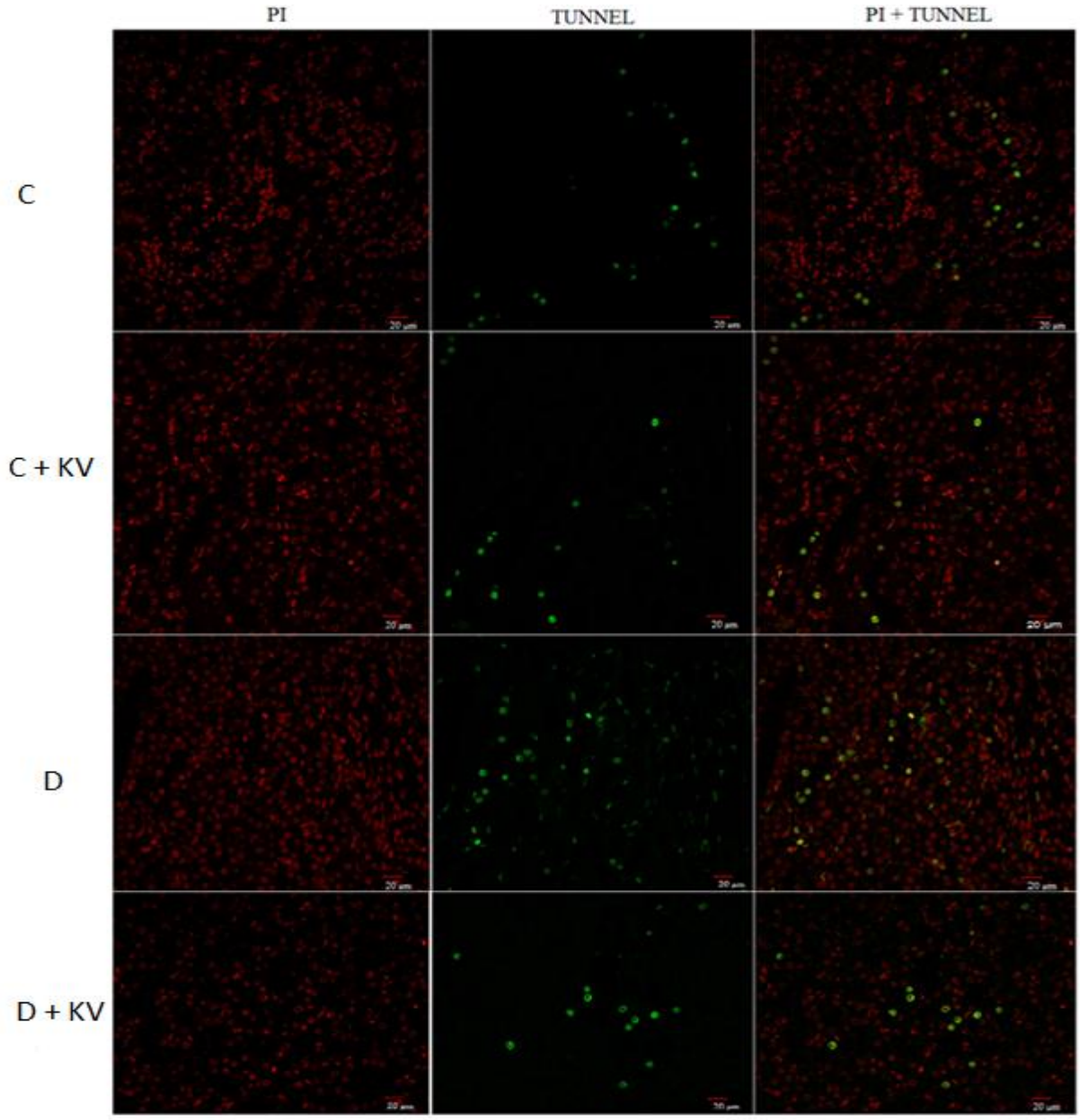


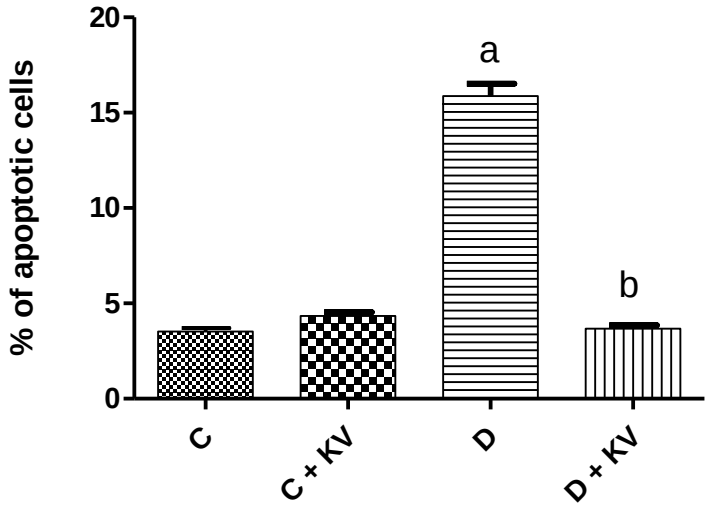
Figure 1: Effect of kolaviron on renal inflammation in diabetic and non-diabetic rats. Data are presented as mean ± S.D. [a]Values differ significantly from non-diabetic control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). C; Non-diabetic control rats, C +KV; control treated with kolaviron, D; untreated diabetic rats, D +KV; diabetic rats treated with kolaviron.

5.3.5 Effect of kolaviron on apoptosis in kidney of normal and STZ-induced diabetic rats

Examination of kidney apoptosis with TUNEL and PI staining revealed an increase in TUNEL positive cells in the kidney sections of diabetes control group as shown in Figure 3. Kolaviron reduced apoptotic cells in the kidney of diabetic rats.



2A



2B

Figures 2A and 2B: Effect of kolaviron on diabetic induced apoptosis. 2A- Representative photomicrographs showing TUNEL-positive cells at 40x magnification in the kidney of (a) control, (b) kolaviron, (c) diabetic, and (d) diabetic +kolaviron rats. The cells emitting a green signal are TUNEL-positive apoptotic cells. 2B- [a]Values differ significantly from control ($p < 0.05$). [b]Values differ significantly from diabetic control ($p < 0.05$). TUNEL; Terminal deoxynucleotidyltransferase mediated dUTP-biotin nick end labeling, PI; Propidium iodide.

5.3.6 Effects of kolaviron on renal structure of rats

No pathological change was observed in kidney sections of rats treated with kolaviron and was comparable to those of control rats showing normal kidney architecture (Figure 4a and 4b). STZ caused degeneration of the glomerulus (Figure 4c) with increased interstitial matrix. Renal sections of diabetic rats treated with kolaviron showed preserved renal glomerulus (Figure 4d).

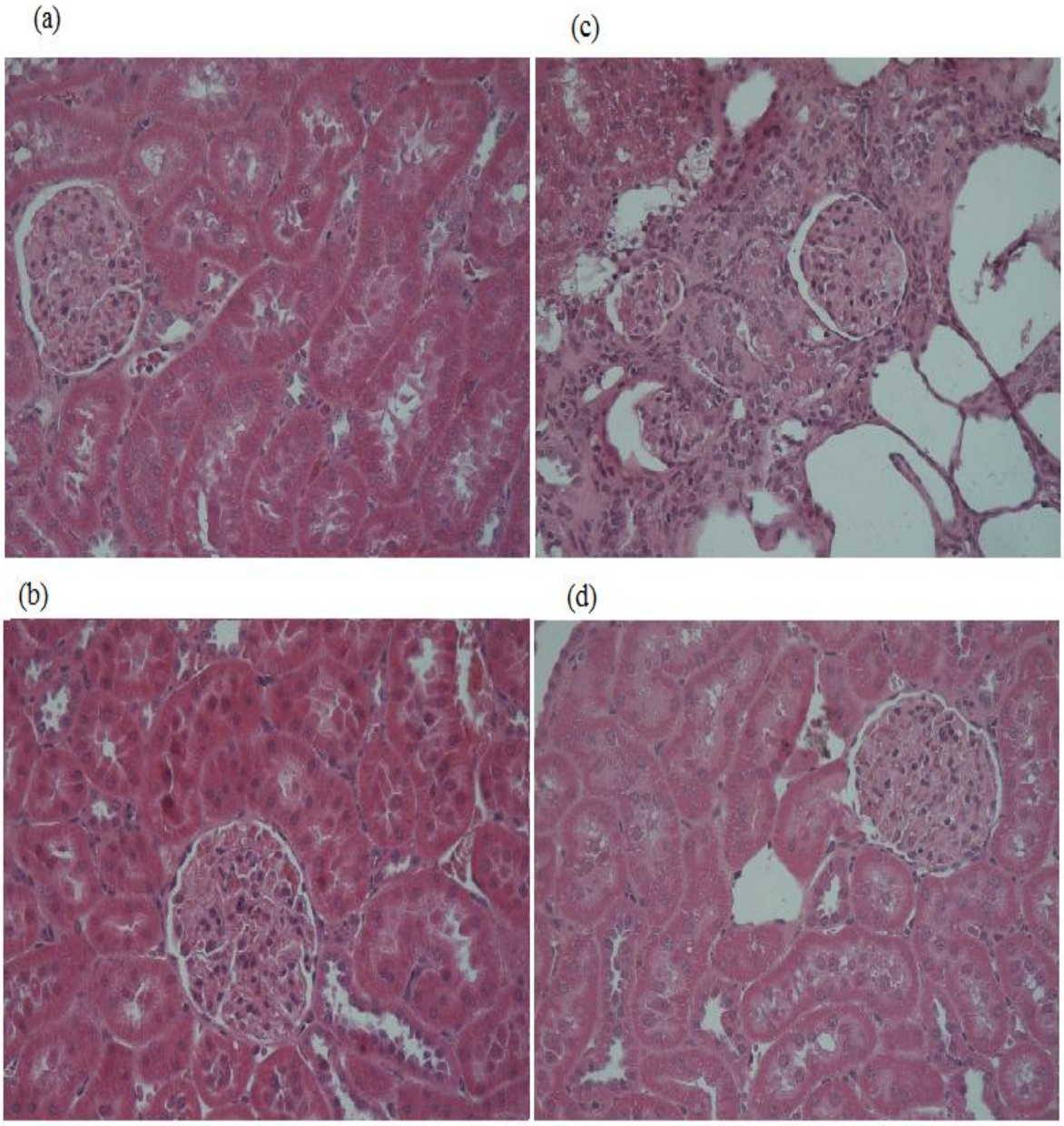


Figure 3: Effects of kolaviron on histopathology of renal sections a) control; (b) KV-treated control rats (c) diabetic rats and (d) kolaviron treated diabetic rats. Haematoxylin-eosin: 200x.

5.4 Discussion

Oxidative stress results from an imbalance between oxidants and antioxidants in favour of oxidants thus leading to cell damage. Accumulation of reactive oxygen species (ROS) which causes oxidative stress in tissues such as the kidney results in damage or toxicity and oxidative stress has been singled as a major cause of diabetic complications including diabetic nephropathy (Rolo and Palmeira, 2006). Cells maintain a variety of defenses in response to oxidative stress through the induction of antioxidant enzymes. Among these are endogenous enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In the present study, GPX activity was increased in the diabetic kidney whereas catalase activity was decreased. However, in kolaviron-treated diabetic rats, CAT and GPX activities normalised. GPX uses the cellular non-enzymatic antioxidant glutathione (GSH) as an electron donor to scavenge ROS generating products that can easily be excreted out of the body (Sahoo *et al*., 2008). Therefore a marked decline in renal GSH concentrations in diabetic rats may reflect an impaired antioxidant defense and thus increased susceptibility to oxidative stress. Kolaviron administered to diabetic and non-diabetic control rats increased kidney GSH concentration. The normalisation of GSH in kolaviron-treated diabetic rats therefore suggests a protective effect of kolaviron against ROS overproduction in the diabetic rat kidney.

ROS degrade membrane polyunsaturated fatty acids through sequential peroxidation processes. The major products of lipid peroxidation are 4-hydroxylnonenal (4-HNE) and malondialdehyde (MDA) (Demir *et al*., 2011). MDA is a highly unstable aldehyde that induces oxidative stress by forming covalent protein adducts collectively referred to as advanced lipoxidation end-products (ALE) (Farmer and Davoine, 2007). The production of MDA serves as a bio-marker to measure tissue oxidative stress levels. Extensive membrane lipid peroxidation impairs membrane fluidity and inactivates some membrane bound enzymes leading to cell death (Goel *et al*., 2005). From our investigation, kolaviron may prevent these deleterious effects by inhibiting the lipid peroxidation process thus reducing the formation of MDA. Previous studies identified some active compounds in kolaviron which include *Garcinia* biflavonoids (GB) 1, GB 2, kolaflavone and kolaflavanone (Iwu, 1985). Recently, we also identified a new compound in kolaviron which may be another biflavonoid-binaringenin (Ayepola *et al*., 2013). The anti-oxidative actions of kolaviron may therefore be attributed to these active constituents.

Kolaviron-treated diabetic rats have reduced IL-1β concentrations with no effect on TNF-α concentrations. IL-1 and TNF-α are pro-inflammatory cytokines produced by a wide range of cell types and can exert their actions in a systemic, paracrine or autocrine manner. IL-1 and TNF-α regulate immunological and inflammatory responses and have been implicated in diabetic kidney disease through diverse biological activities including activation of growth and transcription factors, impairment of insulin secretion and induction of apoptosis and necrosis (Navarro-González *et al*., 2011; Banerjee and Saxena, 2012). IL-10, however, is an anti-inflammatory cytokine which stimulates humoral immune responses and its elevation in the serum of patients with diabetic nephropathy suggests it plays a role in the pathogenesis of the disease (Wong *et al*., 2007; Wu *et al*., 2011). We did not find differences in IL-10 concentrations. The anti-inflammatory action of kolaviron in the diabetic kidney may therefore be related to the suppression of renal IL-1β production, independent of TNF-α.

Apoptosis is a process of physiological cell death that maintains tissue homeostasis; however, excessive or dysregulated apoptosis may lead to various pathological processes including diabetes (Kim *et al*., 2011). Hyperglycemic-induced oxidative stress has been shown to result in pancreatic beta cell dysfunction and apoptosis (Donath *et al*., 1999). *In vitro* and *in vivo* diabetic kidney studies revealed that hyperglycemic-induced oxidative stress also triggers tubular and glomerular cells to undergo apoptosis (Brownlee, 2001; Ha *et al*., 2008). In renal tubular epithelial cells, elevated glucose concentrations induce ROS-dependent apoptosis by an increased Bax protein expression causing mitochondrial permeability and subsequently releasing cytochrome C (Kang *et al*., 2003; Wagener *et al*., 2009). Apoptosis is also associated with inflammation in diabetic nephropathy (Kim *et al*., 2011). Our study demonstrated that renal apoptosis was induced in diabetic rats compared to the non-diabetic control rats while treatment of diabetic rats with kolaviron protected against the renal apoptosis. The ability of kolaviron to inhibit oxidative damage by boosting renal antioxidant status is a possible mechanism of its anti-apoptotic activity. IL-1β has been shown to induce Fas-triggered apoptosis through the activation of the nuclear transcription factor NF-κB. The inhibitory action of kolaviron on IL-1β in the present study potentially contributes to the anti-apoptotic effect of kolaviron.

The increased relative kidney weights of diabetic rats may be due to fatty infiltration, enlargement of tubular cells lining and lymphocyte infiltration in hyperglycemic rats. Elevated concentrations of uric acid and blood urea nitrogen are related to renal dysfunction in diabetes due to metabolic disturbances. Moreover, metabolic disturbances and muscle wasting in diabetes can also result in an increased release of purine, a major source of uric acid. Although uric acid is considered an antioxidant, its excessive generation could result from increased free radical production due to activation of xanthine oxidase (Madianov *et al*., 2000). Kolaviron treatment lowered blood urea nitrogen (BUN) and serum uric acid levels in diabetic rats compared to untreated diabetic rats suggesting an ameliorative effect on kidney function. We also observed reduced serum albumin concentrations in the diabetic rats compared to non-diabetic rats which may be due to glomerular basement membrane damage and increased urinary excretion of albumin which was normalised by kolaviron treatment.

5.5 Conclusion

The results of our study suggest that hyperglycemia caused renal dysfunction and cell death via oxidative stress and inflammation. We demonstrated the beneficial effects of kolaviron on renal dysfunction in diabetic rats via modulation of hyperglycemic-induced inflammation, oxidative damage and apoptosis. Kolaviron should be further explored as a potential candidate in the treatment of diabetic nephropathy.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgment

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CHAPTER SIX

Effects of kolaviron on liver oxidative stress and beta-cell damage in streptozotocin-induced diabetic rats

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Abstract

The liver plays an important role in the regulation of blood glucose and is a target organ of hyperglycaemia. Hyperglycemia plays a crucial role in the onset of various liver diseases and may culminate into hepatopathy if untreated. Alteration in antioxidant defense and increase in oxidative stress that results in tissue injury is characteristic of diabetes. We evaluated the protective effects of kolaviron-a biflavonoid complex, on hepatic antioxidants, lipid peroxidation and apoptosis in the liver of diabetic rats. To induce type I diabetes, rats were injected with streptozotocin intraperitoneally at a single dose of 50 mg/kg. Oral treatment of diabetic rats with kolaviron (100 mg/kg) started on the 6th day after diabetes induction and continued for 6 weeks (5 times weekly). Diabetic rats exhibited a significant increase in the peroxidation of hepatic lipids as observed from the elevated level of malondialdehyde (MDA) estimated by High-Performance Liquid Chromatography. In addition, Oxygen Radical Absorbance Capacity (ORAC), ratio of reduced to oxidized glutathione (GSH/GSSG) and catalase (CAT) activity was decreased in the liver of diabetic rats. TUNEL assay revealed increased apoptotic cell death in the liver of diabetic rats.

Examination of Pancreatic beta-cells by immunohistochemical methods revealed beta cell degeneration and reduction in beta cell/ islet area in the diabetic controls. Kolaviron-treatment increased the area of insulin immunoreactive beta-cells significantly. Kolaviron attenuated lipid peroxidation and apoptosis in the liver of diabetic rats, increased CAT activity GSH levels and the resultant GSH: GSSG. The ORAC of kolaviron-treated diabetic liver was restored to near-normal values. Kolaviron protects the liver against oxidative and apoptotic damage induced by hyperglycemia. The antidiabetic effect of kolaviron may also be related to its beneficial effects on beta-cell function.

6.1 Introduction

Type 1 diabetes mellitus (T1DM) is caused by cellular-mediated autoimmune destruction of pancreatic islet beta-cells leading to loss of insulin production and elevation of blood glucose.
Diabetic patients are more predisposed to microvascular and macrovascular complications. Although the control of blood glucose and dyslipidaemia is beneficial, these therapeutic approaches cannot reverse organ damage. Reports have shown that chronic generation of reactive oxygen species (ROS) due to hyperglycemia play a critical role in the development of diabetic liver injury, hence the use of antioxidant rich plant materials in the control of diabetes has received considerable attention [1; 2]. Due to the involvement of oxidative stress in diabetes, antioxidants might reduce glucose toxicity, improve β-cell function and attenuate or delay the hepatic injury in diabetic patients and in experimental models.

The pharmacological effects of kolaviron, a flavonoid complex isolated from the *Garcinia kola* seed (also known as bitter kola) in animal models are extensive, ranging from protection against *Plasmodium berghei* infection and hepatoxicity by toxins such as carbon tetrachloride [3] to glucose lowering effect [4]. The active compounds so far reported in kolaviron includes *Garcinia* biflavonoid (GB) 1, GB-2, kolaflavone, kolaflavanone, kolaflavone and binaringenin [5; 6]. Kolaviron has been shown to reduce oxidative stress in many studies [7; 8].

In regard to the central role of oxidative stress in the pathogenesis of diabetic liver injury, this study was designed to evaluate the effect of kolaviron on oxidative stress markers in the liver of

streptozotocin-induced diabetes rats. We also investigated the effect of kolaviron on apoptosis in the liver. In addition to immunohistochemical staining of insulin and glucagon, morphological examination of the pancreas was also performed to further substantiate the beneficial effect of kolaviron on pancreatic β-cell function.

6.2 Materials and methods

6.2.1 Animals

The study protocol was approved by the Faculty of Health and Wellness Sciences Research Ethics Committee of the Cape Peninsula University of Technology (Ethics Certificate no: CPUT/HW-REC 2012/AO4). All the animals received humane care in accordance to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Science (NAS) and published by the National Institute of Health (Publication no. 80-23, revised 1978). Male Wistar rats (11-12 weeks, 270 ± 25g), were used for the study. Experiments were performed at the animal facility of the Medical Research Council (MRC) and strictly adhered to the standard operating procedures (SOPs). All animals were housed individually at room temperature (22 ± 2 °C) with 55 ± 5% humidity and an automatically controlled cycle of 12 hr light and 12 hr dark. A standard laboratory diet and water were provided *ad libitum* and rats were habituated to the experimental conditions one week prior to experimentation.

6.2.2 Extraction of kolaviron

Garcinia kola seeds were peeled sliced and air-dried (25–28°C). Kolaviron was isolated according to the method of Iwu *et al.* [4]. Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet for 24 hr. The defatted dried product was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded kolaviron, a golden yellow solid.

6.2.3 Induction of diabetes

Diabetes was induced in rats by a single intraperitoneal injection of freshly prepared solution of 50 mg/kg streptozotocin (Sigma-Aldrich, Johannesburg, SA) in citrate buffer (0.1M, pH 4.5) to overnight fasted rats. Diabetes was confirmed by stable hyperglycemia (>18 mmol/l) in the tail

blood glucose after five days of streptozotocin (STZ) injection using a portable glucometer (Accu-Chek, Roche, Germany).

6.2.4 Study design and tissue collection

The animals were divided into 4 groups (n=10 per group): Normal control (NC group), kolaviron treated normal control (KV), diabetic control (DM group) and kolaviron- treated diabetic group (DM + KV group). Treatment was started on the 6th day post STZ injection and continued for 6 weeks. Kolaviron (100 mg/kg b.wt.), dissolved in dimethylsulphoxide (DMSO) was administered by gastric gavage 5 times a week. The dosage of kolaviron was adjusted every week according to any change in body weight to maintain similar dose over the period of study. At the end of experimental period, The liver was rapidly excised, washed in ice-cold phosphate buffered saline, blotted, frozen in liquid nitrogen, and stored at -80 °C for biochemical estimations.

6.2.5 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity in liver samples with trolox as the antioxidant standard according to the method of Ou *et al.,* [9]. Liver homogenates were deproteinized with 0.5M perchloric acid (1:1, v/v) and centrifuged at 10,000g for 10 min. The supernatant was stored at -80^{o}C prior to analysis. Fluorescein (FL) was used as the fluorescent probe and the peroxyl radicals were generated from AAPH in 75 mM phosphate buffer (pH 7.4). Specifically, 138 µL of 14 µM FL solution was mixed with 12 µL of diluted sample (1:20) with 75 Mm phosphate buffer, pH 7.4) standard, or blank (phosphate buffer, pH 7.4) to a black 96-well fat bottom plate and the plate was incubated at 37°C for 20 min. After incubation, the reaction was started by the addition of 50 µL of AAPH (4.8 mM) to the mixture. Standards and samples were measured in triplicate. The fluorescence of the reaction mixture was monitored and recorded every minute (excitation = 485 nm and emmission = 535 nm) for 2 hr with a Fluoroscan Ascent plate reader (Thermo Fischer Scientific, Waltham, MA, USA). Results were determined by using a regression equation relating trolox concentrations and the net area under the kinetic fluorescein decay curve ($y=ax2 + bx+ c$). The ORAC value was expressed in micromoles of trolox equivalents per gram of tissue (µmol TE/g).

6.2.6 Estimation of Superoxide dismutase (SOD) activity

Superoxide Dismutase (SOD) was determined by the method of Crosti *et al*., [10]. The reaction mixture in a 96-well plate consisted of 15 µL of sample, 170 µL of 0.1mM DETAPAC in 50 Mm sodium phosphate buffer (pH 7.4), and 20 µL of 1.6 mM 6-hydroxydopamine which initiated the reaction. The reaction was measured at 490 nm for 4 min at 30 s intervals and SOD activity expressed as U/mg of protein.

6.2.7 Estimation of glutathione peroxidase (GPX) activity

Activities of antioxidant enzymes were determined in a clear 96-well plate using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Glutathione peroxidase (GPX) activity was determined according to the method of Ellerby & Bredesen [11]. To initiate the reaction, 25 μL of H_2O_2 (15 mM) was added to a final reaction mixture containing 2.5 μL of GSH (0.1 M), 2.5 μL of GR (0.1 U/mL), 5 μL liver homogenate, 5 μL of NADPH (15 mM in 0.1% $NaHCO_3$), 2.5 μL of sodium azide (100 mM) and 210 μL of assay buffer (50 mM potassium phosphate, 1 mM EDTA pH 7.0). The rate of H_2O_2-dependent oxidation of NADPH was monitored at 340 nm at 30 s intervals for 2 min. The activity of GPx was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ and results expressed as nmol NADPH oxidized per min per µg protein.

6.2.8 Estimation of catalase (CAT) activity

Catalase (CAT) activity was determined by the method of Aebi [12] The assay is based on the principle of measurement of decomposition of hydrogen peroxide (H_2O_2) by catalase measured at 240 nm. Assay mixture contained 5 μL of sample, 170 μL of 50 mM potassium phosphate (pH 7.0) and 50 μL of 0.1% hydrogen peroxide in 50mM potassium phosphate (pH 7.0). The rate of decomposition of H_2O_2 was measured at 240 nm for 2 min in 15 s intervals in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Catalase activity is expressed as μmol H_2O_2 consumed/min/mg protein.

6.2.9 Glutathione status analysis

Glutathione (GSH and GSSG) status was determined according to the method of Asensi *et al*. [13]. Liver samples were homogenized (1: 10) in 6% (v/v) PCA containing 1mM EDTA for GSH determination and for GSSG determination, liver samples were homogenized in 6% PCA

containing freshly prepared 3Mm M_2VP and 1Mm EDTA. Homogenates were centrifuged at 15, 000 g for 5 min, 50 μL of supernatant was added to 50 μL of 0.3mM DTNB and 50 μL of glutathione reductase (1U). The reaction was initiated by addition of 50 μL of 1mM NADPH and change in absorbance was monitored at 410 nm for 5 min. GSH and GSSG Levels were calculated using GSH and GSSG as standards.

6.2.10 Estimation of lipid peroxidation

Liver malondialdehyde (MDA) was determined by HPLC using a method adapted from Khoschsorur *et al.* [14]. Briefly, 100 µL of liver homogenates and standard were mixed with 750 µL orthophosphoric acid (0.44 M), 250 µL of aqueous thiobarbituric acid (42 mM), and 450µL distilled water. The mixture was heated in a boiling water bath for 60 min. After cooling on ice, alkaline methanol (50 ml methanol + 4.5 ml 1 M NaOH) was added (1: 1). The samples were centrifuged at 3 500 g for 3 min at 4°C. 1 mL of supernatant was added to 500 µL of n-hexane and the mixture centrifuged at 14000 g for 40 sec. 50 µL of the supernatant was then chromatographed on an Agilent 1200 series HPLC. A 5 µm YMC-PackPro C18 (1 5 0 mm × 4 . 6 mm i.d.) column was used for separation with 60 : 40 (v/v) 50 mM phosphate buffer, pH 6.8-methanol as mobile phase. The flow rate was 1 mL min^{-1}. Fluorometric detection was performed with excitation at 532 nm and emission at 552 nm. The peak of the MDA-TBA adduct was calibrated with the MDA standard.

6.2.11 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

For detection of apoptotic cells, liver sections were stained with the reagents supplied by ApopTag fluorescein *In Situ* Apoptosis Detection Kit (Chemicon, Billerica, CA). Briefly, each slide was deparaffinized, rehydrated, and treated with proteinase K (20 mg/L) for 15 min. Equilibration buffer was applied directly on the slides followed by incubation with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin nucleotide and unlabeled nucleotide for 1 h in a humidified chamber at 37°C. Sections were counter stained with a mounting medium containing 0.5 µg/mL of propidium iodide and viewed by Olympus IX-81 microscope. Apoptotic cell death was quantitatively analyzed by counting

TUNEL positive cells selected randomly from 5 consecutive fields at 10× using the image analysis software 'ImageJ'. TUNEL positive cells were expressed as percentage of total cells.

6.2.12 Histology

Liver tissue was fixed in 10% formalin and embedded in paraffin wax. Fixed liver tissues were cut into 5-µm slices. After being deparaffinized using xylene and ethanol dilutions and rehydration, tissue sections were stained with hematoxylin and eosin (H&E). The pancreata were also excised and embedded in paraffin.

6.2.13 Pancreatic analysis

Immunohistochemical staining of the pancreas was performed on 5 µm-thick paraffin sections. Briefly, each section were dewaxed and immunolabeled for **α**-cells with a polyclonal glucagon antibody (Dako, Carpinteria, CA) thereafter a secondary biotinylated anti-rabbit link antibody was applied and positive labeling was visualised using the peroxidase diaminobenzidine (DAB) method. The β-cells were also immunolabeled with a monoclonal insulin antibody thereafter a secondary biotinylated anti-mouse link antibody was applied and positive labeling was visualised using the alkaline phosphatase (AP method).

The pancreatic image was analysed with Olympus BX50 light microscope comprised of a mounted Nikon DS-Fi1 digital camera. The light microscope was interfaced with a computer via Leica Qwin image analysis software. Pancreatic sections were viewed with a 20× objective. The whole section area was measured and the total areas of the islets were determined. Subsequently, **α**-cell area (brown positive staining) and β-cell area (red positive staining) were measured.

6.2.14 Statistical analysis

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p < 0.05$.

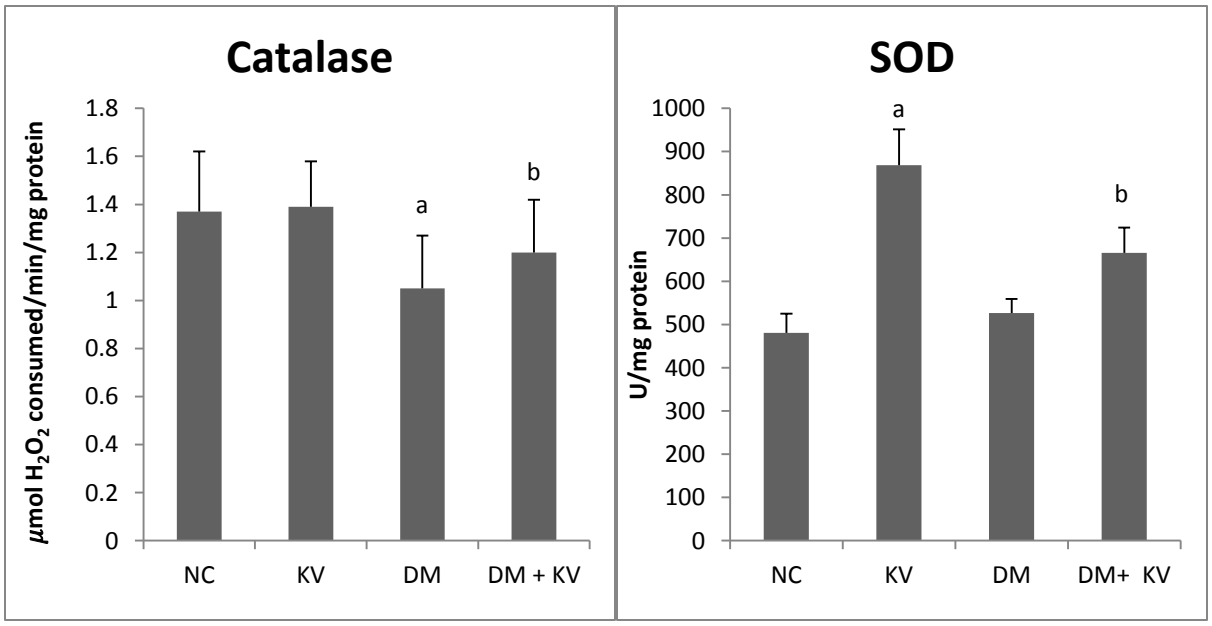
6.3 Results

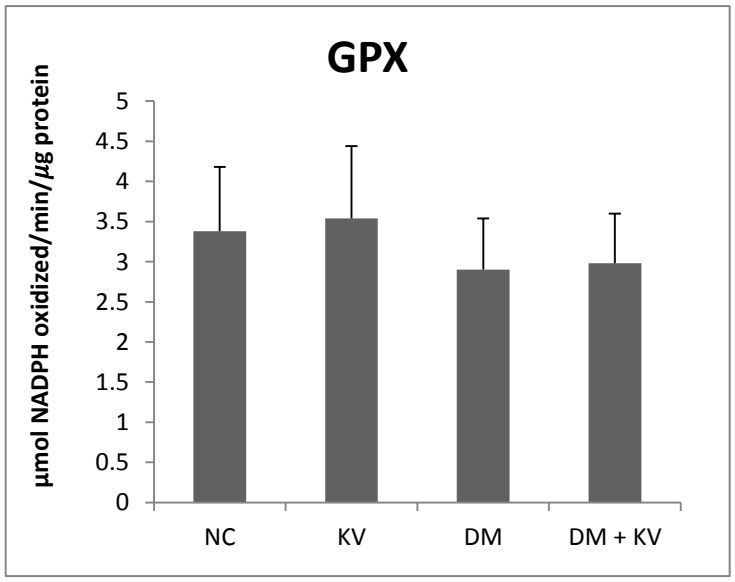
The effects of kolaviron on antioxidant status of experimental rats are shown in Figures 1- 4. The concentration of lipid peroxidation marker, malondialdehyde (MDA) was significantly ($P < 0.05$) higher in diabetic control group. Treatment of diabetic rats with Kolaviron decreased hepatic MDA concentration (Figure 3). The activity of enzymatic antioxidant, catalase was decreased in diabetic tissues and normalized following KV administration (Figure 1a). The increased activity of superoxide dismutase in the hepatic tissues of diabetic and normal rats treated with kolaviron in the present study demonstrates its antioxidant properties and the ability of kolaviron to scavenge highly reactive superoxide radical (Figure 1b). This is further supported by the observed increase in oxygen radical absorbing capacity (ORAC) in the hepatic tissues of diabetic and normal rats treated with KV and a reduction in ORAC of diabetic rats compared to control rats (Figure 4). The ratio of reduced to oxidized glutathione (GSH/GSSG) is an indication of the redox status of the cell. GSH levels and the ratio of GSH/GSSG decreased in hepatic tissues of diabetic rats (Figure 2a–c). Administration of kolaviron improved these alterations as observed from increased levels of GSH and increased ratio of GSH/GSSG in comparison to untreated diabetic control.

Examination of hepatic apoptosis with TUNEL staining revealed an increase of TUNEL positive cells in the liver sections of diabetes control group. The rate of apoptosis was generally very low in normal control and kolaviron treated normal control rats (Figure 5). Kolaviron reduced apoptotic cells in the liver of diabetic rats.

The reduction in β-cell/islet area in the pancreas of control STZ-diabetic rats (35.91 ± 2.68) was demonstrated compared with that of non-diabetic control rats (61.44 ±3.18). Kolaviron treatment of diabetic rats resulted in a significant increase in β-cell area (45.83 ±1.12). An increase in **α**-cell/islet area was also observed after diabetes induction compared to normal control rats (29.16 ±3.78 versus 9.91 ± 2.77). Kolaviron treatment of diabetic rats showed no effects on pancreatic **α**-cell/islet area (25.92 ± 1.09). No effect was observed on the above parameters following kolaviron administration to normal rats in comparison with normal control rats treated with vehicle only.

(A) (B)

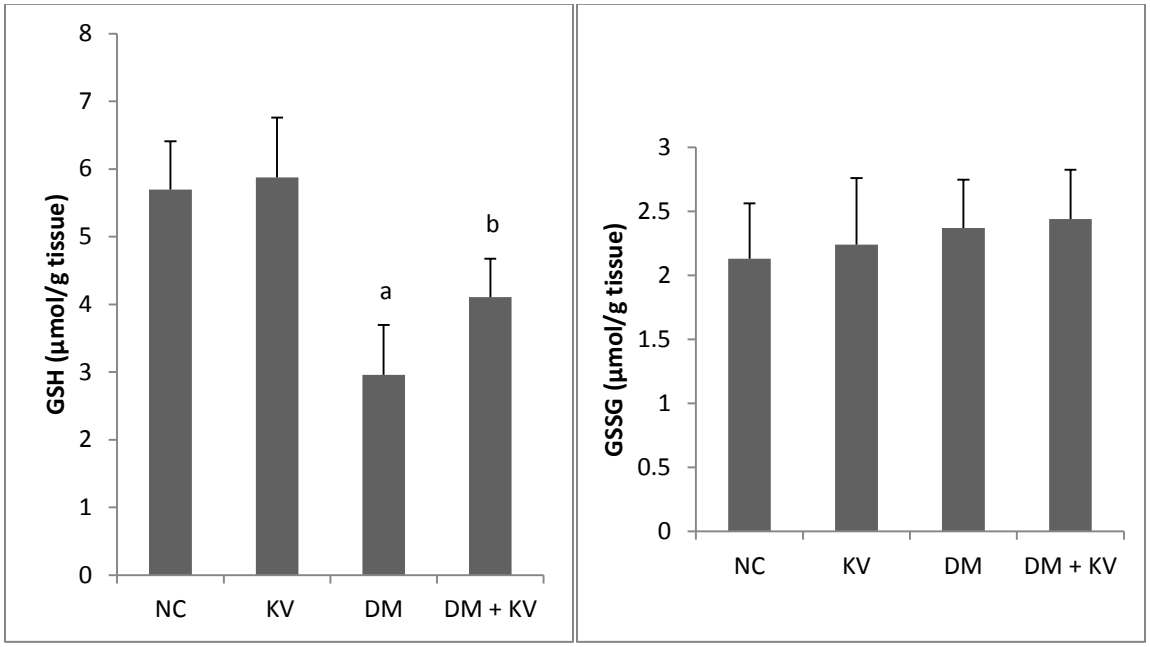


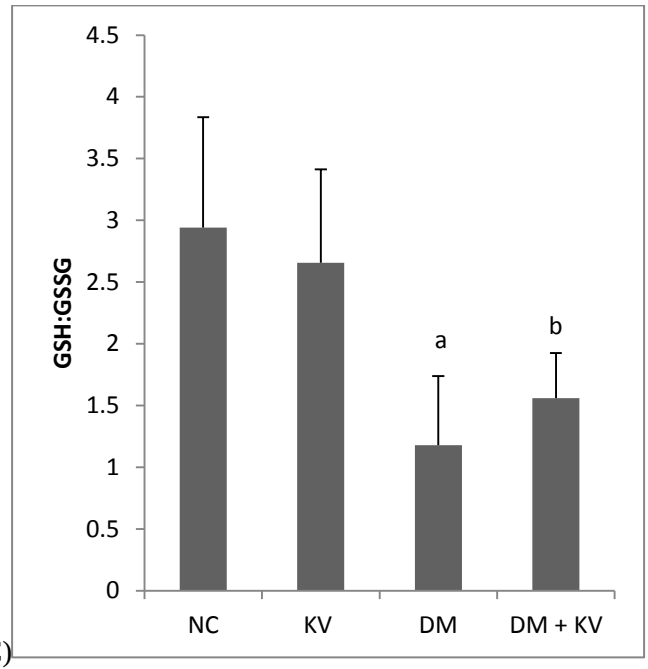


(C)

Figure 1 (A-C): Effects of kolaviron on the activities of antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) in the hepatic tissues of experimental rats. Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$).[b] Values differ significantly from diabetic group ($p<0.05$)

(A) (B)





(C)

Figure 2 (A-C): Effects of kolaviron on GSH levels, GSSH levels and GSH/GSSH ratio in hepatic tissues of experimental rats. Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$).[b] Values differ significantly from diabetic group ($p<0.05$)

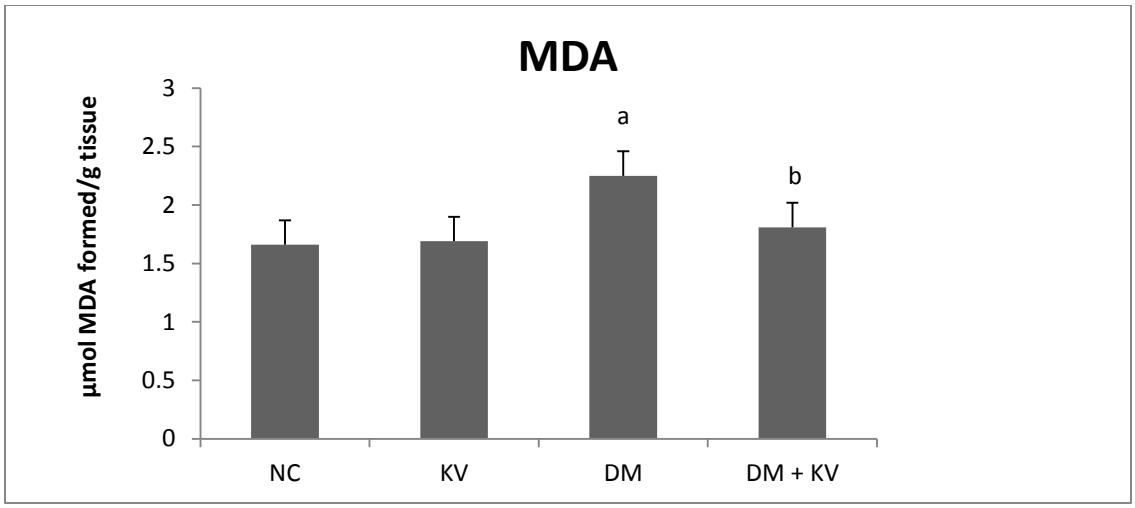


Figure 3: Effects of kolaviron on malondialdehyde (MDA) concentration in hepatic tissues of experimental rats. Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$).[b] Values differ significantly from diabetic group ($p<0.05$)

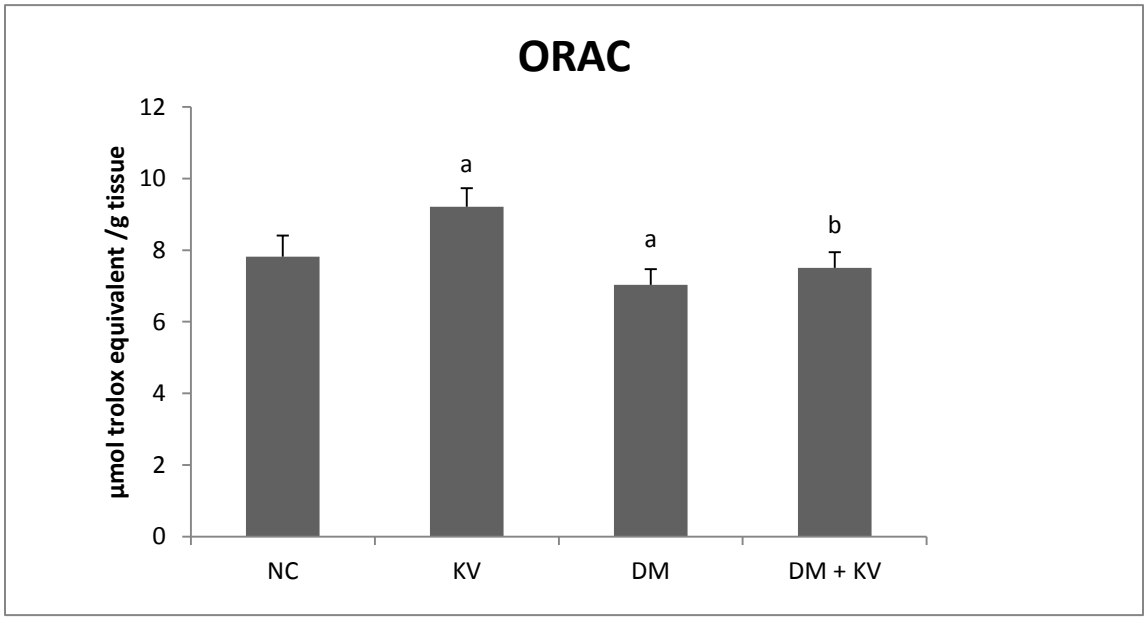
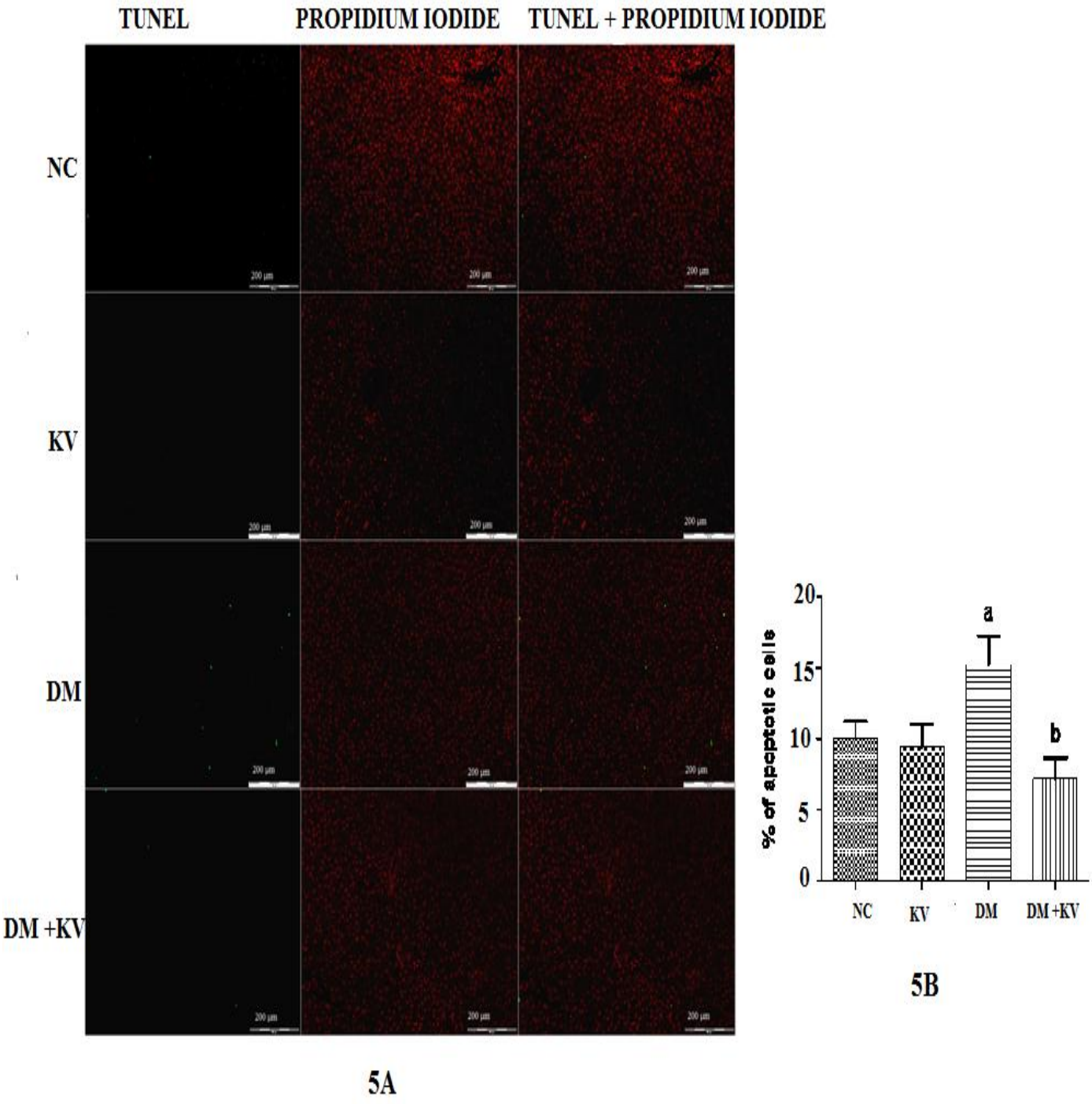


Figure 4: Effects of kolaviron on oxygen radical absorbance capacity (ORAC) in hepatic tissues of experimental rats. Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$).[b] Values differ significantly from diabetic group ($p<0.05$)



Figures 5A and 5B: Effects of kolaviron on apoptosis in the hepatic tissues of experimental rats. Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$). [b] Values differ significantly from diabetic group ($p<0.05$).

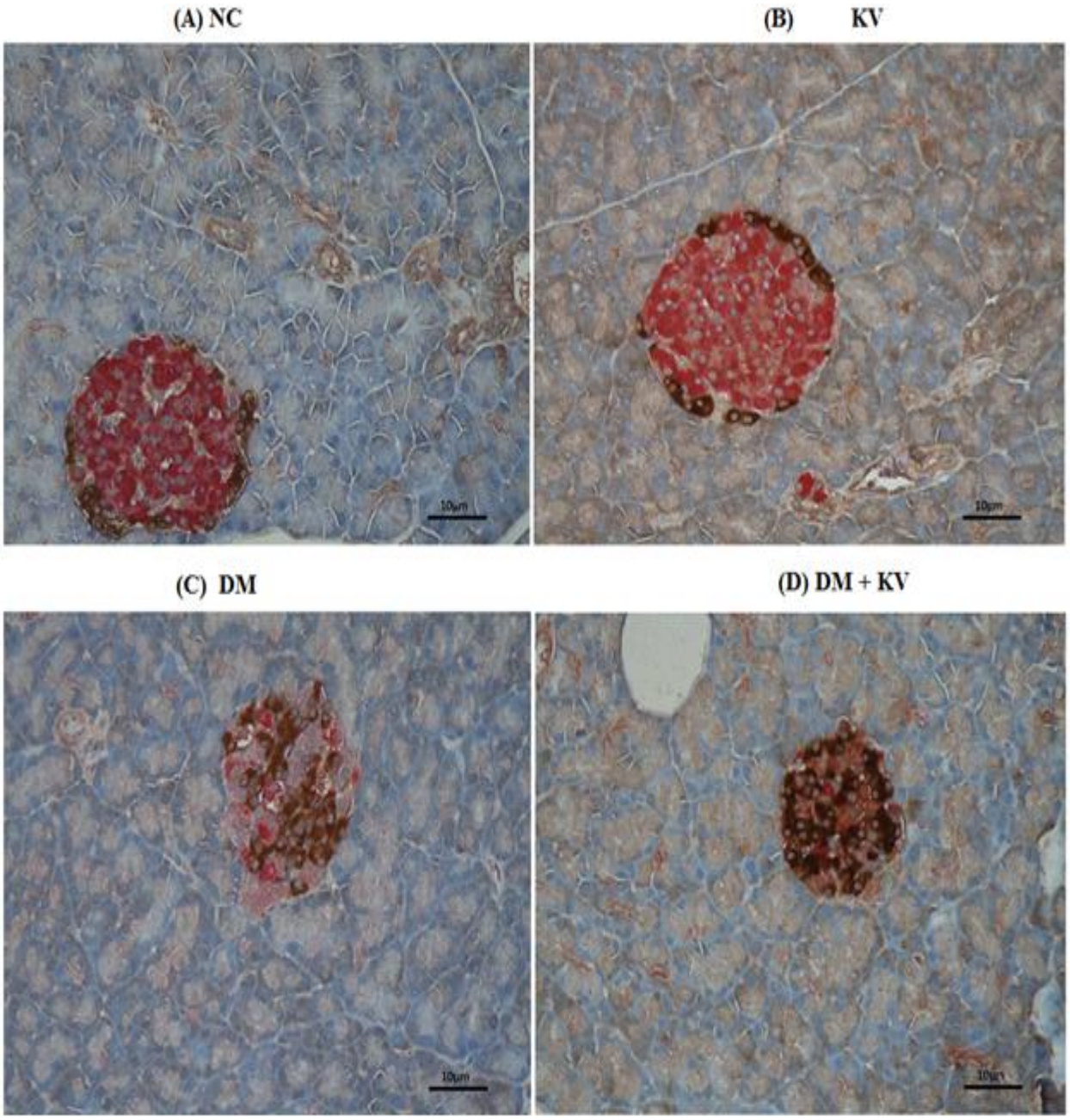
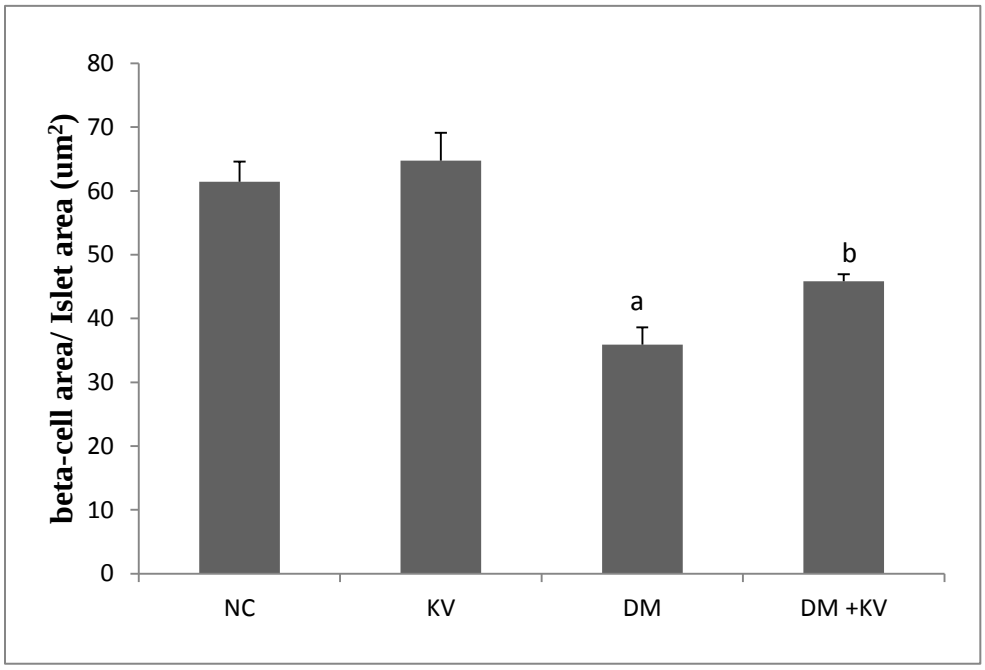


Figure 6: Light microscopy view of pancreatic sections immunohistochemically stained for glucagon-positive α-cells (brown) and insulin-positive β-cells (pink)

(a) Control rats (b) Kolaviron treated control rats (c) Diabetic rats and (d) Kolaviron treated Diabetic rats; Magnification, X20

(7A)



(7B)

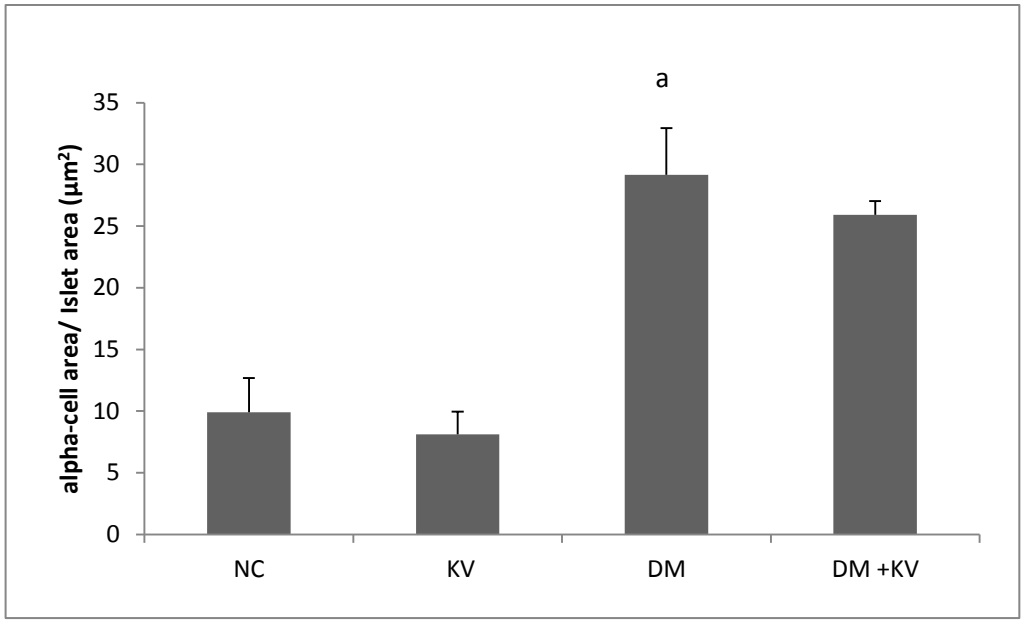


Figure 7A and 7B: Morphometric analysis of the pancreatic islets in the experimental rats.

Data are presented as mean ± S.D. [a] Values differ significantly from those of control ($p<0.05$).[b] Values differ significantly from diabetic group ($p<0.05$)

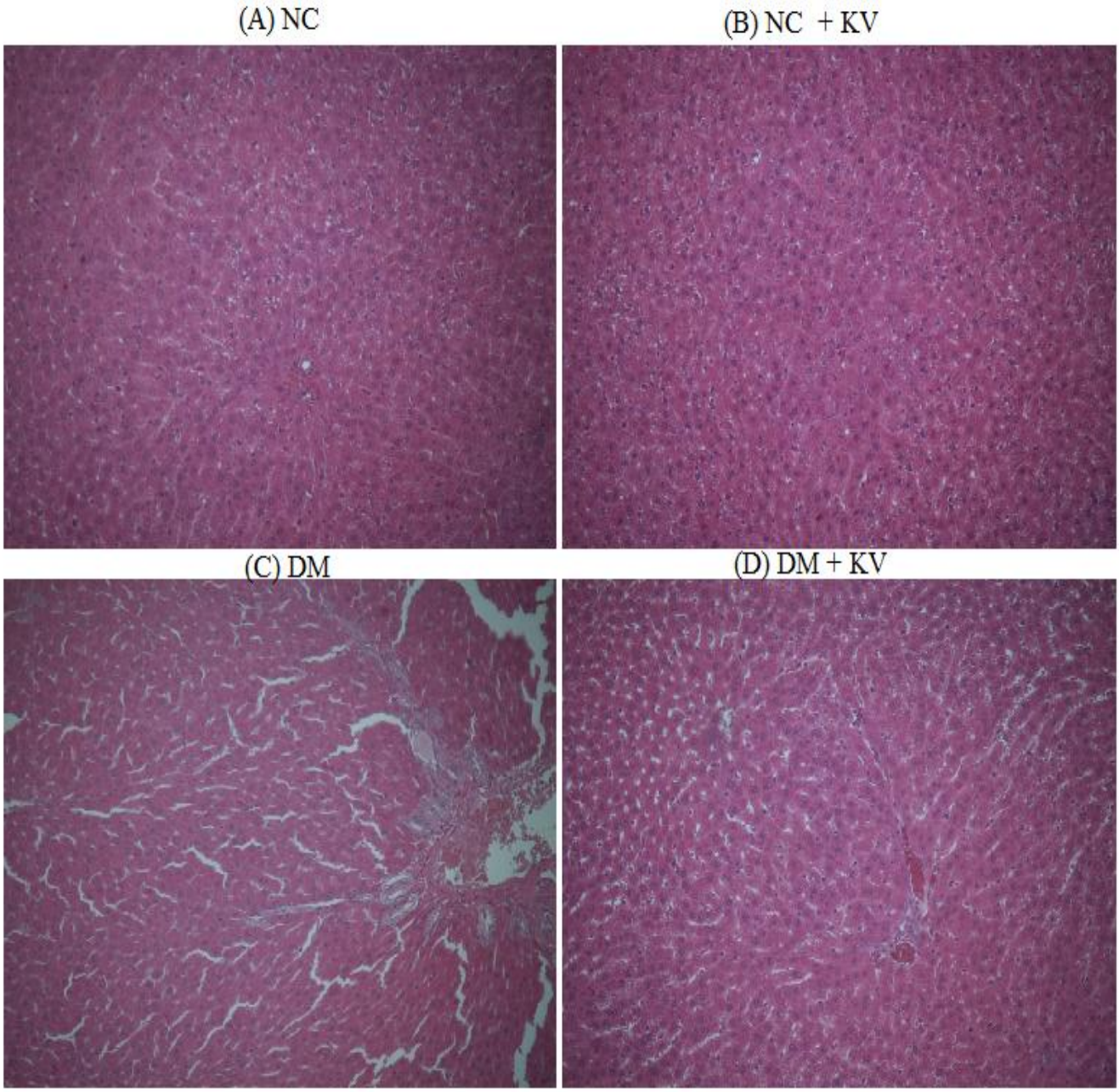


Figure 8: Effects of kolaviron on histopathology of liver sections (a) control; (b) KV-treated control rats (c) diabetic rats and (d) kolaviron treated diabetic rats. Haematoxylin-eosin: X10.

6.4 Discussion

Oxidative stress measured by increases in lipid peroxidation, DNA and protein damage and reduction in antioxidant enzymes activities is a major characteristic of diabetes and diabetic complications in humans and experimental animals [15; 16; 17]. An imbalance between activities of free radicals and antioxidants result in the preponderance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) responsible for the initiation of harmful processes that culminate into the clinical symptoms observed in diabetes [18; 19; 20]. Therefore, it has been proposed that scavengers of oxidative stress may have a positive effect in alleviating diabetes as well as reduce its secondary complications.

Antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and reduced glutathione (GSH) effectively forms a defensive alliance against the onslaught of ROS protecting cells from oxidative damage. In the present study, a significant decrease in CAT activity may reflect the inability of the liver to eliminate hydrogen peroxide in the diabetic rats. No significant difference in SOD and GPX activities was observed in kolaviron (KV)-treated diabetic rats compared to controls. Kolaviron increased CAT activity in diabetic rats and also increased SOD activity in both normal and diabetic rats, demonstrating antioxidative and hepatoprotective effects. Usually, lipid peroxidation occurs via series of processes in a stepwise manner involving several free radicals. Elevated levels of liver MDA, a product of lipid peroxidation, has been reported in diabetic rats [21; 22; 23; 24]. Results from our study corroborate these observations. The attenuation of MDA formation following oral administration of KV is also an indication of its free radical scavenging property.

In the present study the significant elevation of the GSH stores and GSH/GSSG ratio in the liver of diabetic rats treated with KV compared to diabetic control suggests the alleviation of free radical damage by KV. Alteration in the GSH/GSSG status of a biological system usually leads to deleterious effects majorly as a result of the increased activities of reactive oxygen species. Reduced glutathione (GSH) is the main non-enzymatic, non-protein thiol in living cells that has been demonstrated to participate actively in boosting the natural antioxidant defense system by acting as a cofactor for other antioxidants with itself becoming oxidized to GSSG [25, 26]

Apoptosis is a form of cell death that is characterized by several biochemical and morphological changes which are induced through the triggering of either the intrinsic or extrinsic pathways [27]. The permealization of the mitochondrial outer membrane and subsequent release of cytochrome c during apoptosis are strictly regulated by specialized proteins referred to as caspases along with the Bcl-2 family [28]. Although apoptosis occurs normally to maintain tissue homeostasis, it can become uncontrolled or dysregulated leading to deleterious pathological consequences such as diabetes [29]. The positive relationship between oxidative stress and apoptosis in diabetes is well documented [30; 31; 32; 33]. In the liver, increased oxidative stress was shown to induce apoptotic cell death of hepatocytes and endothelial cells [34]. In our experimental model, apoptosis was induced in the liver of untreated diabetic rats as a result of STZ treatment. Treatment with KV however, protected hepatic cells from apoptotic death suggesting the anti-apoptotic property of kolaviron.

The present study revealed that in the pancreas of normal rats, alpha cells (glucagon) were peripherally located while beta cells (insulin), which are numerous, were centrally located On the other hand, in the pancreas of diabetic rats, the glucagon-stained cells were mostly scattered within the centre of the islets. Injection of streptozotocin caused a destruction of beta cell reserve as observed from the decreased ratio of beta cell to islet area. The ratio of alpha cell area in relation to islet area was also increased in the diabetic group versus the control group suggesting an increase in the secretory activity of alpha cells due to reduction in insulin level [35]. KV-treated diabetic group showed an increased beta-cell area but no effect on alpha cell area. Although KV treatment increased β-cell area in diabetic rats, this effect was not observed in our control animals treated with KV. This further supports an extra-pancreatic mechanism of action of KV as previous study from our laboratory has demonstrated the hypoglycemic effect of KV in normal rats with no change in serum insulin concentration. Morphometric findings suggest that the anti-diabetic activity of KV in diabetic rats may be associated with improved beta cell function and enhanced insulin secretion and/or insulin release.

Histological alteration in the liver of diabetic rats was also reversed following kolaviron treatment

In summary, the present study explored the effect of kolaviron on oxidative stress and diabetic liver injury and the results demonstrate that kolaviron treatment impairs free radical damage in the liver of diabetic rats. Kolaviron-treatment of diabetic rats protected against hyperglycemia-induced apoptosis and promoted survival of hepatocytes, perhaps by scavenging oxygen radicals. In addition, the antidiabetic effect of kolaviron may also be related to its beneficial effects on beta-cell function.

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CHAPTER SEVEN

General Discussion and Conclusion

Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which depletes the activity of the antioxidativedefense system and thus promotes the generation of free radicals. The antioxidant systems helps to keep oxidants at a low level to protect against tissue damage and inflammatory cascade; a series of intracellular and intranuclear signaling that results in the release of destructive inflammatory cytokines (Rhee, 2006; Valko *et al*., 2007). The results in this study are presented in four different chapters focusing on the effects of kolaviron on oxidative stress and inflammatory biomarkers in the liver, kidney and blood of diabetic rats compared to healthy control rats and also the effect of this phytochemical on apoptotic cell death in the liver and kidney of diabetic rats. The general discussion of this thesis will therefore be focused on the above parameters.

In addition to consistent elevated blood glucose level, higher levels of HbA_{1c} in diabetic rats in this study confirm a state of hyperglycemia. Haemoglobin (Hb) A_{1c} is used as a marker of cumulative glycaemic exposure over the past three months in diabetic patients and higher level is linked with long-term microvascular complications. Pancreatic islet degeneration was observed post STZ induction and serum insulin level was drastically reduced. Increase in triglyceride level and cholesterol concentrations were observed in the diabetic rats in comparison to controls is also a common feature of DM. Inhibition of lipolysis as a result of reduced action of insulin on adipocytes has been suggested as a cause of the observed dyslipidemic state. STZ administration resulted in a significant decrease in body weight-a typical characteristics of diabetes mellitus. Glucose lowering effect of kolaviron was observed in normoglycemic and hyperglycemic rats. Kolaviron treatment showed beneficial effects on the pancreatic islet and also improved insulin levels in diabetic rats. Treatment of diabetic rats with kolaviron also attenuated the weight loss process, suggesting a possible improvement in energy metabolism.

Increased blood urea nitrogen (BUN) and uric acid along with a decrease in serum albumin is an indication of progressive renal damage. Chronic elevations of serum alanine amino transferases (ALT) and aspartate transaminases (AST) in the present investigation are also a reflection of

hyperglycemia-mediated hepatocyte injury. The present study demonstrated that kolaviron positively affected these parameters demonstrating a beneficial effect on liver and kidney functions.

Antioxidant defense enzymes, against oxidative stress, comprises of superoxide dismutases, glutathione peroxidases and catalase (Klaunig & Kamendulis, 2004). Superoxide dismutases and glutathione peroxidases, which are present in cytosol and mitochondria, reduce the superoxide anion to hydrogen peroxide and water, and remove the majority of hydrogen peroxide, respectively. Meanwhile, catalase, located in peroxisomes, also removes high levels of hydrogen peroxide. Nonenzymatic antioxidants, like vitamin E, vitamin C, β-carotene, glutathione, and coenzyme Q function to quench ROS (Clarkson & Thompson, 2000). ROS can randomly react with lipids, proteins and nucleic acids causing oxidative stress and damage in these macromolecules, leading to pathogenesis of chronic diseases, which include diabetes and cancer. When ROS target lipids, they can initiate the lipid peroxidation (LPO) process, a chain reaction that produces multiple breakdown molecules, such as malonaldehyde (MDA) and 4-Hydroxynonenal (HNE). A significant effect of LPO in all cells is the perturbation of membrane structure and function. LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA resulting in strand breaks and cross-linking (Valko*et al*., 2007).

Renal and hepatic dysfunctions are microvascular complications of diabetes and increased oxidative stress has been proposed as the root cause underlying the progression of long term diabetic complications (Morrow, 2003). There is growing evidence that oxidative stress and inflammation are implicated in cardiac dysfunction, leading to heart failure in diabetes (Somogyi *et al.*, 2005). Enhanced oxidative stress, occurring either locally in the arterial wall or systemically, is one hypothesis to explain the development and progression of atherosclerosis- a major underlying cause of cardiovascular disease (CVD) in humans. Inflammation in large and middle-sized arteries, where activated immune competent cells are abundant, could play a major role to trigger plague rupture which is the immediate cause of CVD (Frostegard, 2013).

In the present study, oxidative damage was assessed in liver, kidney and blood of STZ-induced diabetic rats by assesing the activity of antioxidant enzymes and levels of the non enzymatic antioxidant, GSH. Reduced glutathione (GSH) is the central member of a complex antioxidant system protecting the cell from oxidative stress and also acts as a co-factor for GPx, hence preventing the accumulation of ROS and preventing cellular injury (Livingstone & Davis, 2007). Another indication of oxidative stress was assessed by investigating the levels of malondialdehyde (MDA), a biomarker of lipid oxidation in the tissues and blood of diabetic rats. Antioxidant capacity of diabetic rats was assessed by the oxygen radical absorbing capacity (ORAC) and ferric reducing ability of the plasma (FRAP). Alterations in the antioxidant enzyme activities and antioxidant status were observed in the tissues of diabetic rats in comparison to normoglycemic rats in this study (Table 1). Although no uniform trend in enzyme activity was observed in this study as discrepancies in the activities of antioxidant enzymes have been reported in experimentally diabetic animals (Maritim *et al*., 2003), decreased levels of glutathione and elevated concentrations of thiobarbituric acid reactants (MDA) were however consistently observed in the diabetic control group in our study.

Table 1: Summary of the alteration in antioxidant defense in liver, kidney and blood of diabetic rats

	CAT	GPX	SOD	MDA	GSH	ORAC	FRAP
Liver	↓	NS	NS	↑	↓	↓	----
Kidney	↓	↑	NS	↑	↓	↓	----
Erythrocyte	NS	↑	↓	----	----	------	----
Plasma	-----	----	----	↑	----	NS	NS

Down arrows [↓] indicates a decrease in antioxidant enzyme activity or level of antioxidant makers while upper arrows [↑] indicates a decrease; NS, no significant difference compared to control rats.

Treatment of diabetic rats with kolaviron either normalized or reversed majority of these alterations in antioxidant status (Table 2). Kolaviron either increased the activities of free radical scavenging antioxidant enzymes compared to diabetic control or increased their activities to normal levels (as shown in Table 2). In this study, kolaviron obtained from *Garcinia kola* seeds

also attenuated diabetes-induced MDA formation and increased both hepatic and renal GSH levels, possibly due to its intrinsic antioxidant properties. The different sources of free radicals in diabetes can increase the demands upon the glutathione pool leading to its depletion. Hyperglycaemia-mediated oxidative stress can result in a decline in glutathione concentration through mechanisms such as; excessive glucose oxidation and increased production of superoxide radical in the mitochondrial electron transport chain; increased flux through the polyol pathway causing NADPH depletion, impaired GR activity and a decrease in the GSH:GSSG ratio; increased formation of advanced glycation end-products. Possible mechanism of action of KV includes direct scavenging of ROS, increased synthesis of GSH or interference with any of the aforementioned pathways (Livingstone & Davis, 2007). The results of our study are in agreement with previous studies that reported the protective effect of kolaviron against oxidative stress. The direct free radical scavenging activity of kolaviron and its protection against oxidative damage has been previously documented in human lymphocytes and rat liver cells (Farombi *et al*., 2004). Kolaviron has also been reported to induce the gene expression of some antioxidant enzymes and therefore protect against oxidative stress-induced tissue damage (Abarikwu *et al*., 2011). The mechanisms employed by kolaviron in attenuating oxidative stress in this study can be by depletion of ROS generation, increasing the synthesis of antioxidant enzymes and/or by relieving the inhibition of antioxidant enzymes.

Table 2: Summary of the effects of kolaviron treatment on the alteration in antioxidant defense in the liver, kidney and blood of diabetic rats

	CAT	GPX	SOD	MDA	GSH	ORAC	FRAP
Liver	↑	NS	↑	↓	↑	↑	----
Kidney	Norm	Norm	NS	↓	↑	↑	----
Erythrocyte	NS	↓		----	----	------	----
Plasma	-----	----	----	↓	----	NS	NS

Down arrows [↓] indicates a decrease in antioxidant enzyme activity or level of antioxidant makers while upper arrows [↑] indicates a decrease; NS, no significant difference compared to control rats; Norm; Normalized levels or activity of biomarker.

In addition to oxidative stress biomarkers, the extent of inflammation in the tissues and blood of diabetic rats were also assessed in comparison to normal control rats. Inflammation is one of the manifestations of oxidative stress, and the pathways that generate the mediators of inflammation are all induced by oxidative stress. While inflammation occurring as a consequence of oxidative stress is not the only biological manifestation of excess ROS/RNS (Roberts *et al*., 2010), inflammation resulting from oxidative stress is the cause of much human disease (Martinon, 2010). Cytokines are low-molecular-weight regulatory proteins that are produced during all phases of an immune response. Interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) are the major mediators of the early inflammatory response. They can stimulate the production of acute-phase proteins by the liver, mobilize neutrophils to the site of insult, direct the hypothalamus for a fever response, and increase the adhesion molecules on the vascular epithelium (Wright *et al*., 2006; Margioris, 2009). Hyperglycemia can directly promote an inflammatory state, where the increase in cytokines can lead to destruction of the pancreatic beta cells and malfunction of the endocrine pancreas in both type 1 and type 2 diabetes (Ahrens, 2011).

In agreement with previous findings, the results of the present study have clearly demonstrated a significant elevation in pro-inflammatory cytokines, growth factor and chemokine in the blood of diabetic rats compared to controls. The present study also revealed an association between hepatic and renal function impairment and different mediators of inflammation in diabetes and supports the hypothesis that low-grade systemic inflammation is an underlying factor in the pathogenesis of diabetes. Limited data are available on the effects of kolaviron on the level of cytokines in diabetes. Our study demonstrated that kolaviron reduced the serum levels of MCP-1, VEGF and IL-1β in diabetic rats. Lowered levels of inflammatory mediators (IL-1β, IL-6, TNF-**α** and MCP-1) were also observed in the liver of diabetic rats treated with KV. A decrease was also observed in the levels of IL-6 and TNF-**α** in the liver of normal rats treated with KV. Although kolaviron reduced levels of IL-1β in the kidney of STZ treated rats, we did not observe any changes in the concentrations of TNF-**α** in the kidney suggesting that the antiinflammatory action of kolaviron might be tissue specific.

Apoptosis is a coordinated series of events for the programmed execution of cell death, and plays an important role in the maintenance of tissue homeostasis. Defects in apoptosis regulatory machinery are implicated in a variety of pathological states; inadequate apoptosis may contribute to oncogenesis, while excess apoptosis is the underlying cause for cell loss during HIV/AIDS, neurodegeneration, and diabetes mellitus (Lee & Pervaiz, 2007). Apoptotic destruction of the insulin producing pancreatic β-cells is involved in the aetiology of both type 1 and type 2 diabetes. Oxidative stress and the redox state of a cell play a pivotal role in regulating apoptosis (Curtin *et al*., 2002). The role of ROS in the induction of apoptosis is provided by several studies in which the addition of ROS in low levels induces apoptosis and the observation that various antioxidants can inhibit cell death (Lennon *et al*., 1991; Suzuki *et al*., 1998). Oxidative stress can lead to damage of the mitochondrial inner membrane, resulting in MPTP formation and subsequent release of cytochrome c and apoptosis inducing factor from the mitochondria. In the cytosol, cytochrome c complexes with Apaf-1 to activate procaspase 9, which in turn activates downstream effector caspases 3, 6 and 7 (Bai & Odin, 2003).

Reactive oxygen species (ROS) accumulation leads to considerable cellular damage when insufficient cytoprotective and ROS scavenging molecules are available. Peroxidation or glycation of lipids, proteins, and DNA, reduction of antioxidant defenses and progression of inflammation of tissues are some disturbances, which are induced by oxidative stress (Rains & Jain 2011). Many of the above hyperglycemia-induced pathways converge to activate NF-κB, which in turn contributes to increase pro-inflammatory cytokine productions, oxidative stress and apoptotic processes, exacerbating diabetic-related complications (Singh *et al*., 2011). The involvement of reactive oxygen radicals has been suggested in apoptotic cell death of hepatocytes and endothelial cells (Jaeschke, 2000).

It has been demonstrated in the present study that kolaviron treatment reduced apoptosis in the liver and kidney of diabetic rats as demonstrated by the reduced number of TUNEL-positive cells in diabetic animals treated with kolaviron when compared with diabetic controls. The antioxidative and antiinflammatory effects of kolaviron might be responsible for the observed effects. Apoptosis is initiated by two distinct pathways: an intrinsic pathway involving

mitochondria and an extrinsic pathway leading to the rapid recruitment of Fas-associated protein with death domain and caspase-8 (Sanz *et al*., 2008). A previous study has demonstrated the anti-apoptotic effect of kolaviron against testicular damage induced by carbendazim (CBZ) through its antioxidative and inhibiting effect on both mitochondria- and FasL-mediated apoptotic pathways. The inhibitory effects of kolaviron on these pathways in the present study cannot be ruled out.

7.1 Conclusion

The results of this study provide evidence that activation of oxidative stress and increased activity of the innate immune system is a consequence of excessive excursions of plasma glucose and is very strongly associated with tissue damage. Due to the intrinsic anti-oxidant and anti-inflammatory effects demonstrated by kolaviron in our study, this natural antioxidant and anti-inflammatory phytochemical could therefore be considered as a therapeutic candidate for the complications that accompanies DM especially hepatic and kidney damage.

7.2 Limitation of the study and recommendation

While our study indicated that kolaviron was effective at improving specific markers of oxidative stress, inflammation and apoptotic index in diabetic rats, further studies should focus on the possible effects of kolaviron on specific pathways which have been implicated in increased generation of oxidant's, inflammation and aggravation of apoptotic process in diabetes such as; the advanced glycated end product's (AGE's), protein kinace C (PKC) and the nuclear factor-kappa B (NF-kB) pathways, to elucidate further specific targets in the pathways involved in diabetes and diabetic complications.

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ADDENDUM 1: RESEARCH OUPUT

PUBLISHED ARTICLES:

(1) **Omolola R. Ayepola**, Novel N. Chegou, Nicole, L. Brooks, Oluwafemi O. Oguntibeju (2013). Kolaviron, a *Garcinia* biflavonoid complex ameliorates hyperglycemia-mediated hepatic injury in rats via suppression of inflammatory responses. *BMC Complementary and Alternative Medicine*, 13:363.

(2) **Omolola R. Ayepola**, Nicole L. Brooks and Oluwafemi O. Oguntibeju (2014). Kolaviron improved resistance to oxidative stress and inflammation in the blood (erythrocyte, serum and plasma) of streptozotocin-induced diabetic rats. *In press*. *The Scientific World Journal*

(3) **Omolola R. Ayepola**, Nicole L. Brooks and Oluwafemi O. Oguntibeju (2014). Oxidative Stress and Diabetic Complications: The Role of Antioxidant Vitamins and Flavonoids, Antioxidant-Antidiabetic Agents and Human Health, ISBN: 978-953-51-1215-0, *InTech*, DOI: 10.5772/57282

ARTICLE UNDER REVIEW

(1) Kolaviron modulates apoptosis by suppressing oxidative stress and inflammation in diabetes-induced nephrotoxic rats. Under review in *Phyomedicine (International Journal of Phytotherapy and Phytopharmacology).*

CONFERENCE ATTENDED

(1) 74th Scientific Sessions, American Diabetes association (ADA), June 13-17, San Francisco, California. Omolola R. Ayepola, Nicole L. Brooks, Oluwafemi O. Oguntibeju. Alleviation of Oxidative Damage in the Liver of Diabetic Rats by a *Garcinia kola* Biflavonoid Complex (Kolaviron).

ADDENDUM 2: Ethical Clearance Certificates

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
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2 October 2012

CPUT/HW-REC 2012/A04

Faculty of Health and Wellness Sciences
Biomedical Sciences Department

Dear Ms Ayepola

YOUR APPLICATION TO THE HW-REC FOR ETHICAL CLEARANCE

At the meeting of the Health and Wellness Sciences-REC on 20 September 2012 approval was granted to Omolola Rebecca Ayepola for your application (pending corrections that have been received and reviewed if applicable). This approval is for research activities related to a DTech: Biomedical Technology at this institution.

TITLE: Effects of kolaviron–a *Garcinia kola* biflavonoid on biochemical and histological parameters in streptozotocin - induced diabetes and diabetic complications (nephrotoxicity and hepatotoxicity) in male Wistar rats.

INTERNAL SUPERVISOR: Prof O Oguntibeju

INTERNAL CO-SUPERVISORS: Dr N Brooks

Comment:
Approval will not extend beyond 2 October 2013. An extension should be applied for should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

Note:
The investigator(s) should understand the conditions under which they are authorized to carry out this study and they should be compliant to these conditions. **It is required that the investigator(s) complete an annual progress report that should be submitted to the HW-REC in December of that particular year, for the HW-REC to be kept informed of the progress and of any problems you may encounter.**

Kind Regards

Prof JL Marnewick
CHAIRPERSON: HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE

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HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
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3 October 2013
CPUT/HW-REC 2013/A06

Faculty of Health and Wellness Sciences
Biomedical Sciences Department

Dear Ms Omolola Rebecca Ayepola

YOUR APPLICATION TO THE HW-REC FOR EXTENSION

Approval was granted on 19 September 2013 by the Health and Wellness Sciences-REC to Omolola Rebecca Ayepola for extension of ethical clearance. This approval is for research activities related to a DTech: Biomedical Technology at this Institution.

TITLE: Effects of kolaviron–a *Garcinia kola* biflavonoid on biochemical and histological parameters in streptozotocin - induced diabetes and diabetic complications (nephrotoxicity and hepatotoxicity) in male Wistar rats.

Internal Supervisor: **Prof O Oguntibeju**
Internal Co-supervisor: **Dr N Brooks**

Comment:
Approval will not extend beyond 3 October 2014. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

Note:
The investigator(s) should understand the conditions under which they are authorized to carry out this study and they should be compliant to these conditions. **It is required that the investigator(s) complete an annual progress report that should be submitted to the HW-REC in December of that particular year, for the HW-REC to be kept informed of the progress and of any problems you may encounter.**

Kind Regards

Zuleika Nortjé
CHAIRPERSON – ETHICS RESEARCH COMMITTEE
FACULTY OF HEALTH AND WELLNESS SCIENCES