The effects of naringenin on oxidative stress parameters in cardiac muscles of diabetic rats.

By

Mcobothi Esethu Nosibusiso

218007172

Submitted in partial fulfilment of the requirements for the award of the Degree of

MSc. (Med Sci.) (Pharmacology)

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Molecular and Clinical Pharmacology Laboratory Department of Pharmacology Discipline of Pharmaceutical Sciences, School of Health Sciences University of KwaZulu-Natal, Westville Campus, Durban South Africa.

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As the candidate's **supervisor**, I have approved this dissertation for submission

Name:

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DECLARATION

I, **Mcobothi Esethu Nosibusiso**, declare that:

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PREFACE

This study has been submitted in fulfilment of the academic requirements for obtaining a Masters in Medical Science (Pharmacology). The use of information from other sources in this dissertation has been duly acknowledged in the text and reference section.

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Nosibusiso Mcobothi

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CONFERENCE ABSTRACTS

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1. Presentation at UKZN College of Health Sciences Research Symposium (November 2019), Durban, South Africa, Tittle: Naringenin ameliorates cardiac oxidative stress in streptozotocin-induced diabetes mellitus (Poster presentation).

LIST OFABREVIATIONS

ADA	American Diabetes Association
AGEs	advanced glycation end products
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AREC	Animal Research Ethics committee
AUC	Area under the curve
BHT/TBA	Butylated hydroxytoluene /Thiobarbituric acid
BRU	Biomedical Research Unit
CAT	catalase
CH3+	carbonium ion
CHD	coronary heart disease
Cpr	concentration of protein
CRP	C- reactive protein
CVDs	cardiovascular diseases
DCM	Diabetic cardiomyopathy
DKA	Diabetic ketoacidosis
DPP-4	Dipeptidyl- peptide-4 inhibitors
DTNB	5, 5’-dithio-bis-2-nitrobenzoic acid

ELISA	enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase 1/2
FBG	Fasting blood glucose
FDA	Food and drug administration
FFAs	Fast Fatty acids
FPI	Fasting plasma insulin
G6Pase	Glucose-6-phosphatase
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GLP-1	Glucagon- like peptide -1 agonist
GLUT 1-5	Glucose transporter 1-5
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GPx	Glutathione peroxidase
GSSG	Oxidised glutathione
GTT	Glucose Tolerance Test
HbA1C	Haemoglobin A1c
HDL	High density lipoprotein
HLA-DR3/DR4	Human leucocyte antigens

HNF-1α/4α	Hepatocyte nuclear factor homeobox
HOMA-IR	Homeostasis Model Assessment of Insulin resistance
HRP	Horseradish peroxidase
HW	Heart weight
HW/BW	Heart weight/body weight
ICA	islet cell activation
IDF	International diabetes Federation
IL-1β	Interleukin- 1beta
IL-6	Interleukin- 6
INS	Insulin
IRS	Insulin receptor substrate
I-R	Ischemia-reperfusion
IR	Insulin Resistance
IV	intravenous
JNK	Jun nuclear kinase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPO	Lipid peroxidation
LV	Left ventricle
LVH	Left ventricular hypertrophy

LVW/BW	Left ventricular weight/body weight
MAPK	Mitogen- Activated kinase
MDA	Malondialdehyde
MetS	Metabolic syndrome
MODY 1/2/3	Maturity onset diabetes of the young
NADPH/NADH/NAD+	Nicotinamide adenine dinucleotide phosphate
NAR	Naringenin
NC	Normal control
NFkB	Nuclear factor kappa-light-chain enhancer of activated B cells
PARP-1	poly-ADP-ribose polymerase-1
PBS	phosphate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PKC	Protein kinase C
PPAR-α	peroxisome proliferator activated receptor-α
PPAR-γ	peroxisome proliferator activated receptor -γ
RAAS	Renin- Angiotensin-Aldosterone system
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SGLT2	Sodium glucose cotransporter 2 inhibitor
SOD	Superoxide dismutase

STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinediones
VLDL	Very low density lipoprotein
WHO	World Health Organisation
XOD	xanthine oxidase

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ABSTRACT

Introduction: Diabetic cardiomyopathy (DCM) is defined by hypertrophy, oxidative stress, fibrosis and inflammation of the cardiac muscle. Hyperglycemia-associated oxidative stress plays an important role in the development of cardiac hypertrophy. Naringenin a citrus fruit-derived flavonoid has previously been demonstrated to have antioxidant, anti-diabetic, anti-inflammatory and cardioprotective properties by as yet unknown mechanisms.

Aim: To investigate the effects of naringenin on oxidative stress parameters in cardiac muscles of diabetic rats.

Methods: Wister rats (250-300g) were randomly divided into six groups (n=7). Groups I and IV were orally treated daily for 56 days with 3.0 ml/ kg Body Weight (BW) of distilled water and 60 mg/kg BW of naringenin in distilled water, respectively. Groups II, III, V and VI were made diabetic by a single intraperitoneal injection of 60 mg/kg BW of streptozotocin (STZ) and similarly treated with naringenin, except group VI which was treated with insulin 2.0 U/BW bid. Group V was pre-treated with naringenin for a period of one week before STZ administration. On day 57 the animals were euthanized, blood samples collected, and the hearts were excised, weighed and stored at -80^{O}C. Antioxidant activity (catalase, glutathione peroxidase and superoxide dismutase) was measured using colorimetric commercial kits. Malondialdehyde (MDA) levels were measured using the Thiobarbituric acid reactive substances assay (TBARS) while fasting plasma insulin was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit and insulin resistance was calculated using Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and pro-inflammatory cytokine levels were measured by commercial ELISA kits.

Results: Diabetic animals presented with significant ($p< 0.05$) weight loss, polydipsia, increased fasting blood glucose (FBG) levels and glucose intolerance (GI) compared to control. Naringenin treatment significantly increased antioxidant enzyme levels (cardiac tissue) in diabetic animals compared to the untreated diabetic groups. MDA and TNF-α levels (in cardiac tissue) were significantly increased in the untreated diabetic groups compared to the control. Cardiac mass to body weight ratio was increased in the untreated diabetic rats compared to the naringenin treated diabetic rats.

Conclusion: Naringenin pre-treatment and naringenin post STZ treatment improved diabetic symptoms, antioxidant levels, heart weights and reduced inflammation suggesting its cardioprotective effects in diabetic cardiomyopathy are due to its antioxidant properties.

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CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1. DIABETES MELLITUS

1.1.1. Epidemiology of diabetes mellitus

According to the International Diabetes Federation (IDF), 425 million people were diagnosed with diabetes by the year 2017 and this number is estimated to rise by 48% in 2045, where approximately 629 million people will be diagnosed with diabetes worldwide (International Diabetes Federation, Diabetic Atlas 2017; Cho et al., 2018) (Fig 1). Sedentary lifestyle, genetics and unhealthy food choices are the main risk factors for type 2 diabetes (World health organisation, 2016). Diabetic patients who develop DCM are at a higher risk of increased morbidity and mortality worldwide (Ogurtsova et al., 2017).

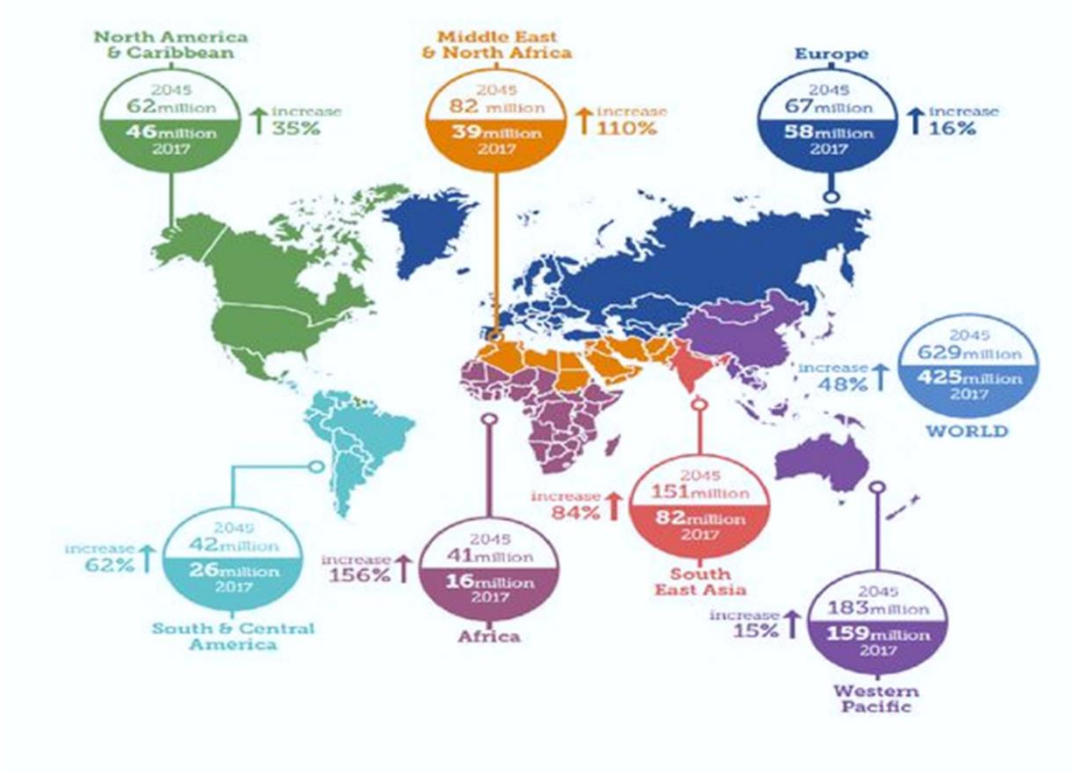


Figure 1. Worldwide prevalence of diabetes (image adapted from the International Diabetes Federation, Diabetic Atlas 2017).

1.1.2. Diabetes mellitus

Diabetes causes microvascular and macrovascular end-point complications (Skyler et al., 2017) (Fig. 2). There are different types of diabetes mainly type I, type II, gestational diabetes and other specific types (monogenic diabetes i.e. MODY), these forms are grouped according to different etiologic classifications (Baynes, 2015). Although there are different classifications of diabetes, the underlying problem is high blood glucose levels (hyperglycaemia) accumulating outside the cells because of insulin defects (Baynes, 2015). Insulin produced by β-cells of the islets of Langerhans in the pancreas, interacts with the insulin receptors to form a cascade of intracellular reactions catalysed by different enzymes. Insulin does not only facilitate glucose entrance into the cells but it also regulates the expression of enzymes that control glucose homeostasis (Guthrie and Guthrie, 2004). In the absence of insulin, the liver produces glucose (gluconeogenesis) to provide the cells with energy, this leads to a further rise and accumulation of blood glucose levels (hyperglycaemia) (Skyler et al., 2017).

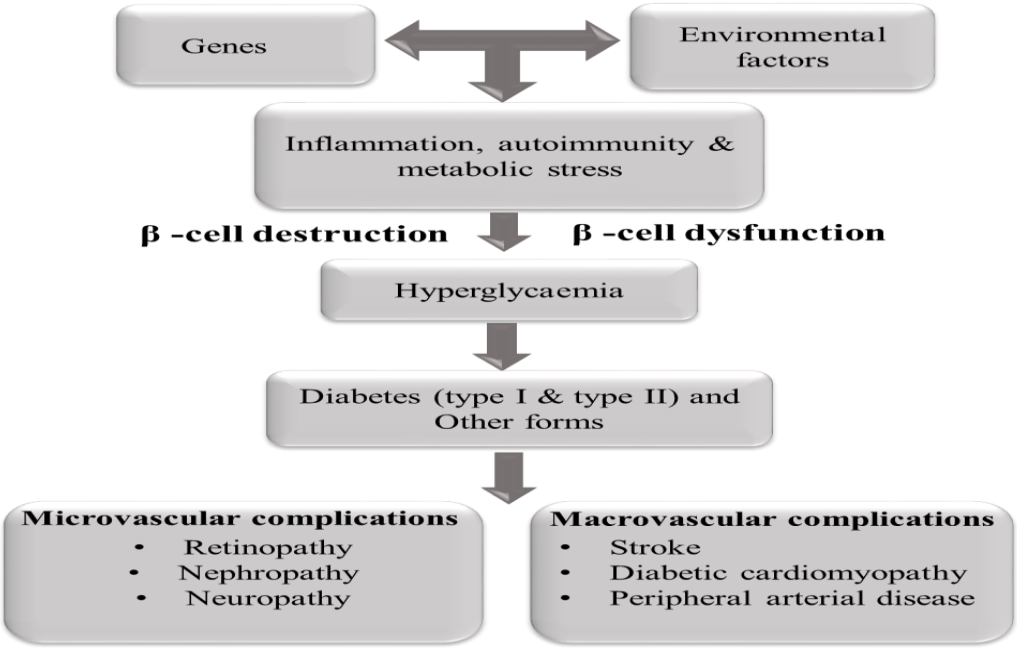


Figure 2. Microvascular and macrovascular complications resulting from different forms of diabetes mellitus (Adapted and modified from Skyler et al., 2017).

1.1.3. Type I diabetes

Type I diabetes mellitus (T1DM) can be a genetic disease resulting from autoimmune destruction of insulin producing pancreatic β- cells leading to progressive and irreversible failure of insulin secretion resulting in hyperglycaemia and a tendency to ketoacidosis (Ogbera et al., 2014; Kahanovitz et al.2017). There are two classes of human leucocyte antigens HLA-DR3 and HLA-DR4 haplotypes located on chromosome number 6 which are responsible for insulin gene expression, responses to viral infections and regulation of T cell activation (DiMeglio et al., 2018). When these genes malfunction, they lead to autoimmune diseases, type 1 diabetes included (Guthrie and Guthrie, 2004) (Paschou et al., 2018).

Chemicals and environmental factors (viruses) can also induce type I diabetes by damaging the pancreatic β-cells continuously over an extended period or rapidly over a short period of time (Baynes, 2015).

Streptozotocin (STZ) is an antibiotic isolated from *streptomyces achromogenes* known to cause pancreatic β-cell destruction and is widely used in experimental diabetes (Wu and Huan, 2008; Atkinson et al., 2015). Once taken up by the pancreatic beta cells via GLUT 2 transporters, STZ induces cell death in three ways: generating free radicals, increasing nitric oxide production by increasing guanyl cyclase activity and DNA methylation, forming carbonium ion ($CH3^{+}$) that activates the nuclear enzyme (poly ADP-ribose synthetase) that is part of the cell repair mechanism (Ventura-Sobrevilla et al., 2011; Tesch, 2007). It causes permanent diabetes in animal models by damaging pancreatic β-cells through generation of superoxide anions that act on the mitochondria causing an increase in the activity of the enzyme xanthine oxidase resulting in complete or partial destruction of the pancreatic β-cells (Atkinson et al.,2015). STZ administered in small doses to induce diabetes and administered in one large single dose to cause a complete necrosis of the pancreatic β-cells (Wu and Huan, 2008). Rats

are usually administered 45-60 mg/kg body weight of STZ to induce T1DM (Wang-Fischer and Garyantes, 2018).

Diabetic animals have increased malondialdehyde (MDA) levels but decreased endogenous antioxidant enzyme levels (glutathione peroxidase, catalase and superoxide dismutase) when compared with control animals in experiments (Wang-Fischer and Garyantes, 2018). The decrease in antioxidant activities and simultaneous increases in MDA activities, indicates increased oxidative stress (Eleazu et al., 2013). Metabolism of STZ results in the degradation of ATP by xanthine oxidase producing uric acid as the final product this reaction generates ROS such as superoxide and hydroxyl radicals emanating from H_2O_2 dismutation during hypoxanthine metabolism, accelerating the process of pancreatic beta cell destruction (Eleazu et al., 2013; Wang-Fischer and Garyantes, 2018).

T1DM is managed by daily injection of insulins such as rapid-acting, short-acting, immediate-acting, long-acting and pre-mixed (IDF, 2017).

1.1.4. Type II diabetes

Type II diabetes mellitus (T2 DM) is characterised by impaired insulin secretion caused by the dysfunction of pancreatic β-cells or impaired insulin action through insulin resistance (Ozougwa et al., 2013). There are many factors that cause insulin resistance (IR) including obesity, sedentary lifestyle, unhealthy dietary intake, age and genetic disorders (Baynes, 2015) (Fig 3).

IR is the initial defect in T2 DM as it can manifest long before any clinical symptoms can be detected and long before blood glucose levels are high enough to make a clinical diagnosis (Taylor, 1994; Guthrie and Guthrie, 2004). Insulin resistance occurs in the skeletal muscles and fat cells (peripheral tissues). IR can occur due to environmental factors (e.g. aging and

obesity) and genetic mutations in the insulin receptors (IRS) and GLUT 1-5, whereby insulin cannot bind to the receptors to activate the required reactions for glucose uptake (Chan et al., 1994; Skyler et al., 2017).

Defects in translocation of GLUT-4 protein is caused by reduced tyrosine phosphorylation of IRS-1 (Colditz et al., 1995). Phosphorylated IRS proteins serves as multisite binding proteins for various effector molecules that have Src homology 2 (SH2) domains, including phosphatidylinositol-3-kinase (PI3K) regulatory subunits, tyrosine kinases and growth factor receptor binding proteins (Wagner et al., 2013). IRS proteins work as essential signaling intermediates of activated cell surface insulin receptors and play an important role in maintaining basic insulin-mediated cellular functions including fatty acid synthesis, glycogen synthesis, cell survival through glucose uptake, protein synthesis and inhibit gluconeogenesis (Albegali et al., 2019)

IR occurs at multiple levels in cells, from the cell surface to the nucleus including insulin receptor desensitization. Inhibition of IRS-1 and insulin receptor substrate-2 (IRS-2) results in the suppression of IRS protein functioning and inhibition of PI3K signaling. Insulin stimulates amino acid uptake, inhibits protein degradation and promotes protein synthesis and a shortage in the diabetic state causes an increase in protein catabolism, rather than a decline in protein synthesis (Colditz et al., 1995).

In the development of IR, insulin production increases in the pancreatic β-cells to maintain homeostasis of blood glucose levels for normal bodily functions (Chan et al., 1994). If IR increases or persists over an extended period, the β-cells undergo genetic changes or exhaustion (Taylor et al., 1994; Van der Zijl et al., 2011). This causes decrease in insulin secretion and continuation of IR resulting in the rise of blood glucose levels favouring the development of T2 DM (Guthrie and Guthrie, 2004). The Homeostatic Model Assessment of Insulin Resistance

(HOMA-IR) is used for the diagnosis of IR or metabolic syndrome (MetS) (Esteghamati et al., 2010).

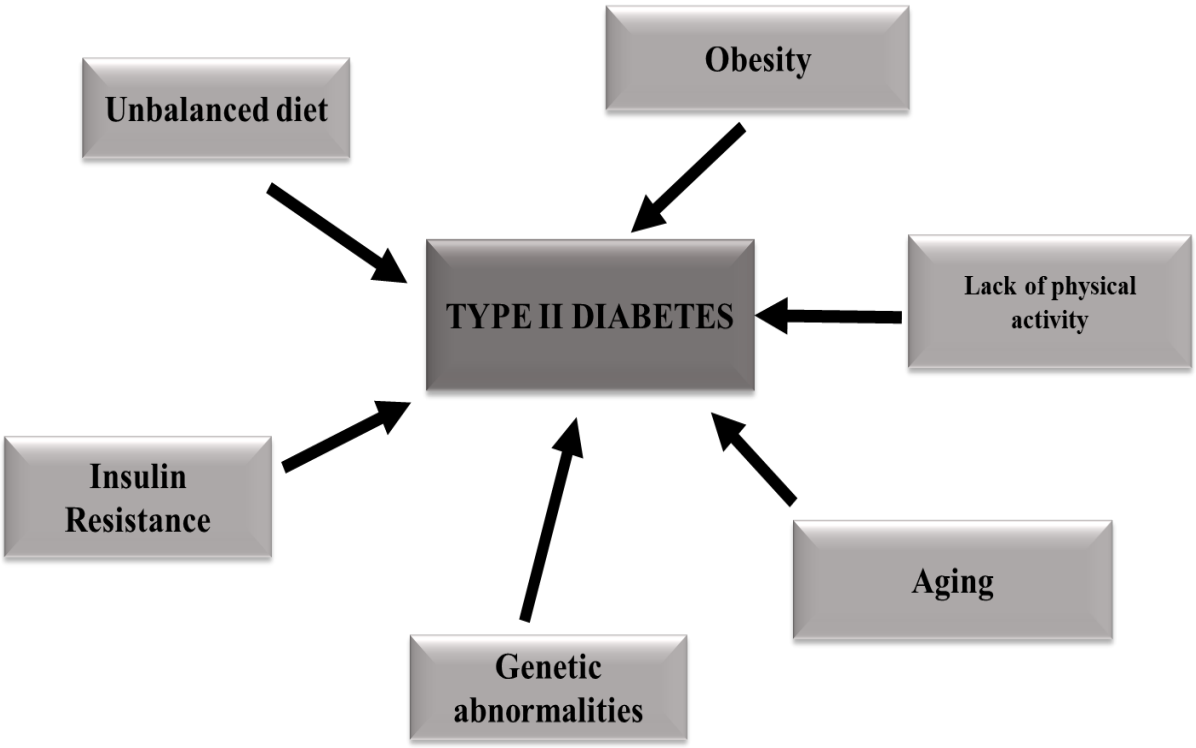


Figure 3. Factors contributing to the development of Type II diabetes mellitus.

1.1.5. Other forms of Diabetes Mellitus

Some forms of diabetes are more common than others, gestational diabetes (GDM) is defined as glucose intolerance with onset of pregnancy and it has become one of the global health concerns in pregnant women, GDM is diagnosed in the second or third trimester of pregnancy (Mishra et al., 2018). Overweight women, women who have had GDM before or have a family history of diabetes are at an increased risk of developing gestational diabetes. GDM left untreated can result in problems with the fetus such as respiratory problems and low blood sugar levels.

GDM is associated with increased risks of maternal and fetal complications (Nien et al.,2007; Morampudi et al., 2017). Fetal macrosomia is a common complication of diabetes in pregnancy it is caused by fetal hyperinsulinemia that occurs as a physiological response to maternal hyperglycemia. Women pregnant with macrosomic fetuses are at increased risk of preeclampsia, labor abnormalities, severe perineal lacerations, risks of preterm birth and cesarean section. The fetus is at risk of stillbirth, intracranial hemorrhage, shoulder dystocia and malformations (Nien et al., 2007). The new born baby is at risk of developing hyperbilirubinemia, hypocalcemia, hypoglycemia, hypomagnesemia, polycythemia vera and neonatal cardiomyopathy (Morampudi et al., 2017). Later in life, the baby may be at an increased risk for obesity and T2DM (Mishra et al., 2018).

Maturity Onset Diabetes of the Young (MODY) is a rare form of diabetes linked with family history (Gerber et al., 2003). It is inherited in the form of an autosomal dominant trait, which is normally a result of mutations in the glucokinase gene on chromosome 7p; glucokinase is the main enzyme of glucose metabolism in the pancreas and the liver (Baynes, 2015). MODY is characterized by a slow onset of symptoms, the absence of obesity, lack of ketosis, and no evidence of β-cells autoimmunity (Ozougwu et al., 2013). There are five different forms of MODY. MODY 1, MODY 3 and MODY 5 are caused by mutations in the hepatocyte nuclear

transcription (HNF) 4α, HNF-1α and HNF-1β factors respectively. These transcription factors are expressed in the liver but also in other tissues including the pancreatic islets and the kidney. They affect islet development or the expression of genes important in glucose-stimulated insulin secretion and the maintenance of β-cells mass (Baynes, 2015).

Individuals with MODY 2 have mutations in the glucokinase gene that plays a key role in glucose metabolism and insulin secretion and MODY 4 is a rare variant form caused by mutations in insulin promoter factor (IPF) 1, which is a transcription factor that regulates pancreatic development and insulin gene transcription together with other genes involved in glucose metabolism (Gerber et al., 2003).

1.1.6. Complications associated with diabetes

There are various complications resulting from T1 and T2 DM affecting various parts of the body, these complications can be microvascular complications (small vascular injury) affecting the retina, kidneys and nerves and macrovascular complications (injury to large blood vessels) affecting the heart, brain and veins (Barret et al., 2017) (Table 1). The mechanisms by which some of these complications develop is similar to that of people who do not have diabetes but diabetic patients have accelerated development due to specific risk factors (Guthrie and Guthrie, 2004) (Table 1).

Table 1. Complications, risk factors & interventions in Microvascular and macrovascular complications of diabetes.

MICROVASCULAR			
	Risk Factors	**Screening Methods**	**Therapeutic Interventions**
Retinopathy	➢ Hyperglycaemia ➢ Hypertension ➢ Dyslipidemia ➢ Oxidative stress	➢ Fundal photography ➢ Ophthalmoscopy	➢ Improved glycaemic control ➢ Laser therapy
Nephropathy	➢ Hypertension ➢ Dyslipidemia ➢ Smoking ➢ Oxidative stress	➢ Urinary albumin/creatinine ratio	➢ Improved glycaemic control ➢ Blood pressure decrease
Neuropathy	➢ Hyperglycaemia ➢ Hypertension ➢ Dyslipidemia ➢ Oxidative stress	➢ History & physical examination	➢ Improved glycaemic control
MACROVASCULAR			
Diabetic cardiomyopathy **Stroke** **Peripheral arterial disease**	➢ Hyperglycaemia ➢ Hypertension ➢ Dyslipidemia ➢ Smoking ➢ Oxidative stress	➢ Lipid profile every 5 years ➢ Blood pressure ➢ Echocardiology ➢ Tissue doppler imaging (TDI) ➢ Magnetic resonance imaging (MRI) ➢ Nuclear Imaging	➢ Improved glycaemic control ➢ Blood pressure control

1.1.7. Diagnosis, management and current treatment for diabetes mellitus

Blood glucose criteria is used to diagnose diabetes either by fasting blood glucose levels (FBG) (normal 5.6 mmol/L), (prediabetes 5.6-6.9 mmol/L) and (diabetes ≥7.0 mmol/L) or random blood glucose levels (normal <11.1 mmol/L) and (diabetes ≥11.1 mmol/L) or the 2 hours blood glucose (2h-BG) value after 75 g oral glucose tolerance test (OGTT) whereby (normal <5.6 mmol/L),(prediabetes 7.8-11.0 mmol/L) and (diabetes ≥ 11.1 mmol/L) (American Diabetes Association, 2018). There are various medications approved by the FDA used in the management of diabetes (Gourgari et al., 2017) (Table 2).

Table 2. Antidiabetic drugs approved by the FDA (Gourgari et al., 2017).

CLASS	MEDICATION (DRUG)	CLINICAL EFFECTS	SIDE EFFECTS
Biguanides	➢ Metformin	➢ Reduces insulin resistance. ➢ Increases skeletal muscle glucose uptake.	Diarrhoea, metallic after taste, nausea
Glucagon-like peptide-1(GLP-1) receptor agonist	➢ Exenatide	➢ Increases secretion of insulin from the pancreas, delays gastric emptying. ➢ Decreases glucagon release after meals.	Nausea, diarrhoea, vomiting
Alpha-glucosidases inhibitors	➢ Acarbose ➢ Miglitol	➢ Decreases digestion and absorption of glucose in the intestines.	Bloating and flatulence
Thiazolidinediones (TZD) (PPAR γ - Agonist)	➢ Pioglitazone ➢ Rosiglitazone	➢ Increase insulin sensitivity of the body cells ➢ Reduces gluconeogenesis in the liver. ➢ Increases GLUT4 expression.	Water retention, weight gain, increased risk of bladder cancer and increased risk of non-fatal heart attack
Sulfonylureas	➢ Glimepiride ➢ Gliclazide ➢ Glyburide	➢ Insulin secretagogues.	Hypoglycaemia (low blood sugar)
Meglitinides	➢ Repaglinide ➢ Nateglinide	➢ Stimulate the pancreas to produce more insulin.	Hypoglycaemia (low blood sugar)
Dipeptidyl-peptidase-4 (DPP-4) inhibitors	➢ Saxagliptine ➢ Sitagliptine	➢ Increases secretion of insulin by the pancreas. ➢ Decreases glucagon release after meals. ➢ Block the degradation of GLP-1, increasing its half-life.	Pharyngitis, headache
Sodium glucose cotransporter 2 (SGLT2) inhibitors	➢ Dapagliflozine ➢ Empagliflozine ➢ Canaglifozine	➢ Inhibits glucose re-absorption in the glomerulus leading to glycosuria.	Genital and urinary infections, frequent urination and diabetic ketoacidosis.

1.2 OXIDATIVE STRESS & HYPERGLYCEMIA

1.2.1 Hyperglycemia induced oxidative stress

Oxidative stress is defined as an imbalance between ROS production and antioxidant defence mechanisms (Shin et al., 2001; Stykal et al., 2017; Poljšak and Fink, 2014). ROS have the ability to damage DNA, lipids and other biomolecules in the body. They can also accelerate the development of diseases such as cardiovascular diseases, cancer and diabetes (Valko *et al.,* 2007). In diabetes, free radicals are produced from uncontrolled hyperglycaemia through a number of mechanisms such as the initiation of the polyol pathway, autoxidation of glucose and enhanced formation of intracellular Advanced- Glycation- End -Products (AGEs) (Giacco and Brownlee 2010). Weight loss, polydipsia, polyuria blurred vision are some of the symptoms associated with diabetes (ADA, 2016).

1.2.2 The Polyol pathway

This polyol pathway is a major contributor of hyperglycaemia-induced oxidative stress, because about 30% of glucose enters this pathway (Cheng and González, 1986) (Fig. 4A). Activation of the aldose reductase pathway increases the rate of NADH/NAD^+ altering the redox potential therefore increasing levels of superoxide ions and decreasing nitric oxide (NO) levels leading to oxidative stress that later results in endothelial dysfunction, hypercoagulability and inflammation implicated in cardiovascular dysfunction (Koya *et. al.,* 1998). This initially starts by a conversion of NAD^+ to NADH by sorbitol dehydrogenase increasing the concentration of NADH which is a substrate for NADH oxidase which can produce large amounts of superoxide radicals in the presence of oxygen and other related oxidant species (Griendling *et. al* 2000) (Fig. 4A). If these superoxide radicals are produced in large amounts, they can form peroxynitrite when they react with NO, thereby causing a decline

in nitric oxide levels (Beckman and Koppenol, 1996; Pacher *et al.,* 2007). NO levels play a role in the formation of other oxidants, if levels of NO are normal other oxidants will not be formed (Fig. 4 A). The rise in glucose results in an increased level of fructose, this excess glucose is transferred into sorbitol by aldose reductase using NADPH as a cofactor. Sorbitol dehydrogenase oxidizes sorbitol to form fructose and produce NADH. The increase in fructose concentrations can activate protein kinase C (PKC) whereby glyceraldehyde-3-phosphate dehydrogenase is inhibited by poly (ADP-ribose) polymerase due to increased ratio of $NADH/NAD^+$, this then favours the production of diacylgycerol which initiates PKC activation (Du *et. al.,* 2003) (Fig. 4 A). PKC participates in redox imbalance thereby causing oxidative stress and a decrease in NO resulting in an inflammatory response causing endothelial dysfunction later leading to cardiovascular dysfunction (Kim et al., 2013) (Fig.4 A).

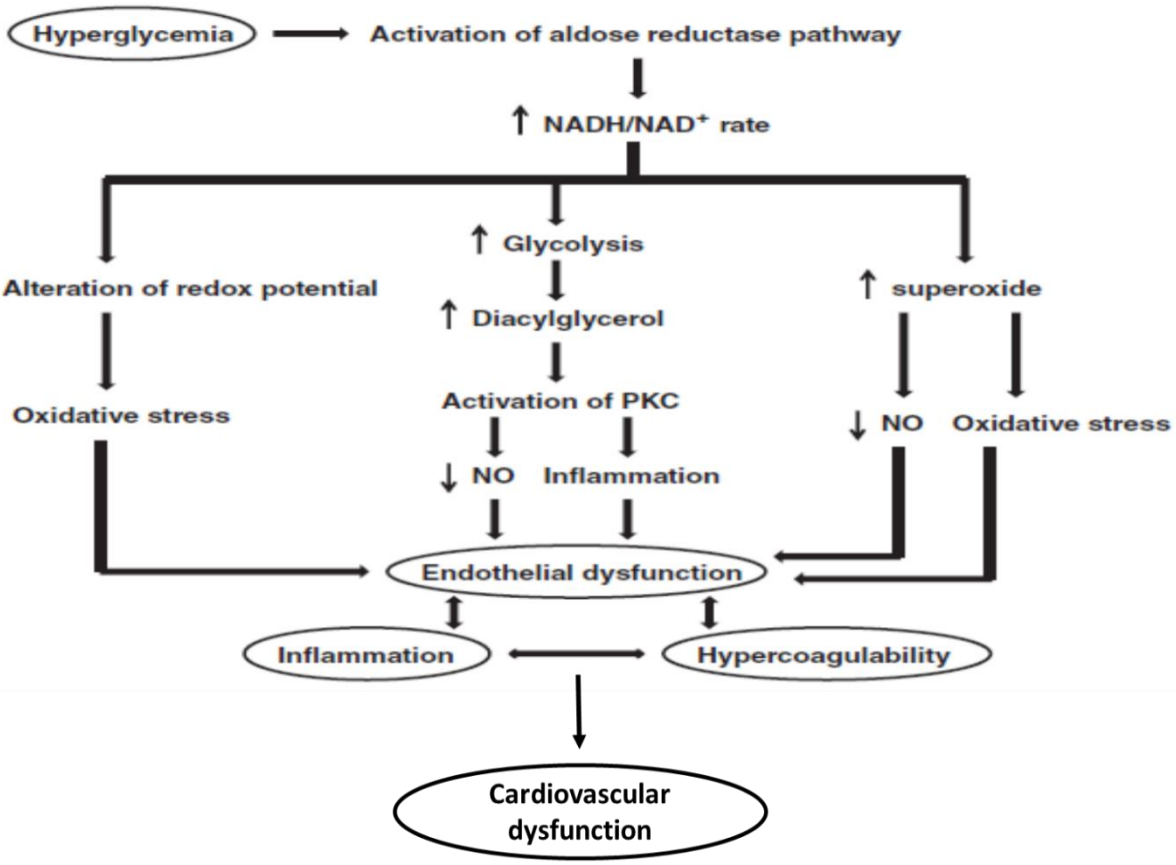


Figure 4 A. Mechanism by which hyperglycaemia leads to oxidative stress, inflammation and cardiovascular dysfunction through activation of polyol pathway (aldose reductase pathway) **(**Adapted and modified from Domingueti et al., 2016).

1.2.3 Autoxidation & Protein glycation

Elevated levels of glucose (hyperglycaemia) lead to the increased production of products such as hydroxyl radicals, hydrogen peroxide, and superoxide (Wolff and Dean 1987) (Fig. 4 B). All these products bind and damage proteins and lipids by fragmentation as they accelerate the formation AGEs (Baynes, 1991; Zhao, 2001) (Fig. 4 B). Protein glycation is a non-enzymatic reaction in glucose and amino acid groups of proteins increasing the production of ROS leading to oxidative stress and decreased NO resulting into endothelial dysfunction, hypercoagulability and inflammation (Aroson and Rayfeild, 2002) (Fig. 4 B). ROS are also are produced when the AGEs interact/bind with their cell receptors RAGEs (Creager et al., 2006) (Fig. 4 B). AGEs produced in this way cause cellular damage by several mechanisms, proteins changed by these precursors attach to the AGE receptors and cause glucotoxicity in cells like endothelial cells, monocots/macrophages (Wright et al., 2006). Once these intracellular ROS are generated, it further activates the nuclear factor-κB (NF-kB) that increases the production of pro-inflammatory cytokines and pro-coagulant molecules resulting into endothelial dysfunction and later cardiovascular dysfunction (Basta et al., 2002; Esposito et al., 2002) (Fig. 4 B).

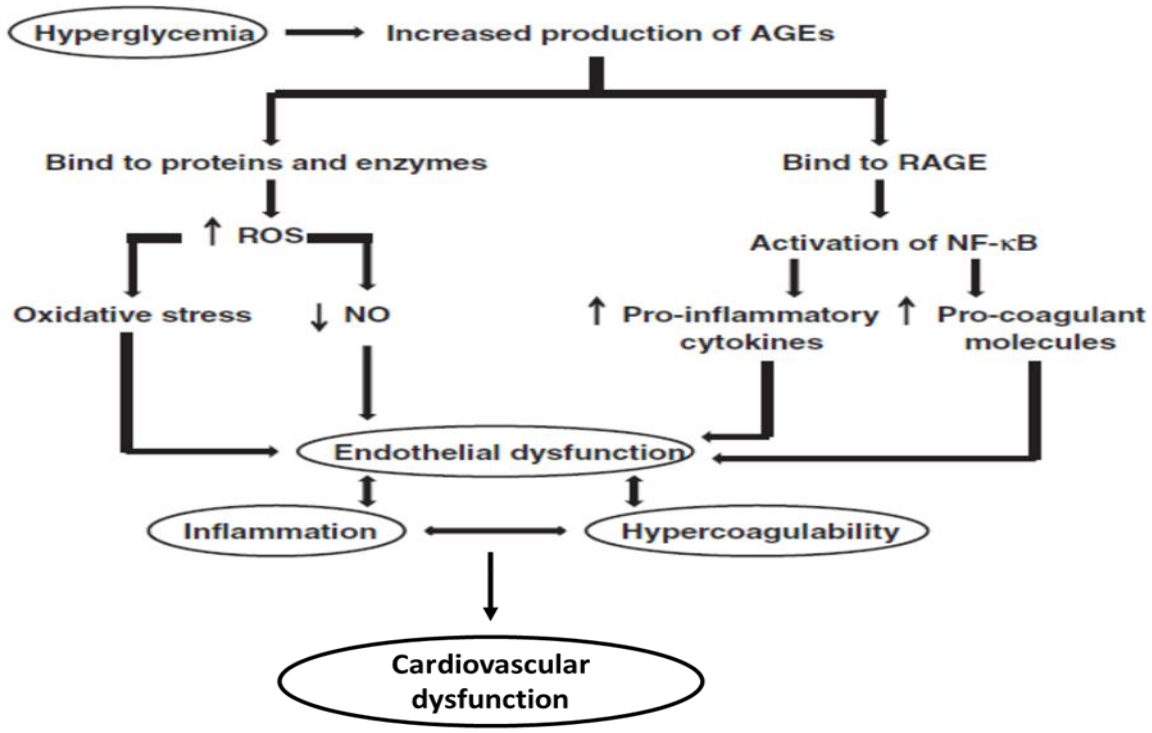


Figure 4 B. Mechanism by which hyperglycaemia leads to oxidative stress, inflammation and cardiovascular dysfunction through the production of AGEs **(**Adapted and modified from Domingueti et al., 2016).

1.3 DIABETIC CARDIOMYOPATHY, INFLAMMATION & SIGNALLING PATHWAYS

1.3.1 Diabetic cardiomyopathy

Diabetes increases the risk of developing cardiovascular diseases (CVDs) such as DCM, diabetic dyslipidaemia, coronary artery disease, ischemia and heart failure (Jia et al., 2018). It has been recorded that approximately 18 million diabetic patients die from CVDs every year (Danaei et al., 2013). DCM affects the myocardium and is characterised by myocardial fibrosis, dysfunctional remodelling, hypertrophy and cardiac stiffness (Jia et al., 2018). DCM is one of

the major cardiac complications in diabetic patients resulting from hyperglycaemia-induced oxidative stress, causing cardiac diastolic dysfunction and systolic dysfunction, later leading to heart failure (Jia et al., 2018; Beran and Yudkin, 2010). High levels of ROS lead to oxidative damage by interacting with lipids and proteins within the myocardium thus affecting the functioning, growth and repairing abilities of cardiomyocytes (Beran and Yudkin, 2010).

1.3.2 Pathophysiology of diabetic cardiomyopathy

Hyperglycemia, fatty acids and IR activate multitude cellular mechanisms in the myocardium and when the cardiomyocytes fail to assimilate glucose it is converted into AGEs, hexosamine and polyols which activate pro-inflammatory and pro-oxidant responses (Milwidsky et al., 2015) (Fig. 5). As a compensatory mechanism fatty acid transporter are increased since ATP generation relies on fatty acid degradation. The free fatty acids (FFA) are taken up in large amounts and some saturate β-oxidation and accumulate in the cytosol as toxic secondary metabolites such as ceramides, diglycerides (DAG) and ROS (Lorenzo-Almoros et al., 2017) (Fig. 5). Lipo/gluco-toxicity and lack of ATP production promote calcium imbalance between the sarcoplasmic reticulum and cytosol, reducing actin-myosin complexes, triggering chronic inflammation, fibrosis and contractile dysfunction. FFA also bind to the Peroxisome Proliferator- Activated receptors (PPAR) to upregulate mitochondrial beta-oxidation enzymes which produce ROS and non-efficient ATP, triggering apoptosis and mitochondrial dysfunction (Bugger et al., 2014). All these stimuli promote the expression of miRNAs, pro-fibrotic and pro-hypertrophic factors which may have paracrine and autocrine effects on the adipocytes and myofibroblasts (Lorenzo-Almoros et al., 2017) (Fig. 5).

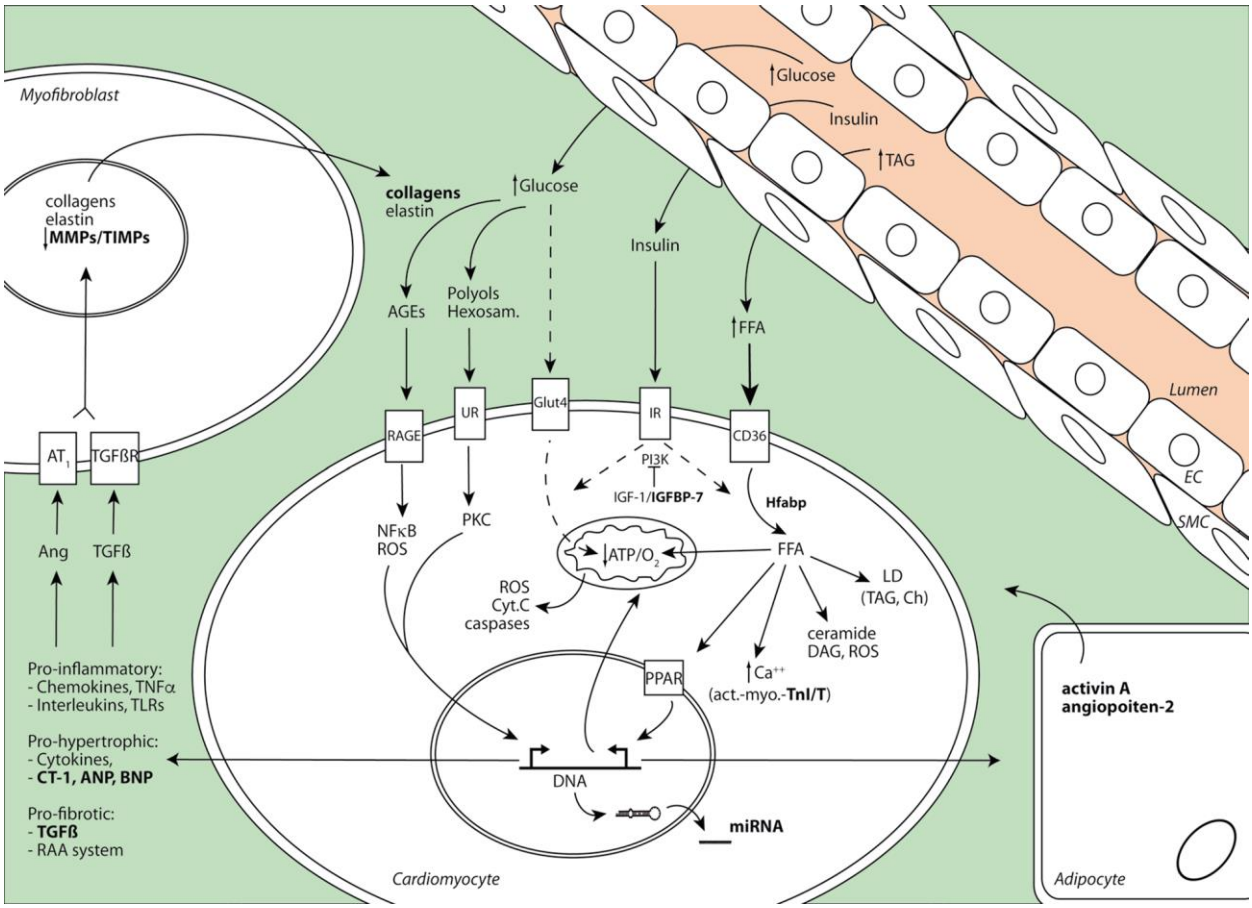


Figure 5. Pathophysiology of diabetic cardiomyopathy (Lorenzo-Almoros et al., 2017). (**UR** Unspecific receptors, **TLRs** Troll like receptors, **IGFBP-7** Insulin-like growth factor binding protein-7, **TGFβ** Transforming growth factor beta, **IGF-1** Insulin growth factor -1, **TnI** Troponin I/T, **CT-1** Cardiotropin-1, **ANP** Atrial natriuretic, **BNP** Brain natriuretic peptide)

1.3.3 Developmental stages of diabetic cardiomyopathy

DCM occurs through progressive metabolic disturbances. In the early stages, structural and functional defects are apparent (Fang et al., 2004). DCM is clinically asymptotic in its early stages due to hyperglycaemia which increases the levels of antioxidants in the heart muscle as a compensatory mechanism (Boudina and Abel, 2010). The early stages of DCM are characterised by increased stiffness and Left Ventricular (LV) diastolic pressure. The

underlying pathological factors include cardiac insulin resistance, increased FFA levels, inflammation, imbalanced calcium levels and oxidative stress (Jia et al., 2018) (Fig. 6).

The later stages of DCM show visible symptoms and antioxidant depletion leaving the myocardium exposed to free radicals (Gilica et al., 2017; Boudina and Abel, 2010). The complications in the later stages of DCM include left ventricular hypertrophy (LVH), impaired diastolic filling, arterial filling and relaxation, cardiac remodelling and fibrosis and as the DCM stages progress ischemic heart diseases develop leading to heart failure (Jia et al., 2018) (Fig. 6).

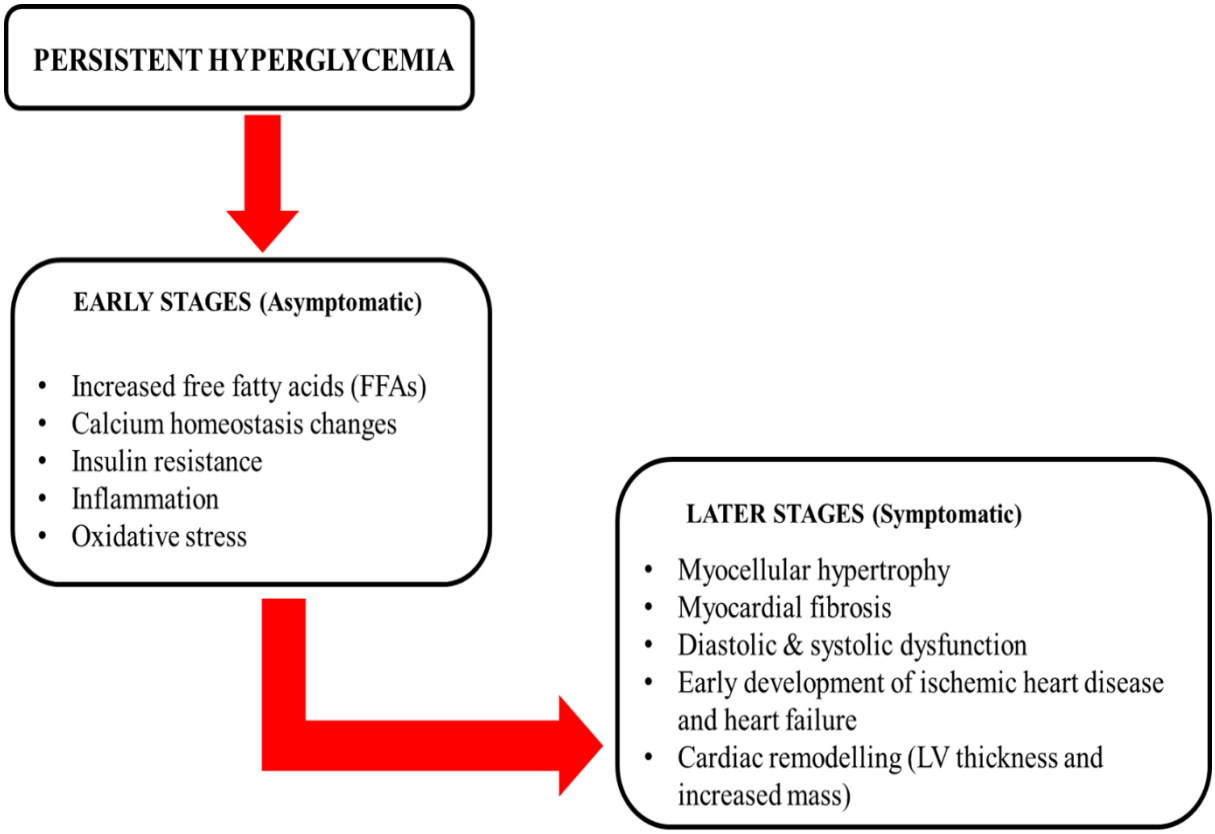


Figure 6. Different stages of the development of diabetic cardiomyopathy (Fang et al., 2004).

1.3.4 Other diabetes associated cardiovascular complications

1.3.4.1 Diabetic dyslipidaemia

Hyperglycaemia and defects in insulin action result in changes in plasma lipoproteins in diabetic patients, leading to increased risk of developing DCM compared to non-diabetic individuals (Adiels et al., 2008). High density lipoproteins (HDL) assists the body in the removal of excess cholesterol preventing atherosclerosis development (Wang et al., 2018). Whereas low density lipoproteins (LDL) favours the build-up of cholesterol resulting into the clogging of arteries causing atherosclerosis (Basta et al.,2004). Dyslipidaemia is defined by overproduction of very low-density lipoprotein (VLDL) particles, lower levels of HDL and higher levels of LDL and triglycerides (TG) (Mulvihill et al., 2009; Schofield et al., 2016).

1.3.4.2 Atherosclerosis and Coronary heart disease

Type 1 & Type 2 diabetes both lead to the development of coronary heart disease, stroke, microvascular disease and accelerated atherosclerosis which have become the main cause of morbidity and mortality in diabetic patients worldwide (Basta et al., 2004). Atherosclerosis affects the functioning of multiple arteries in the body such as coronary arteries, brain arteries, renal arteries and carotid arteries (Orhan et al., 2015; Wang et al., 2018). Atherosclerosis is a chronic inflammatory condition in the blood vessels resulting in the formation of atheromatous plaques in the endothelial lining within the blood vessels causing stiffness and dysfunction of the endothelial cell lining (Chowdhury et al., 2010; Ali et al., 2010). Hypercholesterolemia is considered as one of the major contributors to atherosclerosis, by causing damages to the permeability of the endothelium which allows migration of lipids, such as LDL particles (Biddinger et al., 2008; Bergheanu et al., 2017). Atherogenic dyslipidaemia is one of the major causes of coronary atherosclerosis and coronary heart disease (CHD) and is characterised by

increased plasma concentrations of TG, very low density lipoproteins (VLDL), cholesterol – rich LDL and low levels of high density lipoproteins (Mulvihill et al., 2010).

1.3.4.3 Ischemia and Heart failure

Hypertension, diabetes and hypercholesterolemia increase the risk of ischemia-reperfusion (I-R) injury and heart failure (Collard and Gelman, 2001). Myocardial ischemia is caused by reduced blood flow to the coronary arteries, due to atherosclerosis (Han et al., 2009). The narrowing of blood vessels or plaque build-up cause insufficient flow of blood resulting in decreased cellular oxidative phosphorylation leading to failure in producing ATP and phosphocreatine (Bai et al., 2014) (Aziz and Yadaz, 2016). This affects the functioning of the ionic pump allowing calcium, sodium and water uptake, this cellular oxidative imbalance causes accumulation of ROS (Crossman, 2004) (Kara et al., 2014).

1.3.5 Inflammation associated with diabetes

Inflammation is characterized by swelling, redness and warmth in response to metabolic disturbances (Scott et al., 2004). The most dominant pro-inflammatory mediators responsible for initiating inflammation in various organs in the body include tumour necrosis factor (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6) and other pro-inflammatory cytokines and chemokines (Akash et al., 2018).

Diabetes and hyperglycaemia alter plasma concentrations of pro-inflammatory markers following oxidative stress (Hansen et al., 2010; Brownlee, 2005). Diabetes leads to increased production of a non-specific C-reactive protein (CRP) (Fröhlich et al., 2000). Its production by the liver is increased during inflammation or acute infection, and it has been suggested that type II diabetes causes acute inflammation where there is increased release of adipokines (Schultz and Arnold, 1990; Pickup and Crook, 1998). Increased interlukin 6 and CRP

sometimes predict diabetic complications (Su et al., 2011). Increase in these cytokines especially TNF- α does not only affect insulin action but may also cause beta-cell failure (Moreli et al., 2015; Domingueti et al., 2016).

1.4 PHARMALOGICAL EFFECTS OF NARINGENIN

1.4.1 Medicinal plants vs Modern medicines

Medicinal plants have bioactive compounds which can be extracted from morphological parts and used as medicinal agents. Medicinal plants play a vital role in ethnopharmacological health systems all over the world **(**Hosseinzadeh et al., 2015). They are considered as rich sources of ingredients that can be used in the development and production of drugs. They serve as reservoirs for bioactive compounds that are vital to human health (Mosa et al., 2015). Herbal medications can be suitable alternatives in combination with other fractional replacements for conventional medicines (Zhao et al., 2015).

According to World Health Organisation, about 80% of the world's population is using medicinal plants and currently there is increased interest in the discovery of plant-derived antidiabetic drugs especially in Africa (Piero et al., 2012). Herbal treatments are used mostly in developing countries since they are cheaper (Wadkar et al., 2008). *Galega officinalis* L. (Fabaceae) (also called French lilac) was the first medicinal plant to be described with clear antidiabetic properties, the plant has been used to treat diabetes ever since the Middle Ages and synthetic products made from this plant include metformin (Bedekar et al., 2010).

1.4.2 Naringenin

Phenolic compounds and flavonoids are known as secondary metabolites of plants, they have an aromatic ring consisting of at least one hydroxyl group (Tungmunnithum et al., 2018). Over 8000 phenolic compounds occur naturally as substances from plants and a majority of these

compounds are classified as flavonoids occurring as aglycone, glycosides and methylated derivatives (Yao et al., 2004). Flavonoids are commonly found in vegetables and fruits they are characterized by a benzo-y-pyrone structure and vary in the structure of heterocyclic oxygen ring but all have the same C_6-C_3-C_6 carbon skeleton (Corradini et al., 2011) (Fig. 7).

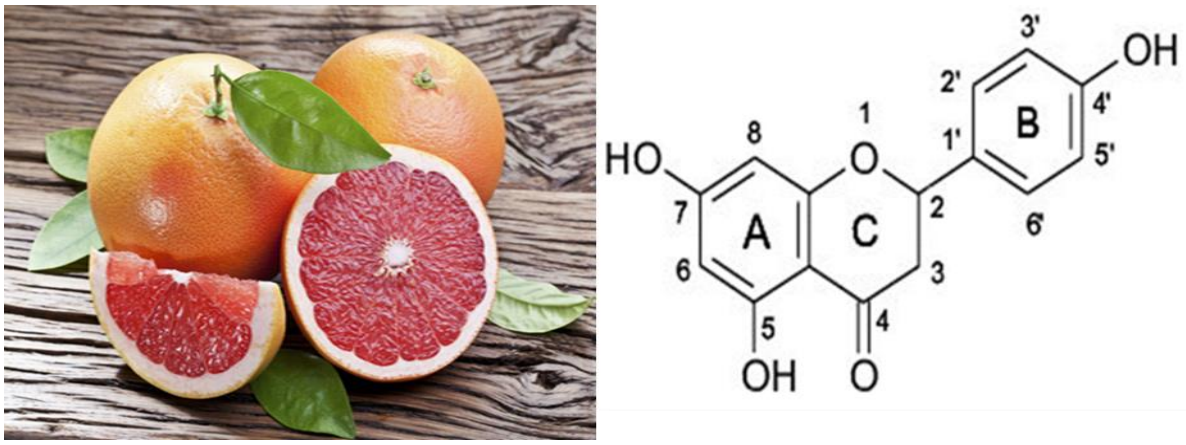


Figure 7. Whole grape fruit and the structure of naringenin (Corradini et al., 2011).

Flavonoids include flavones, flavanones, flavonols, isoflavones, anthocyanidins, and flavanols. They are regarded as potent antioxidants, free radical scavengers, metal chelators and inhibitors of lipid peroxidation (Ramprasath et al., 2014; Cook and Samman, 1996). By scavenging ROS, flavonoids limit the perpetuation of oxidative stress and prevent the formation of ROS (Cavia-Saiz et al., 2010). Naringenin (4, 5, 7-trihydroxyflavonon) is a flavonoid abundant in citrus fruits, and the skin of tomatoes (Fig.7).

Naringenin is an aglycone of naringin following hydrolysis by naringinase. Naringin is hydrolyzed by α-L-rhamnosidase of naringinase to rhamnose and prunin, the prunin formed is then hydrolyzed by β-D-glucosidase forming naringenin and glucose (Ribeiro et al., 2008) (Fig. 8).

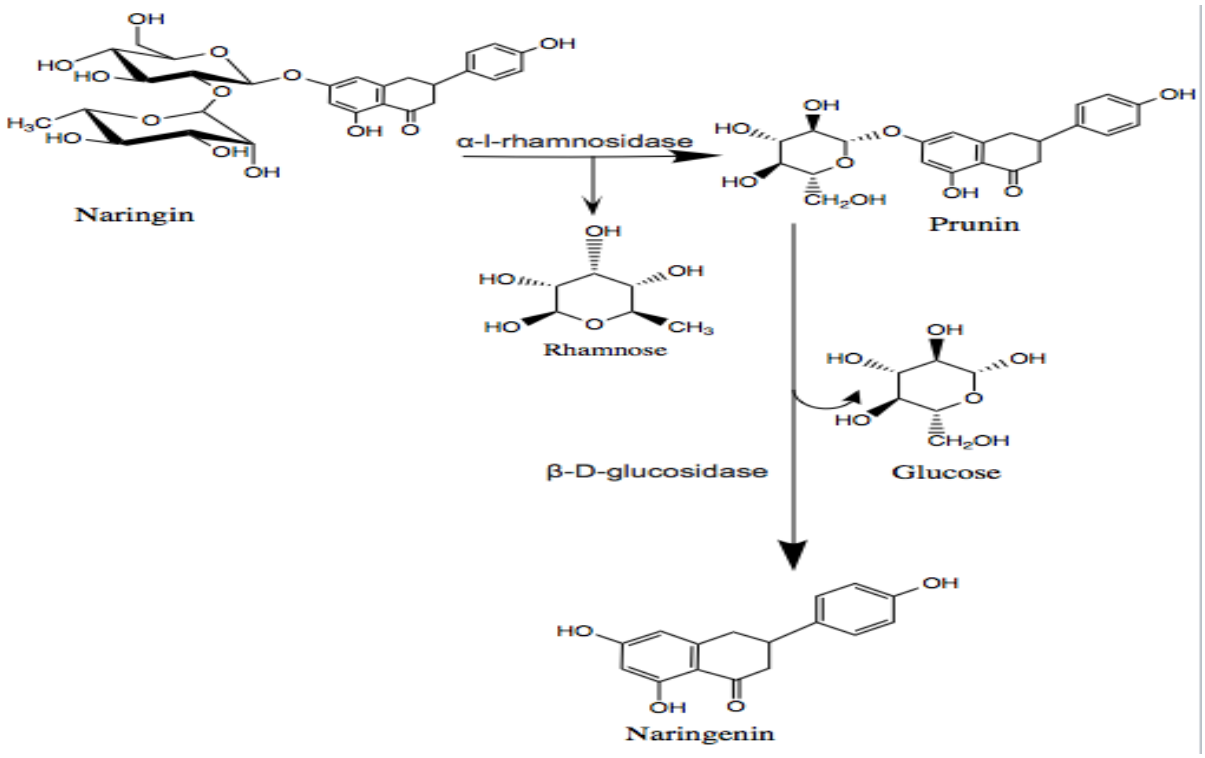


Figure 8. Enzymatic hydrolysis of naringin to produce naringenin (Thomas et al., 1958, Ribeiro et al., 2008)

Naringenin possess pharmacological effects such as anti-hypersensitive, hypolipidemic, anti-diabetic, anti-fibrotic, hepatoprotective and cardioprotective properties (Ramprasath et al., 2014) (Fig. 9). Naringenin increases the expression of PPAR-γ and promotes peripheral glucose uptake by increasing GLUT-4 translocation (Mahmoud and Hussein, 2016) (Fig. 9). As a result, naringenin as a dietary supplement could mitigate hyperglycemia-induced oxidative stress which has been implicated in the development of DCM.

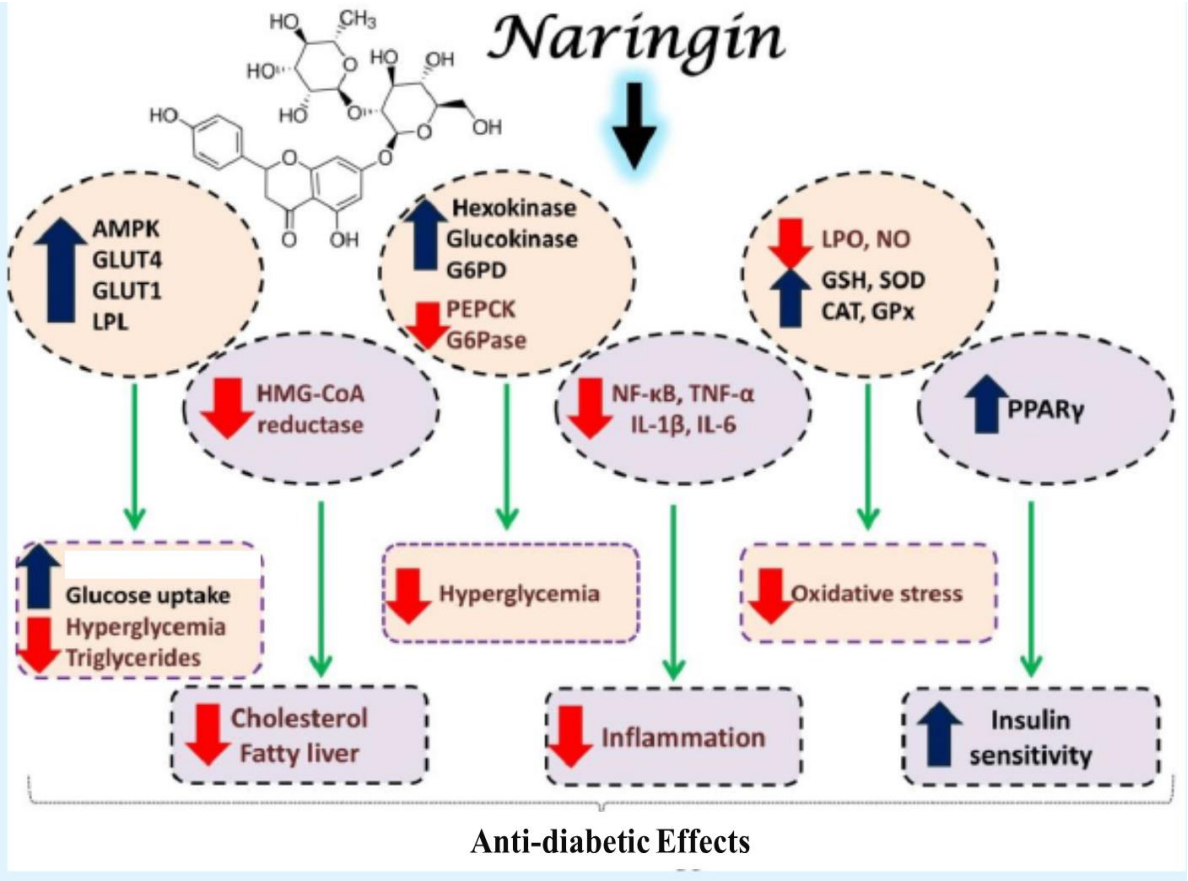


Figure 9. Anti-diabetic mechanism of naringenin (Mahmoud and Hussein, 2016). (**NO** Nitric oxide, **GSH-GPx** Glutathione peroxidase, **SOD** Superoxide dismutase, **LPO** Lipid peroxidation, **CAT** Catalase, **PPARγ** Peroxisome proliferator gamma, **IL** Interleukin)

1.5 HYPOTHESIS, AIM & OBJECTIVES

1.5.1 Hypothesis

Rats with STZ induced diabetes treated with naringenin experience less cardiac oxidative stress than untreated rats.

1.5.2 Aim

To investigate the effects of naringenin on oxidative stress parameters in cardiac muscles of diabetic rats.

1.5.3 Objectives

- To determine the effect of naringenin on the different biochemical (enzymes) and biological (cytokines) parameters that help to explain the oxidative stress that leads to cardiomyopathy.
- To compare the effect of naringenin on heart/body weight ratio and left ventricular weight/heart weight ratio of normal and diabetic rats treated with naringenin and insulin.

CHAPTER 2: MATERIALS & METHODS

2.1 EXPERIMENTAL

2.1.1 Materials and methods

All the Enzyme Linked Immuno-Sorbent Assay (ELISA) and colorimetric assay kits were purchased from Biocom Africa Diagnostics, South Africa (SA). Unless otherwise stated, all the chemicals and reagents were purchased from Sigma Aldrich ™ SA. The glucometer, glucometer test strips and insulin were purchased at the local pharmacy, Durban, SA.

2.1.2 Reagents and chemicals

- Sodium citrate dehydrate
- pH meter (Crison Basic 20, pH-meter, crison instruments, SA)
- Sodium chloride
- DiSodium hydrogen phosphate
- Glucometer and glucometer strips (OneTouch Select®; Lifescan Inc., Milpitas, California, USA)
- EZ 400 Microplate Reader (Biochrom® Ltd, Cambridge, UK)
- Z383k Hermle centrifuge (Labortechnik, Germany)

2.1.2.1 Naringenin preparation

Commercial powdered naringenin extract (3.0 g) was dissolved in 100 ml of distilled water due to its poor solubility it formed a suspension.

2.1.2.2 Preparation of citrate buffer (0.1 M, pH 4.5) solution

Citric acid crystals (1.92 g) and sodium citrate dehydrate (2.94 g) were dissolved in 100 ml of distilled water.

After these two separate solutions were prepared, they were mixed together and the pH was adjusted to 4.5 pH.

2.1.2.3 Preparation of streptozotocin solution

STZ (504 mg) was dissolved in 28 ml of citrate buffer (18 mg/ml) and administered at 60 mg/kg BW to the rats.

2.1.2.4 Preparation of normal saline (0.9 % NaCl) solution

Sodium chloride (0.9 g) was dissolved in 100 ml of distilled water.

2.1.2.5 Preparation of D-glucose

This solution was prepared by dissolving (18.75 g) of D-glucose anhydrous in 50 ml normal saline (0.38 g/ml).

2.1.2.6 Preparation of phosphate buffered saline (PBS)

This solution was prepared by dissolving (8.0 g) sodium chloride, (200 mg) of potassium chloride, diSodium hydrogen phosphate (1.44 g) and (240 mg) of potassium dihydrogen phosphate into distilled water. The volume was brought up to one liter (1 L) and pH was adjusted to 7.4.

2.1.2.7 Preparation of Lysis buffer

Tris-HCL (50 mM) and EDTA (2 mM) were added into distilled water and brought up to 100 ml, pH was adjusted to 7.4.

2.1.3 Experimental animals

Male Wister rats (250-300g) were obtained from the Biomedical Research Unit (BRU) of the University of Kwa-Zulu Natal (UKZN), Durban, SA and they were divided into six groups (n=7). The rats were given one week to acclimatize to their new environment before the study commenced. Temperature at (23-25°C), humidity (55-60%) and 12-hour day-light/dark cycle

throughout the study period were maintained. The rats were given ad libitum access to food and water and they were treated in humane manner following animal treatment guidelines provided by the Animal Research Ethics committee of the UKZN, Ethical clearance number: AREC /067/018M.

2.1.4 Experimental design

Groups I and IV were orally treated with 3.0 ml/ kg Body Weight (BW) of distilled water and 100 mg/kg BW of naringenin in distilled water, respectively. Groups II, III, V and VI were made diabetic by a single intraperitoneal injection of 60 mg/kg BW streptozotocin and similarly treated with naringenin except group VI which was treated with insulin 2.0 U/kg BW (Table 3). Three days after STZ injection, the rats were fasted overnight and the development of diabetes was confirmed using tail pick method to check blood glucose levels using the glucometer and glucometer strips. Rats with fasting blood glucose (FBG) of more than 11.0 mmol/L were included in the study and those with FBG bellow were excluded from the study, the rats were fed and treated for a period of 56 days (Fig. 10). The animals were weighed every week and water intake was measured every day.

On day 56 of the study glucose tolerance tests (GTT) were carried out by overnight fasting followed by intraperitoneal injection of D-glucose (3.0 g/kg BW) dissolved in 0.9% normal saline. Blood droplets mounted on glucometer strips were collected via tail pricks and then glucose levels measured at time intervals of 0, 30, 60, 90, and 120 minutes, by glucometer machine. Blood glucose time plots were done and the Area Under the Curve (AUC), expressed as AUC units [time (min)x blood glucose (mM)].

On day 57, the animals were euthanized by isoflurane overdose, blood samples were collected using cardiac puncture in heparinized tubes then separated into plasma and stored at -20˚C for further biochemical analysis. The hearts were quickly excised and washed in ice-cold

phosphate buffered saline (PBS). LVs were dissected and weighed. The tissues were snap frozen in liquid nitrogen and stored at -80℃ for further analysis (Fig. 10). The LV tissue homogenates were prepared by cutting (100 mg) of tissue and it was inserted into a microcentrifuge tube and 500 µl of lysis buffer was added and an electric homogeniser was used to homogenise the tissue, the tissue samples were immediately snap frozen in -80℃.

Table 3. Animal treatment protocol

(Group III received STZ treatment before naringenin treatment and group V received naringenin treatment before STZ treatment and naringenin treatment again.)

Groups of rats	**Water** (3.0 ml/kg BW)	**Streptozotocin** (60 mg/kg BW)	**Naringenin** (100 mg/kg BW)	**Streptozotocin** (60 mg/kg BW)	**Naringenin** (100 mg/kg BW)	**Insulin** (2 U/kg BW)
I	**X**					
II		**X**				
III		**X**	**X**			
IV			**X**			
V			**X**	**X**	**X**	
VI		**X**				**X**

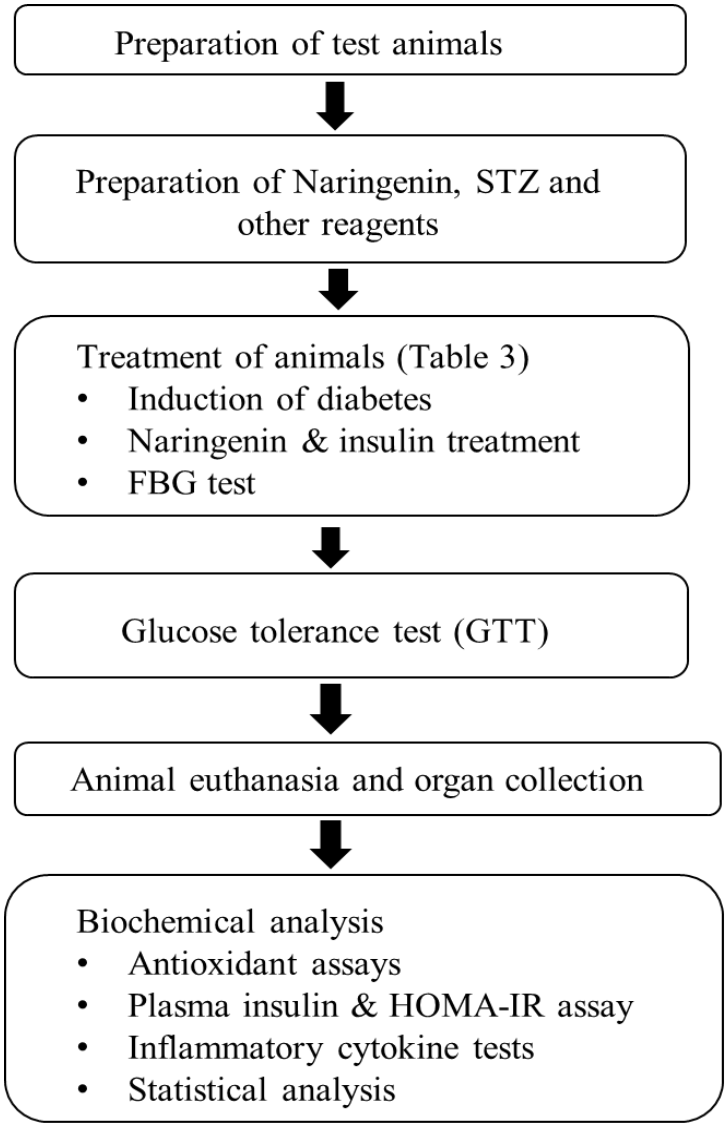


Figure 10. Schematic summary of methodology.

2.1.5 Plasma insulin and HOMA IR

Rat insulin (ELISA) assay was done by using the sandwich-ELISA principle to measure plasma insulin concentrations as per the manufacturer's instructions. In a 96 well plate (100 µl) of the standard and samples was pipetted in triplicates in the wells and the plate was incubated for 90 minutes at 37 ℃. The liquid in the wells was aspirated and (100 µl) of the biotinylated detection

working solution was added, the plate was incubated at 37 ℃ for one hour. The liquid was aspirated again and the plate was washed three times using (350 µl) wash buffer , the plate was blotted dry on absorbent paper, HRP conjugate working solution (100 µl) was added in the wells and incubated for 30 minutes at 37 ℃, the liquid was aspirated and the plate was washed 5 times using the wash buffer. After the final wash (90 µl) of the substrate reagent was added and the plate was incubated for 15 minutes at 37 ℃. The reaction was stopped by the addition of (50 µl) stop solution and optical density (OD) was measured at 450 nm using the EZ 400 microplate reader, fasting plasma insulin is expressed in (µ IU ml/L).

Insulin resistance was calculated using the Homeostasis Model Assessment of Insulin resistance (HOMA-IR) equation (Salgado *et al.*, 2010).

Insulin Resistance $= \frac{\text{FPI x FBG}}{22.5}$, where FPI: Fasting Plasma insulin (µ IU ml/L), and FBG: Fasting Blood Glucose (mM).

2.1.6 Antioxidant Assays

2.1.6.1 Catalase (CAT) Assay

Catalase (CAT) is an antioxidant enzyme and it catalyses the detoxification of hydrogen peroxidase (H_2O_2) (a reactive oxygen species) forming water and oxygen molecule (Kaushal et al., 2018).

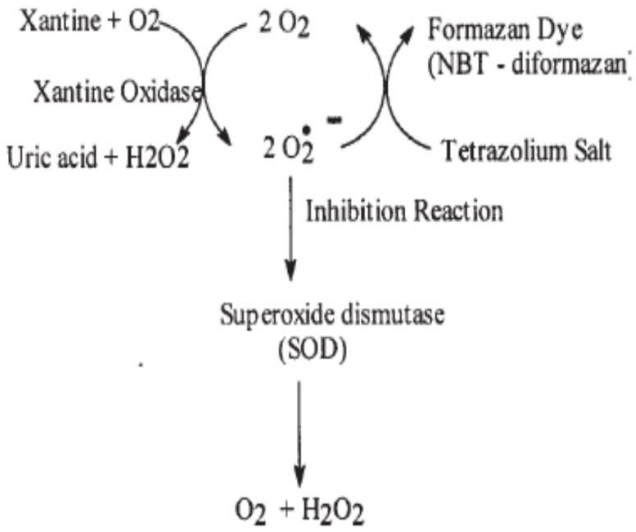
$$2H_2O_2 \xrightarrow{\text{CATALASE}} 2H_2O + O_2$$

The CAT assay kit (Biocom Africa Diagnostics, SA) was used to measure the CAT activity in the tissue homogenate samples of the heart, following the manufacturer's protocol. In this kit, (H_2O_2) reacts with ammonium molybdate used to stop the decomposition reaction above forming a yellowish complex used to calculate CAT activity using the measured OD. The kit

contained reagents such as: buffer solution (1.0 ml), substrate solution (0.1 ml), chromogenic agent (1.0 ml) and clarificant (0.1 ml) ,these reagents were pipetted in appropriate amounts into different tubes separated into control tubes and sample tubes containing the tissue homogenate samples, all the mixtures were incubated for 5 minutes at 37 ℃. Following the protocol, appropriate reagents were added continuously and the mixtures were incubated again for 1 minute at 37 ℃. The tubes containing the mixtures were allowed to stand for 10 minutes at room temperature and OD was measured at 405 nm with 0.5 cm diameter cuvettes using the ThermoSpectronic spectrophotometer. Catalase activity (U/mgprot) was calculated using the following formula: CAT activity (U/mgprot) = (ODControl − ODSample) × (32.5 /1 × the volume of sample) × Dilution factor of sample before tested ÷ Protein concentration of tested sample ($mgprot/mL$).

2.1.6.2 Superoxide Dismutase (SOD) Assay

Superoxide Dismutase (SOD) catalyses the breakdown of superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2) or oxygen (O_2) molecules (Indo et al., 2015). The activity of SOD was measured using the SOD assay kit (Biocom Africa Diagnostics, SA), following the manufacturers protocol. In the SOD assay kit superoxide ions are produced when xanthine and (O_2) are converted into uric acid and (H_2O_2), this reaction is catalysed by xanthine oxidase (XOD). Tetrazolium salt is converted into formation dye by a superoxide anion, superoxide levels are reduced by the addition of SOD into the reaction, this decreases the levels of formazan dye formation. Therefore, in experiments SOD activity is measured as the inhibition percentage of the level of formazan dye production (Tulcan et al., 2013).



In a 96 well plate (20 µl) of the sample (tissue homogenate) was pipetted into the appropriate sample wells. Distilled water (20 µl) was pipetted into the control and blank wells of the plate. Thereafter (20 µl) of the enzyme working solution, enzyme diluent and substrate application solutions were added into the appropriate sample and control wells following the manufacturer's instructions. The mixtures were mixed fully and incubated at 37 ℃ for 20 minutes. Thereafter the OD was measured at 450 nm using the EZ 400 microplate reader (Biochrom Ltd, Cambridge, UK). SOD activity (U/mg prot) was calculated using the formula provided in the kit: Inhibition ratio of SOD (%) = [(A Control-A Blank control) -(A Sample-A Blank sample)/ A Control-A Blank control] ×100%. SOD activity (U/mg prot) = Inhibition ratio of SOD (%) ÷ 50% × (240 µL/20 µL) × Dilution factor of sample before tested ÷ Protein concentration of sample (mgprot/mL)

2.1.6.3 Glutathione Peroxide (GPx) Assay

Glutathione peroxidase (GPx) is an antioxidant that catalyses the breakdown of hydrogen peroxide (H_2O_2). GSH-Px catalyses the reaction of (H_2O_2) and reduced glutathione (GSH) to produce water molecules and oxidised glutathione (GSSG) (Kalpakcioglu and Senel, 2008).

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} 2H_2O + GSSH$$

The amount of GPx was measured using the GPx assay kit (Biocom Africa Diagnostics, SA) following the manufacturer's instructions. Glutathione activity can be calculated by the consumption of reduced glutathione. The appropriate amount of tubes for the samples were used and they were separated into two groups of non-enzyme tubes and enzyme tubes, (0.2 ml) of 1 mmol/L GSH solution was added into the tubes containing the homogenate samples (enzyme tubes) and those without. These tubes were preheated at 37 ℃ in a water bath for 5 minutes. Thereafter (0.1 ml) of the reagent application solution was added into the tubes. The tubes were heated in a 37℃-water bath again for another 5 minutes. An acid reagent (2.0 ml) was added at the end of the enzymatic reaction. The mixtures were centrifuged at 3100 g using the Z383k Hermle centrifuge for 10 minutes and (1.0 ml) of the supernatant was used to initiate the chromogenic reaction. Appropriate tubes were assembled according to the number of samples and the tubes were separated into blank tubes, standard tubes, non-enzyme tubes and enzyme tubes. GSH standard application solution (1.0 ml) was added into the appropriate tubes, (1.0 ml) of 20 μmol/L GSH standard solution was also added. The (1.0 ml) supernatant was added into the appropriate tube and (1.0 ml) of the application, solution was also added into the tubes. DTNB solution (0.25 ml) was added into all the tubes, (0.05 ml) salt reagent application solution was added at the end. The mixtures were allowed to stand for 15 minutes at room temperature, and the OD was measured at 412 nm using the ThermoSpectronic spectrophotometer. The GPx activity (nmol/mgprot) was calculated using the following formula: GSH − PX = (OD Non-enzyme tube-OD Enzyme tube)/ (OD Standard-OD Blank) × 20 μmol/L × f2 × f ÷ (V × Cpr) where: f: dilution factor of sample before tested, f1: dilution factor of serum/plasma in enzymatic reaction (6 times) and Cpr: concentration of protein in sample (mgprot/mL).

2.1.6.4 Thiobarbituric Acid Reactive Substances (TBARS) Assay

Thiobarbituric acid reactive substances assay was carried out in cardiac tissue homogenates as per the modified method previously described by Halliwell and Chrico in 1993 (Halliwell and Chirico, 1993; Adebiyi et al., 2016). Left ventricular tissues (100 mg) were homogenised in (500 µl) of 0.2 % phosphoric acid. This homogenate mixture was centrifuged for 5 minutes at a temperature of 4℃ at 1600 g. Cardiac tissue supernatants (200 µL) were placed in new test tubes and (5 µl) of 2 % phosphoric acid, (400 µl) of 7 % phosphoric acid and (400 µl) of BHT/TBA solutions were added into the tubes as well. Hydrochloric acid 1.0 M (400 µl) was used to initiate the reaction and test tubes were then boiled at 100℃ in a water bath for 15 minutes and the tubes were allowed to cool down at room temperature. n- butanol (1.5 ml) was then added into each test tube and mixed thoroughly and allowed to settle. Top phase (200 µl) was pipetted into a 96 well microplate in triplicates. The OD was measured at 532 nm and 600 nm using the EZ 400 microplate reader. The concentrations MDA were calculated from the differences between the absorbances measured at 532 nm and 600 nm using an extinction coefficient of 1.56 x 10^5 $cm^{-1}M^{-1}$ and the MDA concentrations were expressed in (nmol/mgprot).

2.1.7 Inflammatory cytokine tests

2.1.7.1 Tumor Necrosis Factor Alpha (TNF- α) ELISA Assay

Rat TNF-α (ELISA) kit using the sandwich-ELISA principle was used to determine the plasma TNF-α concentrations following the manufacturer's protocol. In a 96 well plate (100 µl) of the standard and plasma samples was pipetted in triplicates in the wells and the plate was incubated for 90 minutes at 37 ℃. The liquid in the wells was aspirated and (100 µl) of the biotinylated detection working solution was added, the plate was incubated at 37 ℃ for one hour. The liquid was aspirated again and the plate was washed three times using (350 µl) wash buffer , the plate was blotted dry on absorbent paper, HRP conjugate working solution (100 µl) was added in

the wells and incubated for 30 minutes at 37 ℃, the liquid was aspirated and the plate was washed 5 times using the wash buffer. After the final wash (90 µl) of the substrate reagent was added and the plate was incubated for 15 minutes at 37 ℃. The reaction was stopped by the addition of (50 µl) stop solution, optical density (OD) was measured at 450 nm using the EZ 400 microplate reader. TNF-α is expressed in pg/mL.

2.1.8 Cardiac mass estimation

LVs were dissected, weighed and the heart weight/body weight ratio (mg/g) and Left Ventricular Weight (LVW) /heart weight ratio (mg/g) were calculated.

2.1.9 Statistical analysis

Data was expressed as mean ± standard deviation. Unpaired t-tests with Welch's correction or One-way ANOVA was used to determine statistical significance. GraphPad Prism® Software Version 8.0. (Graphpad Prism® Software, Inc. San Diego, CA, USA) was used to compare statistical difference between control and treatment groups. Values of $p < 0.05$ were considered statistically significant.

CHAPTER 3: RESULTS

3.1 EVIDENCE OF DIABETES

3.1.1 Weight gain

The untreated diabetic group showed significantly ($p < 0.0001$) reduced weight gain compared to controls (Fig. 11). However, the naringenin or insulin treatment of diabetic rats significantly ($p < 0.05$) reduced weight loss compared to untreated diabetic group. Naringenin pre-treatment significantly ($p < 0.05$) showed improved body weights compared to naringenin post STZ treatment (Fig. 11). Naringenin treatment significantly ($p < 0.0001$) maintained a steady weight gain compared to controls.

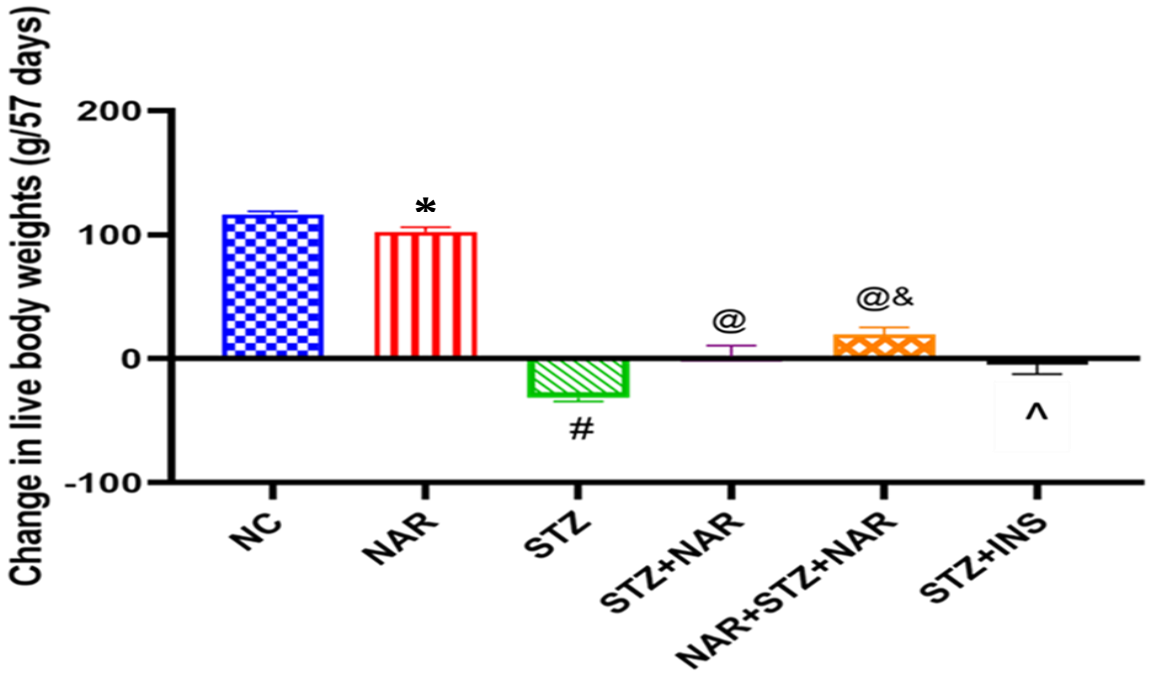


Figure 11. Changes in live body weights between days 0 and 57 of treatment (*, $^{\#}p < 0.0001$ compared to control, $^{\wedge}$, $^{@}$ $p < 0.05$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ+ NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**,Naringenin+Streptozotocin+Narigenin**(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.1.2 Water intake

Average daily water consumption per gram body weight was significantly ($p < 0.0001$) increased in the untreated diabetic group when compared to the controls. However, the naringenin or insulin treated groups showed a significantly ($p < 0.05$) decreased water consumption compared to untreated diabetic group (Fig. 12). Naringenin pre-treatment significantly ($p < 0.05$) showed reduced water intake compared to naringenin post STZ treatment (Fig. 12).

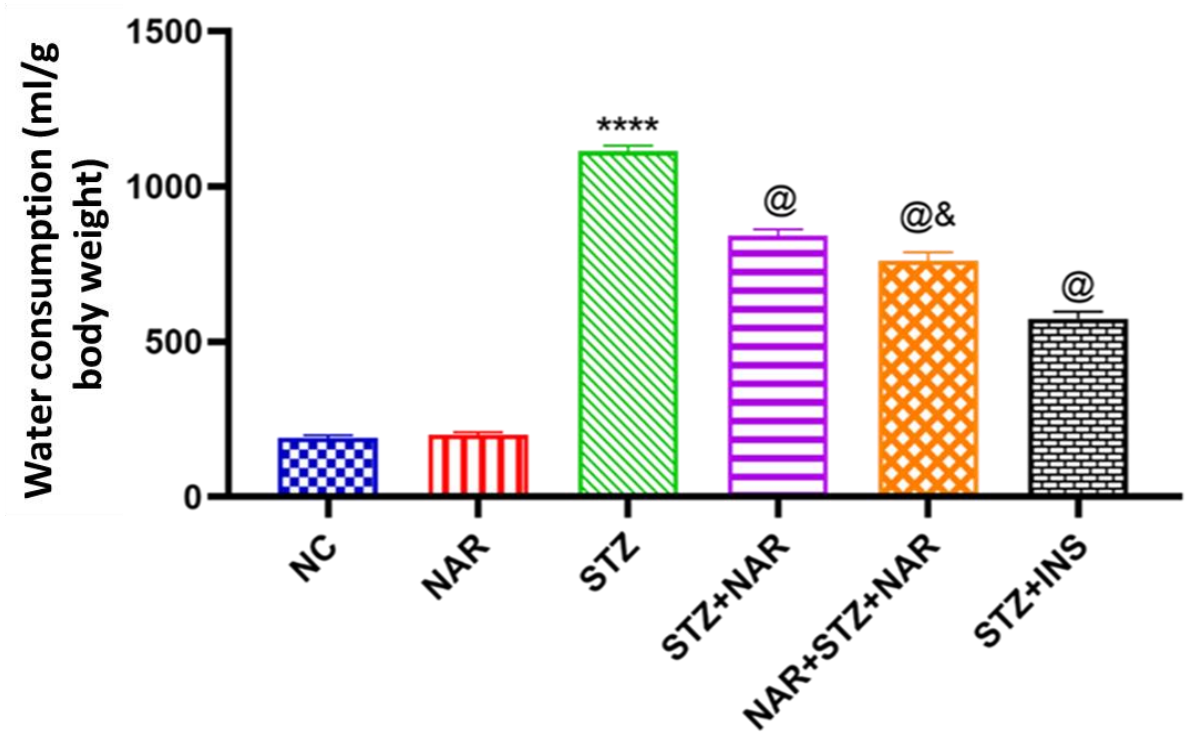


Figure 12. Average daily water intake per gram body weight in all treatment groups (****$p < 0.0001$ compared to control group, $^{@}p < 0.05$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ + NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.1.3 Fasting blood glucose

FBG concentrations were significantly ($p < 0.05$) elevated in the untreated diabetic group compared to the controls. Naringenin or insulin treatment significantly ($p < 0.05$) reduced FBG concentrations compared to untreated diabetic group (Fig. 13). Naringenin pre-treatment had no statistical significance when compared to naringenin post STZ treatment (Fig. 13).

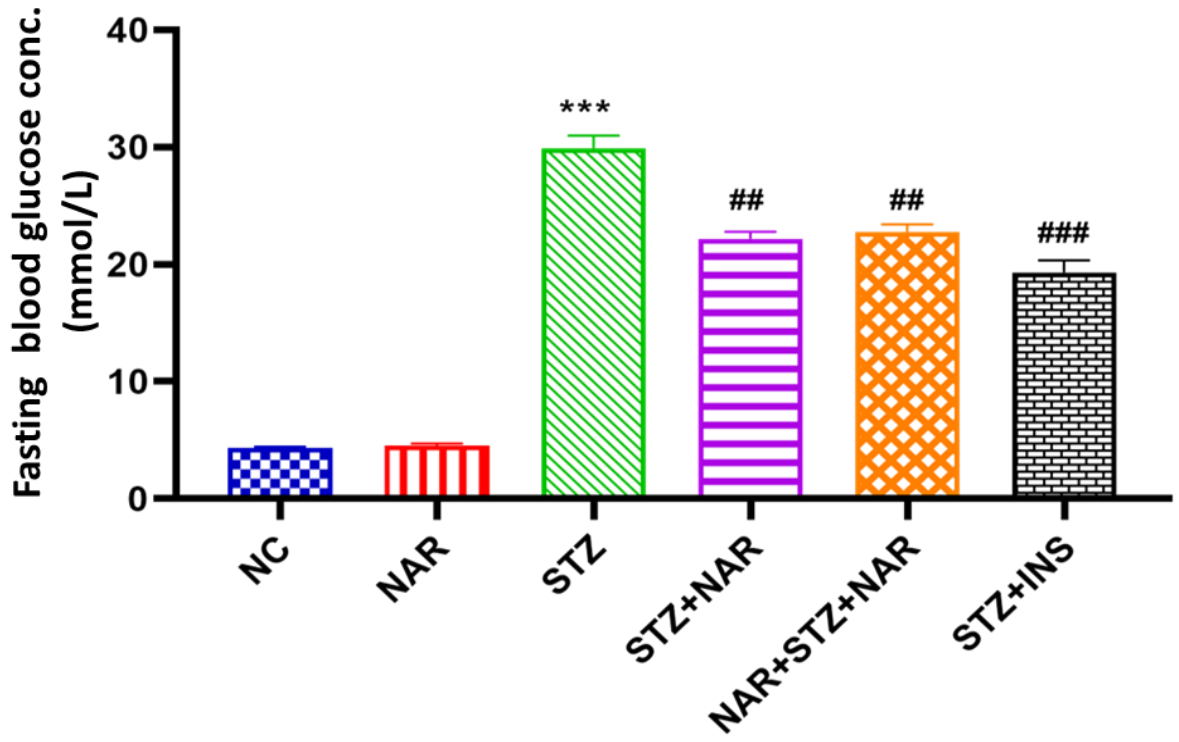


Figure 13. Fasting blood glucose concentrations (***p <0.05 compared to control group, $^{\#\#,\#\#\#}p < 0.05$ compared to STZ group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.1.4 Glucose tolerance test (GTT) & Calculated Area under the curve (AUC)

The untreated diabetic rats presented with impaired glucose tolerance (Fig. 14 A). Calculated area under the curve (AUC) showed untreated diabetic rats significantly ($p<0.0001$) had more glucose intolerance compared to control (Fig. 14 B). Treatment with naringenin did not improve glucose intolerance. However, insulin treatment significantly ($p < 0.05$) improved it (Fig. 14 A & B).

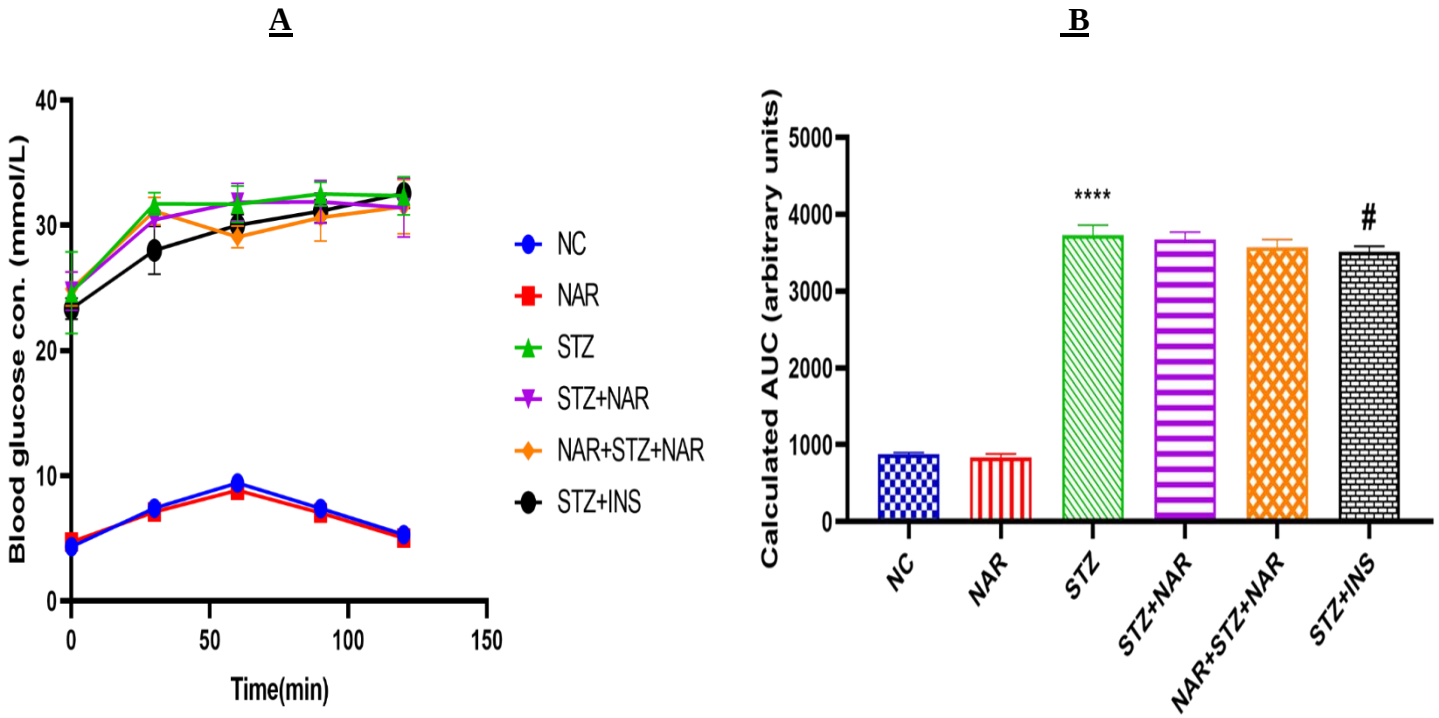


Figure 14. Glucose tolerance tests (GTT) in various treatment groups. **A)** Blood glucose concentrations vs time (GTT curves) **B)** Calculated AUC from the GTT plots (****$p < 0.0001$ compared to control group and $^{\#}p < 0.05$ compared to STZ group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.2 INSULIN RESISTANCE

3.2.1 Fasting plasma insulin

FPI concentrations were significantly ($p<0.05$) reduced in diabetic untreated group compared to the controls, while naringenin or insulin treatment significantly ($p<0.05$) improved FPI concentrations compared to untreated diabetic group (Fig. 15). Naringenin pre-treatment significantly ($p< 0.05$) improved FPI concentrations compared naringenin post STZ treatment (Fig.15).

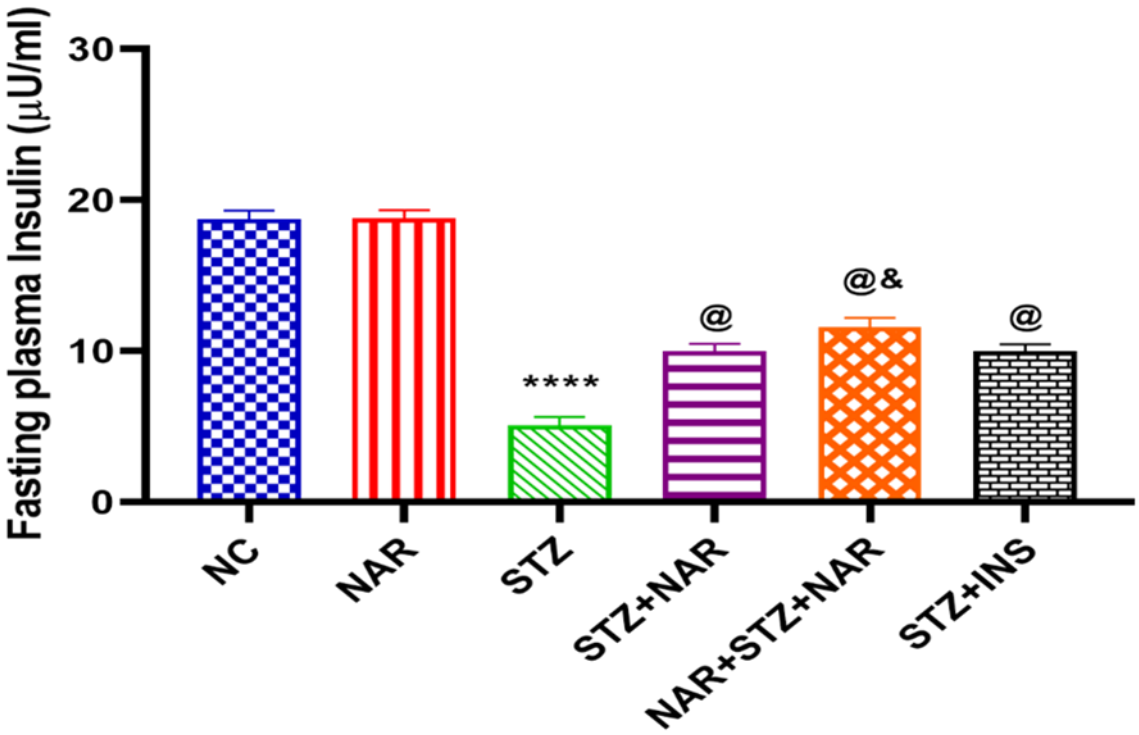


Figure 15. Fasting plasma insulin concentrations (********$p < 0.05$ compared to control group, $^{@}p < 0.05$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ+ NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.2.2 HOMA-IR

Calculated HOMA-IR was significantly ($p<0.0001$) elevated in untreated diabetic group compared to controls (Fig. 16). However, naringenin or insulin treatment of diabetic groups significantly ($p<0.05$) decreased HOMA-IR values compared to untreated diabetic rats, respectively. Naringenin pre-treatment significantly ($p < 0.05$) reduced HOMA-IR compared to naringenin post STZ treatment (Fig. 16).

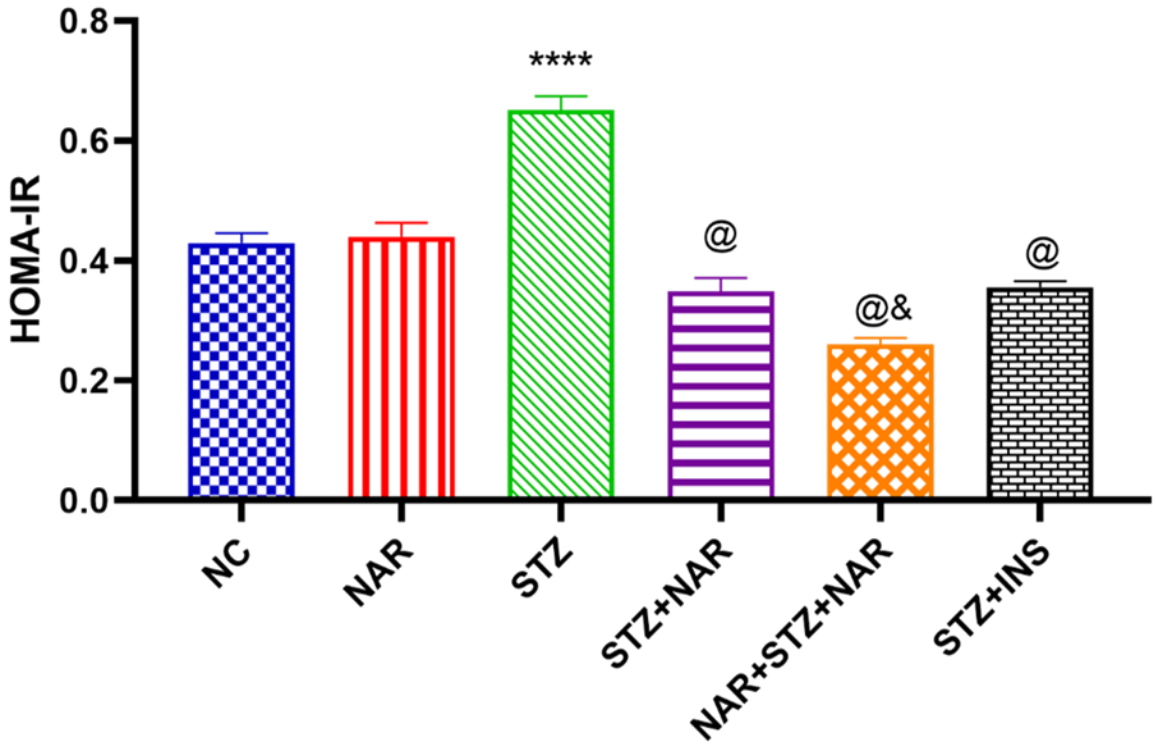


Figure 16. Homeostasis Model Assessment (HOMA) of insulin resistance in all treatment groups (****$p < 0.0001$ compared to control group, $^{@}$ $p < 0.05$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ+ NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.3 ANTIOXIDANT STATUS

3.3.1 Thiobarbituric Acid Reactive Substances (TBARS)

Cardiac tissue MDA (marker of Lipid peroxidation) levels were significantly (P<0.05) increased in the untreated diabetic group compared to the control. Naringenin or insulin treated diabetic groups had significantly (p<0.05) decreased MDA concentrations compared to the untreated diabetic group. Naringenin pre-treatment significantly (p<0.0001) decreased MDA concentrations compared to naringenin post STZ treatment (Fig. 17).

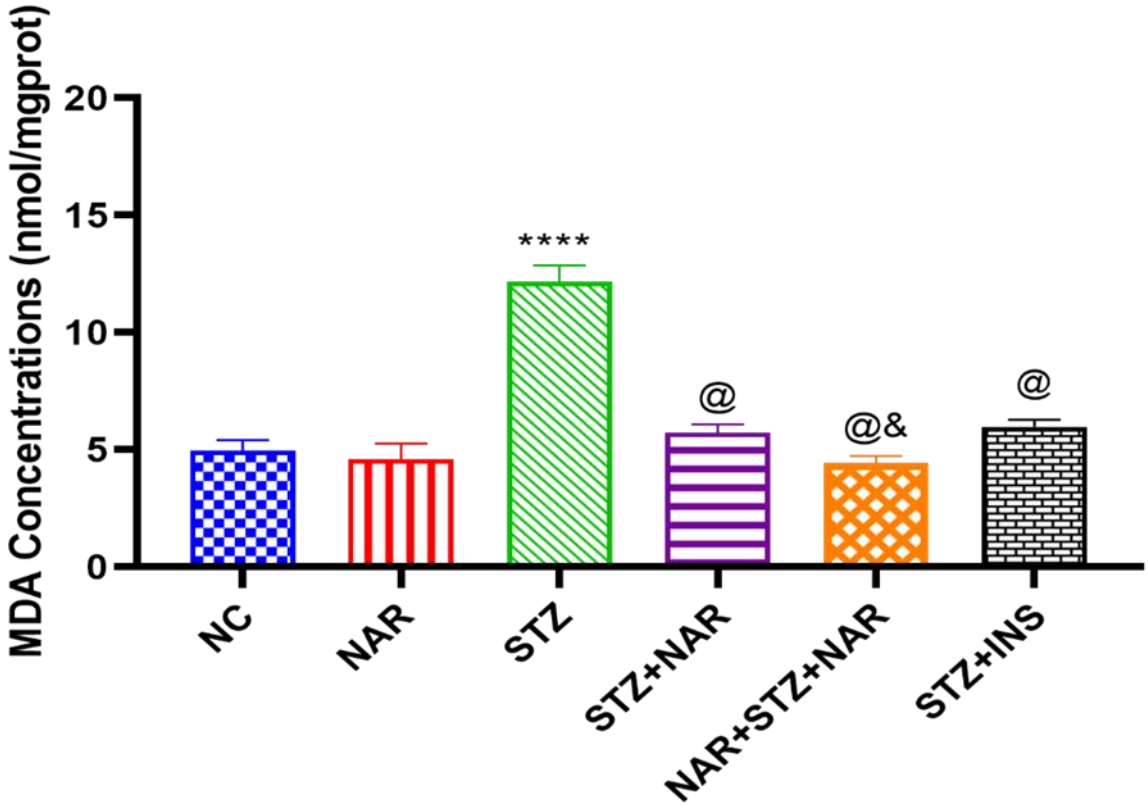


Figure 17. MDA concentrations were measured as an index of lipid peroxidation in all the treatment groups (****p < 0.05 compared to control, $^{@}$p < 0.05 compared to STZ group and $^{\&}$ p < 0.0001 compared to STZ+NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.3.2 Catalase (CAT) activity

Cardiac tissue catalase activity was significantly ($p<0.05$) reduced in the untreated diabetic group compared to the controls (Fig.18). However, naringenin or insulin treatment showed a significantly ($p<0.0001$) increased catalase activity compared to untreated diabetic rats. Naringenin pre-treatment significantly ($p < 0.05$) improved catalase activity compared to naringenin post STZ treatment (Fig. 18).

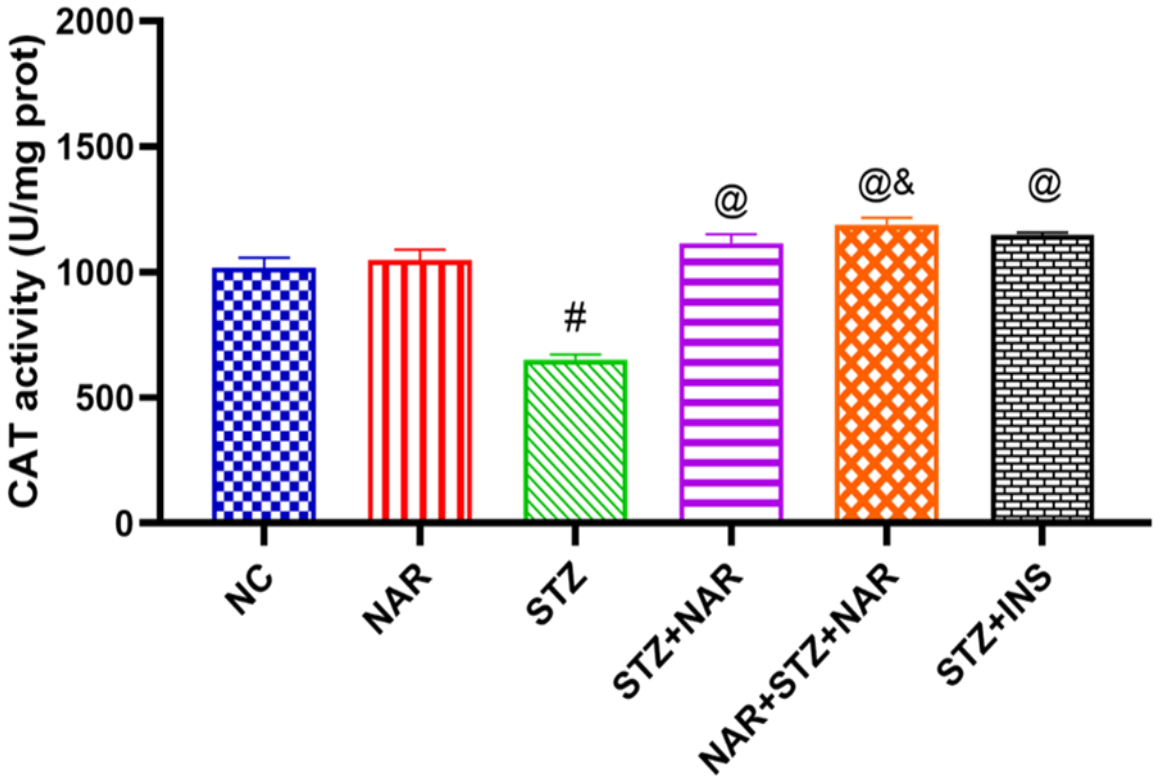


Figure 18. Catalase activity in all treatment groups ($^{\#}p < 0.05$ compared to control group, $^{@}p < 0.0001$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ + NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.3.3 Superoxide dismutase (SOD) activity

Cardiac tissue SOD activity was significantly ($p<0.05$) decreased in the untreated diabetic group compared to the control group (Fig. 19). However, naringenin or insulin treatment significantly ($p<0.05$) increased SOD activity compared to untreated diabetic group. Naringenin pre-treatment significantly ($p<0.05$) improved SOD activity compared to naringenin post STZ treatment (Fig.19). Naringenin treatment of non-diabetic rats significantly ($p < 0.05$) increased SOD activity compared to controls.

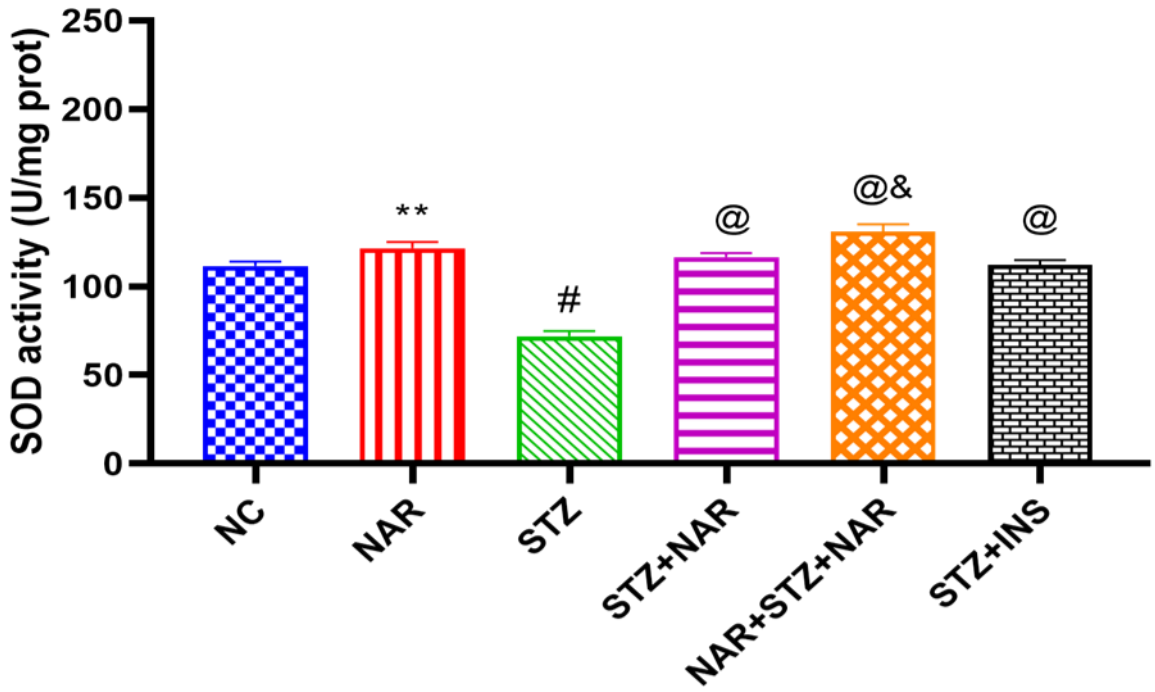


Figure 19. Superoxide dismutase (SOD) activity in all the treatment groups (**, # $p < 0.05$ compared to control group, @ $p < 0.05$ compared to STZ group and & $p< 0.05$ compared to STZ + NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.3.4 Glutathione peroxidase (GPx) activity

Cardiac tissue glutathione peroxidase (GPx) levels significantly ($p<0.0001$) decreased in the untreated diabetic group compared to the control (Fig. 20). However, a significant ($p<0.05$) increase was observed in the naringenin or insulin treated groups compared to the untreated diabetic group. Naringenin pre- treatment significantly ($p<0.0001$) increased GPx activity compared to naringenin post STZ treatment group. Naringenin treatment also showed a significant ($p<0.0001$) increase in GPx activity when compared to control group (Fig. 20).

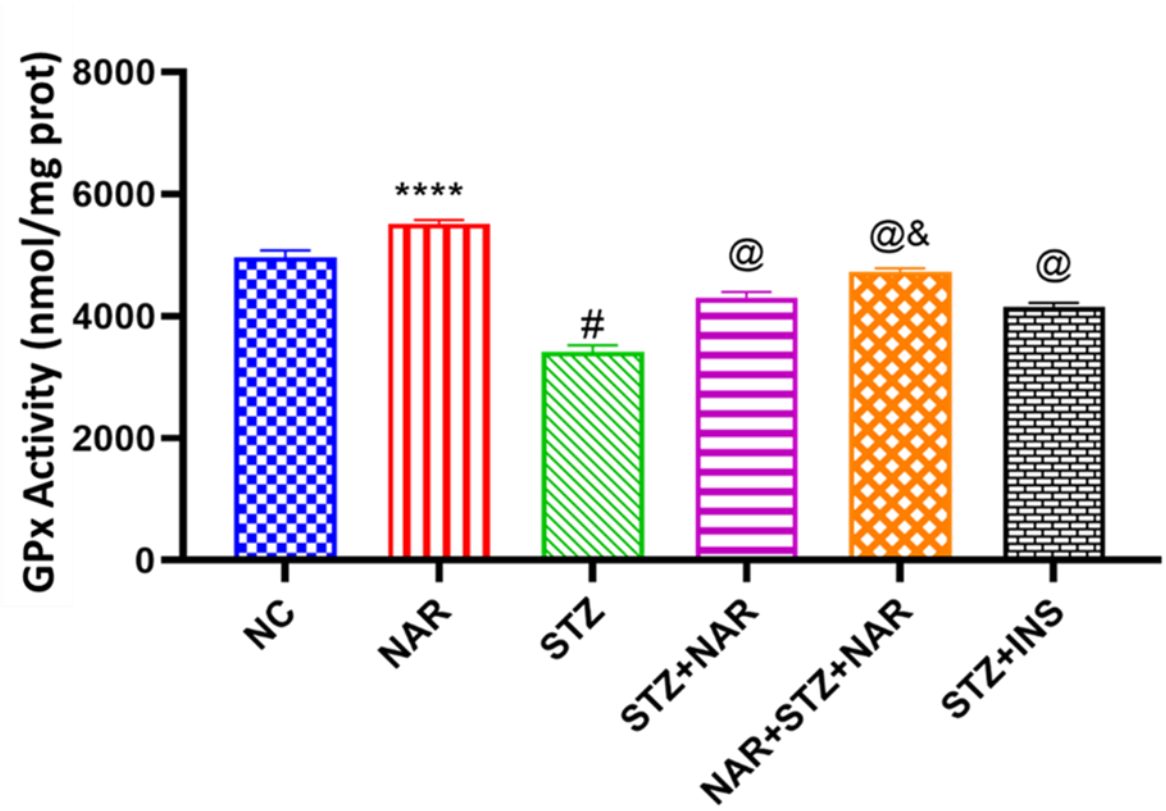


Figure 20. Glutathione peroxide (GPx) activity in all the treatment groups (****, $^{\#}p < 0.0001$ compared to control group, $^{@}p < 0.05$ compared to STZ group and $^{\&}p < 0.0001$ compared to STZ +NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**

3.4 INFLAMMATORY CYTOKINE LEVELS

3.4.1 Tumor Necrosis Factor Alpha (TNF- α) activity

Cardiac tissue TNF-α activity significantly ($p<0.05$) increased in the untreated diabetic group compared to the control. Naringenin or insulin treatment of diabetic rats significantly ($p<0.05$) decreased TNF-α compared to untreated diabetic rats. Naringenin pre-treatment significantly ($p<0.05$) decreased TNF-α activity compared to naringenin post STZ treatment (Fig.21).

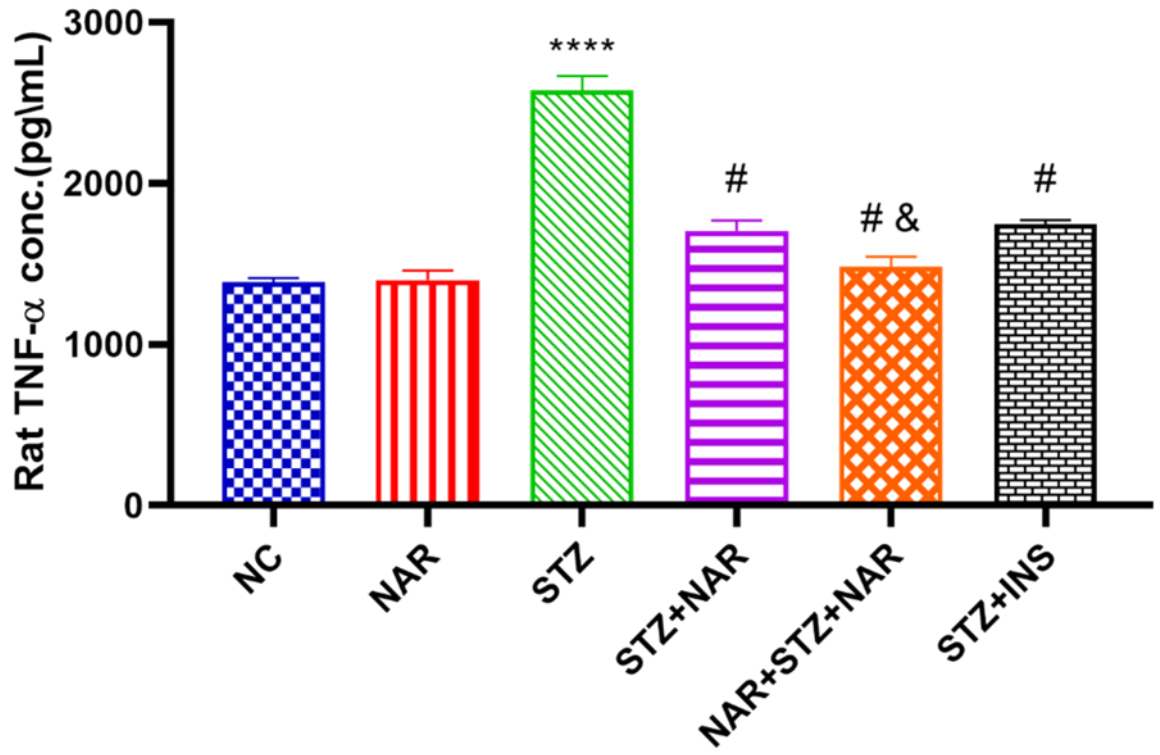


Figure 21. Tumor necrosis factor alpha (TNF-α) activity in all treatment groups (****$p < 0.05$ compared to control group, $^{\#}p< 0.05$ compared to STZ and $^{\&}p< 0.05$ compared to STZ+NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.5 Cardiac mass: Body mass ratio

3.5.1 Heart weight/ Body weight ratio

Untreated diabetic rats showed significant ($p<0.05$) increase in the HW/BW and LVW/HW ratios compared to the control group (Fig. 22 and 23). Naringenin or insulin treated diabetic groups showed a significant ($p<0.05$) decrease in the HW/BW and LVW/HW ratios compared to the untreated diabetic group, respectively. Naringenin pre-treatment significantly ($p<0.05$) decreased the HW/BW ratio compared to naringenin post STZ treatment (Fig.22).

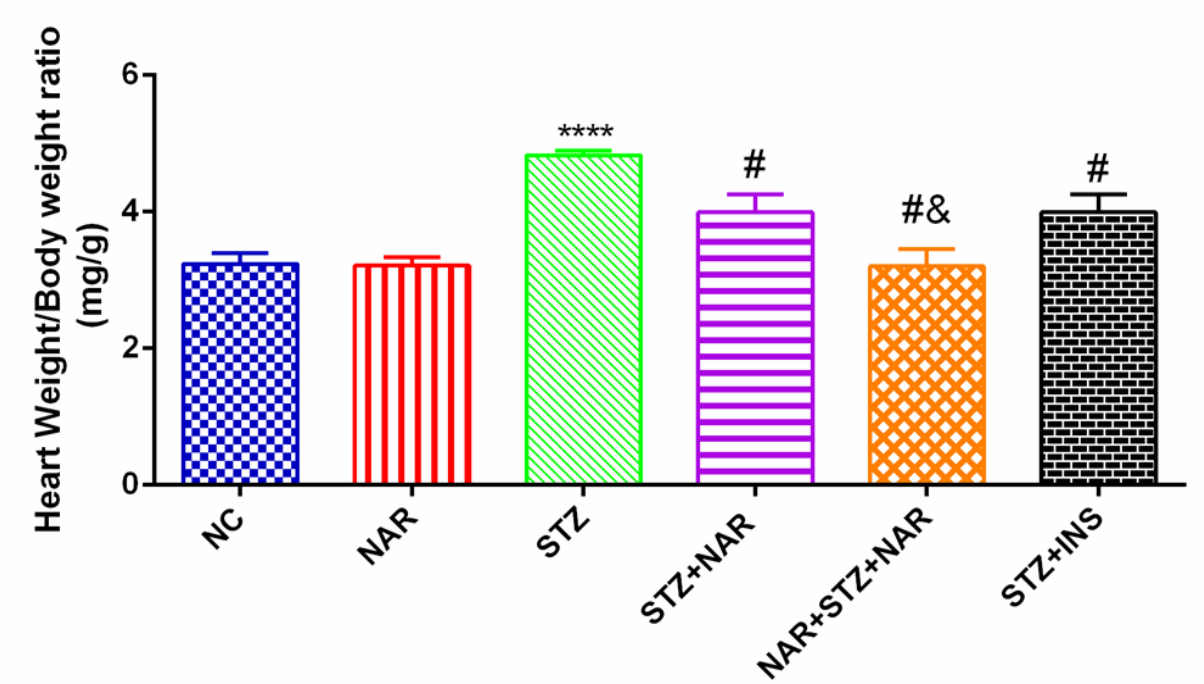


Figure 22. Calculated HW: BW ratio (****$p < 0.05$ compared to control group, $^{\#}p < 0.05$ compared to STZ group and $^{\&}p < 0.05$ compared to STZ+NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

3.5.2 Left ventricular weight/ Heart weight ratio

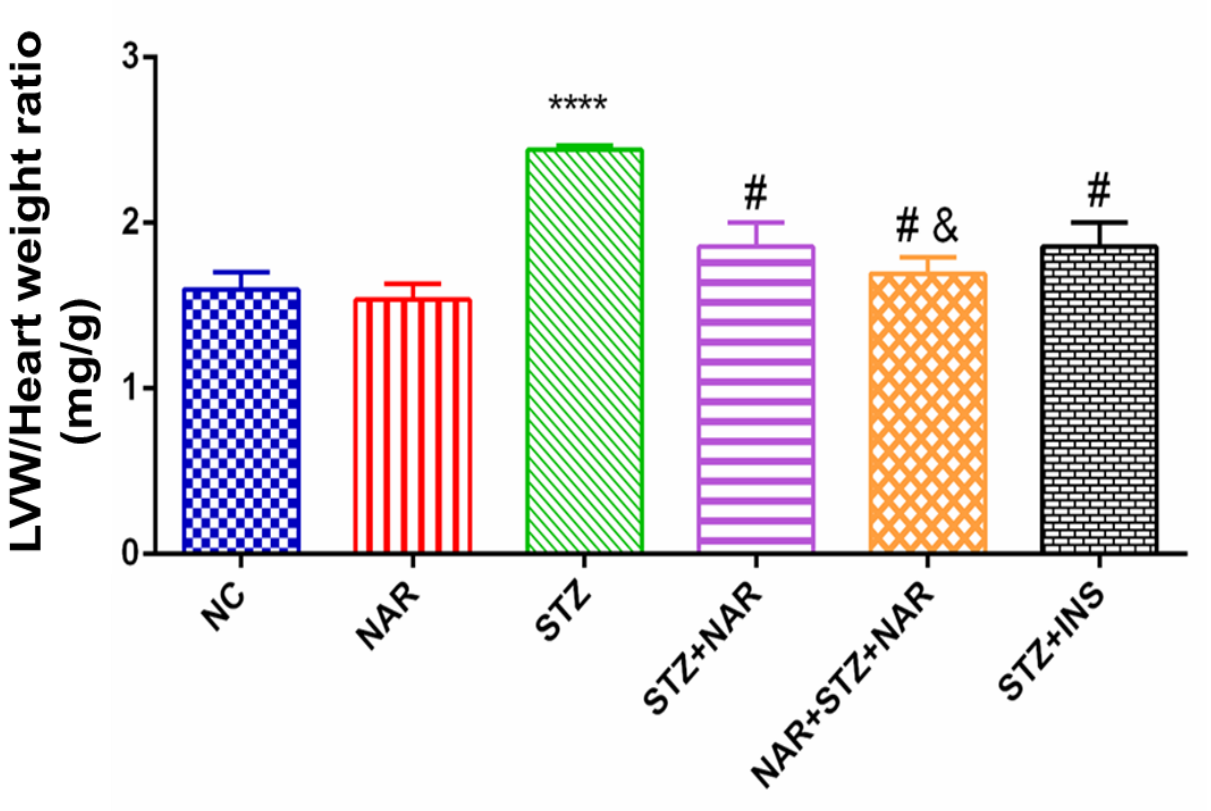


Figure 23. Left ventricular weight to heart weight ratio (LVW/HW), (****$p < 0.05$ compared to control group, $^{\#}p < 0.05$ compared to STZ group and $^{\&}p<0.05$ compared to STZ+NAR group).

Normal control **(NC)**, Naringenin **(NAR)**, Streptozotocin **(STZ)**, Streptozotocin+Naringenin **(STZ+NAR)**, Naringenin+Streptozotocin+Narigenin **(NAR+STZ+NAR)**, Steptozotocin+Insulin **(STZ+INS)**.

CHAPTER 4: DISCUSSION OF RESULTS

4.1 DISCUSSION

Diabetes is a multifaceted metabolic disorder with characteristic changes of glucose metabolism resulting in increased production of ROS (Kapoor and Kakkar, 2014). Increased ROS causes an imbalance between oxidants and antioxidants. When antioxidants are depleted normal bodily functions are disrupted and vital body organs are damaged by exposure to ROS.

Naringenin has been classified as a relatively harmless, safe and non-toxic substance, this is based on the classification of relative toxicity of chemicals (Surampalli e al., 2014). A study done on the preservation of intestine mucosal biochemical composition and cardiac muscle structure and function clarified naringenin as a safe drug (Surampalli et al.,2014, Deferme et al., 2008). These studies support the findings that suggest naringenin the naturally occurring substance has the potential to serve as a safe and novel pharmaceutical adjuvant.

In this current study, we investigated whether naringenin treatment could mitigate some complications caused by myocardium exposure to hyperglycemia, using rat models of diabetes induced with STZ. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg BW), which caused a destruction to the pancreatic β-cells resulting into elevated hyperglycemia and hyperglycemia-induced oxidative stress (Nishigaki et al., 1989; Cheng et al., 2013).

The untreated diabetic group exhibited significant weight loss when compared to the control (Fig. 11), type I diabetic patients normally lose weight regardless the food intake. Insulin deficiency prevents the body from getting glucose to supply the body with energy. As a compensatory mechanism the body utilizes stored fat and muscle energy causing a reduction in the overall body weight of the diabetic individual (McPherson and McEneny, 2012). Naringenin treatment attenuated weight loss by ameliorating lipolysis and proteolysis that is

increased in type I diabetic state. Naringenin pre-treatment improved weight loss compared to naringenin post STZ treatment suggesting that naringenin is a weight stabilizer (Shulman et al., 2011). Insulin treatment did not bring the animal weights to normal this could be due to insulin resistance resulting into glucose build-up forcing kidneys to remove the excess glucose through urine later resulting into weight loss (due to loss of calories) and dehydration.

Naringenin treatment of non-diabetic rats showed a decrease in weight gain compared to control group both these groups had unlimited access to food and water. Naringenin treatment is known to prevent obesity and improve lipid levels in diabetic rats by inducing hepatic fatty acid oxidation, reducing lipid availability (especially triglycerides) allowing for the assembly and secretion of apolipoprotein B- containing lipoproteins, this leads to reduced hepatic lipid accumulation (Mulvihil and Huff, 2012). However, naringenin does not stimulate fatty acid oxidation in skeletal muscles instead it reduces lipid accumulation and promotes glucose uptake, it is also a weight stabilizer like metformin (Assini et al., 2015).

The untreated diabetic group showed increased polydipsia compared to the control group (Fig. 12). Diabetic patients have polydipsia due to hyperglycemia increasing osmotic pressure and diuresis leading to dehydration (Kraut and Madias, 2007). As a compensatory mechanism elevated osmolarity activates osmoreceptors which reduce antidiuretic hormone (ADH), which corrects the state of hyperosmolarity. If this compensatory mechanism fails, thirst is activated causing increased water intake. Naringenin treatment reversed polydipsia in the diabetic groups because there was reduced water consumption. Naringenin pre-treatment reversed polydipsia compared to naringenin post STZ treatment.

Naringenin treatment improved fasting blood glucose compared to the untreated diabetic group we can thereby postulate that naringenin has an antihyperglycemic effect (Fig. 13), naringenin has previously been shown to reduce hepatic expression of the key gluconeogenic enzymes,

glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) which play a pivotal role in gluconeogenesis (Annadurai et al., 2012, Murunga et al., 2016). The untreated diabetic group showed elevated fasting blood glucose levels as expected for diabetic rats. Naringenin pre-treatment did not improve the fasting blood glucose levels compared to naringenin post STZ treatment this is due to insulin deficiency resulting into the inability of naringenin to affect blood glucose levels.

Glucose tolerance tests were similar between the naringenin treated non-diabetic and control rats and so were the calculated AUC (Fig. 14). However, the untreated diabetic rats showed glucose intolerance when compared to the control. The calculated AUC also supported these results, since naringenin and insulin treatment were withheld on the day of GTT, it is therefore expected that glucose levels would not improve in both the naringenin and insulin treated groups. Naringenin pre-treatment did not have any effect on glucose tolerance compared to naringenin post treatment. The untreated diabetic rats showed glucose intolerance and the calculated AUC significantly supported these results (Fig. 14).

The untreated diabetic rats showed lower levels of fasting plasma insulin and highly elevated HOMA-IR levels compared to controls (Fig. 15 and 16), HOMA-IR is a measure of IR and uses fasting plasma insulin levels and fasting blood glucose levels. High levels of fasting plasma insulin and increased resistance to insulin mediated glucose uptake are closely related, an increase in fasting plasma insulin levels is known to be a compensatory attempt to overcome the resistance to glucose uptake (Olefsky et al., 1973). Hence, the results show that the untreated diabetic rats have high levels of insulin resistance and the compensatory mechanism has failed to overcome the resistance of glucose uptake. STZ destroyed pancreatic β-cells but naringenin treatment improved their function by either regeneration or by reducing oxidative stress. Therefore, naringenin treatment significantly improved the fasting plasma insulin levels when compared to the untreated diabetic rats and the HOMA-IR levels were significantly

improved, showing improvement in the level of insulin resistance in the rats. Naringenin pre-treatment significantly improved the fasting plasma insulin and HOMA-IR levels when compared to naringenin post treatment.

Increased ROS leads to oxidative stress that causes lipid peroxidation leading to an overproduction of MDA, which is used as a biomarker of lipid peroxidation. MDA levels were significantly increased in the untreated diabetic rats compared to the control; these results were expected due to hyperglycemia-induced oxidative stress in the diabetic rats (Fig. 17). Naringenin treatment significantly decreased MDA levels compared to the untreated diabetic rats. These results suggest that naringenin treatment decreased lipid peroxidation by scavenging free radicals reducing oxidative stress (Song et al., 2018). Naringenin pre-treatment significantly reduces MDA levels compared to naringenin post-treatment.

SOD, CAT and GPx antioxidants are known as first line defense antioxidants, they suppress or prevent the formation of free radicals or reactive oxygen species, low levels of these antioxidants show the presence of oxidative stress (Ighodaro and Akinloye,2018).

CAT is a common antioxidant enzyme that uses iron or manganese as a cofactor and catalyzes the detoxication of harmful radicals into less harmful molecules. The untreated diabetic rats showed low levels of CAT activity when compared to control (Fig. 18), this suggests that there were high levels of oxidative stress which is expected for diabetic rats. Naringenin treatment significantly increased CAT levels compared to the untreated diabetic group, these results suggest that naringenin either scavenged free radicals causing reduced consumption of the antioxidants or naringenin could be binding free radicals resulting in less production of the antioxidant enzymes. Naringenin pre-treatment significantly improved CAT levels compared to naringenin post treatment.

SOD is an inducible antioxidant enzyme, it catalyzes the dismutation of harmful superoxide anions making less harmful substances. The untreated diabetic rats showed low levels of SOD when compared to control (Fig. 19). These results show that there were increased levels of oxidative stress, naringenin treatment significantly increased the SOD levels, showing that naringenin decreased free radicals or directly enhanced the production of antioxidants in the presence of free radicals. Naringenin treatment in the non-diabetic rats was also increased the antioxidant this could be because oxidative stress was completely reduced leading to an accumulation of the antioxidant enzyme SOD. Naringenin pre-treatment significantly increased SOD levels compared to naringenin post treatment.

GPx is an inducible antioxidant enzyme that also plays a critical role in inhibiting the lipid peroxidation process reducing oxidative stress. The untreated diabetic rats showed significantly low levels of GPx compared to control (Fig. 20), these results show elevated levels of oxidative stress, which is expected in diabetic rats. Naringenin treatment significantly elevated GPx levels compared to the untreated diabetic rats, suggesting that naringenin could have reduced the levels of free radicals by enhancing the antioxidant levels or by binding the free radicals leading to reduced production of the enzyme. Non- diabetic naringenin treated group showed elevated levels of GPx activity compared to control, since GPx is an inducible antioxidant it gets used up when there are free radicals, so once the free radical levels are decreased pressure to detoxify them is reduced and this could resort to an accumulation of the excess GPx antioxidant. Naringenin pre-treatment significantly increased GPx levels compared to naringenin post treatment.

Oxidative stress stimulates the production of pro-inflammatory cytokines such as TNF-α, which can be used as a biomarker of inflammation and/or oxidative stress (Chen et al., 2008). TNF-α injures endothelial cells resulting into endothelial dysfunction, inflammation and insulin resistance, it does this by damaging the mitochondrial chain complex III and this leads

to increased production of oxygen radicals inside the mitochondrion. TNF-α levels were highly elevated in the untreated diabetic rats compared to the control (Fig. 21), naringenin treatment significantly decreased TNF-α levels compared to untreated diabetic rats. The mechanism by which naringenin inhibits cytokine release is not limited to a specific stimulus, naringenin could have inhibited the activation of NF-kB and MAPK signaling pathways which are responsible for the development of inflammation or by scavenging free radicals reducing oxidative stress (Jin et al., 2017). Naringenin pre-treatment significantly reduced TNF-α compared to naringenin post treatment.

Left ventricular remodeling is characterized by inflammation or increased cardiomyocyte diameter/size (Wei et al., 2001; Adebiyi et al., 2016). Untreated diabetic rats showed significantly large heart weights compared to controls (Fig. 22 and 23), this could be due to constant hyperglycemia resulting in cardiomegaly. Cardiac mass was significantly reduced compared to control in the naringenin and insulin treated diabetic rats, this supports a study suggesting that naringenin retarded the development of left ventricular remodeling in diabetic rats by reducing MAPK and PKC levels responsible for cardiac remodeling and inflammation (Sabri et al., 1998; Adebiyi et al.,2016). Prior treatment of naringenin improved the cardiac inflammation and cardiac antioxidant levels of the diabetic rats, this supports the cardio-protective ability of naringenin (Kamel et al., 2016). Naringenin treatment could have activated PPAR- α and PPAR-γ (PPARs are ligand-activated transcription factors that modulate several biological processes implicated in obesity, inflammation and metabolism of lipids and glucose) leading to the down regulation of NF-kB expression, responsible for hypertrophy (Zhang et al., 2010). Naringenin pre-treatment improved the heart weights compared to naringenin post treatment.

CHAPTER 5: CONCLUSION

5.1 CONCLUSION

This study has shown that naringenin treatment has as an antidiabetic, anti-inflammatory and cardioprotective effect on the cardiac muscle this could be attributed to the ability of flavonoids to activate an antioxidant defense system by reducing ROS and increasing antioxidant levels. The findings support the claims of naringenin being a promising anti-diabetic plant extract that can be used to manage diabetes, mitigate diabetic conditions associated with cardiac complications. Prior treatment of naringenin before diabetes induction showed a great improvement in antioxidant, inflammatory cytokine levels and reduced cardiac mass, this supports the cardioprotective claims of naringenin.

5.2 FUTURE STUDIES

The study recommends future work using the same diabetic model and the same animal treatment protocol but the pre-treatment period of naringenin should be extended, to identify the potential ability of naringenin to prevent the onset of diabetes. Pathological studies particularly histology of the cardiac muscle will be included.

5.3 STUDY LIMITATIONS

In this study, pre-exposure time of naringenin treatment was limited, this could have had an impact on the results obtained and maybe the onset of diabetes could have been mitigated completely.

REFERENCES

1. Adebiyi, A.O., Adebiyi, O.O. and Owira, P.M., 2016. Naringin mitigates cardiac hypertrophy by reducing oxidative stress and inactivating c-Jun nuclear kinase-1 protein in type 1 diabetes. Journal of cardiovascular pharmacology, 67(2), 136-144.
2. Adebiyi, O.A., Adebiyi, O.O. and Owira, P.M., 2016. Naringin reduces hyperglycemia-induced cardiac fibrosis by relieving oxidative stress. PloS one, 11(3), e0149890.
3. Adiels, M., Olofsson, S.O., Taskinen, M.R. and Borén, J., 2008. Overproduction of very low–density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome. Arteriosclerosis, thrombosis, and vascular biology, 28(7),.1225-1236.
4. Akash, M.S.H., Rehman, K. and Liaqat, A., 2018. Tumor necrosis factor-alpha: role in development of insulin resistance and pathogenesis of type 2 diabetes mellitus. Journal of cellular biochemistry, 119(1), .105-110.
5. Albegali, A.A., Shahzad, M., Mahmood, S. and Ullah, M.I., 2019. Genetic association of insulin receptor substrate-1 (IRS-1, rs1801278) gene with insulin resistant of type 2 diabetes mellitus in a Pakistani population. *Molecular biology reports*,.1-6.
6. Ali, M.K., Narayan, K.V. and Tandon, N., 2010. Diabetes & coronary heart disease: current perspectives. *The Indian journal of medical research*, *132*(5), .584.
7. American Diabetes Association, 2016. 2. Classification and diagnosis of diabetes. *Diabetes Care*, *39*(Supplement 1), S13-S22.
8. American Diabetes association, 2018
9. Annadurai, T., Muralidharan, A.R., Joseph, T., Hsu, M.J., Thomas, P.A. and Geraldine, P., 2012. Antihyperglycemic and antioxidant effects of a flavanone, naringenin, in streptozotocin–nicotinamide-induced experimental diabetic rats. *Journal of physiology and biochemistry, 68*(3),.307-318.

10. Aronson, D. and Rayfield, E.J., 2002. How hyperglycemia promotes atherosclerosis: molecular mechanisms. *Cardiovascular diabetology*, *1*(1),.1.
11. Assini, J.M., Mulvihill, E.E., Burke, A.C., Sutherland, B.G., Telford, D.E., Chhoker, S.S., Sawyez, C.G., Drangova, M., Adams, A.C., Kharitonenkov, A. and Pin, C.L., 2015. Naringenin prevents obesity, hepatic steatosis, and glucose intolerance in male mice independent of fibroblast growth factor 21. *Endocrinology*, *156*(6),.2087-2102.
12. Atkinson, M.A., von Herrath, M., Powers, A.C. and Clare-Salzler, M., 2015. Current concepts on the pathogenesis of type 1 diabetes—considerations for attempts to prevent and reverse the disease. *Diabetes care*, *38*(6),.979-988.
13. Aziz, M and Yadav, K.S. 2016. Pathogenesis of atherosclerosis, a review, iMedPub Journals, 2(3), 22.
14. Bai, X., Zhang, X., Chen, L., Zhang, J., Zhang, L., Zhao, X., Zhao, T. and Zhao, Y., 2014. Protective effect of naringenin in experimental ischemic stroke: down-regulated NOD2, RIP2, NF-κB, MMP-9 and up-regulated claudin-5 expression. *Neurochemical research*, *39*(8),.1405-1415.
15. Barrett, E.J., Liu, Z., Khamaisi, M., King, G.L., Klein, R., Klein, B.E., Hughes, T.M., Craft, S., Freedman, B.I., Bowden, D.W. and Vinik, A.I., 2017. Diabetic microvascular disease: an endocrine society scientific statement. *The Journal of Clinical Endocrinology & Metabolism*, *102*(12), .4343-4410.
16. Basta, G., Lazzerini, G., Massaro, M., Simoncini, T., Tanganelli, P., Fu, C., Kislinger, T., Stern, D.M., Schmidt, A.M. and De Caterina, R., 2002. Advanced glycation end products activate endothelium through signal-transduction receptor RAGE. *Circulation*, *105*(7), .816-822.

17. Basta, G., Schmidt, A.M. and De Caterina, R., 2004. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovascular research*, *63*(4), .582-592.
18. Baynes, H.W., 2015. Classification, pathophysiology, diagnosis and management of diabetes mellitus. *J diabetes metab*, *6*(5),.1-9.
19. Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. *Diabetes*, *40*(4),.405-412.
20. Beckman, J.S. and Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology-cell physiology*, *271*(5),.C1424-C1437.
21. Bedekar, A., Shah, K. and Koffas, M., 2010. Natural products for type II diabetes treatment. *Advances in applied microbiology*, *71*, .21-73.
22. Beran, D. and Yudkin, J.S., 2010. Looking beyond the issue of access to insulin: what is needed for proper diabetes care in resource poor settings. *diabetes research and clinical practice*, *88*(3), 217-221.
23. Bergheanu, S.C., Bodde, M.C. and Jukema, J.W., 2017. Pathophysiology and treatment of atherosclerosis. *Netherlands Heart Journal*, *25*(4),.231-242.
24. Biddinger, S.B., Hernandez-Ono, A., Rask-Madsen, C., Haas, J.T., Alemán, J.O., Suzuki, R., Scapa, E.F., Agarwal, C., Carey, M.C., Stephanopoulos, G. and Cohen, D.E., 2008. Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. *Cell metabolism*, *7*(2),.125-134.
25. Boudina, S. and Abel, E.D., 2010. Diabetic cardiomyopathy causes and effects. Reviews in Endocrine and Metabolic Disorders, 11(1),.31-39.
26. Brownlee, M., 2005. The pathobiology of diabetic complications. *Diabetes*, *54*(6),.1615-1625.

27. Brownlee, M., Cerami, A. and Vlassara, H., 1988. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *New England Journal of Medicine*, *318*(20),.1315-1321.

28. Bugger, H. and Abel, E.D., 2014. Molecular mechanisms of diabetic cardiomyopathy. *Diabetologia*, *57*(4),.660-671.

29. Cavia-Saiz, M., Busto, M.D., Pilar-Izquierdo, M.C., Ortega, N., Perez-Mateos, M. and Muñiz, P., 2010. Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. Journal of the Science of Food and Agriculture, 90(7),.1238-1244.

30. Chan, J.M., Rimm, E.B., Colditz, G.A., Stampfer, M.J. and Willett, W.C., 1994. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes care*, *17*(9),.961-969.

31. Chen, X., Andresen, B.T., Hill, M., Zhang, J., Booth, F. and Zhang, C., 2008. Role of reactive oxygen species in tumor necrosis factor-alpha induced endothelial dysfunction. *Current hypertension reviews*, *4*(4),.245-255.

32. Cheng, D., Liang, B. and Li, Y., 2013. Antihyperglycemic effect of Ginkgo biloba extract in streptozotocin-induced diabetes in rats. *BioMed research international*, *2013*.

33. Cheng, H.M. and González, R.G., 1986. The effect of high glucose and oxidative stress on lens metabolism, aldose reductase, and senile cataractogenesis. *Metabolism*, *35*(4),.10-14.

34. Cho, N., Shaw, J.E., Karuranga, S., Huang, Y., da Rocha Fernandes, J.D., Ohlrogge, A.W. and Malanda, B., 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes research and clinical practice*, *138*,.271-281.

35. Chowdhury, M., Ghosh, J., Slevin, M., Smyth, J.V., Alexander, M.Y. and Serracino-Inglott, F., 2010. A comparative study of carotid atherosclerotic plaque microvessel density and angiogenic growth factor expression in symptomatic versus asymptomatic patients. *European journal of vascular and endovascular surgery*, *39*(4),.388-395.
36. Colditz, G.A., Willett, W.C., Rotnitzky, A. and Manson, J.E., 1995. Weight gain as a risk factor for clinical diabetes mellitus in women. *Annals of internal medicine*, *122*(7),.481-486.
37. Collard, C.D. and Gelman, S., 2001. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology: The Journal of the American Society of Anesthesiologists*, *94*(6),.1133-1138.
38. Cook, N.C. and Samman, S., 1996. Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of nutritional biochemistry*, *7*(2),.66-76.
39. Corradini, E., Foglia, P., Giansanti, P., Gubbiotti, R., Samperi, R. and Lagana, A., 2011. Flavonoids: chemical properties and analytical methodologies of identification and quantitation in foods and plants. Natural product research, 25(5),.469-495.
40. Creager, M.A., Goldin, A., Beckman, J.A. and Schmidt, A.M., 2006. Advanced glycation end Products-Sparking the development of diabetic vascular injury. *Circulation*, *114*,.597-605.
41. Crossman, D.C., 2004. The pathophysiology of myocardial ischaemia. *Heart*, *90*(5),.576-580.
42. Danaei, G., Singh, G.M., Paciorek, C.J., Lin, J.K., Cowan, M.J., Finucane, M.M., Farzadfar, F., Stevens, G.A., Riley, L.M., Lu, Y. and Rao, M., 2013. The global cardiovascular risk transition: associations of four metabolic risk factors with macroeconomic variables in 1980 and 2008. *Circulation*.

43. Deferme, S., Annaert, P. Augustjns, P., 2008. In vitro screening models to assess intestinal drug absorption and metabolism. In Drug absorption studies (182-215).Springer, Boson, MA.
44. DiMeglio, L.A., Evans-Molina, C. and Oram, R.A., 2018. Type 1 diabetes. *The Lancet*, *391*(10138),.2449-2462.
45. Domingueti, C.P., Dusse, L.M.S.A., das Graças Carvalho, M., de Sousa, L.P., Gomes, K.B. and Fernandes, A.P., 2016. Diabetes mellitus: the linkage between oxidative stress, inflammation, hypercoagulability and vascular complications. Journal of Diabetes and its Complications, 30(4),.738-745.
46. Du, X., Matsumura, T., Edelstein, D., Rossetti, L., Zsengellér, Z., Szabó, C. and Brownlee, M., 2003. Inhibition of GAPDH activity by poly (ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *Journal of Clinical Investigation*, *112*(7),.1049.
47. Eleazu, C.O., Eleazu, K.C., Chukwuma, S. and Essien, U.N., 2013. Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *Journal of Diabetes & Metabolic Disorders*, *12*(1),60.
48. Esposito, K., Nappo, F., Marfella, R., Giugliano, G., Giugliano, F., Ciotola, M., Quagliaro, L., Ceriello, A. and Giugliano, D., 2002. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation*, *106*(16),.2067-2072.
49. Esteghamati, A., Ashraf, H., Khalilzadeh, O., Zandieh, A., Nakhjavani, M., Rashidi, A., Haghazali, M. and Asgari, F., 2010. Optimal cut-off of homeostasis model assessment of insulin resistance (HOMA-IR) for the diagnosis of metabolic syndrome:

third national surveillance of risk factors of non-communicable diseases in Iran (SuRFNCD-2007). *Nutrition & metabolism*, *7*(1),.26.

50. Fang, Z.Y., Prins, J.B. and Marwick, T.H., 2004. Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. Endocrine reviews, 25(4), .543-567.
51. Fröhlich, M., Imhof, A., Berg, G., Hutchinson, W.L., Pepys, M.B., Boeing, H., Muche, R., Brenner, H. and Koenig, W., 2000. Association between C-reactive protein and features of the metabolic syndrome: a population-based study. *Diabetes care*, *23*(12),.1835-1839.
52. Gerber, P., Lübben, G., Heusler, S. and Dodo, A., 2003. Effects of pioglitazone on metabolic control and blood pressure: a randomised study in patients with type 2 diabetes mellitus. *Current medical research and opinion*, *19*(6),.532-539.
53. Giacco, F. and Brownlee, M., 2010. Oxidative stress and diabetic complications. *Circulation research*, *107*(9),.1058-1070.
54. Gilca, G.E., Stefanescu, G., Badulescu, O., Tanase, D.M., Bararu, I. and Ciocoiu, M., 2017. Diabetic cardiomyopathy: current approach and potential diagnostic and therapeutic targets. *Journal of diabetes research*, *2017*.
55. Gourgari, E., Wilhelm, E.E., Hassanzadeh, H., Aroda, V.R. and Shoulson, I., 2017. A comprehensive review of the FDA-approved labels of diabetes drugs: Indications, safety, and emerging cardiovascular safety data. *Journal of Diabetes and its Complications*, *31*(12),.1719-1727.
56. Griendling, K.K. and Ushio-Fukai, M., 2000. Reactive oxygen species as mediators of angiotensin II signaling. *Regulatory peptides*, *91*(1-3),.21-27.
57. Guthrie, R.A. and Guthrie, D.W., 2004. Pathophysiology of diabetes mellitus. *Critical care nursing quarterly*, *27*(2),.113-125.

58. Halliwell, B. and Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. *The American journal of clinical nutrition*, *57*(5),.715S-725S.

59. Han, S., Liang, C.P., Westerterp, M., Senokuchi, T., Welch, C.L., Wang, Q., Matsumoto, M., Accili, D. and Tall, A.R., 2009. Hepatic insulin signaling regulates VLDL secretion and atherogenesis in mice. *The Journal of clinical investigation*, *119*(4),.1029-1041.

60. Hansen, D., Dendale, P., Beelen, M., Jonkers, R.A., Mullens, A., Corluy, L., Meeusen, R. and van Loon, L.J., 2010. Plasma adipokine and inflammatory marker concentrations are altered in obese, as opposed to non-obese, type 2 diabetes patients. *European journal of applied physiology*, *109*(3),.397-404.

61. Hosseinzadeh, S., Jafarikukhdan, A., Hosseini, A. and Armand, R., 2015. The application of medicinal plants in traditional and modern medicine: a review of Thymus vulgaris. International Journal of Clinical Medicine, 6(09),.635.

62. Ighodaro, O.M. and Akinloye, O.A., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4),.287-293.*Immunology*, *199*(10),.3466-3477.

63. Indo, H.P., Yen, H.C., Nakanishi, I., Matsumoto, K.I., Tamura, M., Nagano, Y., Matsui, H., Gusev, O., Cornette, R., Okuda, T. and Minamiyama, Y., 2015. A mitochondrial superoxide theory for oxidative stress diseases and aging. *Journal of clinical biochemistry and nutrition*, *56*(1),.1-7.

64. International Diabetes Federation, Diabetic Atlas 2017

65. Jia, G., Hill, M.A. and Sowers, J.R., 2018. Diabetic cardiomyopathy: an update of mechanisms contributing to this clinical entity. Circulation research, 122(4),.624-638.

66. Jin, L., Zeng, W., Zhang, F., Zhang, C. and Liang, W., 2017. Naringenin ameliorates acute inflammation by regulating intracellular cytokine degradation.

67. Kahanovitz, L., Sluss, P.M. and Russell, S.J., 2017. Type 1 diabetes–a clinical perspective. *Point of care*, *16*(1),.37.

68. Kalpakcioglu, B. and Şenel, K., 2008. The interrelation of glutathione reductase, catalase, glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate in the pathogenesis of rheumatoid arthritis. *Clinical rheumatology*, *27*(2),.141-145.

69. Kamel, E.M., Mahmoud, A.M., Ahmed, S.A. and Lamsabhi, A.M., 2016. A phytochemical and computational study on flavonoids isolated from Trifolium resupinatum L. and their novel hepatoprotective activity. *Food & function, 7*(4),.2094-2106.

70. Kapoor, R. and Kakkar, P., 2014. Naringenin accords hepatoprotection from streptozotocin induced diabetes in vivo by modulating mitochondrial dysfunction and apoptotic signaling cascade. *Toxicology reports*, *1*,.569-581.

71. Kara, S., Gencer, B., Karaca, T., Tufan, H.A., Arikan, S., Ersan, I., Karaboga, I. and Hanci, V., 2014. Protective effect of hesperetin and naringenin against apoptosis in ischemia/reperfusion-induced retinal injury in rats. *The Scientific World Journal*.

72. Kaushal, J., Mehandia, S., Singh, G., Raina, A. and Arya, S.K., 2018. Catalase enzyme: application in bioremediation and food industry. Biocatalysis and agricultural biotechnology, 16,.192-199.

73. Kim, H., Zamel, R., Bai, X.H. and Liu, M., 2013. PKC activation induces inflammatory response and cell death in human bronchial epithelial cells. *PLoS One*, *8*(5),e64182.

74. Koya, D., Haneda, M., Kikkawa, R. and King, G.L., 1998. d-α-tocopherol treatment prevents glomerular dysfunctions in diabetic rats through inhibition of protein kinase C-diacylglycerol pathway. *Biofactors*, *7*(1-2),.69-76.

75. Kraut, J.A. and Madias, N.E., 2007. Serum anion gap: its uses and limitations in clinical medicine. *Clinical Journal of the American Society of Nephrology*, *2*(1),.162-174.

76. Lorenzo-Almoros, A., Tuñón, J., Orejas, M., Cortés, M., Egido, J. and Lorenzo, O., 2017. Diagnostic approaches for diabetic cardiomyopathy. *Cardiovascular diabetology*, *16*(1),.28.

77. Mahmoud AM, Hussein OE, 2016. Anti-diabetic effect of naringenin: Insights into the molecular mechanism, Diabetes and Obesity International Journal 1(5).

78. Mahmoud, A.M., Ahmed, O.M., Ashour, M.B. and Abdel-Moneim, A., 2015. In vivo and in vitro antidiabetic effects of citrus flavonoids; a study on the mechanism of action. *International Journal of Diabetes in Developing Countries*, *35*(3),.250-263.

79. Mahmoud, A.M., Ashour, M.B., Abdel-Moneim, A. and Ahmed, O.M., 2012. Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proinflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. *Journal of Diabetes and its Complications*, *26*(6),.483-490.

80. McPherson, P.A.C. and McEneny, J., 2012. The biochemistry of ketogenesis and its role in weight management, neurological disease and oxidative stress. *Journal of physiology and biochemistry*, *68*(1), .141-151.

81. Milwidsky, A., Maor, E., Kivity, S., Berkovitch, A., Zekry, S.B., Tenenbaum, A., Fisman, E.Z., Erez, A., Segev, S., Sidi, Y. and Goldenberg, I., 2015. Impaired fasting glucose and left ventricular diastolic dysfunction in middle-age adults: a retrospective cross-sectional analysis of 2971 subjects. *Cardiovascular diabetology*, *14*(1), .119.

82. Mishra, S., Bhadoria, A.S., Kishore, S. and Kumar, R., 2018. Gestational diabetes mellitus 2018 guidelines: An update. *Journal of family medicine and primary care*, *7*(6),.1169.

83. Morampudi, S., Balasubramanian, G., Gowda, A., Zomorodi, B. and Patil, A.S., 2017. The challenges and recommendations for gestational diabetes mellitus care in India: A Review. *Frontiers in endocrinology, 8*,.56.

84. Moreli, J.B., Corrêa-Silva, S., Damasceno, D.C., Sinzato, Y.K., Lorenzon-Ojea, A.R., Borbely, A.U., Rudge, M.V., Bevilacqua, E. and Calderon, I.M., 2015. Changes in the TNF-alpha/IL-10 ratio in hyperglycemia-associated pregnancies. Diabetes research and clinical practice, 107(3),.362-369.

85. Mosa, R.A., Cele, N.D., Mabhida, S.E., Shabalala, S.C., Penduka, D. and Opoku, A.R., 2015. In vivo antihyperglycemic activity of a lanosteryl triterpene from Protorhus longifolia. *Molecules, 20*(7),.13374-13383.

86. Mulvihill, E.E. and Huff, M.W., 2012. Protection from metabolic dysregulation, obesity, and atherosclerosis by citrus flavonoids: activation of hepatic PGC1α-mediated fatty acid oxidation. *PPAR research, 2012*.

87. Mulvihill, E.E., Allister, E.M., Sutherland, B.G., Telford, D.E., Sawyez, C.G., Edwards, J.Y., Markle, J.M., Hegele, R.A. and Huff, M.W., 2009. Naringenin prevents dyslipidemia, apoB overproduction and hyperinsulinemia in LDL-receptor null mice with diet-induced insulin resistance. *Diabetes.*

88. Mulvihill, E.E., Assini, J.M., Sutherland, B.G., DiMattia, A.S., Khami, M., Koppes, J.B., Sawyez, C.G., Whitman, S.C. and Huff, M.W., 2010. Naringenin decreases progression of atherosclerosis by improving dyslipidemia in high-fat–fed low-density lipoprotein receptor–null mice. *Arteriosclerosis, thrombosis, and vascular biology, 30*(4),.742-748.

89. Murunga, A.N., Miruka, D.O., Driver, C., Nkomo, F.S., Cobongela, S.Z. and Owira, P.M., 2016. Grapefruit derived flavonoid naringin improves ketoacidosis and lipid peroxidation in type 1 diabetes rat model. *PloS one, 11*(4), e0153241.

90. Nien, J.K., Mazaki-Tovi, S., Romero, R., Kusanovic, J.P., Erez, O., Gotsch, F., Pineles, B.L., Friel, L.A., Espinoza, J., Goncalves, L. and Santolaya, J., 2007. Resistin: a hormone which induces insulin resistance is increased in normal pregnancy. *Journal of perinatal medicine*, *35*(6),.513-521.

91. Nishigaki, A., Noma, H. and Kakizawa, T., 1989. The relations between doses of streptozotocin and pathosis in induced diabetes mellitus. *Shika gakuho. Dental science reports*, *89*(3),.639-662.

92. Ogbera, A.O. and Ekpebegh, C., 2014. Diabetes mellitus in Nigeria: The past, present and future. *World journal of diabetes*, *5*(6),.905.

93. Ogurtsova, K., da Rocha Fernandes, J.D., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N.H., Cavan, D., Shaw, J.E. and Makaroff, L.E., 2017. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes research and clinical practice*, *128*,.40-50.

94. Olefsky, J., Farquhar, J.W. and Reaven, G., 1973. Relationship between fasting plasma insulin level and resistance to insulin-mediated glucose uptake in normal and diabetic subjects. *Diabetes*, *22*(7),.507-513.

95. Orhan E, I., F Nabavi, S., Daglia, M., C Tenore, G., Mansouri, K. and M Nabavi, S., 2015. Naringenin and atherosclerosis: a review of literature. *Current pharmaceutical biotechnology*, *16*(3),.245-251.

96. Ozougwu, J.C., Obimba, K.C., Belonwu, C.D. and Unakalamba, C.B., 2013. The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology*, *4*(4),.46-57.

97. Pacher, P., Beckman, J.S. and Liaudet, L., 2007. Nitric oxide and peroxynitrite in health and disease. *Physiological reviews*, *87*(1),.315-424.

98. Paschou, S.A., Papadopoulou-Marketou, N., Chrousos, G.P. and Kanaka-Gantenbein, C., 2018. On type 1 diabetes mellitus pathogenesis. *Endocrine connections*, *7*(1), R38-R46.

99. Pickup, J.C. and Crook, M.A., 1998. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia*, *41*(10),.1241-1248.

100. Piero, N.M., Njagi, M.J., Kibiti, M.C., Ngeranwa, J.J.N., Njagi, E.N.M., Njue, W.M. and Gathumbi, P.K., 2012. Herbal management of diabetes mellitus: a rapidly expanding research avenue. *International Journal of Current Pharmaceutical Research*, *4*(2),.1-4.

101. Poljšak, B. and Fink, R., 2014. The protective role of antioxidants in the defence against ROS/RNS-mediated environmental pollution. *Oxidative medicine and cellular longevity*, *2014*.

102. Ramprasath, T., Senthamizharasi, M., Vasudevan, V., Sasikumar, S., Yuvaraj, S. and Selvam, G.S., 2014. Naringenin confers protection against oxidative stress through upregulation of Nrf2 target genes in cardiomyoblast cells. *Journal of physiology and biochemistry*, *70*(2),.407-415

103. Ribeiro, I.A., Rocha, J., Sepodes, B., Mota-Filipe, H. and Ribeiro, M.H., 2008. Effect of naringin enzymatic hydrolysis towards naringenin on the anti-inflammatory activity of both compounds. *Journal of Molecular Catalysis B: Enzymatic*, *52*,.13-18

104. Sabri, A., Byron, K.L., Samarel, A.M., Bell, J. and Lucchesi, P.A., 1998. Hydrogen peroxide activates mitogen-activated protein kinases and Na+-H+ exchange in neonatal rat cardiac myocytes. *Circulation research*, *82*(10),.1053-1062.

105. Salgado, A.L.F.D.A., Carvalho, L.D., Oliveira, A.C., Santos, V.N.D., Vieira, J.G. and Parise, E.R., 2010. Insulin resistance index (HOMA-IR) in the differentiation

of patients with non-alcoholic fatty liver disease and healthy individuals. *Arquivos de gastroenterologia*, *47*(2),165-169.

106. Schofield, J.D., Liu, Y., Rao-Balakrishna, P., Malik, R.A. and Soran, H., 2016. Diabetes dyslipidemia. *Diabetes Therapy*, *7*(2),.203-219.

107. Schultz, D.R. and Arnold, P.I., 1990, December. Properties of four acute phase proteins: C-reactive protein, serum amyloid A protein, α1-acid glycoprotein, and fibrinogen. In *Seminars in arthritis and rheumatism* (Vol. 20, No. 3, 129-147).

108. Scott, A., Khan, K.M., Cook, J.L. and Duronio, V., 2004. What is "inflammation"? Are we ready to move beyond Celsus? *British journal of sports medicine*, *38*(3),.248-249.

109. Shin, C.S., Moon, B.S., Park, K.S., Kim, S.Y., Park, S.J., Chung, M.H. and Lee, H.K., 2001. Serum 8-hydroxy-guanine levels are increased in diabetic patients. Diabetes care, 24(4),.733-737.

110. Shulman, M., Cohen, M., Soto-Gutierrez, A., Yagi, H., Wang, H., Goldwasser, J., Lee-Parsons, C.W., Benny-Ratsaby, O., Yarmush, M.L. and Nahmias, Y., 2011. Enhancement of naringenin bioavailability by complexation with hydroxypropoyl-β-cyclodextrin. *PloS one*, *6*(4),.e18033.

111. Skyler, J.S., Bakris, G.L., Bonifacio, E., Darsow, T., Eckel, R.H., Groop, L., Groop, P.H., Handelsman, Y., Insel, R.A., Mathieu, C. and McElvaine, A.T., 2017. Differentiation of diabetes by pathophysiology, natural history, and prognosis. *Diabetes*, *66*(2),.241-255.

112. Song, Y., Ding, W., Bei, Y., Xiao, Y., Tong, H.D., Wang, L.B. and Ai, L.Y., 2018. Insulin is a potential antioxidant for diabetes-associated cognitive decline via regulating Nrf2 dependent antioxidant enzymes. *Biomedicine & Pharmacotherapy*, *104*,.474-484.

113. Su, S.C., Pei, D., Hsieh, C.H., Hsiao, F.C., Wu, C.Z. and Hung, Y.J., 2011. Circulating pro-inflammatory cytokines and adiponectin in young men with type 2 diabetes. *Acta diabetologica*, *48*(2),.113-119.

114. Surampalli, G., Nanjwade, B.K. and Patil, P.A., 2016. Safety evaluation of naringenin upon experimental exposure on rat gastrointestinal epithelium for novel optimal drug delivery. Drug delivery, 23(2).,512-524.

115. Taylor, S.I., Accili, D. and Imai, Y., 1994. Insulin resistance or insulin deficiency: which is the primary cause of NIDDM. *Diabetes*, *43*(6),.735-740.

116. Tesch, G.H. and Allen, T.J., 2007. Rodent models of streptozotocin-induced diabetic nephropathy (Methods in Renal Research). *Nephrology*, *12*(3),.261-266

117. Thomas, D.W., Smythe, C.V. and Labbee, M.D., 1958. ENZYMATIC HYDROLYSIS OF NARINGIN, THE BITTER PRINCIPLE OF GRAPEFRUIT a. *Journal of Food Science*, *23*(6),.591-598.

118. Tulcan, C., Igna, V., Ahmadi, M., Zarcula, S., Motoc, M. and Borza, C., 2013. Quantitative Analysis of Superoxide Ions, Hydroperoxide and Lipoperoxidation Products in Characterization of Fresh Organic Fluid Quality. *REVISTA DE CHIMIE*, *64*(2),.195-197.

119. Tungmunnithum, D., Thongboonyou, A., Pholboon, A. and Yangsabai, A., 2018. Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. *Medicines*, *5*(3),.93.

120. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1),.44-84.

121. van der Zijl, N.J., Goossens, G.H., Moors, C.C., van Raalte, D.H., Muskiet, M.H., Pouwels, P.J., Blaak, E.E. and Diamant, M., 2011. Ectopic fat storage in the

pancreas, liver, and abdominal fat depots: impact on β-cell function in individuals with impaired glucose metabolism. *The Journal of Clinical Endocrinology & Metabolism*, *96*(2),.459-467.

122. Ventura-Sobrevilla, J., Boone-Villa, V.D., Aguilar, C.N., Román-Ramos, R., Vega-Avila, E., Campos-Sepúlveda, E. and Alarcón-Aguilar, F., 2011. Effect of varying dose and administration of streptozotocin on blood sugar in male CD1 mice. In *Proc West Pharmacol Soc* 54(5),.9.

123. Wadkar, K.A., Magdum, C.S., Patil, S.S. and Naikwade, N.S., 2008. Anti-diabetic potential and Indian medicinal plants. *Journal of herbal medicine and toxicology*, *2*(1),.45-50.

124. Wagner, M.J., Stacey, M.M., Liu, B.A. and Pawson, T., 2013. Molecular mechanisms of SH2-and PTB-domain-containing proteins in receptor tyrosine kinase signaling. *Cold Spring Harbor perspectives in biology*, *5*(12),008987.

125. Wang, C., Niimi, M., Watanabe, T., Wang, Y., Liang, J. and Fan, J., 2018. Treatment of atherosclerosis by traditional Chinese medicine: Questions and quandaries. *Atherosclerosis*.

126. Wang-Fischer, Y. and Garyantes, T., 2018. Improving the reliability and utility of streptozotocin-induced rat diabetic model. *Journal of diabetes research*, *2018*.

127. Watanabe, K., A Thandavarayan, R., Harima, M., R Sari, F., Gurusamy, N., T Veeraveedu, P., Mito, S., Arozal, W., Sukumaran, V., Prasath Laksmanan, A. and Soetikno, V., 2010. Role of differential signaling pathways and oxidative stress in diabetic cardiomyopathy. *Current cardiology reviews*, *6*(4),.280-290

128. Wei, S., Rothstein, E.C., Fliegel, L., Dell'Italia, L.J. and Lucchesi, P.A., 2001. Differential MAP kinase activation and Na+/H+ exchanger phosphorylation by H2O2

in rat cardiac myocytes. *American Journal of Physiology-Cell Physiology*, *281*(5),.C1542-C1550.

129. Wolff, S.P. and Dean, R.T., 1987. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation'in diabetes. *Biochemical Journal*, *245*(1),.243-250.

130. World health organisation, 2016

131. Wright, E., Scism-Bacon, J.L. and Glass, L.C., 2006. Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *International journal of clinical practice*, *60*(3),.308-314.

132. Wu, K., Huan, Y., 2008. Streptozotocin-Induced diabetic models in mice and rats. Current protocols in Pharmacology, 2008.

133. Yao, L.H., Jiang, Y.M., Shi, J., Tomas-Barberan, F.A., Datta, N., Singanusong, R. and Chen, S.S., 2004. Flavonoids in food and their health benefits. *Plant foods for human nutrition*, *59*(3),.113-122.

134. Zhang, W., Zhao, J., Wang, J., Pang, X., Zhuang, X., Zhu, X. and Qu, W., 2010. Hypoglycemic effect of aqueous extract of seabuckthorn (Hippophae rhamnoides L.) seed residues in streptozotocin-induced diabetic rats. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *24*(2),.228-232.

135. Zhao, L., 2001. Effects of free radicals in diabetes. *Free Radicals in Biology and Medicine*, *77*,.222.

136. Zhao, Y., Xu, G., Wu, W. and Yi, X., 2015. Type 2 Diabetes Mellitus- Disease, Diagnosis and Treatment. *Journal of Diabetes Metabolism*, 6, 533.

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

01 February 2019

Ms Samukelisiwe Mpanza (218083492)
School of Health Sciences
Westville Campus

Dear Ms Mpanza,

Protocol reference number: AREC/067/018M
Project title: The effects of naringenin on pancreatic beta cells and the hearts of streptozotocin-induced diabetic rats *in vivo*

Full Approval – Research Application

With regards to your revised application received on 29 November 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 01 February 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Dr Sanil D Singh, PhD
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Peter Owira
Cc Academic Leader Research: Professor Pragashnie Govender
Cc Registrar: Mr Simon Mokoena
Cc School Administrator: Ms Phindile Nene

<<MPANZA SM>> OFFICE USE ONLY

Ref Nr:

3. STAFF, RESEARCH ASSOCIATES, STUDENTS AND TECHNICIANS AUTHORISED TO CARRY OUT THE PROPOSED HANDS-ON ANIMAL STUDIES.		
Full name of team members (Exclude animal facility staff and technicians)	Academic qualification	Animal training (If Yes, PROOF* to be attached)
Mcobothi N.E.	**MMed in Pharmacolog**	**No**
Dr Owira Peter	**PhD**	**Yes**

*Proof can be a certificate of animal training, a first authored publication using animals, reference letter from relevant authority etc.

4. EXPERIENCE IN WORKING WITH ANIMALS RELEVANT TO THE APPLICATION

Mpanza S and Mcobothi NE will be attending Biomedical Research Unit training on handling of animals and present attendance certificates. The supervisor Dr Owira has vast experience in in vivo animal experiments

5. ANIMAL HOUSING FACILITIES WHERE WORK WILL BE CARRIED OUT

5.1. University of KwaZulu-Natal Centres

Biomedical Resource Unit (Westville)	√
School of Life Sciences (SLS) (PMB) Animal House	
Ukulinga Research Farm (PMB)	
Other (specify below under 5.2)	

5.2 Non-University of KwaZulu-Natal Centres*

PLEASE SPECIFY in FULL_____

***N.B. If ALL of your work involving animals is performed at a Non-University of KwaZulu-Natal Centre, you need not complete the rest of this form, but you HAVE TO attach a letter of ethical approval obtained from the relevant authority at the Non-University of KwaZulu-Natal Centre.**