UNIVERSITY OF KWAZULU NATAL

NARINGIN REVERSAL OF SOME ASPECTS OF DIABETIC NEPHROPATHY IN RATS TYPE 1 STREPTOZOTOCIN INDUCED DIABETES

NKOMO FEZILE SINETHEMBA (213570378)

BSc. Hons Biochemistry (UNIZULU)

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BY

FEZILE SINETHEMBA NKOMO

213570378

Submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN PHARMACOLOGY

Department of Pharmacology

Discipline of Pharmaceutical Sciences

College of Health Sciences

University of KwaZulu-Natal

Supervisor: Dr. P.M.O. Owira

Date of submission: January 2014

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Science, University of KwaZulu-Natal, Westville from January 2013 to December 2014 under the supervision of Dr. Owira P.M.O.

The study is an original work of the author and has been submitted in partial fulfilment of the academic requirements for obtaining a MSc. Degree in Pharmacology. Information from other sources used in this dissertation has been duly acknowledged in the text and reference section.

Fezile Sinethemba Nkomo

Dr. Owira P.M.O (Supervisor)

DECLARATIONS

DECLARATION 1 – PLAGIARISM

I, **Nkomo Fezile Sinethemba** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) Their words have been re-written but the general information attributed to them has been referenced.
b) Where their exact have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

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First and for most I would like to thank God, the beginner and the finisher of all things, through it all He remained faithful, indeed I can do all through Him who strengthens me. My heartfelt gratitudes to my supervisor Dr PO Owira and Dr David N. Onyango for their assistance throughout this work, to my family (Mrs J Nkomo, Mr Z Nkomo, Zaluleko, Leby and Thuthukani) thank you for believing in me when I less believed in myself, I wouldn't have made it through if it wasn't for your financial and emotional support. I am grateful indeed I am blessed to be a member of such a prayerful and supportive family, my colleagues Alfred, Snazo, Christine and Sane you guys are amazing, it wasn't easy but you always had faith, thank you so much. I would also like to thank the University of KwaZulu Natal for the financial support and the BRU staff for their assistance.

List of Abbreviations

ACE	Angiotensin converting enzymes
AGE	Advance Glycation End Products
AUC	Area under curve
BW	Body weight
Cl^-	Chloride
DKA	Diabetic ketoacidosis
DN	Diabetic Nephropathy
ESRD	End Stage Renal Disease
FBG	Fasting blood glucose
FFA	Free fatty acid
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GK	Glucokinase
GLUT	Glucose transporter
HDL	High Density Lipoproteins
HLA	Human leucocyte antigen
HMG-CoA	Hydroxymethyl glutyryl Coenzyme A
IL	Interleukins

IR	Insulin receptor
IV	Intravenous
K^+	Potassium
LDL	Low Density Lipoproteins
LPL	Lipoprotein Lipase
MA	Microalbumin
MDA	Melaldehyde
Na^+	Sodium
NAD	Nicotinamide adenine dinucleotide (Oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
OGTT	Oral glucose tolerance test
PKC	Protein Kinase C
RAGE	Receptor for Advance Glycation End Products
RAS	Renin Angiotensin System
ROS	Reactive Oxygen Species
SGLT	Sodium glucose linked transporter
SOD	Superoxide Dismutase
STZ	Streptozotocin

TBARS	Thiobarbituric acid-reactive substances
TGF-α	Tubular growth factor alpha
TGF-β	Tubular growth factor beta
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoproteins

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ABSTRACT

The role of naringin on streptozotocin-induced diabetic nephropathy was investigated. Male Wister rats (200-300 g) were divided into six groups (n=7). Group A was treated with a vehicle (0.2 ml of 0.1M citrate buffer pH4.5) by a single intraperitoneal injection (IP) and 3.0 ml/kg/BW of distilled water and group B was treated naringin (50 mg/kg/BW) daily. Groups C, D, E and F were rendered diabetic by a single IP of STZ (60 mg/kg/BW) in 0.1M citrate buffer (pH4.5). Diabetes was confirmed after 2 days (48 hours). Group C was treated with subcutaneous insulin (4 U/kg/BW) twice a day while groups D and F were treated with naringin (50 mg/kg/BW) and ramipril (20 mg/kg/BW) which is the drug that is currently used to treat diabetic nephropathy orally, daily, respectively. On day 55, 24-hours urine samples were collected and on day 56 rats were sacrificed; blood samples were collected by cardiac puncture and kidney and liver samples were excised and snap-frozen in liquid nitrogen for further analysis.

Diabetic groups (C, D, E and F) showed significant ($p<0.001$) hyperglycemia, weight loss, polydipsia, polyuria, impaired glucose tolerance and low fasting plasma insulin compared to the controls. Treatment with naringin improved weight loss, polydipsia, fasting plasma glucose and fasting plasma insulin. Naringin decreased fasting blood glucose but did not improve glucose intolerance and it significantly ($p<0.001$) improved fasting plasma insulin compared to diabetic control. Furthermore, non-treated diabetic groups significantly ($p<0.001$) showed elevated plasma malondialdehyde (MDA) and reduced superoxide dismutase (SOD) activities compared to the controls. Naringin further reduced renal lipid peroxidation and increased SOD activities in diabetic rats. Moreover, naringin reversed electrolytes retention and also increased glomerular filtration rate in diabetic rats.

Naringin therefore ameliorates some aspects of diabetic nephropathy (GFR, serum and urine electrolytes) by reversing oxidative stress associated with DN.

CHAPTER 1

1.0 Introduction and literature review

1. 1 Diabetes Mellitus Epidemiology and Prevalence

Diabetes was estimated in 2013 to affect 382 million people globally with the incidence of 8.3% (IDF, 2009) and type 2 diabetes making up about 90-95% of the cases (Tiwari and Madhusudana *et al*., 2002) whereas type1 diabetes makes up to 5-10% (Raskin and Mohan, 2010). Diabetes has been ranked as a third commonest chronic diseases in the world (Fradkin, 2012) yet many people with diabetes are estimated to be undiagnosed (IDF, 2013).

Even though diabetes is common in developed countries, there is an increase in prevalence rate in Asia and Africa (Wild *et al*., 2004) with highest mortality rates (IDF, 2013). Environmental and genetic factors play an important role in the development of diabetes in varying populations. In South Africa it has been estimated that millions of people will suffer from diabetes due to sedentary lifestyles, urbanization and lack of physical activities (Menghani *et al.,* 2010).

1.2 Diabetes Mellitus definition and classification

Diabetes is a metabolic disorder characterized by dyslipidemia and glucose intolerance. It is an increasingly prevalent condition that contributes to the increased cardiovascular morbidity and mortality (Formiguera and Canton, 2004). Chronic kidney diseases in patients with diabetes add to the morbidity and mortality. There are two major types of diabetes mellitus; Insulin dependent diabetes (Type 1) and Non-Insulin dependent diabetes (Type 2) (Alberti *et al*., 1998).

1.2.1 Type 1 Diabetes (Insulin Dependent Diabetes Mellitus) IDDM

Type 1 diabetes is autoimmune mediated destruction of pancreatic β-cells. The dendritic cells and macrophages in the pancreatic islets process autoantigens and present them to autoreactive $CD4^+$ T cells. $CD4^+$ T cells secret cytokines which can activate β cell-specific cytotoxic T cells ($CD8^+$) which further activate macrophages and other T cells leading to beta cells destruction ultimately absolute insulin deficiency which predisposes individuals to ketoacidosis (Lambert *et al*., 2004). Type 1 usually develops in young children and teenagers (Devendra *et al*., 2004). It has been found that the Human leukocyte antigen chromosome 6 is a genetic portion that is linked with IDDM (Anjos and Polychronakos, 2004) however, it can be due to environmental factors and viruses as well.

1.2.2 Type 2 Diabetes (Non-Insulin Dependent Diabetes Mellitus) NIDDM

The pancreas produces insulin but skeletal muscle cells are resistant to it leading to insulin resistance (American Diabetes Association, 2005). More than 90% of diabetic individuals have type 2 diabetes mellitus (T2DM), a consequence of a sedentary lifestyle and lowered beta-cell mass and genetic predisposition, it is often diagnosed after metabolic dysfunctions have manifested in multiple organ systems. Insulin resistance leads to hyperglycaemia which gives rise to several micro-vascular and macro-vascular complications such as diabetic nephropathy, dyslipidemia, obesity and generation of reactive oxygen species in tissues and of advanced glycation products (Jakus and Rietbrock, 2004). However, pancreas lacks the defence system against reactive oxygen species generated by free radicals hence it is susceptible to oxidative damage due to the absence of glutathione peroxidase-1 gene (Elbein, 2002). In this regard, dyslipidemia is initiated by insulin resistance which activates the activity of lipoprotein lipase (LPL) in adipocytes. The lipoprotein lipases that catalyses release of free fatty acids (FFA) which are taken up by hepatocytes leading to production of triglycerides. Triglycerides then stimulate production of very low density lipoproteins (VLDL) which contributes to the increased risk of cardiovascular diseases (Gennest and Libby, 2011).

1.3 Diabetes mellitus complications

Diabetes mellitus complications are macrovascular (atherosclerosis and coronary heart diseases) and microvascular complications (diabetic nephropathy, retinopathy and neuropathy). These complications are as a result of multiple factors intertwined together (metabolic and hemodynamic factors) (Figure1).

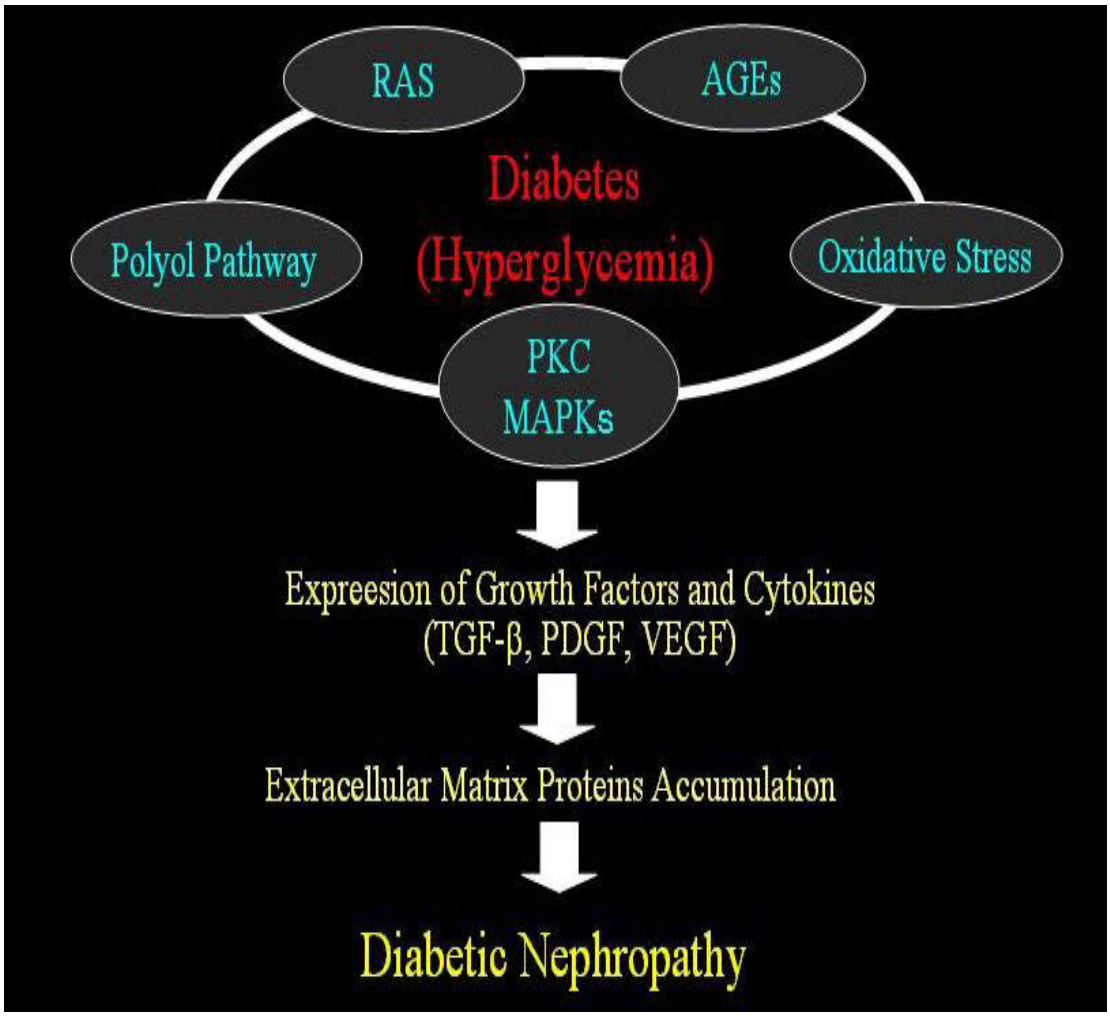


Figure 1: Pathophysiological significance of RAS, AGEs, Polyol, oxidative stress and PKC activation in diabetes (Courtesy of Daisuke Koya *et al*, 2003).

1.3.1 Advanced glycation end products (AGEs)

Advanced glycation end- products are formed by the non-enzymatic reaction of reducing sugars, ascorbate and other carbohydrates with amino acids, lipids and nucleic acids. AGEs have been demonstrated to have wide range of chemical, cellular and tissue effects that are implicated in the development of diabetic complications (Magri and Fava. 2009). AGEs formation occurs continually in the body and is accelerated in diabetes however, external factors such as smoking of tobacco (Nicholl and Bucala, 1998) and western diet (Peppa *et al*, 2002) have been reported to contribute to AGE formation. Peppa (2003) reported that 10% AGEs formed from exogenous factors is absorbed in the gastrointestinal tract and delivered into the liver and tissue but only 1/3 is excreted in the urine while the remaining is involved in AGE derived diabetes complications (Cai *et al*., 2002 and Vlassara *et al*.,2002).

Recent studies, however, demonstrate that interactions of AGE modified proteins with different AGE-receptor complexes not only serve to degrade AGE-proteins, but also activate signal transduction pathways. AGEs induce the synthesis and release of cytokines and growth factors that might initiate tissue repair and protein turnover but also contribute to the development of vascular disease and diabetic complications (Forbes *et al*, 2002 and Kelly *et al*, 2001). In addition, AGEs also react with AGE-specific receptors; activate NADPH oxidase leading to increased formation of reactive oxygen species (ROS) and inflammation. Furthermore, AGE also interact with the renin-angiotensin system, which is another potential mechanism for initiating renal disease (Hollenberg *et al,* 2003).

1.3.2 Renin-angiotensin system (RAS)

In diabetes, renin levels are increased and it has been suggested that it plays a huge role in pathogenesis of diabetic nephropathy. Renin and prorenin receptor are suggested to be involved in the development and progression of kidney disease by enhancing the renal production of inflammatory cytokines which lead to renal cell loss (Kreisberg *et al*, 1994). Renin produces angiotensin I from angiotensinogen after which, it is cleaved by angiotensin-I-converting enzyme (ACE) to release angiotensin II, a potent vasoconstrictor. Ang II has harmful effects on the kidney; it increases the expression of inflammatory cytokines and tubular growth factors hence inducing podocytes injury in the tubules (Kreisberg *et al*., 1994). Increased renin elevates the expression of angiotensinogen gene in proximal tubule cells hence inducing podocytes injury via Ang II receptor gene (Klinik für Innere *et al*., 2006). Ang II is also documented to stimulate the synthesis of VEGF in podocytes through p38 mitogen-activated protein kinase (p38 MAPK) pathway (Kang *et al*., 2006).

(Rabelink, Bakris, 1992) reported that angiotensinogen influences glomerular filtration rate by interacting with autocrine factors such as superoxide and nitric oxide (NO) which is a potent oxidant (peroxynitrite) when it interacts with superoxide. NO is unstable, it can be easily converted to nitrite and its activation by ROS may lead to mesenchymal proliferation, which is an early and characteristic lesion of diabetic nephropathy (Freeman, 1993). Moreover Ang II also mediates reactive oxygen species production by activating NADPH oxidase (James and Soweres, 2002). NADPH oxidase is a key mediator of podocytes apoptosis and subsequent diabetic glomerulopathy in vivo, thus mechanistically linking hyperglycemia, ROS, podocytes apoptosis, podocytes depletion, and diabetic nephropathy (James and Soweres, 2002).

1.3.3 Oxidative stress

Oxidative stress occurs when production of reactive oxygen species (ROS) as a result of free radicals production exceeds local antioxidant capacity (180 to 200 mV) (Trachootham *et al*., 2009). Reactive oxygen species mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. ROS are unstable molecules as a result of the presence of unpaired electrons in their molecular structure; undergo a series of interaction with biological macromolecules such as proteins, lipids, and DNA (Bayir, 2005).

ROS have been reported to be highly involved in the pathogenesis of diabetes-associated vascular complications (Robertson *et al*., 2003; Wang *et al*., 2011). Elevated glucose increases the production of superoxide by mitochondrial electron transport chain with the help of the enzyme NADPH oxidases (Saleem *et al*., 2001). Glucose is reduced to sorbitol at the expense of reduced nicotinamide adenosine dinucleotide phosphate (NADPH) by the enzyme aldose reductase which catalyzes the rate limiting step of the polyol pathway (Figure 1).

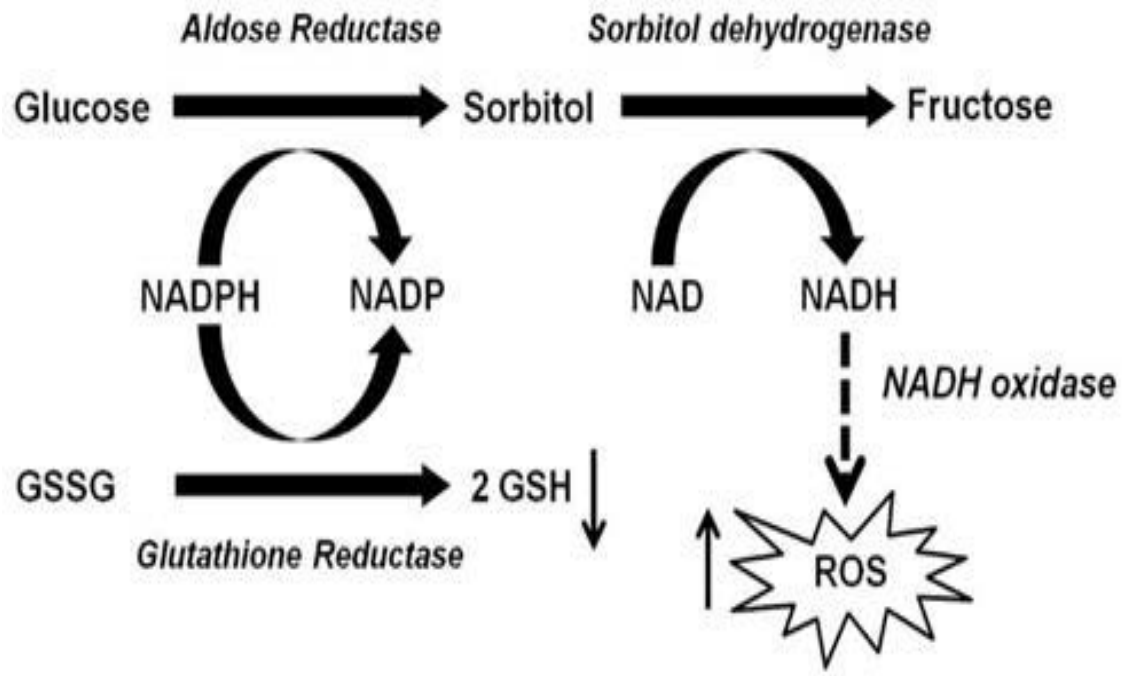


Figure 1: Generation of oxygen reactive species through polyol pathway. (Courtesy of Cumbie and Heemayer, 2007).

Advanced Glycation End- products (AGEs) leads to the production of ROS through Protein Kinase C-depended activation of NAD(P)H oxidase in smooth muscle cells and endothelial cells . AGEs alter normal protein function by modifying the structure and ultimately the function of the protein. Protein modification leads to disruption in redox homeostasis. Disruption in redox homeostasis leads to an excessive generation of ROS leading to an increase in oxidative stress.

The accumulation of AGEs stimulates the release of AGEs proteins which bind to AGE receptors (RAGE) thereby inducing generation of free radicals. An increase in glucose

uptake leads to overproduction of electron donors (NADH and FADH2) from stimulated glycolysis and tricarboxylic acid cycle (Wautier *et al*., 2001).

It is hypothesized that hyperglycemia increases $NADH/NAD^+$ ratio in cell production and NADH is the main electron donor to the mitochondrial electron transport chain. Therefore antioxidants are expected to prevent damage of mitochondria by reducing reactive oxygen species and their role in diabetes complications has been evaluated (Nishikawa *et al*., 2002). Using antioxidant supplements may have chemoprotective effects in diabetes (Oberley, 1998). There are two types of antioxidants; enzymatic antioxidants (catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic antioxidants (Vitamin C and E and reduced glutathione). In diabetic state both types of antioxidants are supressed (Bonnefont-Rousselot *et al*., 2000, Martim *et al*., 2003 and Johansen *et al*., 2005). Glycation impairs the antioxidant effect by inactivating the antioxidant enzymes superoxide dismutase in particular (Arali *et al*., 1987). Cu/Zn-SOD resolves O^2_2 into H_2O_2 and O_2, whereas catalase (CAT) and glutathione peroxidase (GSH-Px) catalyze the reduction of H_2O_2 to $HO+O_2$.

1.3.4 Protein Kinase C activation

Protein Kinase C mediates diverse and important cellular functions; it has a catalytic and a regulatory region in its structure. Protein Kinase C has 11 isoforms, which are activated in the glomeruli of diabetic rats and their activation leads to upregulation of vascular endothelial growth factors (VEGF) in mesangial cells. The activation of PKC-β induces renal dysfunction by increasing tubular growth factor beta (TGF-β) and connective growth factor (CGF) (Aiello *et al*., 1994). Ishii *et al,* 1996 and Koya *et al*., (1997) reported that the treatment of the activated PKC-β with PKC-β inhibitor ameliorated an increase in the urinary

albumin excretion and the structure of the glomeruli (mesangial expansion and extracellular matrix) was restored as well by the treatment).

It is also noted by Ishii *et al*, (1996) that the ruboxistaurin (PKC-β inhibitor) reduced albuminuria and maintain a glomerular filtration rate for 1 year in type 2 diabetic patients with nephropathy. Activation of these factors leads to the accumulation of extracellular matrix proteins in the glomeruli and cell injury as well as apoptosis of the glomerulus cells (podocytes) (Aiello *et al*., 1994).

1.4 Macrovascular complications

1.4.1 Atherosclerosis

In diabetes, AGE/RAGE interaction is accelerated leading to atherosclerosis and cardiovascular diseases. Increased AGEs modify low density lipoproteins (LDLs) in diabetic patients and is associated with increased inflammation, endothelial activation and permeability which increase local oxidative stress and LDL oxidation. Oxidation of LDL particles result in accumulation of endothelial walls of arteries and macrophages and T-lymphocytes proliferation is stimulated (Michael and Owler, 2008). Stimulation of macrophages and T-lymphocytes proliferation further induce proliferation of smooth muscles in arterial walls. This process results in the formation of lipid rich- atherosclerotic lesions which result in vascular infarction (Boylee, 2007). The increased expression of RAGE in macrophages of vulnerable atherosclerotic plaques from diabetes patients, and their co-localization with the inflammatory markers COX-2 and MMPs, support their potential implication in plaque destabilization and rupture.

1.4.2 Cardiovascular Diseases

Although cardiovascular diseases (hypertension, Ischemic heart diseases and cardiomyopathy) are not specific to diabetes, they are more prevalent among patients with diabetes than among those without diabetes (Stemlar *et al*., 1993). Diabetes is associated with alterations in lipid profiles (Pushparaj *et al*., 2007). Changes in concentrations of plasma lipids including cholesterol and lipoprotein are complications frequently observed in patients with diabetes mellitus and certainly contribute to the development of coronary heart disease. Dyslipidemia has a strong co- relation with cardiovascular disease; it is a risk factor of CVD (Ford *et al*., 2009). Moreover, atherosclerosis causes CVD by restricting the blood flow to the heart. Type 1 diabetes, however, it is often associated with coronary heart disease. Ischemic heart disease seen in type 1 diabetes leads to higher mortality in the general population. Different therapeutic agents have been developed over the years in trying to combat such complications.

1.5 Microvascular complications

Hyperglycemia gives rise to reactive oxygen species thus leading to oxidative stress, activates Protein Kinase C, renin-angiotensin aldosterone system, accumulation of nonenzymatally glycosilation of proteins products advanced glycation end products (AGE) and acceleration of Polyol pathway (Figure 3). All these, result in the activation of the vascular endothelial growth factors (VEGF), tubular growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α) and interlukins (IL) 1, 6 and 18. Vascular endothelial growth factor (VEGF) is known to be primarily involved in neoangiogenesis and increased endothelial permeability. These factors converge and promote glomerular and tubular fibrogenesis (Wolf and Ziyadeh 1999).

1.5.1 Diabetic retinopathy (DR)

DR is a diabetic complication that leads to visual impairment and blindness. The microvasculature of the retina is damaged, the blood vessels swell and leak fluid, and if not prevented, new vessels start to grow, and ultimately lead to the detachment of the retina (Sharma *et al*., 2005). The blood vessels of retina have tight junctions that protect them from leaking, but sustained high glucose damages the tight junctions and the vessels become leaky allowing fluid or blood to seep into the retina, thus resulting in the swelling of the retina (Bhavsar *et al*., 2006). Advanced Glycation End-products precursors and PKC activation induce retinal vessel wall thickening and growth arrest and retinal apoptosis of pericytes (Kowluru, 2005). Pericytes apoptosis is a key factor in retinopathy; it triggers endothelial activation of dysfunction which results in neoangiogenesis via activation of vascular endothelial growth factors. Moreover, accumulation of polyols results in elevation of intracellular sorbitol which causes damage in vascular cells causing retinal and neural damage in diabetes. AGEs are involved in the pathogenesis of diabetic retinopathy by altering small vessel wall integrity and structure and by inducing cytokines, growth factors and increased oxidative stress (Yamagishi *et al*., 2002).

1.5.2 Diabetic Neuropathy (DN)

DN is characterized by impaired wound healing due to a decrease in oxygen in tissues because of glycated haemoglobin and altered immune system. It is associated with increased necrosis, impaired wound healing and loss of pain perception (Huijberts *et al*., 2008). AGEs are implicated to be involved in loss of pain perception; they induce the expression of inflammatory genes resulting in neurologic dysfunction and altered pain sensation (Herold *et al*., 2007). AGEs modify axonal cytoskeleton tubulin, neurofilament and actin leading to impaired axonal transport. Moreover, oxidative stress is associated with the development of

apoptosis in neurons and supporting glial cells and so could be the unifying mechanism that leads to nervous system damage in diabetes (Dorbretsov *et al*., 2007).

1.5.3 Kidney

The kidney prevents extra fluids accumulation, keeps the blood and electrolytes stable and it regulates the blood pressure. Kidney receives about 25% of the heart's cardiac output (Brenner, 2001). Nephron is the main functional unit of the kidney (Cushman, 1980). Nephrons are about 0.6-1.4 x 10^6 which makes the kidney an efficient filtration organ (Brownlee, 2001). Nephron is made out of Bowman's capsule, proximal convoluted tubule and distal convoluted tubules (Eroschenko). The glomerulus filters blood; it has got cells (podocytes) and mesangial cells which are responsible for blood filtration. Mesangial cells have phagocytic characteristics and they synthesize the extracellular matrix and secrets interleukin-1 (IL-1) and platelet derived growth factor (Rosenstock *et al*., 2010). It has been noted that during renal dysfunction podocytes fuse and lead to the formation of glomerular basement membrane (GBM) which is thick but leaky leading to proteinuria and glycosuria (Wolf and Ziyadeh 1999) . A prolonged renal dysfunction may lead to the total kidney failure hence dialysis or transplant is needed.

1.5.3.1 Diabetic Nephropathy

Diabetic nephropathy (DN), a frequent and major microvascular complication of diabetes mellitus, is the most common cause of end-stage renal failure disease (ESRD) diabetic patients (Sheetz and King, 2002). DN is characterized by glomerular and tubules hypertrophy thickening of the basement membranes, accumulating of extracellular matrix

components, decrease in glomerular filtration rate (GFR), glomerulosclerosis as well as tubulo-interstitial fibrosis in mesangium and interstitium (Kanwar *et al*., 2008).

Type 1 and type 2 diabetes mellitus can lead to DN (Mauer *et al*, 2001) (United States Renal Data System, 2010). From the year 1991-2001, the number of patients who start renal replacement therapy has doubled (US Renal Data System, 2010). In the last decade there has been a dramatic increase in the proportion of ESRD patients affected by diabetes, and this increase is largely attributed to type 2 diabetes. The basic pathophysiology is similar in both type1 and type 2 diabetes mellitus patients. The natural course of DN is characterized by a mean rate of decline in GFR ranging from 25 - 0 ml/min/ year. Glomerular hyperfiltration is a characteristic of an early diabetic nephropathy in animal models of diabetes. Diabetes contributes to the production of prostaglandin which controls the vascular reactivity of the renal glomerular efferent arterioles allowing autoregulation of glomerular capillary pressure (Forbes *et al*., 2007).

1.5.3.2 Pathogenesis of Diabetic Nephropathy (DN)

Proximal tubules respond to different changes such as hypertrophy and ion transporters. Glucose enters the proximal tubules in an insulin dependent manner (Magri and Fava, 2009). In type 2 diabetes, there are comorbid factors such as metabolic syndrome and hyperinsulinemia and high blood pressure which are likely to affect DN, the most structural renal changes occur in the glomeruli whereas in type 2 diabetes several patients have more advanced tubulo-interstitial and vascular than glomerular lesions (Mauer *et al*., 2001 and Mauer *et al*., 1984). However, there are many similar pathological changes in the kidney in both type 1 and 2 diabetes.

During the early stages, DN is usually not seen at least for 3-5 years after diagnosis. A few changes can be noted at the initial stage such as increase in kidney size, enlarged glomeruli and supranormal glomerular filtration rate (HGFR). Ray *et al*., 1998 reported that the proximal tubule plays a role in kidney mass. Higher filtration rate of the kidney is due to increased blood flow and increased vascular permeability (Chiu *et al*., 2009). However, standard insulin treatment leads to reduction in renal size and an increase in GFR (Ditzel and Schwartz, 1967).

In uncontrolled diabetes, the urinary albumin excretion is elevated. Early detection of DN could reverse or prevent long term outcomes hence preventing the development of ESRD. It is hypothesised that glomerular hypertension is due to provocation of the subsequent demise of diabetic kidney by the early hemodynamic genotype; this glomerular hypertension leads to glomerular hyperfiltration. In an early stage of DN there is an increase in reabsorption of electrolytes and fluids in type 1diabetes (Vallon *et al*., 2003). The proximal tubules reabsorption is enhanced due to the increased glomerular filtration of glucose which in turn increases proximal tubule reabsorption of Na^+ and glucose through sodium glucose transporter proteins SGLT2 and SGLT1 (Vallon *et al*., 1999, and Vestri *et al*., 2001).

As diabetic nephropathy progresses, there is tubular growth which leads to renal damage, tubular growth due to inflammation resulting in increasing proteinuria, decline in glomerulosclerosis and tubulointerstitial (Anderson *et al*., 1988). GFR declines due to the small number of functioning glomeruli. At later stages, DN is characterised by the presence of persistent proteinuria and progressive decline in renal function, which results in renal failure. DN is associated with increased mortality in diabetic patients (Chiu *et al*., 2009).

1.6 Management and Current Treatment for Diabetes

At present, a number of hypoglycemic agents are popularly used to keep blood glucose in normal level such as exogenous insulin in type 1 diabetes. The short comings of insulin are that it cannot be given orally and it needs to be stored in the fridge, injection allergies and weight gain (Hirsch, 2005). Type 2 diabetes is managed by use of oral drugs which are hypoglycemic. They are divided into five classes; **meglitidines**; they stimulate the release of insulin from beta cells e.g. repaglinid and nateglide. **Biguanides**; they reduce hepatic glucose output and, to a lesser extent, enhance insulin sensitivity in hepatic and peripheral tissues e.g. metformin, **thiazolidinedines**; they enhance insulin sensitivity in both muscle and adipose tissue and to a lesser extent by inhibiting hepatic glucose production e.g. pioglitazone, troglitazone and rosiglitazone, **α- glucosidase inhibitors**; they act by inhibiting the enzyme alpha-glucosidase found in the brush border cells that line the small intestine, which cleaves more complex carbohydrates into sugars e.g. acarbose, miglitol and voglibose, **sulfonylureas;** they stimulate insulin release from the beta cells of the pancreas and may slightly improve insulin resistance in peripheral target tissues e.g. gilbenclamide, tolazamide, chlorpropamide, tolbumide and acetohexamide and (Hirsh, 2005). **Incretins mimetics** mimics incretins in the body. Incretins are hormones from glucagon superfamily released in response to nutrient ingestion by regulating the insulin secretory response to the products within the nutrients in the food. Incretins mimetics lower blood sugar levels by preventing the pancreas from giving out too much glucagon e.g. exenatide and liraglutide. These drugs have no direct effect on DN.

Diabetic nephropathy is managed by angiotensin converting enzymes (ACE) inhibitors. ACE inhibitors (ramipril and perindopril) control blood pressure and delay progression of kidney dysfunction. In humans, the rennin-angiotensin system plays a pivotal role in blood pressure

regulation and in the pathophysiology of cardiovascular diseases such as congestive heart failure and hypertension hence rennin-angiotensin system blockers also delays the progression of the ESRD (Erdos and Skidgel, 1987). Ramipril decreases the risk of overt nephropathy by 24% in type 2 patients (DIABHYCAR Study Investigators, 2004). Moreover, as blood pressure decreases, the cells of the juxtaglomerular apparatus release renin and activate the renin- angiotensin-aldosterone pathway. The juxtaglomerular apparatus is a small group of cells situated in the area where renal tubule links up with the efferent and afferent arterioles. Renin converts angiotensinogen into angiotensin I. Angiotensin converting enzymes (ACE) then converts angiotensin I into angiotensin II, a peptide hormone. Angiotensin II has one of its functions being stimulation of sodium reabsorption by the proximal convoluted tubules among others. These changes assist in restoring extracellular fluid volume and stabilizing blood pressure. ACE inhibitors are administered to block the production of angiotensin II in people with elevated blood pressure. Unexpectedly, these agents produce serious side effects in the clinical application, such as weight gain, gastrointestinal disturbances, edema, hypoglycemia and insulin resistance (Vasconcelos *et al*., 2001).

1.7 Basis of the study

Current treatment cannot arrest progression towards end stage renal disease (ESRD) (Lewis *et al*., 1993, Lewis et al., 2001 and Brenner *et al*., 2001). Hypoglycemic drugs present different approaches to control hyperglycemia in different types of diabetes, each with its own limitations and side-effects. Developing various means by which blood glucose can be effectively controlled while minimizing the iatrogenic side-effects remains a continuing task in the diabetic field. However, naringin may thus help ameliorate the situation.

1.8 Medicinal plants used in diabetes management

Medicinal plants have been successfully used by traditional healers to treat many diseases; they play a role in discovery of new therapeutic agents. A number of medicinal plants have been reported to have antidiabetic, antidyslipidemic, anti-inflammatory effects. Antioxidant activities of these medicinal plants have been evaluated and found to play a huge role in diabetes management due to their ability to reduce ROS generated by free radicals leading to superoxide and nitrogen oxides. The ROS affect the activity of glomerular filtration rate thus rendering it dysfunctional. Many plant extracts and products have been shown to possess significant antioxidant activity e.g. *Phyllanthus emblica* (Saleem *et al*., 2001) and *Cichorium intybus* (Ricci *et al*., 2006). Medicinal plants contain bioactive phytochemicals. They may have no nutritional value but are needed by the body because of their ability to prevent diseases (Wang *et al*., 2004). Phytochemicals such as alkaloids, sterols, triterpenes, phenolics, flavonoids and others are found in varying concentrations in these plants hence their use in diabetes management.

1.8.1 Flavonoids

The flavonoids are a diverse group of polyphenolic compounds widely distributed in the plant kingdom. All flavonoids have a common basic structure (Figure 4). There are more than 6400 known flavonoid compounds. In the grapefruit, flavonoids exist in the form of glycosides with naringin being the most abundant among others like narithrine, hesperidin and hesperitin (Kawaii *et al*., 1999).

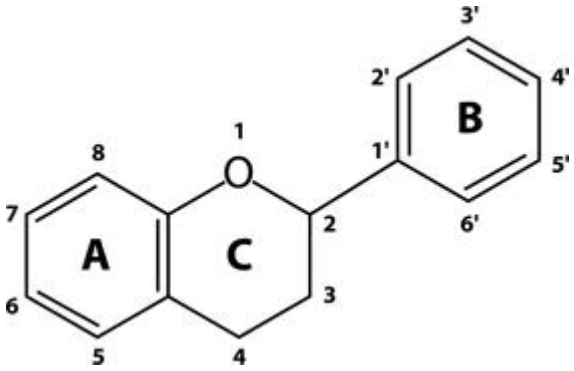


Figure 3: Chemical structure of flavonoids Courtesy of Liu, RH, 2004

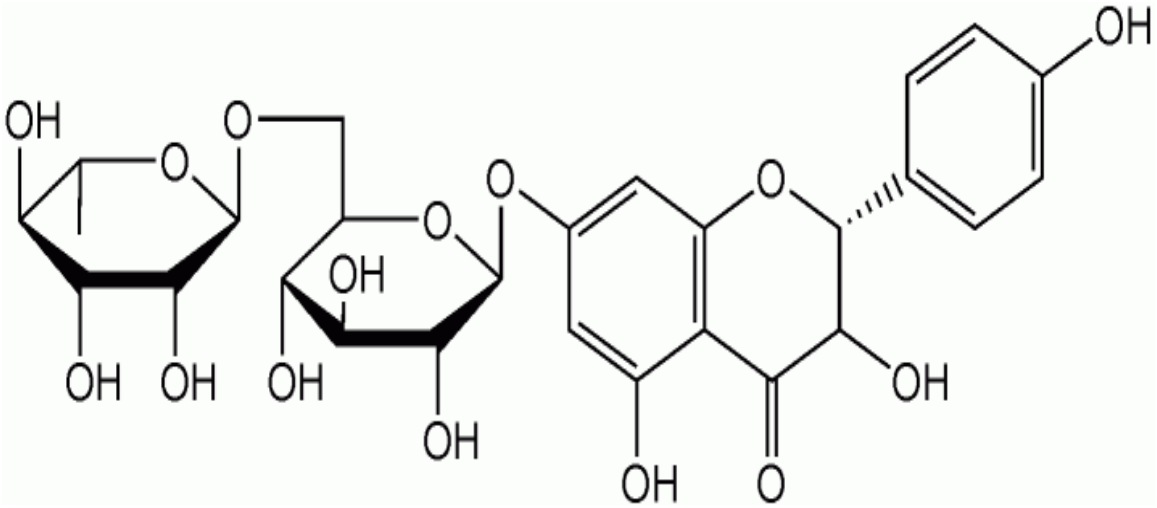


Figure 4: Chemical structure of naringin (4′,5,7- trihydroxy flavonone 7-rhamnoglucoside). Courtesy of Shaddack, 2005.

1.8.2 NARINGIN

Naringin is the predominant flavonone found in grape fruits and related citrus species (Kawaii *et al*., 1999). Naringin has been reported to have antioxidant activities, free radicals scavenging properties (Seon *et al*., 2011) and it is the potent cholesterol lowering agent (Kim *et al*., 2006). Nevertheless, naringin and hesperidin exhibit anti-inflammatory and anti-carcinogenic properties and hypoglycemic effects mediated by hepatic glucose regulating enzymes in C57BL/KsJ-db/db Mice (Jung *et al*., 2014). Punithavathi *et al*, (2008) reported that naringin combined with vitamin C ameliorates streptozotocin- induced diabetes in male Wister rats. Sharma *et al*, (2011) reported that naringin decreases insulin resistance, TNF-α

and IL-6 in type 2 diabetic rats and it increases adiponectin levels hence it ameliorates dyslipidemia and hyperinsulinemia.

Naringin is also known to inhibit hepatic HMG-CoA reductase and ACAT enzymes which are involved in hepatic cholesterol biosynthesis thus improving atherogenic dyslipidemia in rats with type1 diabetes (Xulu and Owira *et al*., 2011). Similarly, it has been shown to exhibit antidiabetic and antioxidant effects in type 2 diabetic rats (Sharma *et al*., 2011). Currently it is not known whether reducing hyperglycemia-induced ROS could have beneficial effects on diabetes end-points. It is therefore hypothesized that naringin may prevent the progression of diabetic nephropathy by reversing or retarding the increased cellular concentration of ROS stimulated by hyperglycemia. This is expected to limit oxidative damage to the podocytes and arrest further deterioration to the kidney. Therefore, this study was set out to determine *in vivo* effects of naringin on STZ-induced diabetic nephropathy in Wistar rats under laboratory conditions.

1.9 Aim

To investigate the effects of naringin on diabetic nephropathy rats *in vivo* with respect to:

Objectives

- developing an animal model of diabetic nephropathy.
- serum and urine electrolytes.
- glomerular filtration rate as an index of renal dysfunction.

CHAPTER 2

2.0 Materials and methods

2.1 Chemical reagents and Equipments

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Aldrich Pty. Ltd., Johannesburg, South Africa. Naringin, D-glucose, Streptozotocin, citrate, sodium sulphate, potassium hydroxide, potassium cyanide, butanol, ethanol, sulphuric acid, phenol, phosphate buffer, hydrochloric acid, phosphoric acid and glycogen were used in the study. Normal saline, insulin, glucometer and glucometer test strips (Ascensia Elite Bayer AG, Leverkusen, Germany) were purchased from a local pharmacy.

2.2 Ethical Clearance

The study was approved by the Animal Ethics Committee of the University of KwaZulu-Natal, reference number 106/13/animal.

2.3 Procedure and animal treatment

Male adult Wistar rats (*Rattus novergicus*, 200-300g body weight) were divided into 6 groups (n=7) and housed, 7 rats per cage, with free access to standard commercial chow and drinking tap water *ad libitum*. Female rats could not be used because they can be affected by hormonal changes during estrous cycle which might influence the outcome. The rats were maintained on a 12- hour dark to light cycle of 08.00 to 20.00 hours of light in an air controlled room (temperature 25 ± 2°C, humidity 55%) and were handled with human care according to the guidelines of the Animal Ethics Committee of the University of KwaZulu-Natal.

Diabetes was induced in groups C, D, E and F (Table 1) by a single intraperitoneal injection of 60 mg/kg body weight (BW) of streptozotocin (STZ) dissolved in 0.2 ml of 0.1 ml citrate buffer, pH 4.5 after an overnight fast. Three days after administration of the STZ, development of diabetes was confirmed by tail pricking to analyse blood glucose levels.

Random glucose levels of less than 11mM were considered not diabetic and thus were excluded from the study. Group B and D were orally treated with 50 mg/kg/ BW of naringin. Group C was treated with regular insulin (4 U/kg/ BW) subcutaneously twice daily. Groups A and E were treated with 1.0 ml distilled water via gastric gavage. Group F was treated with 20 mg/kg/ BW of ramapril. Blood glucose concentrations were measured once in two weeks by tail pricking and a portable glucometer was used to take the readings. Weight gain and water consumption were measured daily. Diabetic and non- diabetic animals were kept in metabolic cages individually and separately under feeding and metabolic control on day 55 for 24 hours for urine collection. On treatment day 56, halothane overdose was used to sacrifice the rats and blood samples were collected by cardiac puncture for plasma insulin analysis. Kidneys and livers were excised after the animals were sacrifised, rinsed in normal saline and snap-frozen in liquid nitrogen and were stored at -80°C for further analysis.

Table 1: Animal treatment schedule

Groups	Treatment				
	Distilled water (1ml)	**STZ (60mg/kg BW)**	**Naringin (50mg/kg BW)**	**Insulin (4U/kg BW)**	**Ramapril (20mg/kg BW)**
A	+	–	–	–	–
B	–	–	+	–	–
C	–	+	–	+	–
D	–	+	+	–	–
E	+	+	–	–	–
F	–	+	–	–	+

2.4 Methods

2.4.1 Blood Glucose Determination

Fasting blood glucose determinations were done on treatment days 0, 14, 21, 28, 56 respectively; whereas oral glucose tolerance determination (GTT) was done on day 52. Blood glucose concentrations were determined after tail pricking and a portable glucometer was used to take the readings. Before GTT, the animals in all treatment groups were starved overnight and fasting blood glucose (FBG) concentrations were determined by administration of intraperitoneal injection of 3.0 mg/kg body weight of glucose in normal saline. Blood glucose concentrations were then measured at times 0, 15, 30, 60, 90 and 120 minutes in all treatment groups. Area under the curve (AUC) was calculated from blood glucose-time curves and presented as AUC units (millimoles per litre × minutes).

2.4.2 Plasma Insulin quantification

Plasma insulin levels were analysed by ultra-sensitive rat insulin enzyme-linked immunoassay kit (DRG Diagnostic Marburg Germany). Twenty five microliters (25 µl) of samples and 100 µl of enzyme conjugate were mixed in a 96-well microtiter plate, then incubated for 2 hours on a plate shaker at 900 rpm at room temperature (25ºC±2º). Seven hundred microliters (700 µl) of wash buffer was used to wash the plate 6 times and 200 µl of the substrate TMB was then added to each well and incubated at room temperature (25ºC±2º) for 15 minutes. Stop solution (50µl) was added to each well and incubated for 5 minutes on a plate shaker then the optical density was read at 450 nm using a Spectrostar micro- plate reader.

2.4.3 Liver Glycogen assay according protocol.

Hepatic glycogen content was measured by the method of Ong and Khoo (2000). The liver tissue (0.5 g) was placed in a test tube. One millilitre (1.0 ml) of 30% KOHw/ Na_2SO_4 was added in the tubes to enable homogenization and boiled at 100ºC for 20 minutes. The tubes with foil (to avoid evaporation) were boiled for 30 minutes and then vortexed and allowed to cool in ice and 95% ethanol (2.0 ml) was added to the mixture to precipitate glycogen from alkaline digestate. The mixture was then incubated in ice for 30 minutes. The tubes were then centrifuged at 550 g for 30 minutes. The supernatant was carefully collected and the tubes were dried upside down for 5 minutes. Pellet was re-suspended in 1ml of distilled water and thoroughly vortexed and 1.0 ml of 5% phenol was then added to the mixture with the preparation being done in duplicates. Five millilitres (5.0 ml) of 98% sulphuric acid was then directly pippeted into the tubes content and the samples incubated on ice bath for 30 min. Three hundred millilitres (300 µl) from the tubes were pippeted to the microtiter plates and the optical density was read at 490 nm within 30 minutes using Spectrostar micro- plate reader. One millilitre (1.0 ml) of water was used as a blank.

2.4.4 Thiobarbituric acid-reactive substances (TARBS) Assay in renal tissue Phulukdaree Chuturgoon AA (2010) protocol.

Plasma TBARS assay was carried out as previously described by Phulukdaree Chuturgoon (2010). Renal cortex (100 mg) was homogenized in 450 µl of 0.2% phosphoric acid (H_3PO_4) and centrifuged at 1000 x g for 10 minutes at 4ºC. The supernatant was then collected; 200 µl of 7% phosphoric acid (H_3PO_4) and 400 µ of BHT/TBA solution was added as well as 100 µl of 1M HCL. The solution was incubated in boiling water bath (100ºC) for 15 minutes and later on cooled at room temperature (25±2). Butanol (1.5ml) was then added to all the tubes and they were thoroughly vortexed before 200 µl of the top phase of the solution was then

transferred to a 96 well microplate in triplicates. The absorbance was read at 532 nm and 600 nm using a Spectrostar micro-plate reader. The kidney MDA was calculated using the formula below.

$$\text{Concentration} = \frac{\text{A532} - \text{A600}}{156\text{Mm}^{-1}}$$

2.4.5 Superoxide Dismutase Assay

SOD activity of the renal tissue (mitochondrial) was measured using commercially available kit. The tissue was homogenized at 10,000 x g in 5 ml cold buffer for 15 minutes at 4ºC and the supernatant (upper layer) was discarded; the pellet was used. Potassium cyanide (1 mM) was added to inhibit other enzymes (Cu/Zn-SOD) and extracellular SOD. Radical detector (200 µl) was pipetted to each well of a microtiter plate and 10 µl of sample was then added. To initiate the reaction, 20 µl of diluted xanthine oxidase was added as quickly as possible to all wells. 96 -well plate was shaken carefully and incubated on the shaker for 20 minutes at room temperature (25±2º). The activity of SOD was calculated as the amount of SOD that produces a 50% dismutation of superoxide ion by reading absorbance at 450 nm by Spectrostar, micro-plate reader.

2.4.6 Urine and serum electrolytes analysis

The samples were removed from the freezer and thawed at room temperature for analysis of electrolytes. Briefly, 40 ml of samples were mixed with a buffered solution. The ratio used was one part sample to 33 parts buffer (1:33). The higher molar strength buffer was added. Electrolytes sodium (Na^+), potassium (K^+) and chloride (Cl^-) constant coefficients were analysed using the Beckman Coulter Synchron LX20 clinical Systems, Fullerton, California, USA).

2.4.7 Glomerular filtration rate (GFR)

The urinary clearance of creatinine and plasma creatinine were used to calculate the glomerular filtration rate using the formula below:

$$\text{Creatinine clearance (ml/min)} \frac{\text{urinary creatinine}}{\text{Plasma creatinine}} \text{mmol/L x urine volume (ml)}$$

2.4.8 Statistical analysis

All data were presented as means ± standard error of mean (SEM). Statistical comparisons in different groups were performed by using One Way Analysis of Variances (ANOVA) and student t test. All statistical analysis were performed with Graph-pad InStat Software (version 5, GraphPad Software, San Diego, California USA). A value of $p<0.05$ was considered statistically significant.

Chapter 3

3.0 Results

3.1 Weight gain

Untreated diabetic group exhibited significant ($p<0.001$) weight loss compared to the normal control groups (Figure 5) however, diabetic rat groups that were treated with naringin and insulin significantly improved weight gain ($p<0.001$) compared to the diabetic control group. Naringin did not affect normal growth in non-diabetic rats (Figure 5).

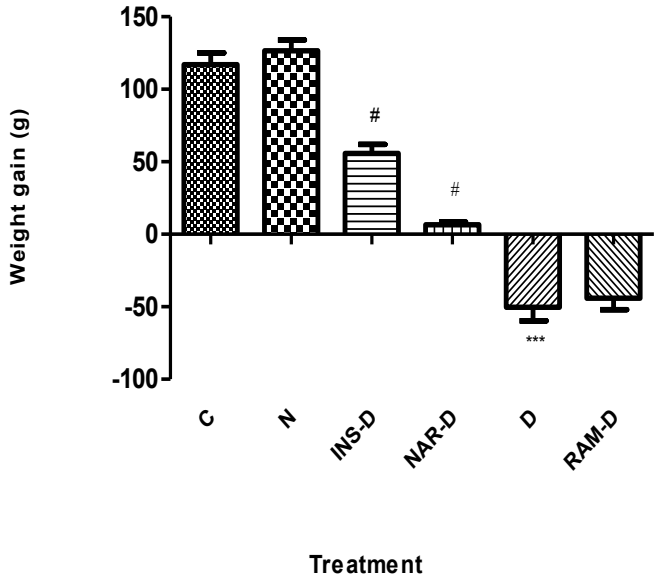


Figure 5: The animal weights were recorded at baseline and at the end of the treatment period and the differences were recorded as means per treatment group as an indication of growth. C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$compared to diabetic control (n=7).

3.2 Water consumption

The average daily water consumption was consistently and significantly ($p< 0.001$) higher in all diabetic rats compared to normal control groups (Figure 6). However, polydipsia was significantly ($p<0.05$) less pronounced in the naringin, ramapril and insulin treated groups compared to the non-treated diabetic rats, respectively (Figure 6).

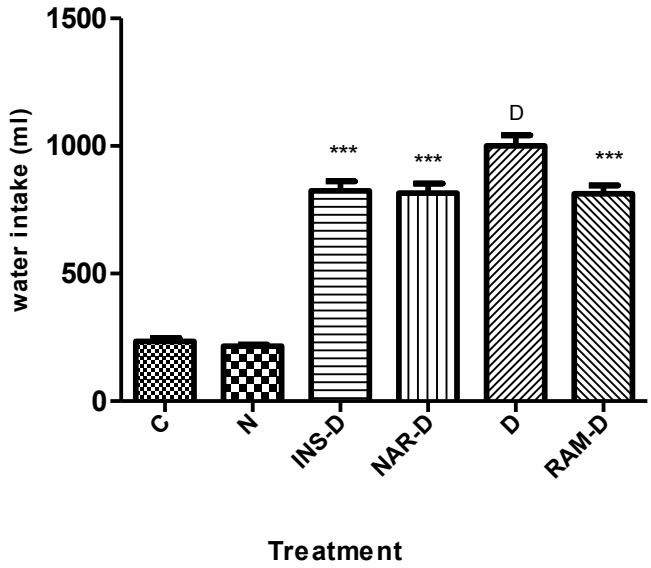


Figure 6: Water consumption in ml was recorded by measuring the volume of water in the drinking bottle each morning before a fresh one was added. (C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{D}p<0.001$ compared to normal control and $^{***}p<0.001$compared to diabetic control (n=7).

3.3 Urinary output

Urinary output was significantly higher ($p<0.001$) in diabetic rats compared to normal control groups but the treatment with insulin and ramapril significantly ($p<0.05$) reduced urine output compared to the non-treated diabetic rats. Naringin did not significantly reduce the urinary output compared to diabetic control (Figure 7).

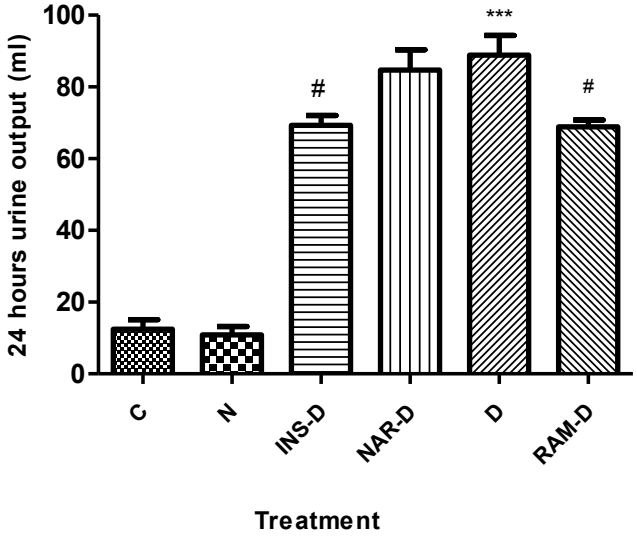


Figure 7: Urinary output C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$compared to diabetic control (n=7).

3.4 Fasting blood glucose

Diabetic rats had elevated fasting blood glucose ($p<0.001$) (from day 14-56) compared to the normal controls however, the treatment with naringin, insulin and ramipril significantly decreased FBG (Figure 8).

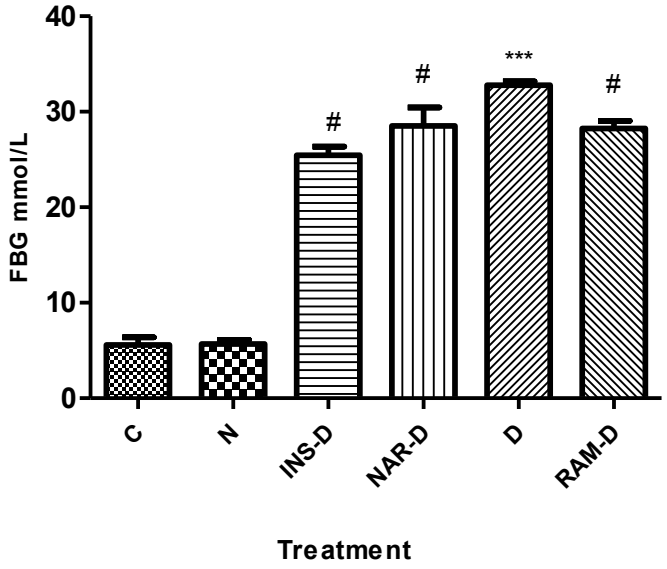
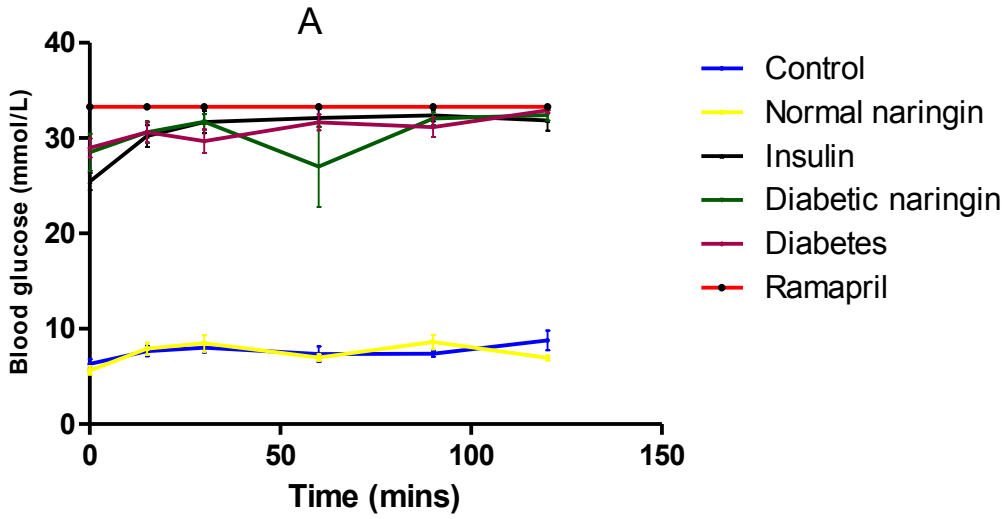


Figure 8: Fasting blood glucose C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.05$ compared to diabetic control (n=7).

3.5 GTT and Area under the curve

The effect of naringin was evaluated by GTT on overnight fasted animals. Diabetic groups showed glucose intolerance compared to normal controls ($p<0.001$). Naringin or insulin treatment did not improve glucose intolerance compared to diabetic control (Figure 9A) and area under the curve was calculated from OGTT (Figure 9B).



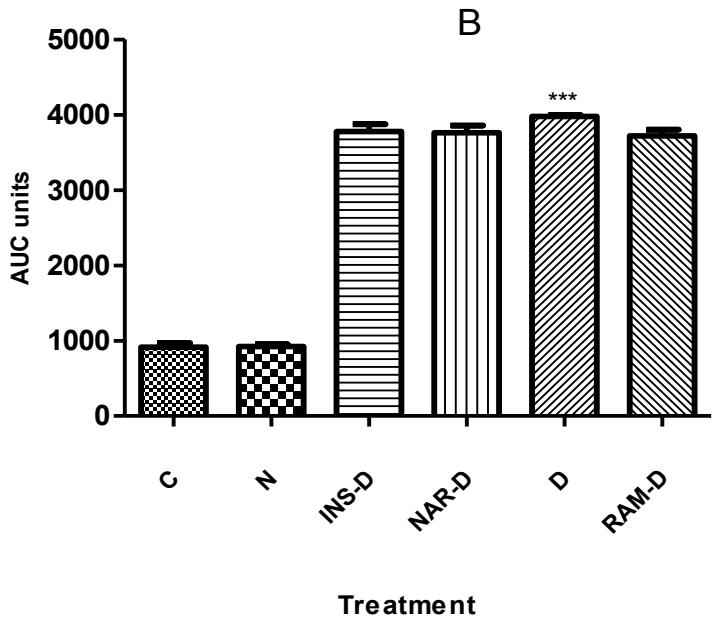


Figure 9: OGTT and Area under the curve. Ramipril treated group had blood glucose which was above what the glucometer could measure (>33.3mmol/L). C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control (n=7).

3.6 Fasting plasma insulin

The fasting plasma insulin was significantly lower ($p<0.001$) in diabetic groups compared to normal controls. Treatment with naringin or insulin improved plasma insulin secretion in both diabetic and normal rats. Ramapril did not significantly improve plasma insulin secretion compared to diabetic control (Figure 10).

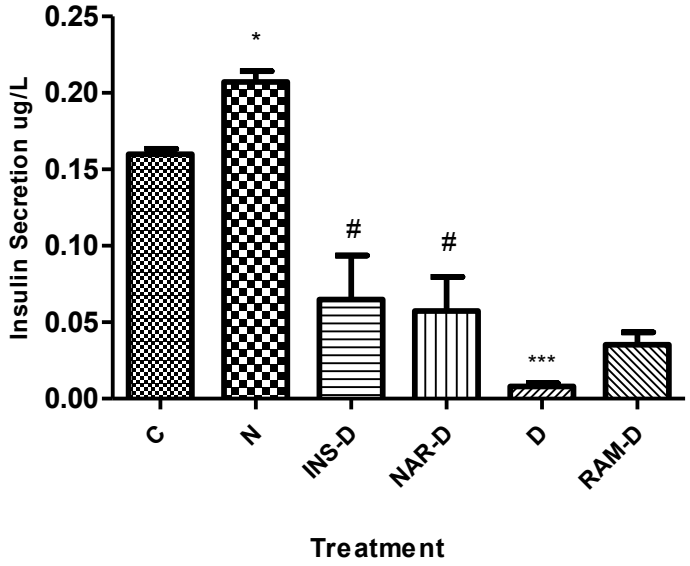


Figure 10: Fasting plasma insulin C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ and $^{*}p<0.05$ compared to normal control, and $^{\#}p<0.001$compared to diabetic control (n=7).

3.7 Hepatic glycogen

The diabetic groups had a significantly ($p<0.001$) reduced hepatic glycogen content compared to the non-treated diabetic control group. However, treatment with insulin or naringin significantly ($p<0.01$ and $p<0.05$) increased hepatic glycogen content as compared to the non-treated diabetic control respectively. Ramipril did not significantly increase glycogen content (Figure 11).

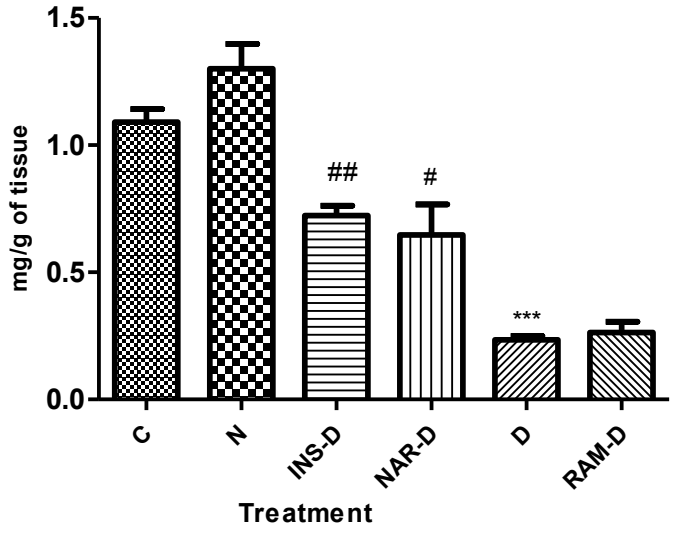


Figure 11: Hepatic glycogen C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$compared to diabetic control (n=7).

3.8 Lipid peroxidation

MDA concentrations in the kidney were elevated in diabetic groups when compared with normal controls but were significantly reduced ($p<0.05$ and $p<0.01$) when treated with naringin or insulin respectively. Ramipril did not significantly reduce MDA levels (Figure 12).

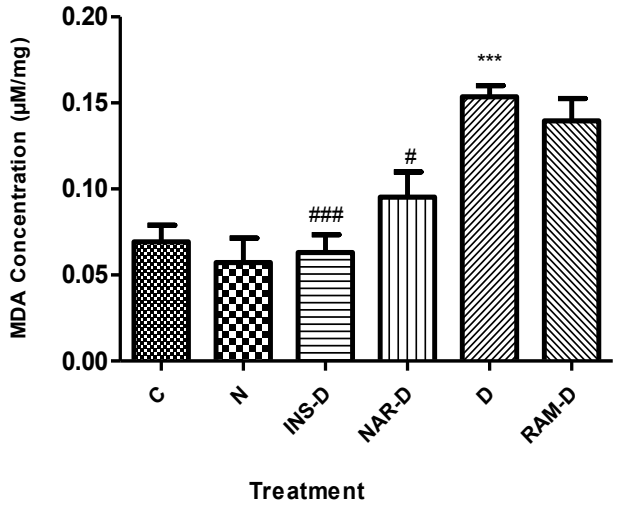


Figure 12: Lipid peroxidation C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$ compared to diabetic control (n=7).

3.9 Superoxide Dismutase

Diabetic rats had significantly ($p<0.001$) reduced activities of SOD compared to normal controls however, chronic treatment with naringin, insulin or ramipril significantly ($p<0.01$, $p<0.001$ and $p<0.0054$) increased SOD activities compared to diabetic control respectively (Figure 13).

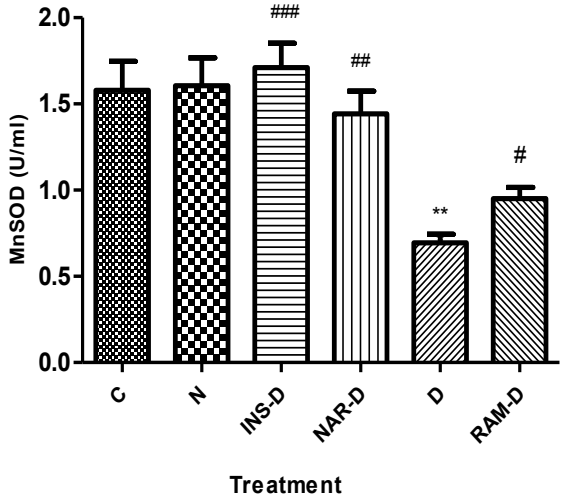
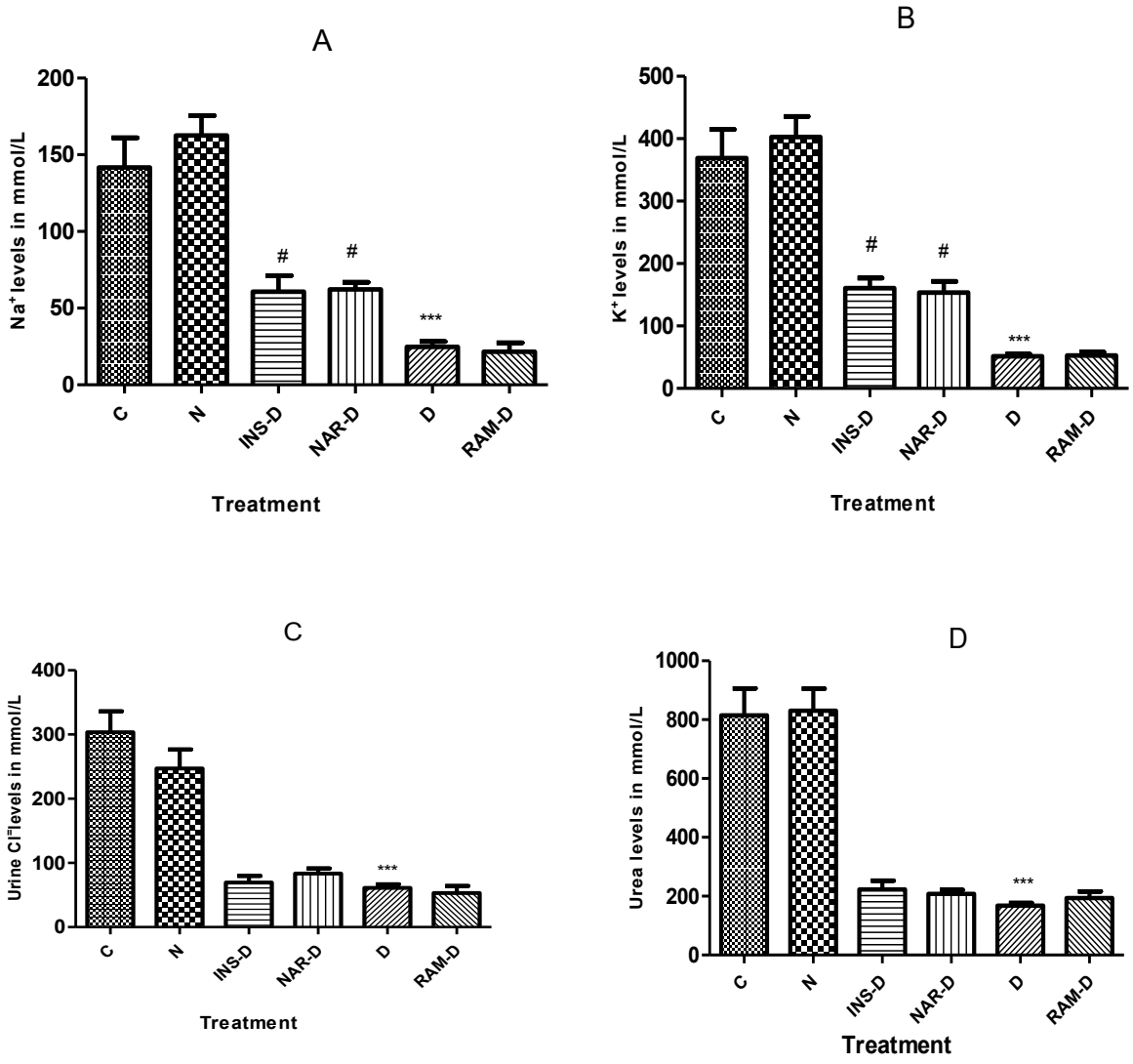


Figure 13: Lipid peroxidation C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril). $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$compared to diabetic control (n=7).

3. 10 Urine biomarkers

Electrolytes (Na^+, K^+ and Cl^-), urea and creatinine levels were significantly ($p<0.001$) decreased in the urine of diabetic groups compared to normal controls however, naringin or insulin significantly ($p<0.05$) reversed sodium and potassium retention compared to diabetic control (Figures 14A and B). Naringin showed no effect on urinary chloride and urea (Figures 14 C & D). Treatment with naringin, insulin or ramipril significantly ($p<0.001$) increased urinary creatinine (Figure 14 E).



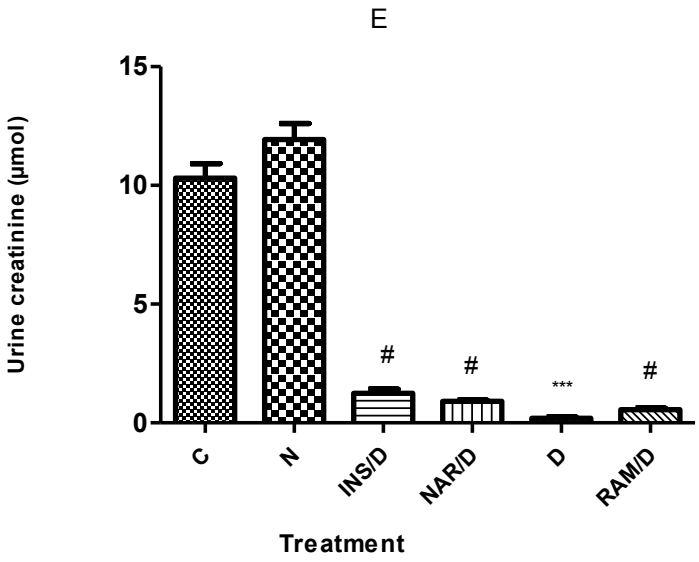


Figure 14: Urine electrolytes A (sodium), B (potassium), C (chloride), D (urea) and E (creatinine) C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril) $^{***}p<0.001$ compared to normal control and $^{\#}p<0.001$compared to diabetic control (n=7).

3.11 Serum electrolytes

Serum electrolytes were similar between diabetic rats and normal controls however, serum creatinine of diabetic group was significantly higher ($p<0.04$) compared to normal control (Figure 15 A, B, C, D) and Figure 15.

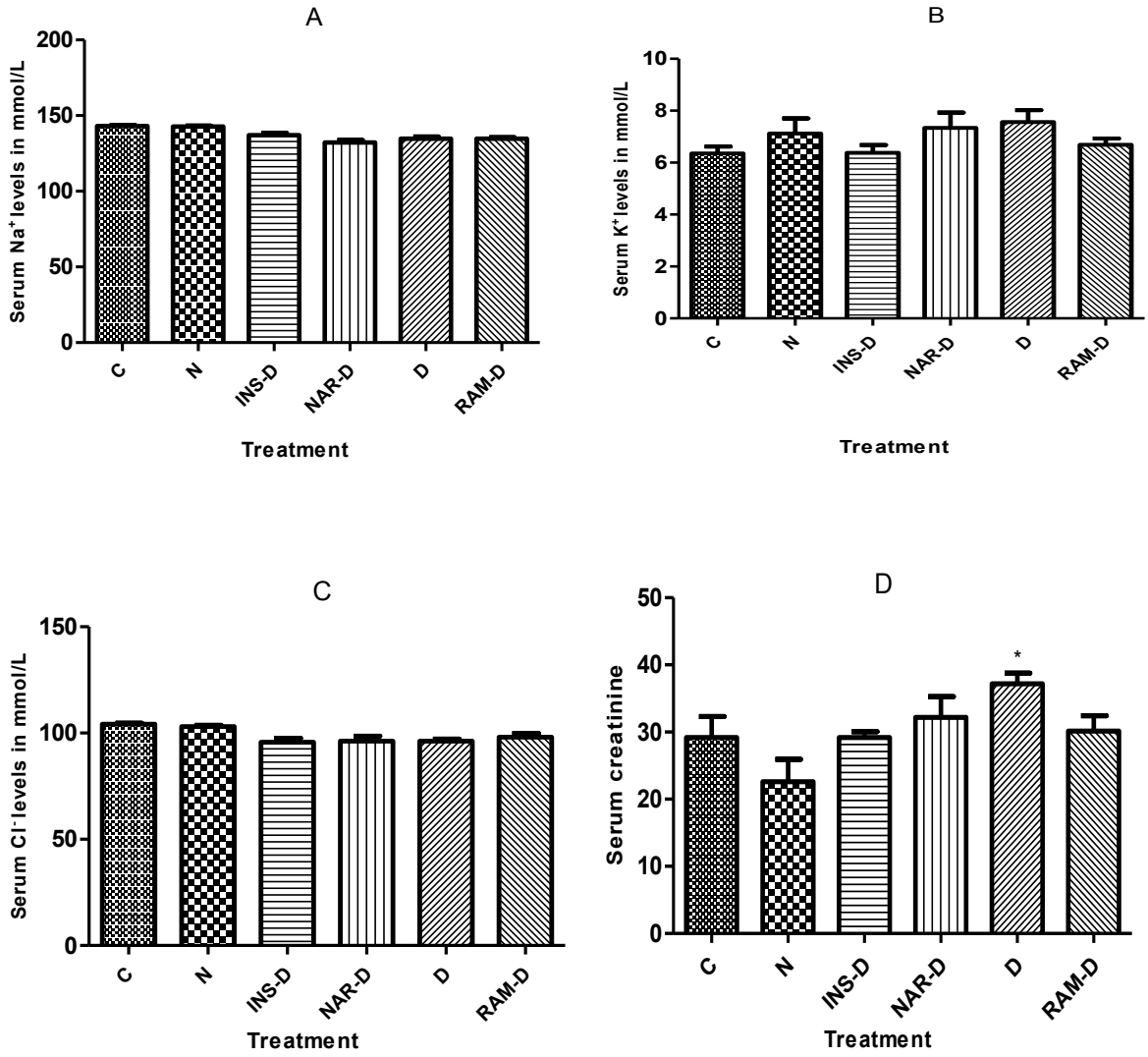


Figure 15: Urine electrolytes A (sodium), B (potassium), C (chloride) and D (creatinine) (n=7).

3.12 Glomerular Filtration Rate (GFR)

GFR was calculated using the formula in 2.4.7. In STZ-induced groups GFR was significantly ($p<0.001$) decreased compared to normal control groups however chronic treatment with naringin, insulin and ramipril significantly ($p<0.05$) increased glomerular filtration rate (Table 5).

Table 2: Showing expressed mean ± of treatment(s) against Creatinine and urinary output in Wistar rats. $p<0.05 compared to normal controls (n=7).

Treatment	**Serum Creatinine (µmol)**	**Urine Creatinine (µmol)**	**Urinary output (ml)**
Normal control	30.17±5.565	10.81±2.403	12,43±7.208
Normal naringin	29.20±6.979	13.27±1.260	10,86±6.309
STZ-induced and Insulin	32.25±5.123	1.504±0.47$	69.29±7.365
STZ- induced and naringin	32.20±6.834	0.9773±0.130$	84.71±7.14.95 $
STZ-induced	37.60±7.436	0.262±0.275	88.88±15.63 $
STZ-induced and ramapril	33.67±10.86	0.666±0.33$	68.86±5.14

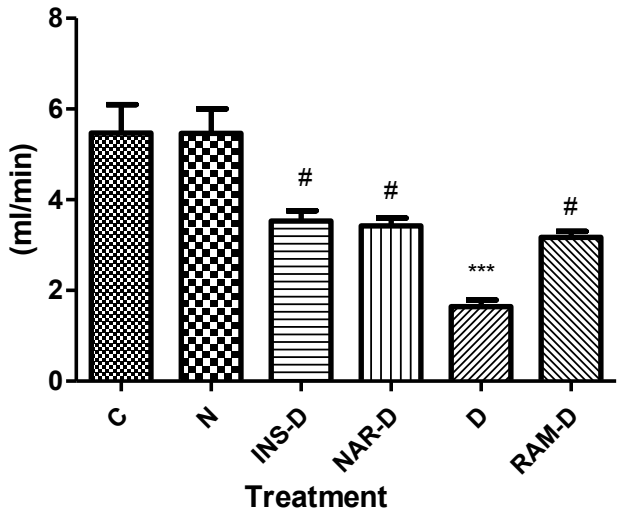


Figure 16: GFR C (normal control), N (normal naringin), INS-D (diabetic mellitus and insulin), NAR-D (diabetic naringin), D (diabetic mellitus), RAM-D (diabetic ramapril) $^{***}p<0.001$ compared to normal control and $^{\#}p<0.05$ compared to diabetic control (n=7).

Chapter 4

4.0 Discussion

Type1 diabetes is characterized by low insulin due to predisposition to cell autoimmunity and it is associated with high blood glucose, low insulin, weight loss, polydipsia and polyuria. In order to induce diabetes in this study; beta cells were destroyed by using (STZ). Low doses (40 mg/kg) of STZ destroy the pancreatic beta cells partially but the rats become diabetic permanently (Aybar *et al*., 2001). Using 60 mg/kg of STZ destroy beta cells completely leading to clinical diabetes within 2-4 days (Weiss *et al*., 1982). Streptozotocin selectively destroys pancreatic beta cells by inducing toxicitity by DNA alkylating activity and induction of free radicals production (Nukatsuka *et al*., 1990 and Yamamoto *et al.,* 1981). In the present study, Type1 diabetes mellitus model was successfully created after 3 days of diabetes induction; elevated blood glucose, polydipsia, polyuria and weight loss that indicated deficiency of insulin biosynthesis and secretion in Wister rats.

In type 1 diabetes low insulin or infectiveness leads to drop in body weight since it is an anabolic hormone; Low insulin leads to elevation of counter regulatory hormones; glucagon, cortisol, growth hormone and catetholimines. These hormones are catabolic and contribute to weight loss. Elevation of these hormones results in lipolysis and production of ketone bodies (Gerich *et al*., 1996). These hormones activate hormone sensitive lipase in the adipose tissue which in turn causes breakdown of triglyceride into glycerol and free fatty acids. In this study, weight loss was pronounced in diabetic rats compared to normal groups ($p<0.001$) (Figure 5). However, naringin improved weight loss ($p<0.05$) which means naringin through its known antioxidant and free radical scavenging effects could have inhibited the release of counter regulatory hormones as reported by Kitabchi *et al*., (2001) that reactive oxygen species are involved in the provocation of counter regulatory hormones. Weight loss in

untreated type 1 diabetes is also due to the inability of cells to utilize glucose for energy production, but rather there is a remarkable activation of the gluconeogenic pathway. The excessive utilization of muscle protein and excessive mobilization of fats from the adipose tissues for energy production in the gluconeogenic pathway accounts for the weight loss in type 1 diabetes. Also frequent episodes of urinating (polyuria) due to hyperglycemia result in dehydration are possible cause of weight loss.

In this study, water consumption was significantly higher in diabetic rats compared to the normal controls (Table 3). Hyperglycemia changes the blood osmotic potential and the receptor in the hypothalamus reacts to the shift in osmotic pressure and triggers dehydration and electrolytes disturbances. Interestingly, diabetic groups treated with naringin significantly reduced polydipsia ($p<0.001$) compared to diabetic control. Similarly ramipril reduced polydipsia (Figure 6) and urine output (Figure 7) which was expected because ramipril is known to have reno-protective effects; however, naringin did not reduce urine output which could mean naringin does not have antidiuretic effects. In DM, the renal threshold (11mM) for glucose is exceeded (i.e. plasma glucose concentration is above the normal range) and glucose is excreted in the urine. Glucose then acts as osmotic solute in the urine, producing an osmotic diuresis (polyuria).

4.1 Effects of naringin on glucose homeostasis

Individuals with DM have hyperglycemia due to lack of insulin hence glucose accumulates in bloodstream due to decreased uptake by skeletal muscles. In this study fasting blood glucose was significantly elevated in diabetic groups compared to normal controls suggesting hyperglycemia and insulin deficiency (Figure 8). However, naringin reduced fasting blood glucose and did not improve glucose intolerance suggesting that naringin might require

insulin to exhibit its hypoglycemic effects just like metformin (Pari, 2010). Metformin is a biguanide that activates adenosine monophosphate- activated protein kinase (AMPK). AMPK is a multisubunit enzyme that is recognized as a major regulator of lipid biosynthetic pathways due to its role in the phosphorylation and inactivation of key enzymes such as acetyl-CoA carboxylase and it also regulates expression of cAMP-stimulated gluconeogenic genes such as PEPCK and G6Pase (Lochhead *et al*., 2000). In the previous study in our lab, it was reported that naringin does not ameliorate hyperglycemia in type 1 diabetes (Xulu, Owira, 2012). Conversely, naringin improved insulin secretion in normal rats and diabetic rats compared to normal control. This therefore means that naringin functions through its free radical scavenging activities to boost mitochondrial antioxidant capacity leading to the increase of ATP production ultimately insulin secretion. Previous studies (Pari and Suman, 2010, Jung *et al*., 2004 and Heart *et al*., 2012) reported that naringin improves plasma insulin secretion which is consistent with our findings (Figure 10). In diabetic state there is an increase in hepatic glucose production and decreased hepatic glycogen synthesis which might be due to decreased activity of glucokinase ultimately glycolysis (Felig and McGarry *et al*., 1992). Glucokinase is an enzyme that acts as a glucose sensor, facilitates phosphorylation of glucose to glucose-6-phosphatase. In the current study hepatic glycogen was significantly decreased in diabetic groups ($p<0.001$) (Figure 11). However, treatment with insulin and naringin significantly ($p<0.01$ and $p<0.05$) increased hepatic glycogen respectively. This is supported by Punithavathi *et al*., (2008), who reported that naringin increased hepatic glycogen storage by regulating gluconeogenesis by lowering the activities of glucose-6-phosphatase and PEPCK. The increase in hepatic glycogen storage may improve the utilization of blood glucose ultimately decrease chances of diabetes.

4.2 Effects of naringin on oxidative stress

Sustained hyperglycemia is associated with alterations in oxidation state due to the increase in production of reactive oxygen species (ROS) and the reduction of antioxidant concentrations. Wang *et al*, (2011) reported that oxidative stress contributes to the relationship between acute hyperglycemia and its complications including nephropathy (Lehmann and Schleicher, 2000). Oxidative stress induces the expression of tubular growth factors, tumor necrosis factors, interleukins (1, 6 and 18) and vascular endothelial growth factors which give rise to extracellular matrix proteins and promote glomerular and tubular fibrogenesis. Vascular endothelial growth factors are primarily involved in neoangiogenesis and increased endothelial permeability due to apoptosis of podocytes. Apoptosis of the cells might contribute to the structural changes that occur in the diabetic kidney which lead to the formation of glomerular basement membrane becoming leaky leading to proteinuria ultimately total kidney failure (Wolf and Ziyadeh 1999).

Thiobarbituric acid reactive substance (TBARS) is a biomarker for oxidative stress; it measures lipid peroxidation which is induced by increased free radicals. Lipid peroxidation is higher in patients with diabetic nephropathy (Griesmacher *et al*., 1995; Kedziora-Kornatowska *et al*., 1998). Lipid peroxides can stimulate prostaglandin production, which is a well-recognized source of free radicals. Prostaglandins also alter renal hemodynamics in various stages of diabetic nephropathy (Salahudeen *et al.,* 1997). In this study, diabetic rats exhibited higher lipid peroxidation ($p<0.001$) compared to non-diabetic controls, suggesting increased oxidative stress (Figure 12). Interestingly, naringin significantly reduced ($p<0.05$) renal lipid peroxidation in diabetic rats compared to normal controls. Naringin has been previously reported by Sharma *et al*., (2011) to have antioxidant activities and free radicals scavenging properties which may decrease the cell viability through suppressing the

excessive production of ROS in the kidney. Moreover, natural antioxidants (enzymatic and non-enzymatic) are expected to prevent damage by reducing reactive oxygen species. MnSOD was significantly reduced in diabetic groups suggesting the increase in oxidative stress (Figure 13). This is supported by other studies which reported that in diabetic state both types of antioxidants are supressed by AGEs (Arali *et al*., 1997). However chronic naringin treatment significantly increased the activity of mitochondrial superoxide dismutase (Figure 13). These results then indicate that naringin acts as a superoxide scavenger and an antioxidant just as it has been previously reported (Sharma *et al*., 2011). Ramipril and insulin showed similar effects as naringin ($p<0.05$ and $p<0.001$) respectively. ACE inhibitors are currently used to treat diabetic nephropathy; they reduce the circulating angiotensin II. Ang II is involved in reactive oxygen species production by activating NADPH oxidase, an enzyme known to be overactive in diabetes, hence contributing to the increased oxidative stress in renal tissue.

4.3 Effects of naringin on renal function

Sustained hyperglycemia leads to renal dysfunction such as proteinuria, imbalance in electrolytes and fluids, reduction in GFR and elevated blood pressure (Magri and Flava, 2009) which lead to renal failure. Hannedouche et al (1990) have previously reported that there is electrolyte retention (Na^+, K^+ and Cl^-) in insulin-dependent diabetes mellitus in experimental animals. Retention of Na^+ in proximal tubules is associated with decreased GFR via sodium/glucose transporter (Vallon *et al*., 1999). Na^+ and glucose are transported by SLGT2 in the proximal tubules in concert with facilitative glucose transporters (GLUTs) (Chao and Henry, 2010). SGLT2 has been detected in high levels in the renal cortex of a diabetic rat and is described as the major Na+/glucose cotransporter in the kidney (Rosenstock *et al*., 2010). Glucose efflux from the tubules relies on the gradient created by

Na^+- dependent influx due to outwardly directed glucose gradient. About 90% of plasma glucose is filtered in the glomerulus of the kidney and is reabsorbed by SGLTs (symporters) in the proximal tubules of the kidney. In this study, retention of electrolytes was increased in diabetic rats, this was confirmed by the low levels of electrolytes in urine (Figure 14 A, B and C) suggesting hyperkalaemia and hypernatremia in diabetic rats (Hannedouche *et al*., 1990). It is noteworthy that naringin led to the increase in levels of electrolytes in urine (Figure 14). Naringenin (naringin aglycones) has been reported to have inhibitory effects on renal and intestinal SLGTs (Jian *et al*., 2005).

Na^+ handling is very important in understating renal function as it is a major electrolyte that controls osmolality in blood and, therefore extracellular fluid (ECF) volume. Na^+ retention is associated with low GFR (Vallon *et al*., 1999). Rebsomen et al (2006) reported that glomerular filtration rate (GFR) and creatinine clearance (CrCl) are good markers for renal function, they measure how well the kidneys filter the DN-induced build-up of blood creatinine. In this study glomerular filtration rate was significantly reduced ($p<0.001$) in STZ-induced diabetic rats compared to normal controls, interestingly treatment with naringin significantly increased GFR compared to diabetic control (Figure 16). Ramipril showed similar effects as naringin as expected. Renal impairment is suspected to be due to the hyperfiltration rate during early stages of the disease. However, as the disease progresses, renal function deteriorates and GFR declines drastically (Rudberg *et al*., 1997; Mogensen, 1997). Hyperglycemia causes osmotic diuresis that leads to hypovolemia and decreased glomerular filtration rate. Moreover, chronic exposure to hyperglycemia is associated with decrease in urine urea and creatinine. Urea and creatinine are waste products which are normally excreted by the kidneys in urine; however, in diabetic state there is low clearance hence they accumulate in blood and hence their low concentration in urine (Lau *et al*., 2007).

Low urine creatinine and urea levels were observed in this study in diabetic rats and high levels in serum (Figure 14 E and 15 D). Naringin however, significantly improved creatinine excretion compared to diabetic control.

Chapter 5

5.1 Conclusion

These results demonstrate that in the diabetic nephropathy model created diabetic state is characterised by hyperglycemia, polyuria, polydipsia, weight loss, low insulin, reduced glomerular filtration rate and electrolytes disturbances. However, naringin improved renal function in diabetic rats through increased GFR and urinary electrolytes output despite not improving glucose intolerance. This suggests that naringin exhibits reno-protective effects independently of glucose hemeostatis in diabetic rats by reversing oxidative stress associated with DN. These results therefore suggest that naringin supplementation could have positive implications to diabetic end-points such as DN.

5.2 Limitations of the study

Histology and glucose concentrations in urine were not measured and renal regulating hormones like aldosterone were not measured to relate the effects of naringin on renal tissue with biological functioning of hormones.

5.3 Recommendations

Future studies should investigate

- The effect of naringin on Na^+/glucose transporters (STLGs) expression in the kidney and urine glucose.
- Hormones (aldosterone and vasopressin) should be measured.
- Structural changes associated with DN should be evaluated in the in the presence of naringin supplemental in diabetic state
- Clinical investigation in diabetic patients could shed more light on the finding of this study

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Appendix I

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research Office
Animal Ethics Research Committee
Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email:

4 September 2013

Reference: 106/13/Animal

Dr P Owira
Discipline of Pharmaceutical
Sciences
School of Health Sciences
WESTVILLE Campus

Dear Dr Owira

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for **2012/2013** on the following two-part project:

"1. Potential modulation of the expression and activity of organic cation transporter protein (OCT1), in the rat liver by grapefruit juice and/or naringin in vivo.

2. Effects of grapefruit juice and naringin on serum lipid profile, glucose intolerance and cardiovascular function in diabetic rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Head of School – Prof. S Essack
BRU – Dr S Singh

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Appendix II

Poster no:**1266**

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

NARINGIN ATTENUATES OXIDATIVE STRESS IN TYPE 1 DIABETES MELLITUS

ADEBIYI OA, ADEBIYI OO, NKOMO FS, MURUNGA AN, COBONGELA SZZ, OWIRA PMO

MOLECULAR AND CLINICAL PHARMACOLOGY LABORATORY, DEPARTMENT OF PHARMACOLOGY, DISCIPLINE OF PHARMACEUTICAL SCIENCES, SCHOOL OF HEALTH SCIENCES, UNIVERSITY OF KWAZULU-NATAL P.O.BOX X5401, DURBAN, SOUTH AFRICA.