ANTI-DIABETIC AND ANTI-DYSLIPIDEMIC EFFECTS OF NARINGIN

BY

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213570376

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

MASTER OF SCIENCE IN CLINICAL 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: 2015

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As the candidate's supervisor, I have approved this thesis/dissertation for submission.

Signed: Name: Date:

PREFACE

The experimental work described in this dissertation was carried out in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban from February 2013 to March 2015 under the supervision of Dr. Owira P.M.O.

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

Cobongela Sinazo Zezethu Zongeziwe

Dr. Owira P.M.O (Supervisor)

DECLARATIONS

DECLARATION 1 – PLAGIARISM

I, **Cobongela Sinazo Zezethu Zongeziwe** 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 words 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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- The staff at Breeding Research Unit (BRU) for all their support.
- The College of Health Sciences for the financial support granted during my study.

Above all, thanks to God Almighty

LIST OF ABBREVIATIONS

ACAT	Acyl-CoA: cholesterol acyltransferase
AGEs	Advanced glycation end products
AI	Atherogenic index
ALT	Alanine aminotransferase
Apo	Apolipoprotein
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
CAD	Coronary artery disease
CETP	Cholesterol ester transfer protein
CM	Chylomicrons
CRI	Coronary risk index
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
FBG	Fasting blood glucose
FFA	Free fatty acids
GLUT	Glucose transporter
GTT	Glucose tolerance test
HDL	High density lipoprotein
HMGCoA-R	3-hydroxy-3-methyl-glutaryl-CoA reductase
HMIT1	H+/myoinositiol co-transporter 1

HSL	Hormone sensitive lipase
IDF	International Diabetes Federation
IDL	Intermediate density lipoproteins
LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
LPL	Lipoprotein lipase
OGTT	Oral Glucose Tolerance Test
PPAR	peroxisome proliferator-activated receptor
sdLDL	Small dense lipoprotein lipase
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
T1D	Type 1 diabetes
TBS-T	Tris-buffered saline Tween-20
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low density lipoprotein

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

ABSTRACT

The incidence of diabetes is expected to dramatically increase over the next decade. Dyslipidemia is the greatest risk factor of coronary heart diseases in patients with diabetes. Anti-diabetic and anti-dyslipidemic effects of naringin were investigated in type 1 diabetes.

Male Sprague-Dawley rats (n = 7) were treated daily with 3.0 ml/kg body weight (BW) of water (group 1), naringin (50 mg/kg BW) (groups 2, 4 and 7, respectively), regular insulin (4 U/kg BW, subcutaneously, twice daily) (group 3 and 7), and simvastatin (20 mg/kg BW) in group 6. On treatment day 45, halothane overdose was used to sacrifice the animals and blood samples were collected via cardiac puncture for plasma insulin and lipid profile analysis. Rat livers were excised, rinsed in normal saline and stored at -80^{o}C for glycogen content analysis.

Group 3, 4, 5, 6 and 7 exhibited weight loss, polydipsia and hyperglycemia after injection with 60 mg/kg body weight of streptozotocin. Naringin with or without insulin significantly prevented weight loss in diabetic animals compared to non-treated diabetic animals. Insulin with/without naringin, but not naringin, significantly lowered fasting blood glucose levels in diabetic rats. Naringin with/without insulin significantly improved hepatic glycogen content compared to non-treated diabetic rats. Naringin with/without insulin significantly increased the plasma insulin levels in diabetic animals compared to non-treated diabetic animals. Plasma total cholesterol, triglycerides, very low density lipoprotein, low density lipoprotein cholesterol concentrations were significantly higher in non-treated diabetic rats compared to non-diabetic controls. High density lipoprotein cholesterol was significantly higher in non-treated diabetic rats compared to non-diabetic control. Naringin with/without insulin improved lipid profile in diabetic animals,

whereas simvastatin decreased only total cholesterol and triglycerides compared to non-treated diabetic animals. Naringin with/without insulin significantly decreased coronary risk index in diabetic animals compared to non-treated diabetic animals. Atherogenic index was significantly decreased by insulin or naringin with/without insulin in diabetic rats compared to non-treated rats. Naringin is not hypoglycemic but improves coronary risk index and atherogenic index in type 1 diabetes. However, naringin with insulin showed synergistic effects.

This study was conducted to investigate the effect of naringin on blood glucose regulation dyslipidemia in type 1 diabetes. The results showed that naringin is not hypoglycemic, however, it improved fasting plasma insulin and hepatic glycogen. Naringin also showed anti-dyslipidemic effects by decreasing the antherogenic lipids and increasing the high density lipoprotein cholesterol. The findings suggests that naringin can be used as a dietary supplement to ameliorate diabetic dyslipidemia.

CHAPTER 1

1.0. Literature review

1.1. Introduction to Diabetes

1.1.1. Epidemiology

The International Diabetes Federation (IDF) estimates that diabetes affects close to 300 million people worldwide and 552 million people are predicted to have diabetes by 2030, of which 90-95% will be type 2 diabetes. In Africa, 12.1 million people were estimated to be living with diabetes in 2010, and this is expected to increase to 23.9 million by 2030 (Sicree *et al*., 2009). Globally, Africa has a prevalence of 5.1% and 62.5% of undiagnosed people (IDF, 2014). In South Africa, 11.5 million people were diagnosed with diabetes (Bradshaw *et al.*, 2007). Type 1 diabetes (T1D) accounts for 5-10% of the total cases of diabetes worldwide (Diagnosis and classification of diabetes mellitus).

1.1.2. Risk factors and symptoms

Risk factors for diabetes are family history of diabetes (genotype), age, sex, ethnicity and others. Common complications associated with diabetes are cardiovascular diseases such as stroke and heart failure, blindness, kidney failure and amputation of lower limbs (Danaei *et al.*, 2006, Nakagami, 2004). Symptoms such excessive polydipsia, dehydration, increased urination, tiredness, weight loss/ weight gain, poor healing of wounds especially the extremities and vision changes may also manifest in diabetic affected people. Diabetes develops due to failure of the body to maintain glucose homeostasis.

1.2. Glucose

Glucose is an essential biological energy source that produces ATP. Glucose is absorbed by the intestinal microvilli facilitated by sodium- glucose co-transporter carriers, an active transport process, to portal circulation. Glucose from portal circulation diffuses to the hepatocytes (Voet *et al*., 2002). The diffusion of glucose is facilitated by glucose transporter (GLUT) molecules. Glucose transporter family includes GLUT1–12, GLUT14 and H+/myoinositiol co-transporter 1 (HMIT1) (Scheepers *et al*., 2004).

GLUT-1 has high affinity for glucose uptake even in hypoglycemic conditions and it is found in red blood cells, blood brain barrier and placenta (Paula *et al.,* 2000). GLUT-2 mediates uptake and release of glucose in the hepatocytes, regulates glucose stimulated insulin secretion in the pancreas and it is also expressed in the intestines and kidneys (Thorens B, 1992). GLUT-3 is the neuronal glucose transporter present in the brain and nerves (McCall *et al*., 1994; Simpson *et al*., 2008). GLUT-4 is essentially present in insulin-sensitive cells such as adipose tissue and skeletal muscle cells and cardiac muscle cells (Ploug *et al.,* 1988). Blood glucose concentrations are homeostatically maintained at 3.1- 7.8 mmol/L and a minimum concentration of 2.2-3.3 mmol/L is required for tissues that use glucose as the primary energy source. Glucose is converted and stored in the liver as glycogen. The extracellular circulating glucose levels are controlled by insulin and glucagon (Nair and Wilding, 2010).

1.3. Pathogenesis of diabetes

Diabetes is a chronic disease resulting from the pancreas not producing enough insulin or when the body cells are resistant to the insulin produced (American Diabetes Association, 2014).

Diabetes is characterized by hyperglycemia resulting from insulin deficiency or lack of insulin action (American Diabetes Association, 2014). There are two major types of diabetes, type 1 and type 2 diabetes. Type 1 diabetes is the result of an autoimmune reaction and/or chemical damage to proteins of the β-cells and the cells cannot produce enough insulin, hence it requires daily administration of insulin. Type 2 diabetes is characterized by insulin resistance or relative insulin deficiency as a result of environmental factors such as obesity. However, type 2 diabetes affects 90% of diabetic patients worldwide (International Diabetes Federation, 2013) and increases the number of patients at risk of diabetes-related complications such as heart failure and stroke.

Altered glucose, protein and lipid metabolism due to relative or absolute insulin deficiency leads to chronic hyperglycemia (Bardini *et al*., 2012). Insulin binds to membrane bound insulin receptor which in turn starts insulin signaling cascades such as translocation of GLUT-4 to the plasma membrane, influx of glucose through GLUT-4, glycogen synthesis, and fatty acid synthesis. The insulin signaling cascade and glucose uptake is altered in the absence of insulin or insulin resistance leading to abnormal glucose metabolism (Saltiel and Kahn, 2001). The imbalance between insulin/glucagon ratio causes an increase hepatic glucose production (basal hyperglycemia), whereas the decrease in plasma insulin concentration causes decrease in peripheral tissue glucose utilization (postpandrial hyperglycemia) (Giugliano *et al*., 2008). Oral Glucose Tolerance Test (OGTT) is a standardized test for glucose tolerance (Lindholm *et al*, 2001). Diabetes is diagnosed when blood fasting plasma glucose is ≥7 mmol/L or when the glucose concentration is ≥11 mmol/L after 2 hours of OGTT (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

The increase in counter-regulatory hormones (i.e. glucagon, epinephrine, cortisol, growth hormone) which is secondary to absolute or relative insulin deficiency leads to increased hepatic glycogenolysis, gluconeogenesis and lipolysis (Kitabchi and Nyenwe, 2006). This hormonal imbalance results in hyperglycemia, amino and nucleic acids and increased serum free fatty acids leading to the formation of advanced glycation end products (AGEs) (Fraser and Hansen, 2005). AGEs have a wide range of chemical, cellular and tissue effects and are thought to be a major cause of diabetic complications (Negre-Salvayre *et al.,* 2009). AGEs acts as stimuli for activating and modifying the intracellular signaling pathways (Brownlee, 1995). The accumulation of AGEs in the tissues leads to activation of receptor-mediated cell signaling pathways and increased oxidative stress (Yonekura *et al*., 2005). AGEs contribute to the development of vascular disease in diabetic by promoting oxidative stress generation through the interaction with the AGEs receptor which subsequently promote inflammation thrombogenic reactions leading to development and progression of vascular complications such as nephropathy, cardiomyopathy and atherosclerosis in diabetes (Sten *et al.,* 2002).

1.4.Complications of diabetes

Consistently high blood glucose levels may cause damage to various organs in the body. This situation may lead to delayed diagnosis thus increasing the prevalence of diabetes. Diabetes can lead to a series of microvascular and macrovasular complications. Microvascular complications such as neuropathy, nephropathy, ketoacidosis and retinopathy and macrovascular complications include cardiomyopathy, stroke and peripheral vascular diseases (Deshpande *et al*., 2008).

1.4.1. Diabetic ketoacidosis

Diabetic ketoacidosis (DKA) is an acute and life-threatening diabetes complication occurring mostly in type 1 diabetes. It is characterized by the increase hyperketonemia, hyperglycemia and metabolic acidosis (Laffel, 1999). Elevated plasma concentrations of ketone bodies i.e. β-hydroxybutyrate, acetoacetate and acetone result in hyperketonemia. Ketone bodies serve as a major source of energy in hypoglycemic state in vital organs such as brain (Sass, 2012). Increased free fatty acids are metabolized in the liver as an alternative source of energy (ketogenesis) resulting in accumulation of a ketone bodies. The increased proteolysis and decreased protein synthesis leads to more gluconeogenesis precursors. Meanwhile, there is a decrease in glucose uptake by peripheral tissues due to insulin deficiency or insulin resistance leading to severe hyperglycemia

Ketogenesis causes an increase in blood concentrations of these acidic metabolites (ketonemia) resulting in significant drop in blood pH and bicarbonates (ketoacidosis) (Mrozik and Yung, 2009). Ketonuria develops due to excessive production and accumulation of ketone bodies while the respiratory compensation for ketoacidosis results in rapid shallow breathing. Ketones induce nausea and vomiting leading to fluid and electrolyte loss. Osmotic diuresis, dehydration and hyperosmolarity secondary to glucosuria leads to severe dehydration, if not properly compensated may lead to renal dysfunction.

1.4.2. Diabetic nephropathy

Diabetic nephropathy is a progressive kidney disease in diabetes and a leading cause of chronic renal impairment in patients with diabetes (Jorge *et al*., 2005). In the early stages, diabetic nephropathy is characterized by hyperfiltration and glomerulus hypertrophy resulting in renal enlargement and higher glomerular filtrate rate (Magee *et al*., 2009). Morphological lesions develop over years and diabetic nephropathy progresses from microalbuminuria to macroalbuminuria and finally to the advanced diabetic nephropathy.

Glomerular high blood pressure develops in the later stages of diabetic nephropathy and if left untreated it leads to renal function decline. Glomerular hypertension is associated with the degree of hyperglycemia state (Hall, 2006) which sensitizes the endothelial layer to injury from elevated blood pressure and leads to secretion of intra-renal vasoactive hormones that constricts efferent arterioles (Susztak *et al*., 2006). The early stages of diabetic nephropathy is similar in type 1 and type 2 diabetes, however, the differences have been described between diabetic nephropathy caused by type 1 and type 2 diabetes (Biesenbach *et al.*, 1994). Diabetic nephropathy due to type 1 diabetes coincides with diabetic retinopathy in contrast to diabetic nephropathy due to type 2 diabetes in which this phenomenon is less clear. Furthermore, some claim that diabetic nephropathy develops more often in type 1 diabetes than in type 2 diabetes and many patients die due to cardiovascular diseases before reaching the clinical stage of diabetic nephropathy (Nelson *et al*., 1993).

1.4.3. Diabetic cardiomyopathy

Diabetic cardiomyopathy is defined as dialation and hypertrophy of myocardial tissue (Voulgari *et al*., 2010). This further leads to a decrease in systolic and diastolic function of the left ventricle. Hyperglycemia is the main pathogenic factor in the development of cardiomyopathy in diabetes. Hyperglycemia causes abnormalities to the myocytes leading to structural and functional abnormalities (Hayat *et al.,* 2004). The small coronary vessels and myocardial endothelium alterations increases cell adhesiveness, impaired fibronolysis, impaired relaxation and increase permeability (Popov, 2010). This is accelerated by increased tissue AGEs. As diabetic cardiomyopathy progresses, left ventricle hypertrophy is observed due to myocardial hypertrophy, fibrosis and thickening of capillary basement membrane (Palmieri *et al*., 2003).

Reduced systolic function is defined as a loss of the heart's ability to pump blood to the peripheral circulation. Reduced diastolic function is determined by delayed and extended diastolic phase. Diabetes is known to modify the action of calcium ions and sensitivity of cardiomyocyte contractile element (Gallego *et al*., 2009) which activates calcineurin, leading to interstitial fibrosis and ventricular contractile dysfunction.

1.4.4. Dyslipidemia in Diabetes

Type 2 diabetes is associated with obesity, insulin resistance, β-cell dysfunction, increase hepatic glucose production, hypertension and dyslipidemia presenting with high plasma triglycerides and triglyceride rich lipoproteins, low levels of high density lipoprotein (HDL) cholesterol, increase in very low density lipoprotein (VLDL) normal or slightly increased low density lipoprotein (LDL) cholesterol and presence of small dense LDL (sdLDL) particles which are cholesterol

depleted (Schwartz, 2006). In type 2 diabetes, dyslipidemia is initiated by insulin resistance which activates the activity of lipoprotein lipase (LPL) in the adipocytes that catalyzes release of free fatty acids (FFA) which are taken up by hepatocytes leading to production of triglycerides which then stimulates production of VLDL. Increased concentration of VLDL promotes cholesteryl ester transfer protein (CETP) to transfer of LDL cholesteryl esters or HDL cholesteryl esters in exchange for triglycerides leading to decrease in HDL and increase in sdLDL. This may contribute to the increased risk of atherosclerotic cardiovascular disease (Gennest and Libby, 2011) and increased mortality in patients with type 2 diabetes. (Harder *et al*., 2004). LDL comprises of a large buoyant LDL and sdLDL. This small dense LDL is depleted in cholesterol and is considered to be more atherogenic than its normal counterpart because it is more easily oxidized, penetrates the arterial wall more freely and has higher affinity for proteoglycans (Jiang *et al*., 2004). The sdLDL is the sub-fraction of LDL which is particularly related to coronary artery risk and is frequently raised in diabetics (Jiang *et al*., 2004).

1.4.4.1.Diabetic dyslipidemia on apolipoproteins

Apolipoprotein B (Apo B) and apolipoprotein A-I (Apo A-I) are the main structural proteins of atherogenic lipoproteins (VLDL, IDL and LDL) and HDL particles, respectively. Apo B is present as one molecule per each LDL particle, and Apo A-I is present in about 70% of HDL particles (Walldius and Jungner, 2004; Walldius and Jungner, 2005). Apo B100 is involved in endogenous LDL metabolism and Apo B48 is involved in chylomicron (CM) formation from the small intestines. After CM have been digested, Apo B48 returns to the liver as part of CM remnants where it is degraded by endocytosis. Apo B100 facilitates the transport of LDL-

cholesterol (LDL-c) to/ from the liver to peripheral tissues (Hermans *et al.*, 2011). After binding to the receptors, LDL is internalized via receptor-mediated endocytosis and degraded in lysosomes (Brown and Goldstein, 1986) and the cholesterol is liberated for use in the synthesis of steroid hormones and new plasma membranes. Upregulation of liver LDL receptor (LDL-R) expression is effective in decreasing plasma cholesterol (Kong *et al*., 2006). Consequently, measurement of Apo B100 provides direct information as to the number of circulating LDL-c particles (Hermans *et al.,* 2011). Although the mechanisms regulating the synthesis of ApoB-100 are incompletely understood, the production of Apo B100 messenger RNA (mRNA) has been shown to be altered by insulin and unaffected by changes in lipid metabolism *in vitro*.

Atherogenic particles, collectively known as non-HDL lipoproteins, are associated with atherogenic dyslipidemia, insulin resistance, portal hyperinsulinemia and the metabolic syndrome phenotype (Levinson *et al*., 2007). Serum Apo A-I, which is attached to the HDL particle, correlates to the concentration of HDL in plasma. HDL particles transport cholesterol from peripheral tissue and vessels to the liver. Acyl-CoA: cholesterol acyltransferase (ACAT) is an enzyme that converts cholesterol into cholesteryl esters for HDL-mediated transport in the circulation to the liver for excretion in bile (Rader *et al*., 2009). Apo A-I in HDL particles mainly stimulates the ACAT enzyme to enhance reverse cholesterol transport (Kontush and Chapman, 2006). Thus, the balance between the pro-atherogenic Apo-B and the anti-atherogenic Apo-A-I has been suggested to be more predictive compared with conventional lipids in the estimation of cardiovascular risk (Walldius *et al*., 2001)

1.4.4.2. Enzymes involved in cholesterol synthesis

Enzymes involved in cholesterol synthesis are ACAT and 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCoA-R) (Jung *et al*., 2003). ACAT is an endoplasmic reticulum enzyme that catalyzes the esterification of cholesterol in the liver and contributes to the formation the cholesterol ester pool to be used as storage or as a source of lipoprotein cholesterol (Chang *et al*., 1997). Its activity is generally reciprocally coordinated with HMGCoA-R, the key enzyme of cholesterol biosynthesis. HMGCoA-R is regulated at the transcriptional level and their transcriptional regulation is controlled by a family of transcription factors known as Sterol Regulatory Element Binding Proteins (SREBPs) (Sever *et al*., 2003).

1.4.4.3.Regulation of cholesterol synthesis

Three forms of Sterol Regulatory Element Binding Protein (SREBP) have been characterized, SREBP- 1a, -1c, and -2. SREBP-1c retains some ability to stimulate cholesterol synthesis (Shimano *et al*., 1997). SREBP-1a and SREBP-2 were elevated by higher potential for cholesterol synthesis. The SREBPs also regulate the LDL-R, which supplies cholesterol through receptor-mediated endocytosis (Horton *et al*., 2002). Both ACAT and HMGCoA-R enzymes are stimulated in type 2 diabetes. Evidence from clinical trials suggests that aggressive LDL-lowering therapy with statins, which are the inhibitors of HMGCoA-R, reduces cholesterol synthesis and decreases coronary heart disease events in diabetic patients (Borghi *et al*., 2002).

SREBP's are encoded by genes SREBF-1 and SREBF-2. SREBF-1 regulates genes that are required for de-novo lipogenesis while SREBF-2 regulates genes of cholesterol metabolism. When levels of sterol are high in the cells, SREBP's (inactive) are bound to the nuclear envelope

and endoplasmic reticulum (Horton *et al.,* 2002). However, low levels of sterols activate water soluble N-terminal domain of SREBP's (active) translocate to the nucleus. Inside the nucleus, SREBP's binds to sterol regulatory element (SRE) DNA sequence and upregulate the synthesis of HMGCoA-R. Also, SREBP's activates transcription of LDL-R target genes. Insulin stimulates cholesterol synthesis in liver by increasing the mRNA and the processed nuclear form of SREBP-1c, a transcription factor that activates all the genes needed to produce FFAs and Triglycerides (TGs) in liver (Horton *et al*., 2002). Of the three SREBP isoforms, SREBP-1c is the one whose expression is highest in liver, and it is the only one that is controlled primarily by insulin. Insulin increases SREBP-1c processing to liberate the nuclear form, and it increases transcription of the SREBP-1c gene, leading to increased SREBP-1c mRNA and precursor protein (Horton *et al.,* 2002; Ferré and Foufelle, 2010).

Caballero *et al*., (2009) identified increased cholesterol and also found increased hepatic SREBP-2 transcript expression in NASH (non-alcoholic steatohepatitis) patients which is caused by obesity, type 2 diabetes and dyslipidemia. SREBP-2 is responsible for transcriptional regulation of a number of target genes involved in cholesterol biosynthesis, the most important being HMGCoA-R (the rate-limiting enzyme), and LDL-R, the plasma membrane transporter responsible for receptor-mediated endocytosis of serum LDL cholesterol (Radhakrishnan *et al*., 2008). Active SREBP-2 fragments are also able to increase the expression of SREBP-2.

For hepatic lipid metabolism, insulin increases the expression levels of SREBP-1c (Shimomura *et al*., 1999) a member of sterol regulatory element-binding proteins (SREBPs) which are critical

transcription activators for hepatic cholesterol and fatty acid biosynthesis, and their homeostasis (Horton *et al*., 2002).

1.4.4.4.Other enzymes involved in lipid metabolism

Insulin activates LPL but inhibits hormone sensitive lipase (HSL). LPL regulates the supply of fatty acids from CM and VLDLs, producing chylomicron remnants and intermediate density lipoproteins (IDLs), to various tissues for either storage or oxidation. HSL is an intracellular neutral lipase that hydrolyses triglycerides into free fatty acids (Sweetman, 2009). In insulin resistance HSL is enhanced and LPL is inhibited, HSL releases FFA that are transported to the liver for atherogenic lipoproteins synthesis and LPL that catalyzes uptake of lipids in the blood to peripheral tissues thus increasing plasma lipids. The increase in synthesis and accumulation of cholesterol in the liver causes non-alcoholic liver disease; during this condition liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) tend to be elevated in the blood. The abnormalities in circulating lipids and lipoproteins are considered to be important risk factors for cardiovascular disease in diabetic individuals (Betteridge, 2011). Reversal of these abnormalities in lipid profile may reduce the accelerated atherosclerotic cardiovascular complications in patients with DM (Betteridge, 2011).

1.4.4.5.Hyperlipidemic indices

The atherogenic index (AI) is a logarithmical ratio of Total LDL and HDL expressed in molar concentration (Dobiasova and Frohloch, 2001). It predicts the risk of developing atherosclerosis. AI has a strong correlation with lipoprotein particle size which explains its high predictive value. Coronary risk index (CRI) is also a logarithmical ratio of Total Cholesterol (TC) and HDL for screening ischemic heart disease (da Luz *et al*., 2008). Lipoprotein ratios are an attempt to optimize the predictive capacity of lipid profile, they provide information on risk factors that are difficult to quantify by routine analyzes and are a better mirror for metabolic and interactions between lipid fractions.

1.5. Management and treatment of diabetic dyslipidemia

The contemporary treatment of diabetes is focused on decreasing hyperglycemia. Diabetic dyslipidemia can be prevented in various ways including regular exercise and eating diet with low cholesterol and total fats. However dietary and lifestyle interventions which are generally the first steps taken towards reducing lipid levels often have small and inconsistent effects. Therefore, pharmacological measures are necessary for those people who continue to have dyslipidemia following diet and lifestyle interventions. Current management strategies for diabetic dyslipidemia focus on the use of statins as first-line lipid modifying therapy American (Diabetes Association, 2004; Cholesterol Treatment Trialists' (CTT) Collaborators, 2008). Fibrates and nicotinic acid have therapeutic roles in the prevention of cardiovascular disease in patients with diabetic dyslipidemia (Shepherd *et al.,* 2005). Therefore, use of hypolipidemic, drugs is suggested to be an alternative treatment for diabetic induced dyslipidemia.

Synthetic hypolipidemic drugs are known of their numerous side effects, statins are known to cause rhabdomyolysis and myopathy (Brucken *et al.,* 2005; Backes *et al.,* 2009) such as neuropathy, nausea, gastric irritation, headache, stomach ache, vomiting, heart attack, diarrhea, abnormal liver function, and pancreatic dysfunction (Kim *et al*., 2011). In correlation with the synthetic drugs known for their adverse side effects, alternative medicine such as plant-derived chemicals which are known to be efficient, effective and have less or no side effects are now sought after.

1.5.1. Alternative treatment

Medicinal plants, fruits and vegetables with various active principles and properties have been used since ancient times to treat a variety of human diseases. It has been suggested that compounds present in medicinal plants, either alone or in combination, possess beneficial effects in chronic diseases like diabetes and its complications (Iyer *et al*., 2009). 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 and others, these chemicals are found in varying concentrations in the plants.

1.6.Flavonoids

Flavonoids are phenolic compounds; plant derived secondary metabolites, they have a common basic structure (Figure 1) and are subdivided into several groups based on their structural components. The structure of flavonoids consists of 2 aromatic rings (Figure 1: A and B) linked together by a 3-carbon chain that forms an oxygenated heterocyclic ring (Figure 1: C ring) (Barnes and Prasain, 2005; Benavente *et al.,* 2008). There are several flavonoids that has been identified such as flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavan-3-ols,

their C rings differ in generic structure, state of oxidation and functional groups on the C ring (Mazza and Miniati, 1993). Other flavonoids such as isoflavones have a β-ring at position 2 instead of position 3, proanthocyanidins are oligomers of flavan-3-ols and anthocyanidins form a flavylium cation.

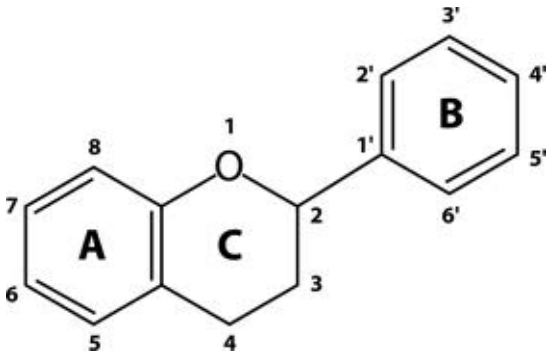

Figure 1: Basic structure of flavonoids (Lazarus and Schmitz, 2000).

The most prominent flavonols are quercetin, myricetin and few others. Quercetin was found to control dyslipidemia and improve lipid profiles in pregnant rats with diabetes mellitus and it was also found to have anti-dyslipidemic effect that resulted in the amelioration of streptozotocin (STZ) induced diabetes in rats through regulation of lipid metabolism-related genes (Pereira *et al.*, 2013; Wang *et al.*, 2012). Myricetin on the other hand has been found to stimulate glucose transport and improved insulin-stimulated lipogenesis (Ong and Khoo, 1996).

Flavones are known of their beneficial effects against atherosclerosis and diabetic mellitus (Cermak , 2008). Chrysin and luteolin are one of the flavones that showed preventive effect on diabetic dyslipidemia by decreasing TG and LDL in diabetic rats (El-Bassossy, 2003). Apigenin which is a natural flavone also prevented lipid accumulation that occurs in HepG2 cells exposed to high glucose (Zang *et al.*, 2006).

1.7.Naringin

Flavanone is another class of flavonoids which abundant in citrus fruits. Naringin (4′,5,7-trihydroxyflavone 7-rhamnoglucoside), one of the Flavanones found in grapefruit, decreased plasma LDL to HDL ratio in type 1 diabetes and also decrease hepatic HMGCoA-R and ACAT enzymes (Xulu and Owira, 2012). Significant reduction of blood glucose levels and beneficial effect on dyslipidemia were observed in diabetic rats treated with trans-chalcone a core structure of naringenin chalcone (Najafian *et al.,* 2010).

There are other flavonoids that show anti-dyslipidemic effect in diabetic rats such as rutin (Fernandes *et al.*, 2010), formononectin, genistein and others (Shen *et al.,* 2006). Also, other plant polyphenols have shown anti-atherosclerotic, anti-hyperglycemic and anti-dyslipidemic effect in diabetic induced rats though the inhibition of CETP, uptake of oxidized LDL, decrease in atherogenic lipoproteins and increase in HDL-c and also decrease the hepatic HMGCoA-R and ACAT enzymes(Kim *et al.,* 2012; Zhang *et al.,* 2010; Sefi *et al.,* 2010). Table 1 shows the effects of 2 flavanones as anti-dyslipidemic agents.

Table 1: Anti-dyslipidemic effects of 2 flavanones.

Flavonoid subclass		Effects	References
Flavanones	Hesperetin	Inhibits HMGCoA-R and ACAT	Lee *et al.*, 1999
		Decreases TG synthesis by inhibiting phosphatidate phosphohydrolase	Cha *et al*., 2001

		Inhibits HMGCoA-R, and ACAT, decreases TG and TC	Kim *et al.*, 2003
	Naringenin	Decreases TC and Increases HDL-c	Santos *et al.*, 1999
		Decreases VLDL, LDL and TG	Kurowska and Manthey , 2004
		Decreases TG and TC	Cho *et al.*, 2011

1.8. Motivation

Naringin has been shown to be an antioxidant, also downregulate HMGCoA-R and ACAT in diabetic animals. However, studies to determine anti-dyslipidemic effects of naringin in diabetic dyslipidemia have not been done.

1.9.Hypothesis

Naringin has anti-dyslipidemic effects in diabetic dyslipidemia.

1.10. Aim

To evaluate the effects of naringin on blood glucose control and plasma lipids in a diabetic state.

1.10.1. Objectives

- To investigate the effects of naringin on blood glucose regulation in diabetic rats.
- To investigate the effects of naringin on diabetic dyslipidemia.

CHAPTER 2

2.0.Material and methods

2.1. Chemicals and reagents

The reagents and drugs used included naringin, D-glucose, streptozotocin, simvastatin, citrate and phosphate buffers, anthrone reagent, bovine serum albumin (BSA), potassium chloride, potassium hydroxide, sodium chloride, sulphuric acid, hydrochloric acid, glycerol, TRIS, sodium dodecyl sulphate and ammonium per sulphate were purchased from Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa.

Regular insulin, normal saline, portable glucometers and glucose test strips (Ascencia Elite™, Bayer Leverkusen, Germany) were purchased from a local pharmacy. Halothane and other accessories were provided by the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

2.2. Study approach

2.2.1. Ethics approval

The experimental protocol for the study was approved by the Animal Ethics Committee of the University of KwaZulu-Natal, reference number 106/13/animal (Appendix).

2.2.2. Procedure and animal treatment

Male *Sprague-Dawley* rats (250-300g body weight) were randomly assigned into 7 groups (n=5) and housed, 5 rats per cage, with free access to standard commercial chow and drinking tap

water *ad libitum*. 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.

3.2.3. Induction of diabetes

Diabetes was induced in groups 3, 4, 5, 6 and 7 by a single intraperitoneal (IP) 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 starvation of the rats. Three days after administration of the STZ, development of diabetes was confirmed by tail pricking to analyze blood glucose levels. Random glucose levels above 11 mmol/ L were considered diabetic and thus included in the study (Babu *et al.,* 2006).

3.2.4. Experimental design

Group 2 and 4 were orally treated with 50 mg/kg BW of naringin. Group 3 was treated with regular insulin (4 U/kg BW) subcutaneously twice daily. Group 1 and 5 were treated with 3.0 ml/kg BW distilled water via gastric gavage. Group 7 was treated with a combination of 4 U/kg insulin and 50 mg/kg BW of naringin. Naringin treatment was initiated one week after STZ treatment and continued for group 2 and 4. Insulin treatment was initiated after confirmation of the presence of diabetes. Group 6 was treated with 20 mg/kg BW of simvastatin. On treatment day 45, halothane overdose was used to sacrifice the rats and blood samples were collected via cardiac puncture for plasma insulin and lipid profile analysis respectively. Rat livers were excised, rinsed in normal saline, weighed and snap frozen in liquid nitrogen and was stored at -80°C for further analysis.

2.3. Methods

2.3.1. Blood glucose testing

Fasting and random blood glucose tests were done on treatment days 0, 3, 14, 21, 28 and 35 respectively whereas glucose tolerance test (GTT) was done on day 41. Blood glucose concentrations were determined after tail pricking and was analyzed by a portable glucometer. Prior to GTT, all animals in all treatment groups were starved overnight and fasting blood glucose (FBG) was determined by IP administration of 3.0 mg/kg body weight of glucose in normal saline. Blood glucose concentrations were 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 (mmol/L×minutes).

2.3.2. Glycogen assay

Hepatic glycogen content was measured by the modified method of Seifter *et al.,* (1949). The liver tissues were homogenized using 5 volumes of 4 M KOH (ice cold). The homogenate was then dissolved by boiling in a water bath (100° C) for 30 minutes. The glycogen was then precipitated with ethanol, thereafter pelleted, washed, and resolubilized in distilled water. Treatment with anthrone reagent followed to assay the glycogen concentration of the livers (92 g/L anthrone in 95% (v/v) H_2SO_4), and the absorbance measured at 620 nm. Glycogen content was expressed as mg/g liver protein.

2.3.3. Plasma insulin

An ultra-sensitive rat insulin enzyme-linked immunoassay kit (DRG Diagnostics, Marburg, Germany) was used to analyze the plasma insulin levels. 100µL of the sample was added into appropriate wells in a 96 well plate and incubated at 4ºC for 2 hours. The solution was washed

four times with wash solution. 100 µL of 1X Biotinylated Insulin Detection Antibody was added and incubated for one hour in a shaker at room temperature. After washing, 100 µL of 1X HRP-Streptavidin solution was added and incubated for 45 minutes in a shaker at room temperature. 100 µL of TMB One-Step Substrate Reagent was added and incubated for 30 minutes at room temperature followed by addition of 50 µL of Stop Solution and the absorbance was read at 450 nm.

2.3.4. Plasma lipid profile

Fasting plasma total cholesterol, HDL cholesterol and triglycerides were analyzed by an automated chemistry analyzer (Labmax 240) using commercial kits. The HDL fractions was separated by the heparin-manganese procedure as previously described (Burstein *et al*., 1970) whereas LDL cholesterol was calculated according to the Friedwald formula: LDL = total cholesterol –(HDL cholesterol +VLDL cholesterol) (Firedwald *et al*., 1972). VLDL was calculated as VLDL = Triglycerides/ 5 (Rjadurai and Prince, 2006). AI was calculated as LDL/HDL and CRI as TC/HDL ratio.

2.4. Statistical analysis

Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by two-tailed t-test analysis of variance using the GraphPad Prism 5.0. Results with $p < 0.05$ were considered statistically significant and were expressed as the mean ± SEM of 5 animals.

CHAPTER 3

3.0. Results

3.1. Natural growth

Diabetic rats showed significant ($p<0.001$) weight loss compared to non-diabetic control group. Oral administration of naringin at a dose of 50 mg/kg BW significantly ($p<0.001$) prevented weight loss in diabetic rats compared to non-treated diabetic ones (Figure 3.1). Insulin with/without treatment significantly ($p<0.001$) reversed the weight loss compared to non-treated diabetic animals. Simvastatin did not significantly prevent weight loss in diabetic animals compared to non-treated diabetic rats.

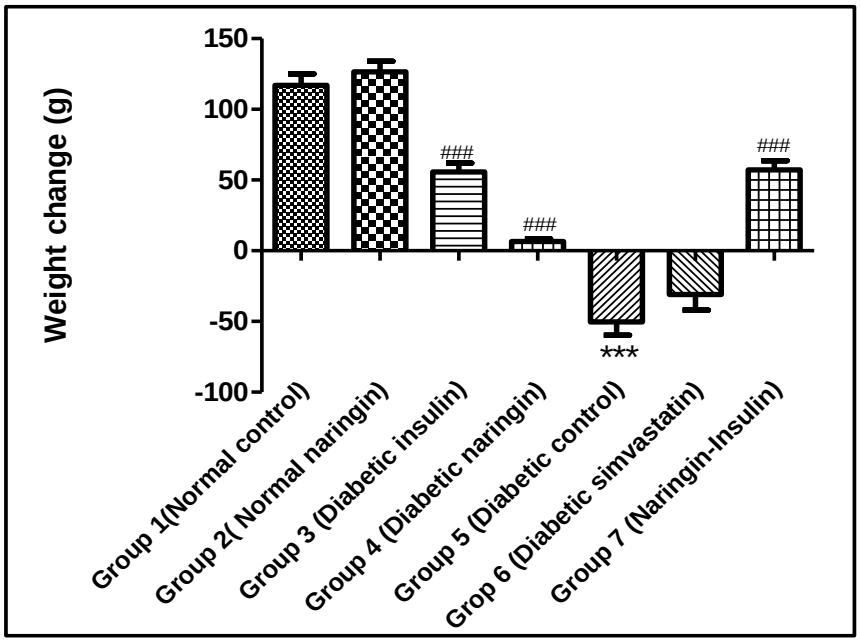

Figure 3.1: Average weight change in grams. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.2. Water consumption

All diabetic rats showed significant ($p<0.001$) increase in average water consumption compared to non-diabetic group. However, oral administration of naringin with subcutaneous injection of insulin in the same group (naringin-insulin) significantly ($p<0.05$) decreased water consumption in diabetic induced rats compared to un-treated diabetic group (Figure 3.2). Each of the 7 groups with 5 animals was allowed free access to water. The average water consumption was determined by the milliliters consumed by each group per day.

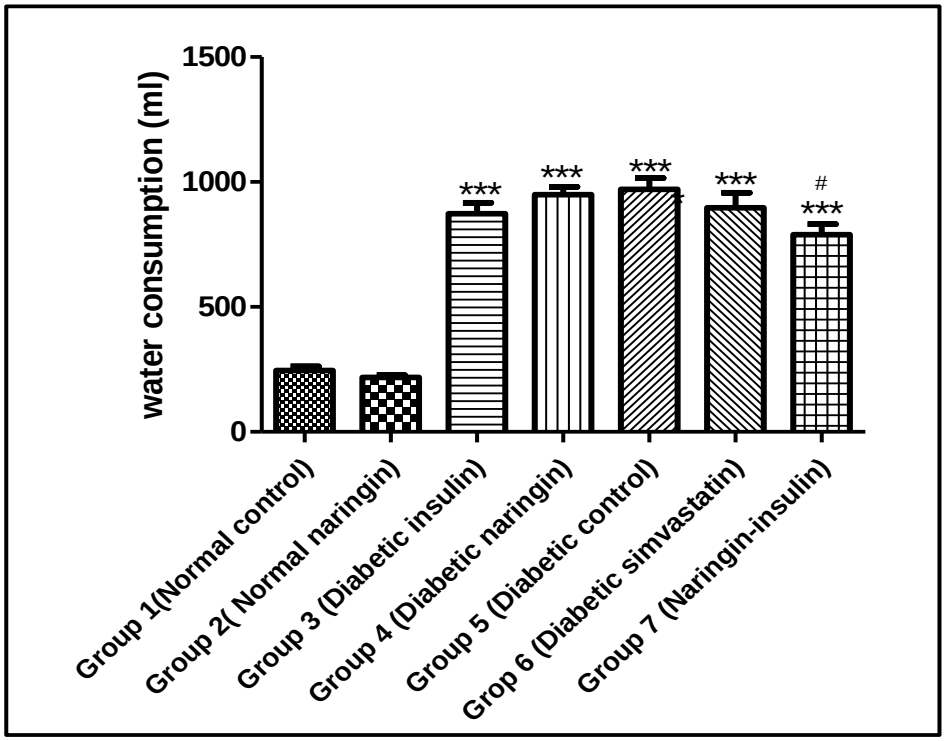

Figure 3.2: Average water consumption in milliliters per day. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.3. Fasting blood glucose

All diabetic groups showed significantly ($p<0.001$) elevated FBG compared to control. FBG was similar between diabetic naringin-treated and diabetic un-treated rats. Insulin with/without naringin treatment significantly ($p<0.05$) improved the FBG levels compared to the diabetic control. Simvastatin showed significant ($p<0.001$) increase in FBG levels compared to non-treated diabetic rats (Figure 3.3).

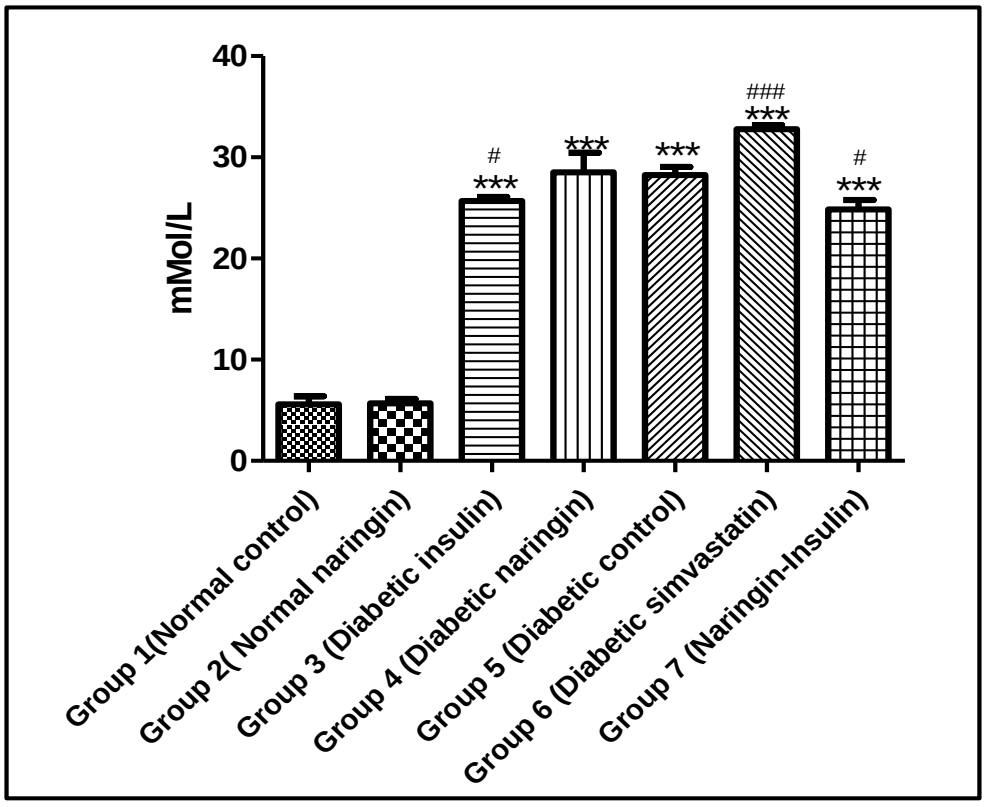

Figure 3.3: Fasting blood glucose levels. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic animals.

3.4. Glucose tolerance test

All diabetic rats (treated and non-treated) groups showed significant ($p<0.001$) increase in AUC compared to non-diabetic groups (Figure 3.4.2). Neither of the treatments administered significantly improved glucose tolerance in diabetic rats (Figure 3.4.1). The animals were severely hyperglycemic with blood glucose concentration beyond the measurable concentrations with a glucometer.

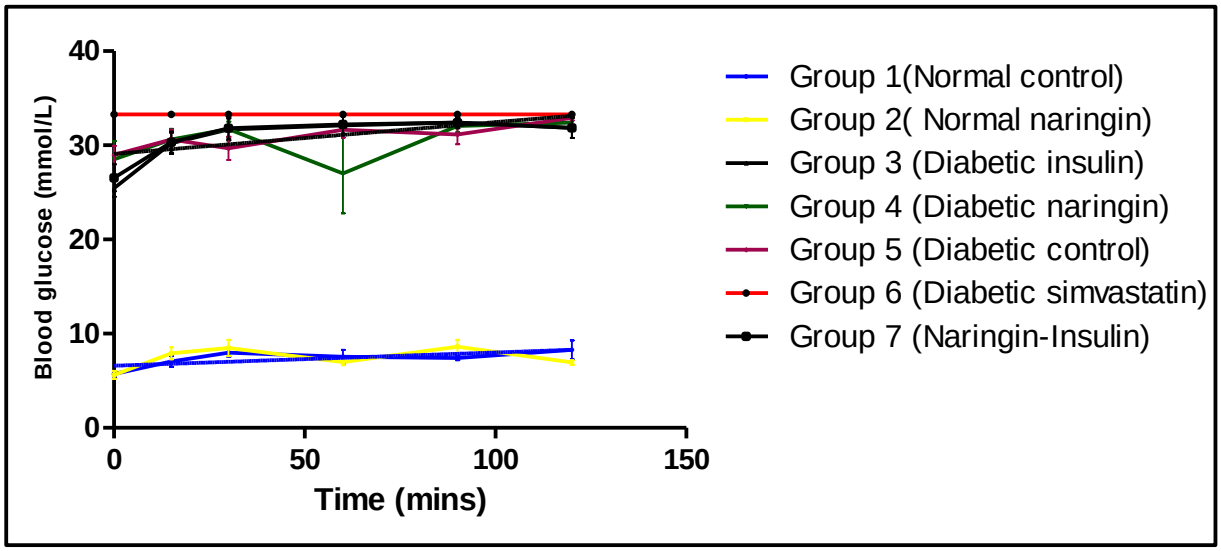

Figure 3.4.1: Glucose tolerance test after 15, 30, 60, and 90 minute intervals of treatment. The data is expressed as the mean ± SEM of 5 animals.

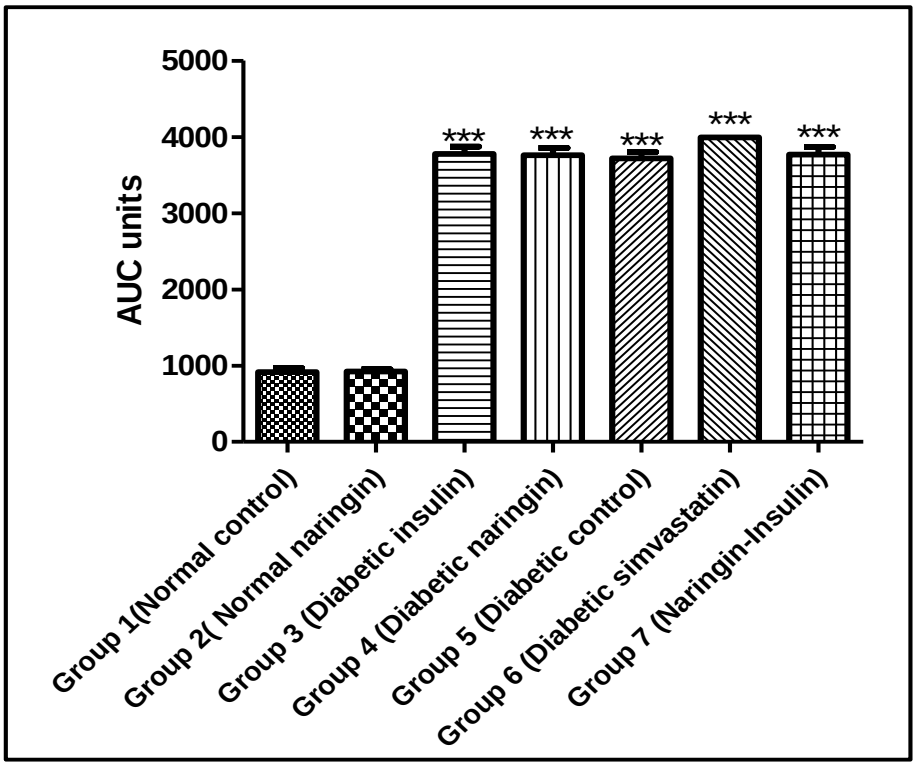

Figure 3.4.2: Calculated AUC for GTT. The data is expressed as the mean ± SEM of 5 animals.

***p<0.001 compared to non-diabetic control.

3.5. Hepatic glycogen content

Un-treated diabetic rats showed a significant ($p<0.001$) decrease in hepatic glycogen content compared to non-diabetic control. Administration of naringin to diabetic rats significantly ($p<0.05$) increased glycogen content compared to non-treated diabetic rats. Naringin with significantly ($p<0.001$) increased hepatic glycogen content compared to diabetic control. Insulin significantly ($p<0.001$) increased hepatic glycogen content compared to diabetic control. Simvastatin did not significantly improve hepatic glycogen content compared to untreated diabetic group (Figure 3.5).

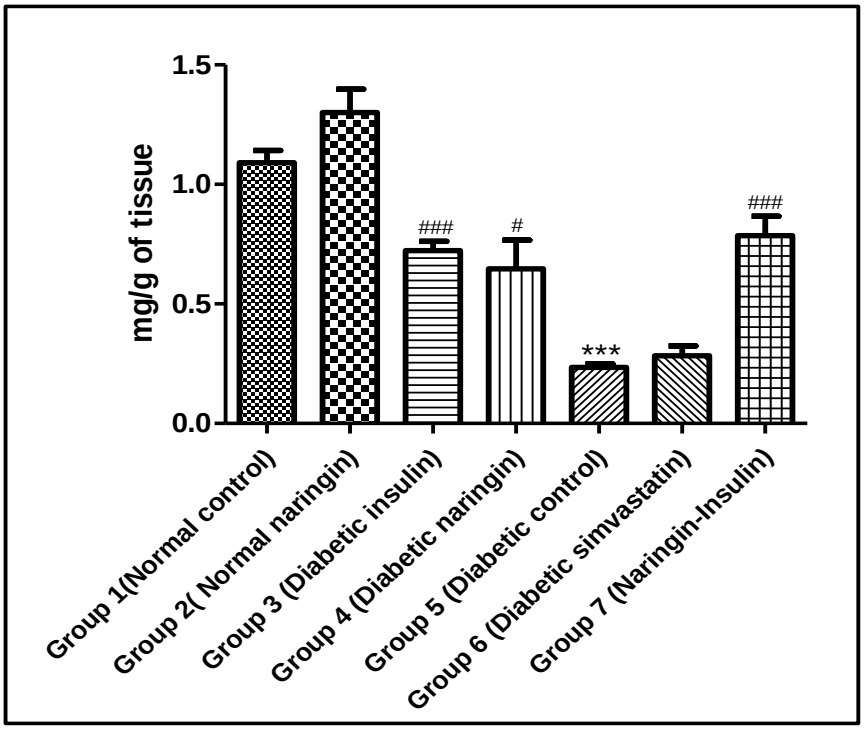

Figure 3.5: Hepatic glycogen in mg/g of tissue. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.6. Fasting plasma insulin

The results showed significant ($p<0.001$) decrease in plasma insulin in diabetic groups compared to non-diabetic control. Naringin or insulin significantly ($p<0.001$) improved fasting plasma insulin levels compared to un-treated diabetic group. Naringin with insulin treatment significantly ($p<0.001$) increased in fasting plasma insulin levels compared to diabetic control. Simvastatin did not improve the fasting plasma insulin. Non-diabetic animals treated with naringin showed significant ($p<0.001$) increase in insulin levels in comparison to non-diabetic control (Figure 3.6).

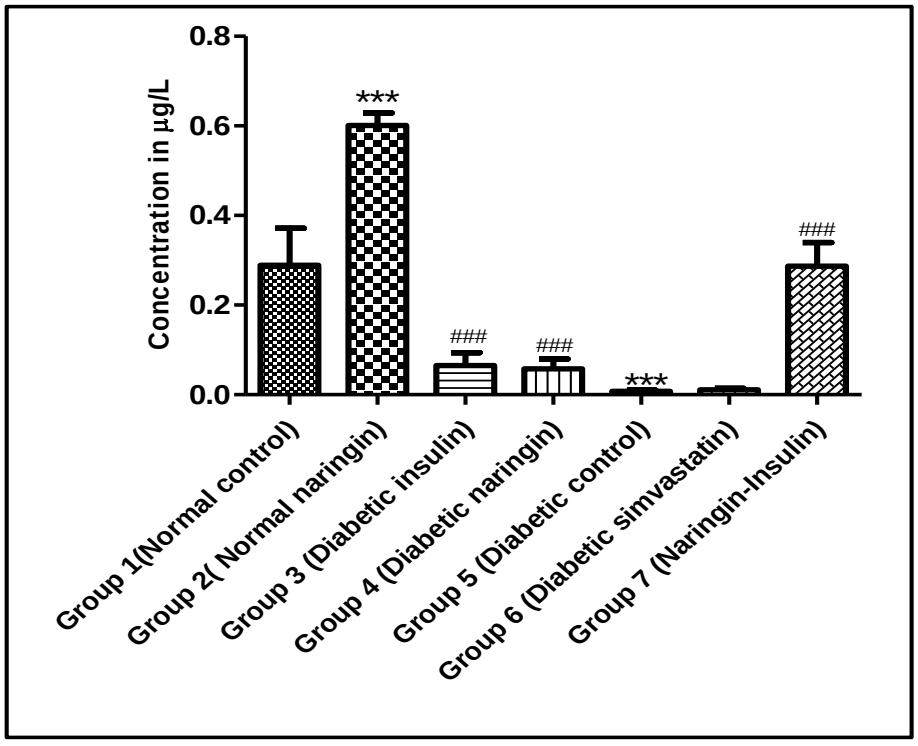

Figure 3.6: Plasma insulin concentration. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.7. Lipid analysis

3.7.1. Total cholesterol

TC was significantly ($p<0.005$) increased in non-treated diabetic animals compared to non-diabetic control. Naringin or simvastatin showed a significant ($p<0.05$) decrease in TC compared to diabetic control. Naringin with insulin showed significant ($p<0.001$) decrease in TC levels compared to diabetic control. Insulin did not decrease TC levels compared to diabetic untreated animals. Naringin significantly ($p<0.005$) increased the levels of TC compared to non-diabetic control (Figure 3.7.1).

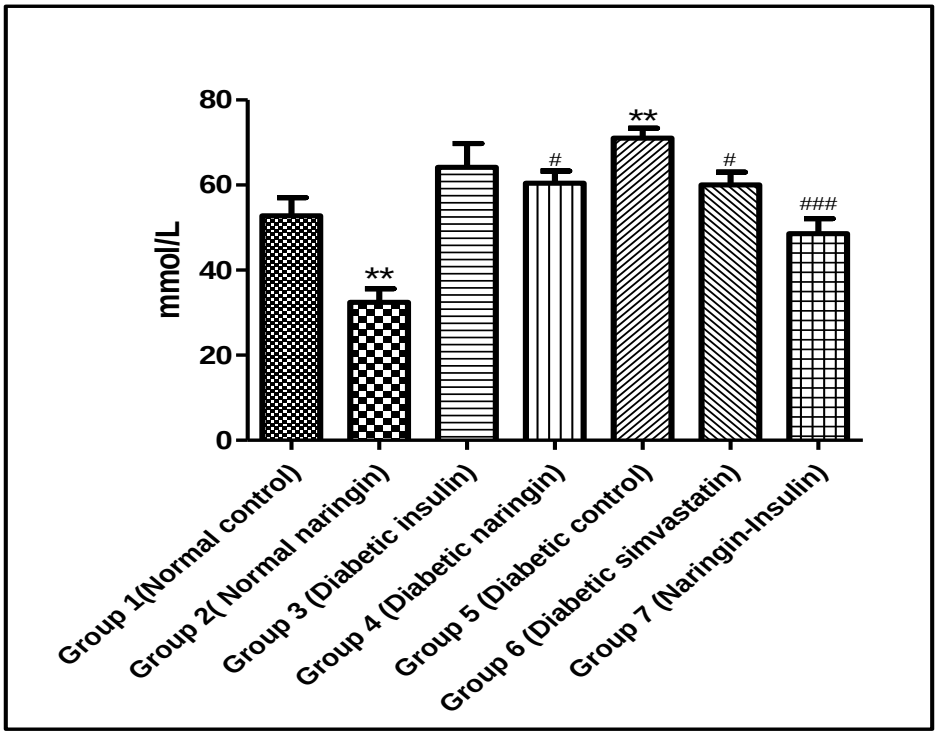

Figure 3.7.1: TC expressed in mmol/L. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.7.2. Triglycerides

TG were significantly ($p<0.001$) increased in non-treated diabetic animals compared to non-diabetic control. Naringin significantly ($p<0.005$) decreased the levels of TG compared to diabetic non-treated animals. Naringin with insulin showed significant ($p<0.001$) decrease in TG levels compared to diabetic non-treated animals. Insulin did not decrease TG levels compared to diabetic control. Simvastatin significantly ($p<0.05$) decreased TG level compared to diabetic control (Figure4.7.2).

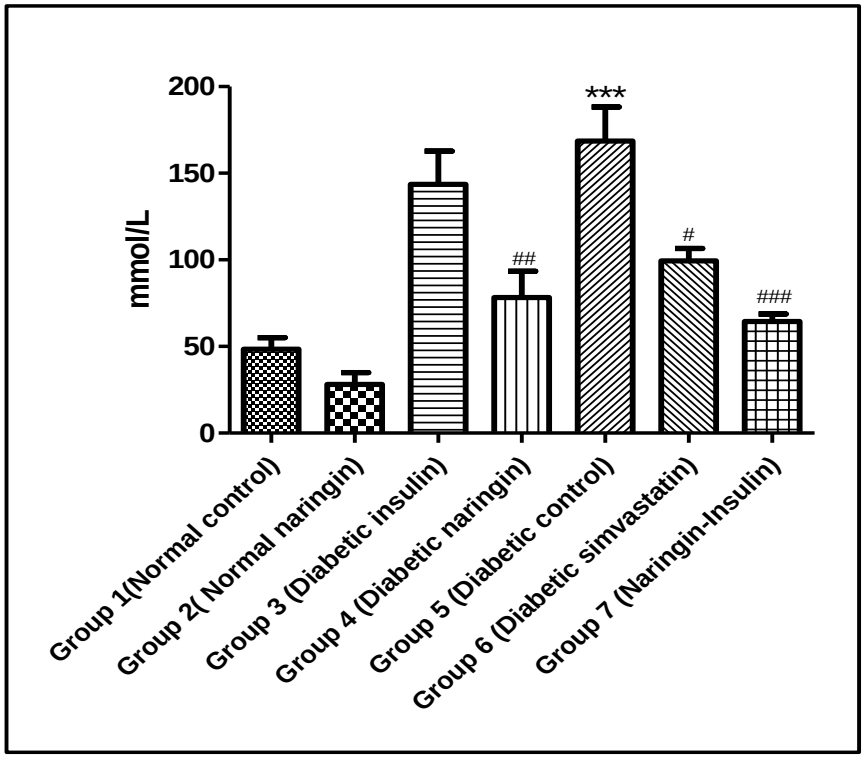

Figure 3.7.2: TG expressed in mmol/L. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic animals.

3.7.2. Very low density lipoprotein

Non-treated diabetic animals showed significant ($p<0.001$) increase in VLDL levels compared to non-diabetic control. Naringin did not decrease plasma VLDL levels compared to diabetic non-treated group. Naringin with insulin significantly ($p<0.05$) decreased VLDL levels compared to non-treated diabetic rats. Simvastatin or insulin did not have an effect on VLDL levels. Naringin showed significant ($p<0.05$) decrease in VLDL compared to non-diabetic control animals (Figure 3.7.3).

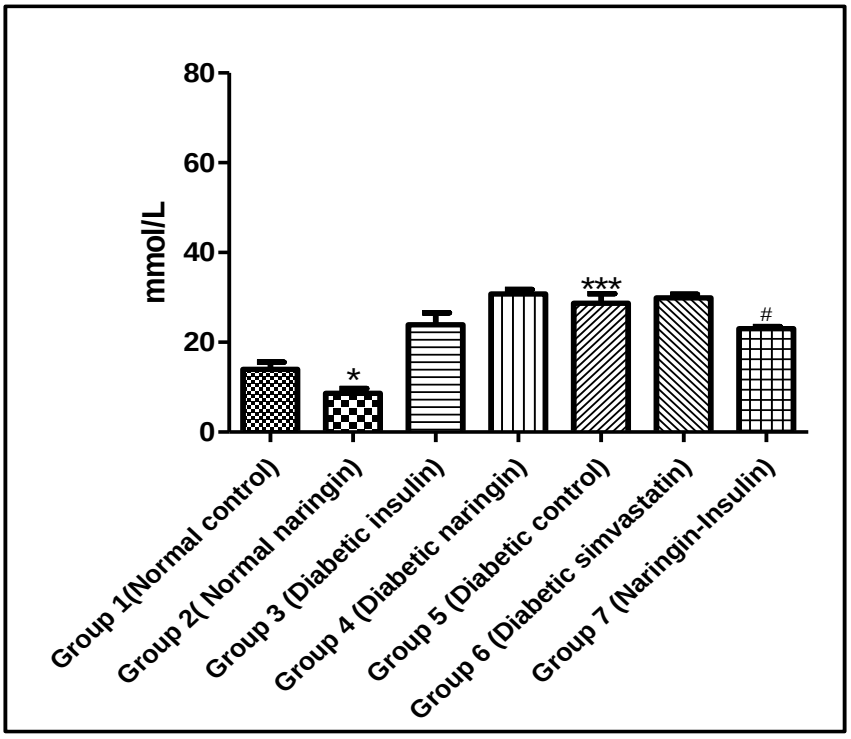

Figure 3.7.3: VLDL expressed in mmol/L. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.7.3. Low density lipoprotein

Non-treated diabetic animals showed significant ($p<0.001$) increase in LDL levels compared to non-diabetic control. Naringin did not decrease LDL levels compared to non-treated diabetic animals. Naringin with insulin showed significant ($p<0.05$) decrease in LDL levels compared to diabetic control. Simvastatin or insulin did not decrease LDL levels compared to diabetic control. Naringin treated non-diabetic animals showed significant ($p<0.05$) decrease in LDL levels compared to non-diabetic control.

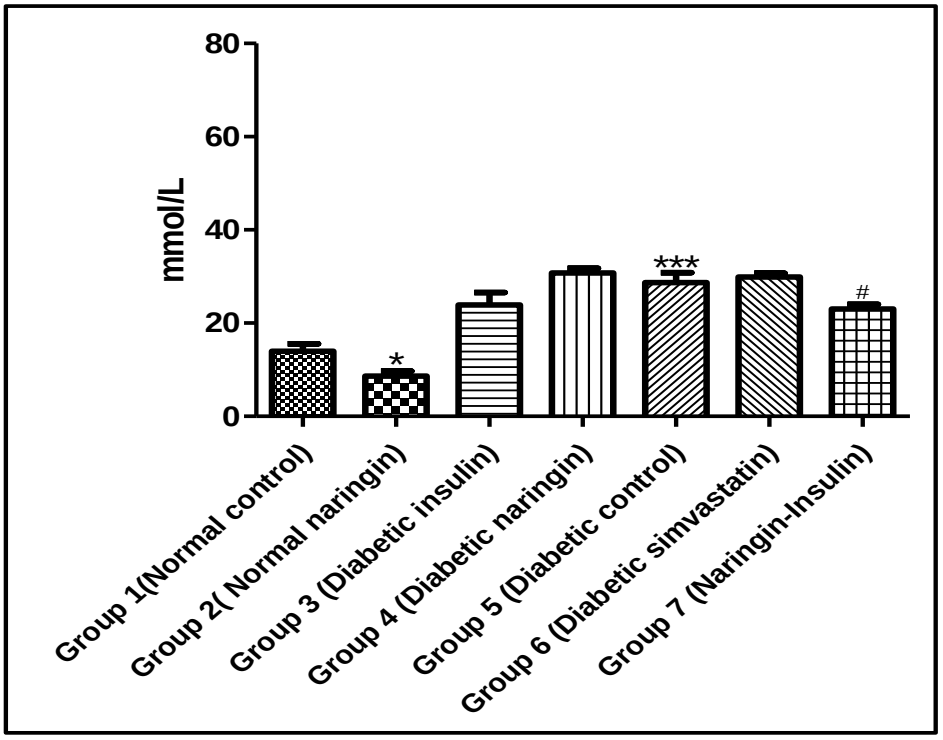

Figure 3.7.4: LDL expressed in mmol/L. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to diabetic control.

3.7.4. High density lipoprotein

HDL was significantly ($p<0.001$) decreased in non-treated diabetic rats compared to non-diabetic control. Naringin significantly ($p<0.005$) increased HDL levels compared to non-treated diabetic group. Naringin with insulin showed significant ($p<0.001$) increase in HDL levels compared to non-treated diabetic rats. Insulin significantly ($p<0.05$) decreased HDL levels compared to non-treated diabetic animals. Simvastatin did not have an effect of HDL levels (Figure 3.7.4).

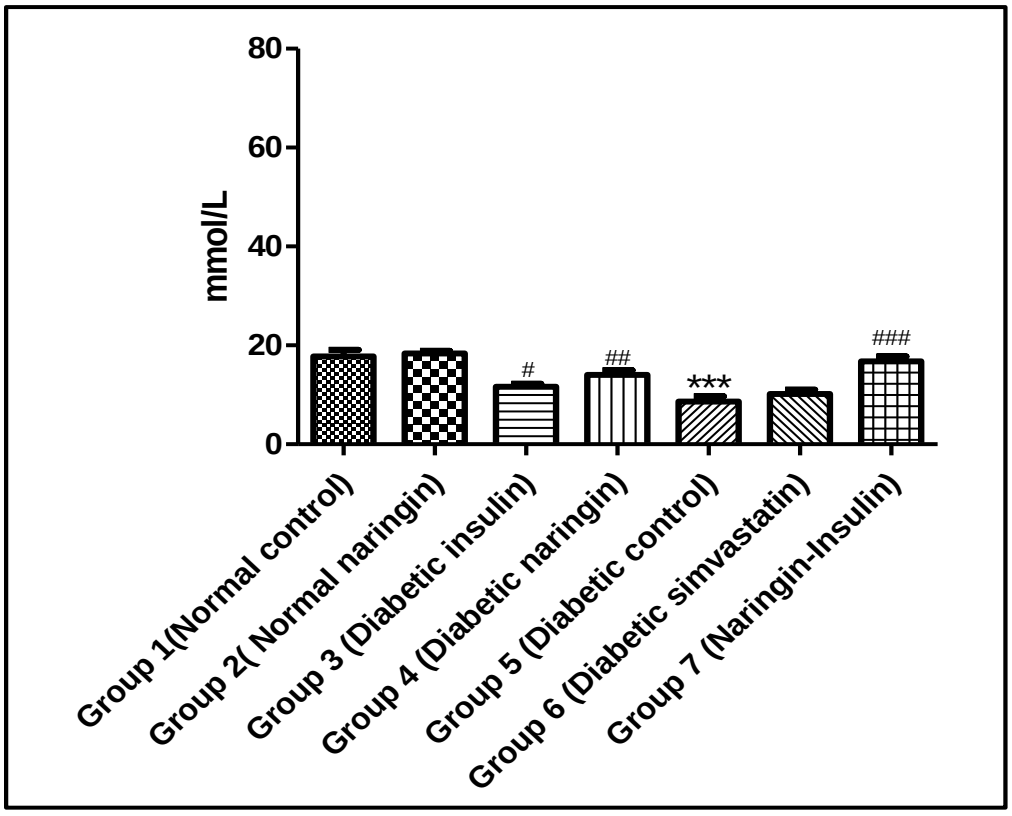

Figure 3.7.5: HDL expressed in mmol/L. The data is expressed as the mean ±SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic animals.

3.8. Hyperlipidemic indices

3.8.1. Coronary risk index

The CRI was significantly ($p<0.001$) increased in none-treated diabetic group compared to non-diabetic control. Naringin or naringin with insulin showed significant ($p<0.001$) decrease in CRI compared to non-treated diabetic animals. Simvastatin did not reduce CRI compared to non-treated diabetic rats group. Naringin administration to non-diabetic rats significantly ($p<0.001$) decreased CRI compared to non-diabetic control (Figure 3.8.1).

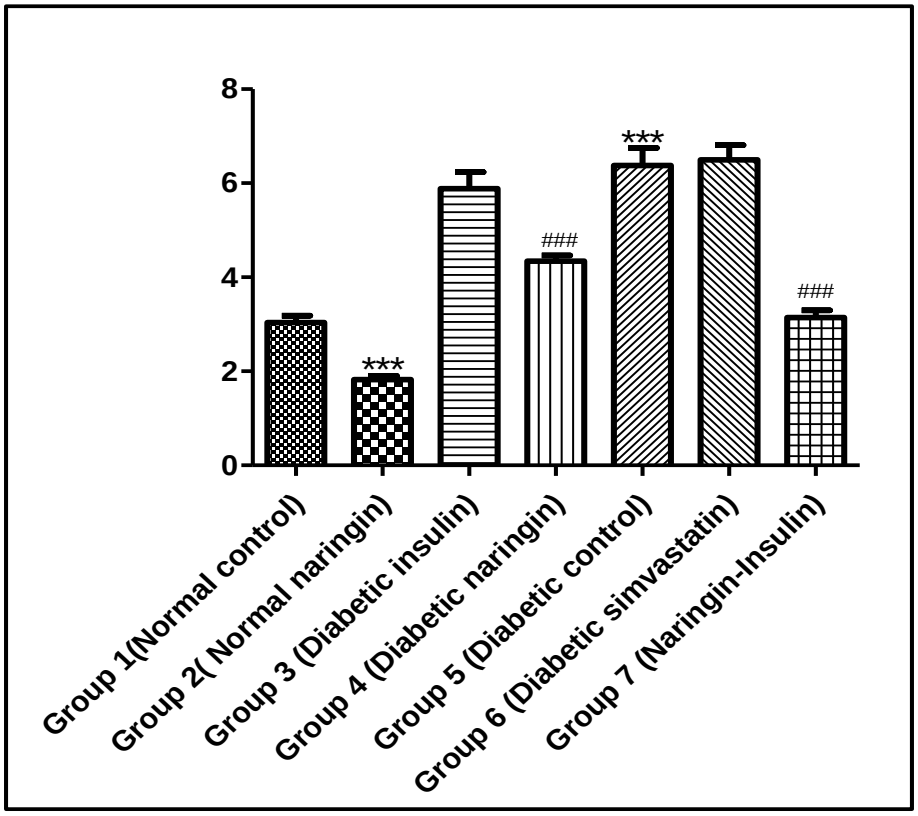

Figure 3.8.1: CRI. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic rats.

3.8.2. Atherogenic index

AI was significantly ($p<0.001$) increased in non-treated diabetic group compared to non-diabetic control. Naringin with/without insulin showed significant ($p<0.005$ and $p<0.001$, respectively) decrease in AI compared to non-treated diabetic rats. Simvastatin did not reduce AI compared to non-treated diabetic group. Naringin administration to non-diabetic rats significantly ($p<0.001$) decreased AI compared to non-diabetic control (Figure 3.8.2).

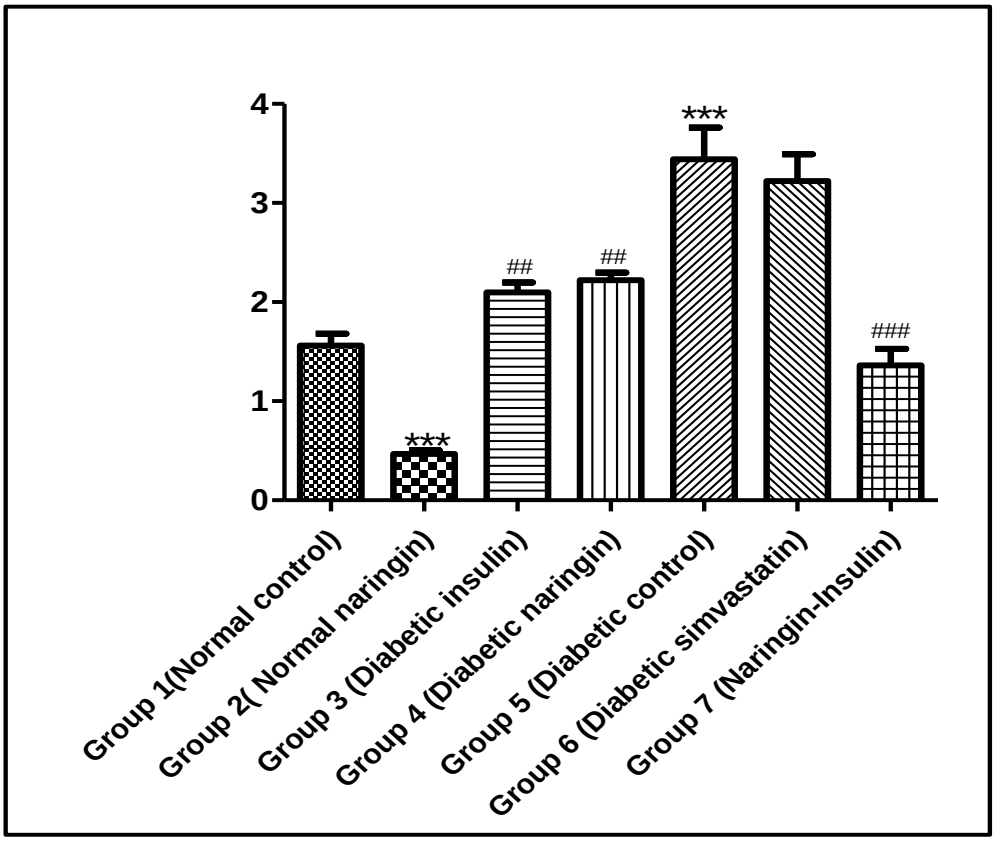

Figure 3.8.2: AI. The data is expressed as the mean ± SEM of 5 animals. ***$p<0.001$ compared to non-diabetic control. $^{###}p<0.001$ compared to non-treated diabetic animals.

CHAPTER 4

4.0.Discussion

In this study, naringin was investigated for *in vivo* anti-diabetic and anti-dyslipidemic effects. Diabetic animals showed significant weight loss compared to non-diabetic (Figure 3.1). Type 1 diabetes patients are often underweight due to osmotic diuresis, glucosuria and catabolism caused by insulin deficiency (Purnell *et al*., 1998; Liamis *et al*., 2014). Naringin, at a concentration of 50 mg/kg BW, prevented weight loss in diabetic animals compared to non-treated diabetic animals. These results are supported by the study of Jung *et al.,* (2004) suggesting that naringin has an effect on preventing proteolytic and lipolytic events associated with diabetes. Insulin significantly prevented weight loss in diabetic animals compared to non-treated diabetic animals. Type 1 diabetes patients receiving insulin treatment generally gain weight due to anabolic effects of insulin (Betts *et al.,* 2005). Insulin inhibits lipolysis and promotes lipogenesis and protein metabolism thus promoting weight gain. Naringin with insulin was more effective in preventing weight loss in diabetic animals compared to non-treated animals due to possible synergistic effects of naringin with insulin. Simvastatin is known to prevent weight gain (Knapik-Czajka, 2014), this was also observed in this study where simvastatin did not significantly prevent weight loss in diabetic animals compared to non-treated diabetic animals. Studies in animal models suggest that statin treatment may possibly impair peripheral insulin signaling (Preiss and Sattar, 2011) and increases concentration of adiponectin which accelerates fatty acid breakdown thus encourages weight loss in (Arnaboldi and Corsini, 2015).

All diabetic rats showed significant increase in average water consumption compared to non-diabetic group (Figure 3.2). This is due to raised blood osmolarity secondary to increased blood glucose concentration, furthermore insulin deficiency causes an increase in plasma vasopressin due to the loss of osmoregulation, which in turn increases water consumption (Vokes *et al*., 1989). Insulin, naringin or simvastatin did not decrease water consumption in diabetic animals compared to non-treated diabetic animals suggesting failure to decrease blood osmolarity and blood vasopressin concentrations. The combination of naringin and insulin significantly decreased water consumption compared to non-treated diabetic rats. Naringin might be accelerating the effect of insulin in decreasing blood glucose concentration thus decreasing osmolarity. This suggests possible synergistic effects of naringin with insulin are more effective than either naringin or insulin alone.

All diabetic animals showed a significant increase in FBG levels compared to non-diabetic control (Figure 3.3). Previous studies have indicated a marked increase in blood glucose levels compared to non-diabetic rats caused by administration of STZ which destroys pancreatic β-cells leading to impaired glucose stimulated insulin release (Akbarzadeh *et al.,* 2007; Schaalan *et* al., 2009; Mahmoud *et al*., 2012). Hyperglycemia is due to reduced glucose entry in the peripheral tissues and increased gluconeogenesis. Insulin with/without naringin treatment significantly improved FBG levels compared to non-treated diabetic rats. Naringin did not improve FBG in diabetic animals compared to non-treated diabetic animals, these results are supported by studies of Akondi *et al*., (2011) and Xulu and Owira (2012) suggesting that naringin does not have an effect on FBG in Tpype 1 diabetes model. Simvastatin had significantly increased FBG concentrations than non-treated diabetic animals suggesting that statins may cause

hyperglycemia by decreasing GLUT-4 mediated uptake of peripheral glucose in patients with or without diabetes (Sukhija *et al*., 2009). All diabetic rats (treated and non-treated) groups showed glucose intolerance compared to non-diabetic groups (Figure 3.4.1 and Figure 3.4.2). Neither of the treatments administered improved glucose tolerance in diabetic rats. Satoh *et al.,* (2005) proved that statins not improve GTT in diabetic rats.

Hepatic glycogen content is one of the markers to assess antihyperglycemic effects of any drug. An increase in hepatic glucose production and decrease in Insulin, naringin or insulin-naringin significantly increased the glycogen concentration in comparison to non-treated diabetic animals (Figure 3.5). Insulin is known to stimulate hepatic glycogen synthesis by promoting a net decrease in the extent of phosphorylation of glycogen synthase, a rate limiting enzyme in the pathway of glycogen synthesis, to increase its activity (Bouskila *et al*., 2008; Liu and Brautigans, 2000). Naringin significantly increased hepatic glycogen content in diabetic animals compared to non-treated diabetic group. Naringin is said to increase hepatic glycolysis and glycogen concentration by lowering hepatic gluconeogenesis in mice (Jung *et al*., 2004).

Results showed significant decrease in plasma insulin in diabetic groups compared to non-diabetic control. Naringin or insulin significantly increased plasma insulin concentration in diabetic animals compared to non-treated diabetic rats. Naringin is not an insulin secretagogue (Ali *et al.,* 2012), however due to its antioxidant effects (Thangavel *et al*., 2012), it might be protecting the pancreatic β-cells from oxidative damage induced by STZ whereas insulin increases ATP production in the pancreatic β-cells thus improving insulin production. Simvastatin did not significantly increase plasma insulin levels in diabetic animals compared to non-treated diabetic animals. These results are in accordance with those of Paolisso *et al*.,

(2000), suggesting that statins reduce insulin secretion by inhibiting glucose-induced elevation of calcium ions. Naringin with insulin treatment restored the blood insulin levels in the diabetic animals to the level of the non-diabetic untreated animals, with a significant increase compared to non-treated diabetic animals. Jung *et al.*, (2004) also found that naringin increased plasma insulin levels and the combination of naringin with vitamin C improved plasma insulin levels in STZ induced diabetes (Punithavathi *et al*., 2008). This futher suggest that naringin is more effective when combined with other treatments.

TC was significantly increased in diabetic control animals compared to the control animals, also demonstrated by Gylling *et al*., (2004) (Figure 3.7.1). Naringin significantly decreased TC levels in non-diabetic naringin treated animals compared to non-diabetic control, suggesting that naringin is effective in decreasing TC in non-diabetic animals. Naringin is said decrease plasma cholesterol by reducing HMG-CoA activity (Xulu and Owira, 2012; Choi *et al* ., 2001).

Simvastatin significantly decreased TC levels in diabetic animals compared to diabetic control. Statins are known to reduce TC levels in the blood and this was also observed by Sheng *et al*., (2012). Insulin did not significantly reduce TC levels in diabetic rats compared to non-treated diabetic rats. Insulin induces SREBP-1c transcription which increases the production of cholesterol (Ye and BeBose-Boyd, 2011), however naringin with insulin showed significant decrease TC levels in diabetic rats compared to un-treated diabetic animals, this suggests that naringin combined with insulin prevents the insulin from raising total cholesterol.

TG levels were significantly high in the non-treated diabetic in comparison to the non-treated animals (Figure 3.7.2). With type 1 diabetes there is an increase in TG leading to

hypertriglyceridemia following the decrease in lipoprotein lipase (Dullaart, 1995) which leads to decreased catabolism of TG. Naringin or simvastatin significantly decreased the TG levels in diabetic rats compared to non-treated diabetic animals respectively. However, insulin did not significantly decrease TG in diabetic animals compared to non-treated animals. These results are in agreement with the findings of Choe *et al*., (2001) and Kim *et al.,* (2006), who reported that naringin decreases plasma TG. This might be the effect on naringin inducing peroxisome proliferator-activated receptor (PPAR) alpha (Liu *et al*., 2008) which activates the uptake of triglyceride rich lipoproteins. Furthermore, the effect of simvastatin in decreasing TG was also observed by Isley *et al.,* (2006) where they suggested that simvastatin improve TG clearance and stimulates intravascular TG lipolysis while not affecting TG production. Insulin treatment did not significantly decrease TG levels in diabetic animals compared to non-treated diabetic animals, it is suggested that continuous infusion of insulin leads to rapid reduction of plasma TG levels, however, this method eventually leads to severe hypoglycemia (Poonuru *et al*., 2011). Whereas insulin with naringin treatment significantly decreased plasma TG levels in diabetic animals compared to non-treated animals which suggests a synergistic effect of the two treatments.

Naringin significantly reduced VLDL concentration in non-diabetic animals (Figure 3.7.3). All diabetic animals showed a significant increase on VLDL concentrations, this might be due to the decrease in lipoprotein lipase activity and increased lipolysis (Verges, 2001). Under non-diabetic conditions, insulin inhibits hormone sensitive lipase resulting in decreased plasma free fatty acid concentration. Insulin treatment did not significantly decrease plasma VLDL levels compared to non-treated diabetic control. In a diabetic state, it is suggested that cells are resistant to the effect

of insulin suppressing free fatty acid production thus leading to increased plasma VLDL production (Lewis and Steiner, 1996). However insulin with naringin significantly reduced VLDL concentration in diabetic animals compared to diabetic control. Naringin might be enhancing the effect of insulin in inhibiting hormone sensitive lipase or naringin encourages the effect of insulin in preventing lipolysis in the hepatocytes, hence naringin alone did not significantly decreased VLDL concentrations. This further confirms that combination treatment is effective than the individual administration of naringin or insulin.

Gylling *et al*., (2004) observed a significant increase in LDL levels in diabetic induced animals compared to non-diabetic animals and this was also witnessed in this study (Figure 3.7.4). Niesen *etal*., (2008) suggested that LDL-R protein levels were not affected but there was a significant decrease in LDL-R mRNA in STZ induced diabetic animals. This could be assumed that it decreases responsiveness of LDL-R protein in a diabetic state compared to a non-diabetic individual leading to increase LDL. Increased peripheral lipolysis and hepatic lipogenesis also leads to an increase in plasma lipoprotein particles. Naringin or insulin did not significantly decreased plasma LDL concentrations, however, naringin with insulin treatment in diabetic animals significantly reduced LDL levels against non-treated diabetic animals. Demonty *et al*., (2010) suggested that naringin does not have affect LDL levels. Naringin combined with insulin might be increasing the sensitivity of the LDL-R protein. The dose of simvastatin used in this study did not significantly reduce plasma LDL. Other studies used high-dose simvastatin to achieve maximum results (Szendroedi *et al*., 2009), however simvastatin are associated with dose-limiting side-effects. Insulin did not significantly decrease LDL levels in diabetic animals compared to non-treated diabetic animals (Posadas *et al*., 2004).

Significant decrease in HDL concentrations were observed in diabetic control, diabetic treated with insulin, diabetic treated with naringin and diabetic treated with simvastatin (Figure 4.7.5). It is known that in diabetic state, plasma HDL decreases compared to non-diabetic individuals (Weidman *et al*., 1982). Insulin showed significant increase in HDL levels compared to non-diabetic rats, Agardh *et al*., (1982) suggested that insulin treatment increase plasma HDL concentrations by altering cholesterol transfer to and from HDL particle (Sadur and Eckel., 1983). Naringin treatment significantly increase plasma HDL concentrations, these results are in accordance with those of Jeon *et al*., (2004). Rats lack cholesteryl ester transfer protein (CEPT) (Tall, 1993) which is involved in HDL synthesis therefore naringin might be increasing plasma HLD levels by enhancing cholesterol acyltranferase (LCAT). Naringin with insulin showed significant increase in HDL levels compared to non-treated diabetic animals. Simvastatin did not significantly increase HDL in diabetic animals compared to non-treated diabetic rats, due to the dose-limiting side-effects of simvastatin. There was no significance difference in naringin with or without insulin compared to non-diabetic rats, suggesting the preventive effect of this combined treatment in increasing HDL.

Patients with diabetes are prone to develop cardiovascular diseases (Roper *et al*., 2002). This study also investigated the CRI in none-treated and treated diabetes. Diabetic animals, excluding naringin with insulin group, showed a significant increase in CRI compared to non-diabetic control (Figure 3.8.1). This is further supported by Schnell and Standl, (2010) suggesting the increase in cardiovascular diseases in type 1 diabetes. Naringin with/without insulin treatments both significantly decreased CRI compared to diabetic control. Naringin administration to non-diabetic rats significantly decreased CRI compared to non-treated non-diabetic rats. These results

suggest that naringin decreases CRI in both diabetic and non-diabetic state, however, it showed even greater results in diabetic animals when it was combined with insulin. This suggests that naringin can be used as a supplement in type 1 diabetic patients on insulin treatment to decrease the risk of developing coronary complications. Although simvastatin significantly reduced TC levels in diabetic animals, HDL levels were not significantly increased, thus animals diabetic animals treated with simvastatin were at risk of developing coronary artery diseases.

The AI was significantly increased in the diabetic induced animals, excluding naringin with insulin group, compared to non-diabetic control (figure 3.8.2). Insulin or naringin with/without insulin showed a significant decrease in AI compared to diabetic control. Thus, insulin, naringin or naringin with insulin decreased the risk of developing atherosclerosis in animals with diabetes. Simvastatin did not significantly reduce LDL or significantly increased HDL thus diabetic animals treated with simvastatin were at risk of developing atherosclerosis.

5.0.Conclusion

The main focus on this study was to evaluate anti-diabetic and anti-dyslipidemic potential of naringin, an abundant flavonoid in grapefruit. This study has been undertaken to evaluate the activities of naringin with/without insulin on weight change, water consumption, FBG, hepatic glycogen storage, GTT, plasma insulin, TC, TG, VLDL, LDL, HDL, CRI and AI in streptozotocin (STZ) induced Wistar rats.

Results suggest that naringin was not hypoglycemic, however prevented weight loss and improves glycogen storage. Moreover, when naringin was combined with insulin, it ameliorated diabetes by preventing weight loss, decreasing water consumption, decreasing FBG, improving glycogen storage and increasing plasma insulin levels. This indicates that naringin improves the effect of insulin treatment in diabetic individuals. Concurrently naringin with/without insulin had anti-dyslipidemic effect and also showed a potential in preventing cardiovascular diseases and the risk of developing atherosclerosis. This might be due to its anti-oxidant effect or other unknown mechanisms. The results also suggest that naringin offers potential promise to clinically enhance the effect of insulin treatment in ameliorating diabetic dyslipidemia.

5.1. Suggestion for further studies

- Effects of naringin on SREBP
- Effects of naringin on Apolipoproteins.

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