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THE INFLUENCE OF OLEANOLIC ACID ON HIGH FRUCTOSE INDUCED OXIDATIVE STRESS AND INFLAMMATION IN SPRAGUE DAWLEY RATS

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Dissertation submitted in *partial* fulfillment of the requirements for the degree *Master of Science* in Biology at the Mafikeng Campus of the North-West University

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DECLARATION

I, declare that this dissertation submitted to the North-West University, Mafikeng Campus, for the Master`s degree in Biology and the work contained therein is my own work in design and execution and has not previously been submitted to another university for a degree, and that all the materials contained therein have been duly acknowledged.

Signed at ..... on this..... Day of ....., 2017

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Dr Ademola Ayeleso (Co-Supervisor)

DEDICATION

This thesis is dedicated to my GOD.

ACKNOWLEDGEMETS

I thank the God almighty for making it possible for me to undertake this research study and for the grace and strength bestowed unto me to successfully complete my Master's degree.

My deepest gratitude to my supervisor and co-supervisor, Prof Emmanuel Mukwevho and Dr Ademola Ayeleso for their patience, support and instructive guidance throughout the programme.

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ABSTRACT

Oxidative stress is associated with pathogenesis of many diseases, causing cellular injury by reaction of free radicals with cellular proteins, lipids and DNA. It has been implicated in the pathogenesis of diabetes, hypertension and atherosclerosis. High levels of inflammatory markers have been shown in numerous studies to be independently related to the risk of future cardiovascular events. It has been reported that an interrelation between inflammation, oxidative stress and metabolic abnormalities in diabetes leads to endothelial injury and the development of cardiovascular complications. Chronic fructose intake has been reported to increase oxidative stress. Oleanolic acid is a pentacyclic triterpenoid that can be found in the form of free acid or aglycones in various medicinal plants and fruits. The main aim of the study was to investigate the modulatory effects of oleanolic acid on oxidative stress and inflammatory responses in high fructose-fed male rats. Four groups of pups heart tissues were used. Group A (normal control) was only given normal diet (breast milk) and group B was given breast milk and oleanolic acid, (OA), group C was given breast milk and high fructose diet (HFD) and group D was given breast milk, high fructose diet and oleanolic acid (HFD+OA). Established laboratory techniques were used to carry out the experiments. Trolox Equivalent Antioxidant Capacity (TEAC) values showed no significant differences ($p>0.05$) in all the treatment groups of the heart tissue. In a similar vein, there were no significant differences ($p>0.05$) in Ferric Reducing Antioxidant Power (FRAP) values in all the treatment groups of the heart tissues. The level of glutathione was decreased, although not significantly, in HFD treatment group while treatment with OA showed the tendency to increase GSH concentration. Malonaldehyde (MDA), a marker of lipid peroxidation, analysed in the heart tissue was elevated in the HFD group while treatment with OA slightly inhibited lipid peroxidation significantly ($p>0.05$). There were no significant differences in both the gene expression and activity of catalase in all the four treatment groups. Elevation of pro-inflammatory molecules IL-1b, IL-6, IL-12, IFN-g, TNF-alpha and MCP-1 in HFD treatment group was observed when compared to normal control groups, but it was not significantly different ($p>0.05$). Treatment with OA consistently decreased the levels of pro-inflammatory cytokines to levels that are almost the same as normal controls. The level of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 were increased in HFD when compared with normal controls though not significant. The study revealed that the consumption of high fructose in the early stages of life could initiate oxidative stress and inflammation in the heart. Oleanolic acid may be beneficial in reducing the risk of oxidative stress and inflammation in progression to heart diseases and related diseases.

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ABBREVIATIONS

ANGII	ANGIOTENSIN II
ATP	ADENOSINE TRIPHOSPHATE
CAD	CORONARY ARTERY DISEASE
CHD	CORONARY HEART DISEASE
CRP	C-REACTIVE PROTEIN
CVDs	CARDIOVASCULAR DISEASES
DNA	DEOXYRIBONUCLEIC ACID
DPPH	2, 2-DIPHENYL-1-PICRYLHYDRAZYL
DUOX	DUAL OXIDASE
$_{e}$NOS	ENZYMATIC NITRIC OXIDE SYNTHASE
$FADH_2$	FLAVIN ADENINE DINUCLEOTIDE
FFA_S	FREE FATTY ACIDS
H_2O_2	HYDROGEN PEROXIDE
HDL	HIGH DENSITY LIPOPROTEIN
HF	HEART FAILURE
HFD	HIGH FRUCTOSE DIET
ICAM-1	INTERCELLULAR ADHESION MOLECULE 1
IHD	ISCHEMIC HEART DISEASE
IL-12345678	INTERLEUKINS 1,2,3,4

INF-G	INTERFERON-G
LDL	LOW DENSITY LIPOPROTEIN
MCP-1	MONOCYTE CHEMOATTRACTANT PROTEIN-1
MDA	MALONALDEHYDE
MET_S	METABOLIC SYNDROME
MI	MYOCARDIAL INFARCTION
NO	NITRIC OXIDE
NO_2	NITRIC DIOXIDE
NOx	OXIDES OF NITROGEN
N	NITROGEN
OH^-	HYDROXIDE
$ONOO^-$	PEROXYNITRITE
$_{OX}$LDL	OXIDISED LOW DENSITY LIPOPROTEIN
qPCR	QUANTITATIVE REAL TIME PCR
RNS	REACTIVE NITROGEN SPECIES
ROS	REACTIVE OXYGEN SPECIES
TBA	THIOBABITURIC ACID
TEAC	TROLOX EQUIVALENT ANTIOXIDANT CAPACITY
TNF-α	TUMOR NECROSIS FACTOR- ALPHA
VSMCS	VASCULAR SMOOTH MUSCLES
XDH	XANTHINE DEHYDROGENASE
XO	XANTHINE OXIDASE

XOR	XANTHINE OXIREDUCTASE

CHAPTER 1

1.0 INTRODUCTION

Oxidative stress refers to the cytopathological consequences of an imbalance between production of free radicals and the inability of the cell to defend against them (Franco and Panayiotidis, 2009). Free radicals are highly reactive compounds with an unpaired electron. This makes them capable of causing oxidative damage to major macromolecules in cells such as lipids, proteins and nucleic acids (Franco and Panayiotidis, 2009). Major families of free radicals include the reactive oxygen species (ROS) and reactive nitrogen species (RNS). In humans, oxidative stress is involved in many diseases such as neurodegenerative disorders including Parkinson's and Alzheimer's diseases, cardiovascular diseases, diabetes mellitus, cancers and ischemic cascade due to oxygen reperfusion injury followed by hypoxia (Johansen *et al.*, 2005; Dalle-Donne *et al.*, 2006; Blum, 2009).

Oxidative stress is the common factor underlying insulin resistance, type 2 diabetes mellitus and cardiovascular diseases (Montuschi *et al.*, 2004). Inflammation is one of the manifestations of oxidative stress. The pathways that generate the mediators of inflammation, such as adhesion molecules and interleukins are all induced by oxidative stress (Ceriello and Motz, 2004; Roebuck, 2003). Inflammation is a key feature of obesity, insulin resistance and type 2 diabetes characterized by abnormal cytokine production, increased acute phase of reactants and activation of network of inflammatory signalling pathways (Wellen and Hotamisligil, 2005). The sub-clinical pro-inflammatory state observable in conditions including atherosclerosis, cancer, and ageing is caused through various processes which include mitochondrial over-generation of free radicals (Lane, 2005; Ceriello and Motz, 2004).

Poor dietary choices have led to an increase in the proportion of people consuming artificially sweetened foods rich in high fructose (Weiss *et al.,* 2004; Rutledge and Adeli, 2007). Fructose is a sugar / common sweetener used in food processing, soda and fruit flavoured drinks (Dai and McNeill, 1995). The administration of a fructose-rich-diet has been used as a model for investigating the development of metabolic syndrome (Steyn and Temple, 2012). The increasing role of fructose in diet has prompted a myriad of research into its metabolic fate and consequences to human health.

Natural products have been major sources of chemical compounds for new drugs development in the treatment of infectious diseases, lipid disorders, neurological diseases, cardiovascular and metabolic diseases, and immunological, inflammatory and related diseases without showing any side effects (Liu, 2005; Dzubak *et al.,* 2006; Castellano *et al.*, 2013). Triterpenes are a large and diverse group of compounds that can be found in medicinal plants, fruits and vegetables. Medicinal plants containing triterpenoids have been used for centuries in traditional medicine for the treatment of various diseases (Nikiwa *et al*, 1993; Gao *et al*, 2006; Cui *et al.*, 2006).

Oleanolic acid is a naturally occurring pentocyclic triterpene widely distributed in foods and medicinal plants (Gurib-Fakim, 2006). In numerous epidemiological investigations, oleanolic acid has been associated with decreased risk of chronic diseases such as cancer, cardiovascular disease (CVD), asthma and Type II diabetes (Feskanich *et al.*, 2000; Knekt *et al.*, 2000; Le Marchand, *et al.,* 2000; Knekt, *et al.*, 2002; Boyer & Liu, 2004; Gossé *et al.*, 2005). Oleanolic acid has been shown to attenuate fructose-induced hyperglycemia by modulating enzymes involved in carbohydrate digestion, insulin secretion and signalling (Liu *et al.,* 2013). It has been shown to possess beneficial effects against the development of diabetes and metabolic syndrome (MetS) by improving β-cell functionality and insulin sensitivity (Castellano *et al.,* 2013). In an *in vitro* study, oleanolic acid has been shown to protect hepatocytes against oxidative stress induced by tert-butyl-hydroperoxide (Wang *et al.,* 2010).

1.1 PROBLEM STATEMENT

Oxidative stress and chronic inflammation are inseparably interconnected, and both have been implicated in the aetiology of many disease states. The role of oxidative stress and inflammation in potentiating fructose toxicity is important in discovering novel strategies for combating and preventing fructose-induced toxicity. The use of medicinal plants for disease prevention and therapeutic purposes is gaining more attention because of the health benefits of their bioactive phytochemicals (Gurib-Fakim, 2006). Therefore, there is a need for the discovery and development of safe and effective drugs to combat the oxidative stress and inflammation which lead to the progression of risk factors associated with cardiovascular diseases. One of such plant phytochemicals is oleanolic acid.

1.2 AIM OF THE STUDY

The main of the study was to investigate the modulatory effects of oleanolic acid on oxidative stress and inflammation in the heart of high fructose-fed male rats.

1.3 OBJECTIVES OF THE STUDY

To achieve the aim of the study, the following objectives were set:

(i) To determine the antioxidant capacity i.e. Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC)

(ii) To measure the indicators of oxidative stress such as lipid peroxidation, glutathione status and, catalase activity and its gene expression.

(iii) To measure pro and anti-inflammatory biomarkers such as interleukins (IL-1β, IL-6, IL-12, IL-4, IL-6 and IL-10), monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor-alpha (TNF-alpha).

CHAPTER 2

2.0. LITERATURE REVIEW

2.1 OXIDATIVE STRESS

Oxidative stress occurs when the amount of substances responsible for oxidative damage exceeds the capacity of neutralization by the antioxidant system (Macdonald *et al.*, 2003). Reactive oxygen species (ROS), which are very unstable molecules, have potent oxidative effects on many cellular constituents (protein, lipids and DNA) and lead to the impairment of various cellular functions (Berg *et al.*, 2004). The cells are protected against the harmful effects of ROS by antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase) and other antioxidant substances (vitamin C, vitamin E and glutathione). However, when ROS generation overwhelms antioxidant capacity, the resulting accumulation of ROS, which is recognized as oxidative stress, contributes to the development of many pathological conditions (Johansen, *et al.*, 2005).

Oxidative stress is associated with pathogenesis of many diseases, causing cellular injury caused by the reaction of free radicals with cellular proteins, lipids and DNA (Berg *et al.*, 2004). It has been implicated in the pathogenesis of diabetes, hypertension and atherosclerosis (Rosen *et al.*, 2001; Philips, 2004; Johansen *et al.*, 2005; Niedowicz and Daleke, 2005). Oxidation of DNA and proteins may take place along with membrane damage due to lipid peroxidation, leading to alterations in membrane permeability, modification of protein structure and functional changes (Fridovichi, 1999). Oxidative damage to the mitochondrial membrane can also occur, resulting in membrane depolarization and the uncoupling of oxidative phosphorylation, with altered cellular respiration (Ambrosio *et al.*, 1993; Kadenbach, 2003).

Several reports have shown that oxidative stress is closely linked with obesity, diabetes and cardiovascular diseases (CVD) and coronary disease risk factors such as endothelial dysfunction, myocardial ischaemic injury, atherosclerosis, and hypertension (Moreno and Fuster, 2004; Dzau *et al.*, 2006; Vaziri, 2008; Blum, 2009; Shahbaz *et al.*, 2010), chronic kidney disease (Gosmanova and Le, 2011; Small *et al.,* 2012; Tang *et al.*, 2012), cancer (Valko *et al.*, 2006, 2007; Shah *et al.*, 2009; Gupta-Elera *et al.*, 2011) and diabetes (Giacco and Brownlee, 2010;

Matough *et al.*, 2012). Clinical evidence suggests that oxidative stress and inflammatory processes may be the key in the development of insulin resistance, diabetes, cardiovascular disease and other chronic diseases (Valko *et al.*, 2006, 2007; Shah *et al.*, 2009).

The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by a mechanism called "redox regulation". The process of "redox regulation" protects living organisms from various oxidative stresses and maintains "redox homeostasis" by controlling the redox status in vivo (Dröge, 2002).

2.2 SOURCES OF REACTIVE OXYGEN SPECIES

2.2.1 MITOCHONDRIA

Mitochondria constitute a major source of cellular ROS which are produced during oxidative phosphorylation, the essential cellular process by which ATP is generated by the electron transfer chain from NADH (nicotinamide adenine dinucleotide, reduced form)) or $FADH_2$ (flavin adenine dinucleotide) to molecular oxygen (Murphy, 2009). The process results in the formation of superoxides (Maechler *et al.* 1999) which have been implicated in the pathophysiology of a variety of diseases including cardiovascular diseases (CVDs), diabetes as well as the aging process itself (Muller *et al.*, 2004). The oxygen production occurs primarily through a series of electron transport carriers localized on the matrix side of mitochondrial membrane (Vries, 1986; Ott *et al.*, 2007). The electron transport chain carriers include; complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome c reductase), and complex IV (cytochrome c oxidase). In addition, complex I is the most likely site of electron leakage leading to formation of molecule O_2 (Madamanchi *et al.*, 2005). Oxygen (O_2) production by complex I was found to be markedly stimulated in the presence of succinate, the substrate of complex II (Vries, 1986; Ott *et al.*, 2007). Ubiquinone, a component of the mitochondrial respiratory chain, linking Complex I with III, and II with III, is regarded as a major player in the formation of O_2 by Complex III (Ott *et al.*, 2007; Turrens *et al.*, 1985; Muller *et al.*, 2003).

Previous studies have identified the ubiquinone of complex III and an unknown component of complex I as the major respiratory chain sites of ROS generation (Liu *et al.*, 2002; Barja, 1999; Yaffe, 1999; Lenaz, 1998; Dineley *et al.*, 2005). The mitochondrial respiratory chain is the major source of intracellular ROS generation and, at the same time, an important target for the damaging effects of ROS (Madamanchi *et al.*, 2005). Cellular metabolism depends on the continuous supply of ATP from the mitochondria, which in most tissues account for the bulk of ATP produced (Muller, 2000). Hence, any damage that impairs the function of the respiratory chain might also have an impact on cell viability (Madamanchi *et al.*, 2005; Ott *et al.*, 2007).

2.2.2 NADPH OXIDASE

The enzymatic activity of the membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is another major source of ROS, whose primary function is to catalyse the transfer of electrons from NADPH to molecular oxygen, resulting in the production of ROS, specifically superoxide (O_2^-) or hydrogen peroxide (H_2O_2) (Asaba *et al.*, 2005; Cai, 2005; Dröge, 2002). NADPH oxidases are abundantly expressed in the vasculature, where they are emerging as the key producers of ROS (Griendling *et al.*, 2000; Lambeth, 2004; Lassègue and Griendling, 2010). Overproduction of ROS arising from mitochondrial electron transport chain or stimulation of NADPH result in oxidative stress.

The enzymes NADPH oxidase (NOX) and dual oxidase (DUOX) generate ROS in a regulated manner, producing reactive oxygen in various cells and tissues in response to growth factors, cytokines and calcium signals (Lambeth, 2004). In vascular smooth muscle cells (VSMCs), recent studies have reported that there are membrane-bound and intracellular nonmitochondrial NADPH oxidases, which are all capable of producing oxygen molecule in these cells (Cai, 2005; Griendling *et al.*, 2000). With various existing techniques, oxygen molecules have been demonstrated to be accumulated within Vascular smooth muscle cells (VSMCs) when NAD (P)H oxidase is activated by different agonists such Angiotensin II (Griendling *et al.*, 1994; Gupte *et al.*, 2005). This intracellular accumulation of O_2^- has led to an assumption that a plasma membrane bound NADPH oxidase may produce and release O_2^- into cells, which is different from the orientation of phagocyte NADPH oxidases (Griendling *et al.*, 2000; Brandes and Kreuzer, 2005; Mohazzab *et al.*, 1994).

Reactive oxygen species, including superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are important determinants of vascular function, acting not only as signalling intermediates to promote growth and differentiation but also as modulators in pathological processes such as hypertension, atherosclerosis, and diabetic microvascular disease. Major sources of ROS in the vascular wall are NADPH oxidases which are multi-subunit enzymes that differ structurally and biochemically from the prototypical phagocyte NADPH oxidase active in host defense (Kleniewska *et al.*, 2012; Dworakowski *et al.*, 2008).

NADPH oxidase (NOX) consists of seven catalytic subunits, four of which are (Nox1, Nox2, Nox4, and Nox5) found in the vasculature. These enzymes transfer electrons from NADPH to molecular oxygen, thus producing superoxide (Lassègue and Griendling, 2010; Lassègue and Clempus, 2003).

Nox1 is expressed in endothelium, smooth muscle, and adventitial fibroblasts (Lassègue and Clempus, 2003; Csányi *et al.*, 2009), plasma membrane (Zhang *et al.*, 2007), caveolae (Hilenski *et al.*, 2004) and endosomes (Ranayhossaini *et al.*, 2013). The role of Nox1 in cardiovascular disease has been studied mainly in hypertension. Vascular or kidney Nox1 has been shown to be upregulated in experimental models, such as renin transgenic (Drummond *et al.*, 2011).

Nox2 is found in all vascular wall cells, except VSMC from large arteries, and produces intracellular or extracellular superoxide (Chamseddine and Miller, 2003; Petry *et al.*, 2006). These results suggest that Nox2 may affect blood pressure. A correlation between Nox2 expression and hypertension has been observed repeatedly (Park *et al.*, 2008). Hypertension is worsened by Nox2 overexpression in endothelial-targeted transgenic after acute or chronic angiotensin II treatment (Liu *et al.*, 2006). Accumulating evidence suggests that Nox2 adventitial fibroblasts as well as recruited macrophages promote inflammation and atherosclerosis (Brasier, 2010; Sun *et al.*, 2002; Drummond *et al.*, 2011).

Nox4 mRNA is present in all vascular wall cells and is significantly more abundant than other Nox enzymes, it is expressed in the kidney and blood vessels. In the kidney, NADPH oxidase components are expressed abundantly in the renal vessels and in the glomerular mesangial and podocyte cells (Chabrashvili *et al.*, 2002). The pro-inflammatory mediators tumor necrosis factor- and oxidized phospholipids increase Nox4 expression or activity (Yoshida and Tsunawaki, 2008; Datla *et al.*, 2007).

Within cells, Nox5 can be found in the cytoskeletal fraction, endoplasmic reticulum, or plasma membrane (Selemidis *et al.*, 2008; BelAiba *et al.*, 2007). Multiple isoforms are expressed in the vasculature (Selemidis *et al.*, 2008). Previous studies showed that atherosclerosis increased Nox5 expression and calcium-sensitive oxidase activity in coronary arteries (Lassègue and Clempus, 2003). Nox5 is detected in the endothelium of control arteries, in the neointima of diseased arteries, and in smooth muscle underlying complex lesions in arteries with advanced atherosclerosis (Guzik *et al.*, 2008; Guzik *et al.*, 2006). The NOX isoforms not only vary in their expression pattern, but also in their sub-cellular localizations (Brown and Griendling, 2009; Lambeth, 2007). Evidence for the involvement of NOX in CVD can be found in patients with chronic granulomatous disease, who have an inherited mutation in genes encoding the components of NOX2. These individuals have early mortality due to impaired innate immunity, and have increased production of pro-inflammatory mediators and recurrent infection (Serrander *et al.*, 2007).

2.2.3 XANTHINE OXIDOREDUCTASE (XOR)

Xanthine oxidase is an enzyme present in the cytoplasm which catalyzes oxidation of its substrates hypoxanthine and xanthine to uric acid using O_2 as an electron receptor and, in the process, produces O_2^- and H_2O_2. The ability of XOR to generate a diverse range of reactive oxygen nitrogen species (RONS) has been previously described by (Godber *et al.*, 2000). These RONS are usually eliminated by antioxidant enzymes abundantly present in the cytoplasm. The mechanism of ischemia and tissue injury during reperfusion has been extensively studied, especially in the heart, thus, it is a good example of the involvement of oxidative stress in a frequent pathology (Ceconi *et al.*, 2003). Xanthine oxidoreductase (XOR) is part of the molybdoflavin enzyme family, and exists in two inter-covertible forms known as xanthine oxidase (XO) and dehydrogenase (XDH). Recent work has indicated a role for XO and XOR related oxidant species in the pathogenesis of chronic heart failure (CHF) (Berry and Hare, 2004). *In vitro* studies on isolated hearts have demonstrated that the progressive development of heart failure is associated with increased myocardial XOR levels, and is negatively correlated with endothelial dysfunction which leads to an enhancement of oxidative stress in the heart (Ferdinandy *et al.*, 2000; Viazzi *et al.*, 2005). During oxidation of hypoxanthine or xanthine, electrons are donated first to the molybdenum site, then transferred via the FAD^+ site, to reduce either molecular oxygen or NAD^+ (Xia *et.al.*, 1999). Xanthine dehydrogenase (XDH) preferentially reduces NAD^+, aside from in settings of NAD^+ deficiency, which can occur in the setting of inflammation. The reduction of molecular oxygen results in the production of O_2^- and H_2O_2. In the past, much focus has been on O_2^- production by XOR, but more recent research has suggested that of the two forms of ROS and H_2O_2 is produced in far greater quantities (Kelley *et al.*, 2010). Inflammatory molecules such as IL-I, IL-6, TNF-a, and IFN-g have been shown to increase the activity of XOR, these cytokines upregulate the gene expression of this enzyme (Matsui *et al.*, 2001). Furthermore, XO-derived ROS have been proposed to act as mediators of inflammatory signal transduction pathways and pro-inflammatory gene expression (Pacher *et al.*, 2006).

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2.3 TARGET OF FREE RADICAL DAMAGE

High concentration of ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al.*, 2007a). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell, 2007). Changes in genetic material resulting from these oxidative damage incidents represent the first step involved in mutagenesis, carcinogenesis, and ageing (Pala and Gürkan, 2008; De Groote *et al.*, 2012; Valko *et al.*, 2007b).

2.3.1 OXIDATIVE DAMAGE TO DNA

DNA can be damaged by ROS either directly or indirectly via lipid peroxidation products (Møller *et al.*, 2000; Collins *et al.*, 1998). DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating damage in the development of cancer (Leonard *et al.*, 2004; Waalkes *et al.*, 2004). Reactive oxygen species (ROS) induced DNA damage involves single or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis (Valko *et al.*, 2007a; Marnett, 2000). DNA damage, mutations, and altered gene expression are thus all key players in the process of carcinogens. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as peroxynitrites and nitrogen oxides have also been implicated in DNA damage (Hehner *et al.*, 2000). In addition to the extensive studies devoted to the role of oxidative nuclear DNA damage in neoplasia, there exists evidence about the involvement of mitochondrial oxidative DNA damage in the carcinogenesis process (Valko *et al.*, 2006). Mutations and altered expression in mitochondrial genes encoding for complexes I, III, IV and V, and in the hypervariable regions of mitochondrial DNA have been identified in various human cancers (Valko *et al.*, 2007a).

2.3.2 LIPID PEROXIDATION

The high concentration of unsaturated fatty acids in the cellular membrane makes the membranes particularly prone to oxidation by ROS, called lipid peroxidation. The peroxidation leads to alterations in the biological properties of the membrane and could be extremely harmful. Once peroxyl radicals (ROO) are formed, they can be rearranged *via* a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). The major aldehyde product of lipid peroxidation is MDA. The level of MDA, a thiobarbituric acid (TBA) reacting substance, is a widely used biomarker for lipid peroxidation (Mao *et al.*, 1999).

2.3.3 PROTEIN OXIDATION

The level of nitrotyrosine can be measured to estimate oxidative damage to proteins. Nitrotyrosine is generated from the oxidation of tyrosine and is a biomarker for RNS. Oxidative stress is associated with increased formation of ROS that modifies phospholipids and proteins leading to peroxidation and oxidation of thiol groups (Molavi and Mehta, 2004) . The damage by ROS leads to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins (Catalá, 2009). In addition to cellular protein and lipid damage, abnormalities in myocyte function due to increased oxidative stress are associated with the effects of ROS on subcellular organelles (Powers and Jackson, 2008).

2.4 ANTIOXIDANT DEFENCE SYSTEM

2.4.1 ENZYMATIC ANTIOXIDANTS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Valko *et al.*, 2007a). Defence mechanisms against free radical-induced oxidative stress involve; (a) preventative mechanisms, (b) mechanisms to repair oxidative damage, (c) physical defences, and (d) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants.

The antioxidant defences come from endogenous systems including uncoupling proteins of the respiratory chain, mitochondrial, cytoplasmic and peroxisomal enzymes, which exist to reduce oxidative stress (Harrison *et al.*, 2003). Antioxidant defence is the conversion of O_2- to H_2O_2 by superoxide dismutase (SOD) in the mitochondria and then the further detoxification of H_2O_2 to form H_2O by the action of glutathione peroxidase (GPX) in the cytoplasm or the action of catalase in the peroxisome (Trachootham *et al.*, 2008). The transformation of reduced glutathione (GSH) to oxidized glutathione (GSSG), which is catalysed by GPX, is a major antioxidant system that reduces a broad range of hydroperoxides. GSH, in addition to being a cofactor of various antioxidant enzymes, is the most abundant peptide in cells and possesses an excess of functions. These include direct scavenging of OH-, singlet oxygen, and regeneration of other antioxidants such as vitamin C and E to their active forms (Nakamura *et al.*, 1997).

Superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins are examples of antioxidant enzymes preventing damage by direct removal of ROS. Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion to O_2 and H_2O_2. The human classes of this enzyme include CuZn-SOD and are present in the cytosol as well as in the intermembrane space of the mitochondria and Mn-SOD present in the mitochondria. In addition to mitochondrial SOD, the other SOD isoforms include extracellular SOD, which is produced and secreted by vascular smooth muscle cells and binds to components of the extracellular matrix to regulate interstitial redox status (Laufs *et al.*, 2004).

Catalase and glutathione peroxidase are responsible for the reduction of hydrogen peroxide to water (Sindhu *et al.,* 2005). Catalase protects cells from hydrogen peroxides generated intracellular and plays a major role in the adaptive response of cells to oxidative stress (Mates, 2000). However, in myocardium, catalase is present at relatively low concentrations, while in GSH-Px is present at higher concentrations (Kalogeris *et al.*, 2014; Currie *et al.*, 1988; Fabiani *et al.*, 1993).

Thioredoxins are a ubiquitous group of small ubiquitous proteins which are important intracellular antioxidants. Thioredoxins contain reduced cysteine residues which allow enzymatic repair of oxidative damage to cellular proteins by reducing thiol disulphide bonds which develop in the context of oxidative. The enzyme thioredoxin reductase reduces thioredoxins to recycle them for their participation in these reactions (Ebrahimian and Touyz, 2008). Overexpression of thioredoxins increases the lifespan of transgenic mice (Yoshida *et al.*,

2005), and reduces the development of diabetic nephropathy in a mouse model of diabetes (Hamada et al., 2007).

2.4.2 NON - ENZYMATIC ANTIOXIDANTS

2.4.2.1 VITAMIN C

The water-soluble vitamin C (ascorbic acid) is one of the key aqueous phase antioxidants. Vitamin C is capable of scavenging ROS such as superoxide, hydrogen peroxide and singlet oxygen (Young and Woodside, 2001). Vitamin C might also reduce oxidised tocopherol. Ascorbic acid itself is oxidised to dehydro-ascorbate and reduced back to ascorbic acid by glutathione (Pelley, 2011). The concentration of ascorbic acid in plasma is clearly related to the dietary intake (May and Harrison, 2013).

2.4.2.2 VITAMIN E

Vitamin E is a chain breaking antioxidant that protects lipids against peroxidation by donating hydrogen from the phenolic groups, reacting with peroxyl radicals and forming a relatively stable tocopheroxyl radical (Sies and Stahl, 1995). Vitamin E may have other properties than being an antioxidant; for example, tocopherol has been shown to possess anti-inflammatory properties (Peh *et al.*, 2016; Grammas *et al.*, 2004). Vitamin E protects cell membranes and low-density lipoproteins (Raederstorff *et al.*, 2015). Dietary vitamin E is mainly found in vegetable oils with elevated levels of polyunsatured fatty acids. Rich sources of tocopherol are wheat germ and sunflower oil, sunflower seeds and almonds (Shahidi and de Camargo, 2016).

2.4.2.3 CARETONOIDS

The carotenoids are scavenger antioxidants, inactivating ROS, especially singlet oxygen (Sies and Stahl, 1995). The activated carotenoid is regenerated by emitting energy. Carotene is considered to be an effective antioxidant at the oxygen tension found in most tissues. However, at higher oxygen tension, such as the one found in lungs, carotene exhibits pro-oxidative character (Clark, 2002). Un-refined palm oil is one of the most carotenoid rich foods in nature. The carotenoids are also found in bright coloured fruits and vegetables (Khoo *et al.*, 2011), with the orange ones being rich in carotene (e.g. carrot, apricot) (Mortensen, 2006), the red ones are rich in lycopene (e.g. tomato) (Khoo *et al.*, 2011) and the green ones containing elevated levels of lutein and zeaxanthin (e.g. Spinach, broccoli) (Guo *et al.*, 2008).

2.4.2.4 FLAVONOIDS

Flavonoids have been shown to work through three different antioxidative mechanisms. They are suggested to be effective scavengers of free radicals, to have inhibitory effects on enzymes responsible for superoxide production, and to have metal binding (chelating) characteristics (Korkina and Afanas' Ev, 1996; Bravo, 1998). The most ubiquitous flavonoid subclasses found in western diets are the flavonols and flavones. Flavonols are present in onions, leeks, broccoli, red wine and tea. Flavones are less common than flavonols in fruit and vegetables(Manach *et al.*, 2004).Celery and parsley are the most important dietary sources. Isoflavonoids are predominantly found in leguminous plants, e.g. soya. Flavanones are present in high concentrations in citrus fruit, especially in the membranes separating the segments(D Archivio *et al.*, 2007). Anthocyanins gives the pink, red, blue and purple colour in the skin of different berries and fruits. Green tea and chocolate are rich sources of flavans (Manach *et al.*, 2004).

2.5 OXIDATIVE STRESS AND VASCULAR COMPLICATIONS

Several reports have shown increased levels of circulating markers of free radical-induced damage and reduced antioxidant defences in patients with type 2 diabetes (Farahmand *et al.*, 2003; Davì *et al.*, 2005; Aneja *et al.*, 2008). Factors such as hyperglycemia, glucose autoxidation, and elevated levels of free fatty acids and leptin have been reported to contribute to increased ROS generation in diabetes (Jay *et al.*, 2006). Evidence also exists for increased production of ROS from nonmitochondrial sources such as NADPH oxidase (NOX), or reduced neuronal nitric oxide synthase (nNOS) activity coupled with increased activation of xanthine oxidoreductase (Li *et al.*, 2006). Activity of NOX, an important enzyme involved in superoxide generation, has been found to be significantly higher in vascular tissue (saphenous vein and internal mammary artery) obtained from diabetic patients (Guzik *et al.*, 2002). Multiple pathways have been linked with hyperglycemia-induced oxidative stress, inflammation, and cardiovascular disease vascular complications (Son, 2007). Atherogenic mechanisms also appear to be involved in pathophysiology of diabetes-associated heart disease. Several atherogenic mechanisms, such as low-density lipoprotein (LDL) oxidation, endothelial dysfunction, and vascular smooth muscle proliferation and migration, could be induced by oxidative stress, leading to cardiomyopathy. Increased oxidative stress may lead to the formation of gene products that cause cellular damage, thereby contributing to the pathogenesis of various diabetic vascular complications (Newsholme *et al.*, 2007; Nishio *et al.*, 2004; Son, 2007).

Oxidative stress and subsequent damage are considered the main components of the common soil hypothesis for the pathogenesis of CVD and type 2 diabetes (Ceriello and Motz, 2004). High oxidative stress is associated with many of the risk factors implicated in the pathophysiology of atherosclerosis and diabetes, such as hypercholesterolaemia, renal failure, hypertension, smoking and ageing(Harrison *et al.*, 2003). Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, the oxidation of LDL, endothelial dysfunction, plaque rupture, myocardial ischemic injury, and recurrent thrombosis (i.e., the secondary, or subsequent clot that often occurs after initial thrombolysis). The role of oxidative stress in the connection between the various coronary disease risk factors such as elevated blood pressure, diabetes and cigarette smoking, and the clinical sequelae of disease associated with vasoconstriction, thrombosis, plaque rupture, and vascular remodelling has been recognized by Moreno and Fuster (Pashkow, 2011). Oxidative stress has been implicated as well in diabetic cardiomyopathy (Khullar *et al.*, 2010; Shahbaz *et al.*, 2010), congestive cardiomyopathy (Shahbaz *et al.*, 2010), and hypertensive heart disease.

It is also known that oxidative stress and inflammation are closely associated with the pathogenesis and progression of diabetes induced CVD where the overproduction of reactive oxygen species (ROS) and increased expression of inflammatory proteins or cytokines such as C-reactive protein (CRP) are shown to serve as biomarkers for the onset of CVD and HF (Braunwald, 2008; Rajesh *et al.*, 2010). ROS overproduction is associated with hyperglycemia and metabolic disorders, such as impaired antioxidant function in conjunction with impaired antioxidant activity. Long-term exposure to oxidative stress in diabetes induces chronic inflammation and fibrosis in a range of tissues, leading to formation and progression of disease states in these tissues. Thus, it is of great concern that CVD is far more common in patients with DM than the non-diabetic population(Braunwald, 2008; Kayama *et al.*, 2015). Diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs in diabetic patients independent of a recognized cause, such as coronary artery disease (CAD) or hypertension (Boudina and Abel, 2007; Zarich and Nesto, 1989). The hallmark characteristic of diabetic cardiomyopathy is a subclinical phase associated with cellular structural abnormalities leading initially to diastolic dysfunction, later to systolic dysfunction, and eventually to heart failure.(Braunwald, 2008). Oxidative stress induced by hyperglycemia is implicated as a source of altered endothelium relaxation in diabetes (Giugliano *et al.*, 1995). Previous studies have

shown the possibility that oxygen derived free radicals play a role in the pathogenesis of vascular complications of diabetes.

Many recent studies have suggested that oxygen-derived free radicals may be important participants in a wide array of cardiac conditions (Wattanapitayakul and Bauer, 2001). Several recent studies have demonstrated that altered oxygen utilization and/or increased formation of reactive oxygen species (ROS) contributes to CVD progression (Wattanapitayakul and Bauer, 2001). The initial suggestions of oxidative mechanisms during CVD were described in the acute settings of ischemia/ reperfusion injury and MI. These conditions are associated with a sudden reduction of coronary perfusion and oxygen availability, leading to altered myocardial metabolism, ROS production, and cell death. Interestingly, ROS production and associated cellular damage is higher in cardiac tissue during tissue reperfusion relative to ischemic conditions (Wattanapitayakul and Bauer, 2001). ROS may contribute to the progression of other more chronic cardiovascular conditions that are not related to acute oxygen deprivation. In addition to cellular and/or tissue evidence of oxidative damage, elevated levels of oxidative stress markers are detected in several pathologic conditions of cardiovascular disorders, including hypertension, ventricular hypertrophy and atherosclerosis(Sugamura and Keaney, 2011; Panth *et al.*, 2016; Drozdz and Kawecka-Jaszcz, 2014).

Oxidative stress in the heart is increased in response to hypertrophy and heart failure. It has been suggested that increases in oxidative stress in the failing heart are primarily due to the functional uncoupling of the respiratory chain caused by inactivation of complex I (Ide *et al.*, 1999; Li *et al.*, 2002). However, increased ROS in the failing myocardium may also be due to impaired antioxidant capacity, such as reduced activity of Cu/Zn superoxide dismutase and catalase (Kuroda *et al.*, 2010).

2.6 INFLAMMATION

Inflammation is a biological process initiated by the immune system in response to tissue injury caused by microbial infection and other noxious stimuli. Acute inflammatory response is characterized by vasodilation, leakage of the vasculature, and infiltration of leukocytes into the site of infection to destroy invading pathogens and is followed by a rapid resolution phase and repair of the damaged tissue (Kundu and Surh, 2012). Although, acute inflammation plays a beneficial role against infection and injury, when there is inadequate resolution of such inflammatory responses, inflammation may occur (Schottenfeld and Beebe-Dimmer, 2006;

Aggarwal *et al.*, 2006; Kundu and Surh, 2008). Several studies have shown a strong relationship between low-grade systemic (chronic) inflammation and disease conditions including insulin resistance, diabetes, cardiovascular diseases, metabolic syndrome, Alzheimer's disease and cancers (Achike *et al.*, 2011; Callewaert, 2012; Kundu and Surh, 2012). Substantial evidence suggests that chronic inflammation is associated with enhanced production of reactive oxygen and nitrogen species (RONS) resulting in deregulated inflammatory responses including, but not limited to pro-inflammatory cytokine production (Reuter *et al.*, 2010; Bulua *et al.*, 2011; Naik and Dixit, 2011).

Chronic inflammation is a pathological condition characterized by continued active inflammatory responses and tissue destruction. Scientific evidence has indicated that chronic inflammation and oxidative stress are interconnected as inflammatory processes induce oxidative stress and deplete cellular antioxidant capacity (Vaziri, 2008; Khansari *et al.*, 2009). Several studies have confirmed the strong relationship between chronic inflammation and chronic conditions, such as metabolic syndrome, CVD, diabetes, cancer and other serious disease states (Kundu and Surh, 2008; Armitage *et al.*, 2009; Dinarello *et al.*, 2010; Grivennikov *et al.*, 2010; Achike *et al.*, 2011). Nuclear Factor-Kappa B (NF-κB) has been implicated as a major mediator of inflammation in most chronic disease conditions and it has been established that its inhibition can prevent / delay the onset of chronic diseases (Aggarwal *et al.*, 2012).

It has been reported that an interrelation between inflammation and metabolic abnormalities in diabetes leads to endothelial injury and the development of cardiovascular complications (Goldberg, 2009). The increased expression of inflammatory cytokines and adhesion molecules can amplify the inflammatory responses leading to aggravation of diabetic vascular complications (Magertic, 2012). Vascular injury, oxidative stress, inflammation and chronic alterations in the thermodynamic balance deriving from alterations underlying hyperglycemia may initiate a process of atherosclerosis and the arterial thrombus (Annichino-Bizzacchi, 2004).

The first mechanistic link between obesity and low-grade inflammation was the finding that white adipose tissue synthesizes and releases TNF-α. It was later shown that the obese adipose tissue is infiltrated by macrophages which are an important source of the inflammation in this tissue (Vandesompele *et al.*, 2002; Suzuki *et al.*, 2011). An imbalance or dysfunction of adipose tissue contributes to obesity-induced chronic inflammation, which in turn results in energy metabolism disorders such as insulin resistance, type 2 diabetes, inflammation and

cardiovascular disease (Scherer, 2006; Olefsky and Glass, 2010). There is emerging evidence that certain leukocytes, such as eosinophils, seem to have a protective metabolic effect (Wu *et al.*, 2011) while other leukocytes, such as macrophages, cause metabolic deregulation (Olefsky and Glass, 2010; Oh *et al.*, 2012). It has been reported that additional inflammatory cytokines including interleukin (IL)-6, interleukin-1 beta (IL-1β), and monocyte chemotactic protein-1 (MCP-1) are increased in obese adipose tissue (Berg and Scherer, 2005). Furthermore, it is now appreciated that other tissues, including the liver, pancreas, and even the brain, exhibit increased inflammatory cytokine expression and inflammation in obesity (Hotamisligil *et al*, 1995; Donath, 2014).

Inflammation is a complex process generally caused by tissue injury which in turn is caused by bacteria, ischemia/reperfusion, chemicals or heat. It is characterised by vasodilatation of local blood vessels, migration of leucocytes, increased permeability of the capillaries and tissue oedema (Markiewski and Lambris, 2007). In addition, there is also a systemic reaction with increased concentrations of acute phase cytokines and proteins, and cyclooxygenase (COX) mediated prostaglandin formation (Mittal *et al.*, 2014; Lacroix and Rivest, 1998). If the elimination of the acute phase reaction products is disturbed or if the tissue injury is of chronic nature, chronic inflammation might occur. Low-grade inflammation is detected in type 2 diabetes mellitus (Pickup, 2004), the metabolic syndrome (Ford, 2003) and obesity (O'Rourke, 2009) and is believed to be of importance in the pathogenesis of these diseases.

2.6.1 CHRONIC INFLAMMATION

Chronic systemic inflammation arises due to the persistence of a pro-inflammatory environment. This could be as a result of persistent pro-inflammatory stimulus or a result of inadequate resolution of the inflammatory response (Baniyash, 2006). Conditions that are often associated with chronic systemic inflammation include obesity, diabetes and metabolic syndrome (Das, 2001; Das, 2002; Karin *et al.*, 2006). Metabolic syndrome is characterized by a host of systemic inflammatory conditions including abdominal obesity, insulin resistance / hyperinsulinemia, dyslipidemia, impaired glucose tolerance / type 2 diabetes mellitus, and hypertension (Karin *et al.*, 2006). This syndrome often leads to coronary heart disease, which also features systemic inflammation. Each of these conditions, among other inflammatory diseases, contains features that can promote and support mutagenic changes as well as create a self-sustaining environment, which may potentiate the development of a tumor (Coussens and Werb, 2002; Lowe and Storkus, 2011).

The chronic local inflammation present during atherogenesis is mediated by specific cellular responses that affect arterial endothelial function and vascular structure (Van Lente, 2000). The response is mediated by macrophages and specific T lymphocytes at the site of the lesion (Springer and Cybulsky, 1996; Van der Wal *et al.*, 1989), which produce a collection of cytokines, chemokines, growth factors, and enzymes and their actions participate in the development of the mature lesion (Jonasson *et al.*, 1986). The response of the arterial endothelium to injury includes expression of specific adhesion molecules that provide binding sites for monocytes and T cells (Van Lente, 2000). Other molecules that are active in promoting migration of circulating monocytes and T cells into the artery include monocyte chemotactic protein 1, and modified low density lipoprotein (LDL) (Ross, 1999).

Inflammation leads to the localised recruitment of neutrophils and monocytes, and the presence of activated macrophages in the cap of the atherosclerotic plaque has led to suggestions that they contribute to plaque rupture through effects on matrix metalloproteinases (Yudkin *et al.*, 2000). Both monocytes and macrophages produce interleukin 1 (IL-1) in their potentiation of inflammation. Increased IL-1 synthesis has been demonstrated in human arterial plaques (Tipping and Hancock, 1993).

The molecules intracellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1) have been shown to be extensively expressed on endothelial cells overlying plaque during accelerated, post-transplant atherosclerosis. Increased ICAM-1 expression has been associates with chronic vascular disease after cardiac transplantation and also similar changes have been identified in patients with failing myocardium (Van Lente, 2000). It is known that local inflammation occurs in the formation plaques, as macrophages and other immuno-competent cells are present in the lesions from an early stage. It is also known that inflammation plays an important role in the weakening of the fibrous cap of the advanced plaque, eventually leading to plaque rupture and acute coronary syndromes (Lind, 2003).

C-reactive protein (CRP) is a pentameric protein synthesised in the liver. Its main action is to activate complement and to counteract infections. The main stimuli for secretion of CRP are interleukin-1 and -6, and tumour necrosis factor alpha. Circulating levels of CRP have been found to be related to a number of well-known cardiovascular risk factors, such as obesity, smoking, serum fibrinogen, HDL-cholesterol, heart rate, blood pressure, serum triglycerides, fasting blood glucose, apolipoprotein B and inversely to HDL-cholesterol levels both in children

and in adults (Cook *et al.*, 2000; Mendall *et al.*, 1996). Healthy subjects with at least one parent with myocardial infarction have elevated CRP levels compared with those without heredity for myocardial infarction (Margaglione *et al.*, 2000; Kuller *et al.*, 1996).

The main pro-inflammatory cytokines are interleukin 1 and 6 and tumour necrosis factor alpha (TNF-α). Tumour necrosis factor alpha (TNF-α) is a multifunctional circulating cytokine derived from endothelial and smooth muscle cells, as well as macrophages (Akdis *et al.*, 2016). Tumour necrosis factor alpha (TNF- α) plays a key role in the cytokine cascade as it stimulates the synthesis of the other cytokines. Also, the other two pro-inflammatory cytokines, interleukin 1 and 6, are pleotropic cytokines with a broad range of humoral and cellular immune effects relating to inflammation, host defence and tissue injury (Stenvinkel *et al.*, 2005).

The evolution of coronary heart diseases (CHD) to the stage with vulnerable plaque and acute coronary syndromes results in a more vigorous involvement of inflammation in the course of disease (Van Lente, 2000). The instability and rupture of atherosclerotic lesions with subsequent thrombus formation and possible myocardial necrosis are likely to have a significant inflammatory component (Van Lente, 2000; Ambrose and Singh, 2015). In the population studies, it has consistently been shown that high levels of CRP (Ridker *et al.*, 2000a), an increased number of leukocytes in peripheral blood (Ridker *et al.*, 1998; Ridker *et al.*, 2000c) and elevated levels of serum fibrinogen (Ma *et al.*, 1999) are found to lead to the prrogression to atherosclerotic diseases, in particular coronary heart disease. Also in the prospective studies using patients with coronary heart disease, a number of other biomarkers of inflammation have been investigated. It has then been shown that elevated levels of interleukin-6, TNF-a, soluble receptors for interleukin-1, the adhesion molecules ICAM-1, VCAM-1, ELAM-1 and E-selectin, as well as PLA-2 and the monocyte derived neopterin, all have been associated with a poor outcome in this patient group (Kugiyama *et al.*, 2000; Ridker *et al.*, 2000b).

2.7 OXIDATIVE STRESS AND INFLAMMATION IN HEART DISEASES

Inflammation has a key role in the pathogenesis and progression of many forms of cardiovascular disease. Chronic inflammatory process induced by pro-inflammatory cytokines and chemokines also contribute to the pathogenesis of diabetic cardiomyopathy (De Luca and Olefsky, 2008). Obesity and the associated development of inflammation are major components of insulin resistance. Studies in human obesity and insulin resistance have revealed a clear

association between the chronic activation of pro-inflammatory signalling pathways and decreased insulin sensitivity. For example, elevated levels of tumor necrosis factor (TNF-a), interleukin-6 (IL-6) and interleukin (IL-8) have all been reported in various diabetic and insulin resistant states (Hotamisligil and Spiegelman, 1994; Roytblat *et al.*, 2000; Straczkowski *et al.*, 2002).

Inflammation resulting from oxidative stress is the cause of much human diseases namely diabetes, metabolic syndrome and hypertension (Martinon, 2010) (Pashkow, 2011). Inflammation is also a key factor in all aspects of coronary disease including the initiation and progression of atherosclerotic plaque, plaque rupture, and thrombosis (atherothrombotic), especially in recurrent thrombosis where oxidative stress is known to play a significant role. Pro-inflammatory cytokines are also involved in cardiac muscle dysfunction and in the complex syndrome of heart failure (Mann and Young, 1994; Mann, 2002; Torre-Amione, 2005).

Emerging evidence suggests that diabetic conditions induce an inflammatory response by oxidative mechanisms, which may contribute to the development of diabetic cardiomyopathy (Khansari *et al.*, 2009). Hyperglycemia has been observed to be associated with greater systemic inflammation that may be an important contributor to cardiovascular risk among diabetic patients (Farahmand *et al.*, 2003). A number of inflammatory cells accumulating at vulnerable sites along the vessel wall, thus triggering plaque instability type 1 and type 2 diabetes mellitus have been experimentally characterized by intramyocardial inflammation, including increased tumor necrosis factor (TNF)-α expression, oxidative stress, and myocardial fibrosis (Helmersson *et al.*, 2004).Tumour necrosis factor alpha (TNF- α) is known to reduce myocyte contractility and increase tissue fibrosis, contributing to cardiac failure. Anti-TNF-a antibody treatment has been shown to significantly ameliorate intramyocardial inflammation and myocardial fibrosis associated with diabetic cardiomyopathy (Westermann *et al.*, 2007).

Type I and type II diabetes mellitus has experimentally been characterized by intramyocardial inflammation, including increased TNF-α expression, oxidative stress and myocardial fibrosis. Excessive TNF-α levels can induce left ventricular (LV) dysfunction and cardiomyopathy increased cardiac TNF-α expression negatively affects cardiac contractility (Westermann *et al.*, 2007). Clinical and experimental studies have demonstrated the importance of tumor necrosis factor (TNF)- α in the pathogenesis of atherosclerosis dilated cardiomyopathy, viral myocarditis preconditioning , myocardial infarction and heart failure (Bryant *et al.*, 1998; Mann, 2003).

TNF-α is an important molecule that triggers inflammation and cell injury in the heart ultimately inducing cardiac fibrosis. Suppression of TNF-α has been shown to improve diabetic cardiomyopathy, reduce cardiac fibrosis, and enhance cardiac function. NF-κB has also been implicated as a key mediator of the inflammatory process in the diabetic heart. NF-κB is a major transcription factor that controls the expression of many genes including pro-inflammatory pro-fibrotic and hypertrophy-related genes (Braunwald, 2008).

An increase in inflammatory cytokines IL-6 (Interleukin-6), IL-18 (Interleukin -18), IL-1 (Interleukin -1) and TNF-α has been reported in the blood of patients with diabetes mellitus (Cabric *et al.*, 2007). Hyperglycemia can also activate nuclear factor kappa-light-chain-enhancer of activated B cells) (NF-Kb), a transcription factor leading to an increase in the expression of inducible nitric oxide synthase (iNOS) which is accompanied by an increased generation of nitric oxide (Spitaler and Graier, 2002). Nitric oxide can react with superoxide to produce the strong oxidant peroxynitrite, which can in turn increase lipid peroxidation, protein nitration, and low-density lipoprotein (LDL) oxidation, affecting many signal transduction pathways (Griendling and FitzGerald, 2003).

Elevated levels of inflammatory markers including high-sensitivity C-reactive protein (hs-CRP), a marker of systemic inflammation, as well tumour necrosis factor-alpha (TNF-α), a measure of vascular inflammation, have been shown in numerous studies to be independently related to the risk of future cardiovascular events (Morrow and Ridker, 2000; Libby, 2006). Interleukin-6, other interleukins, myeloperoxidase, and tumour necrosis factor-alpha (TNF- α) also have inflammatory activity and have been shown to be associated with cardiovascular events in some studies (Greenland *et al.*, 2010).

Congestive heart failure has been defined in many ways, and many of these definitions imply it to be "the pathophysiological state in which an abnormality of cardiac function is responsible for failure of the heart to pump blood at a rate commensurate with the demands of the metabolizing tissues (Dhalla *et al.*, 1996). Evidence showed that congestive heart failure is a common end of many abnormal cardiac conditions, including hypertrophy. Heart failure, subsequent to cardiac hypertrophy, remains a major clinical problem, and the progression of a well-compensated hypertrophied heart to that of a decompensated stage is poorly understood. It has been shown that an increase in oxidative stress due to an increase in free radicals or a relative deficit in the endogenous antioxidant reserve, or both, can cause contractile dysfunction (Dhalla *et al.*, 1996).

IL-6 might play a key role in the development of coronary disease through a number of different mechanisms; metabolic, endothelial and coagulant. IL-6 increases basal glucose uptake, alters insulin sensitivity, increases the release of adhesion molecules by the endothelium and increases the hepatic release of fibrinogen, as well as having pro-coagulant effects on platelets (Yudkin *et al.*, 2000). There is a close relationship between circulating concentrations of CRP, IL-6 and TNF-a, with the components of the insulin resistance syndrome — insulin sensitivity, high triglyceride and low HDL-cholesterol concentration, and elevated blood pressure (Tangvarasittichai *et al.*, 2016).

2.8 OXIDATIVE STRESSS IN PROGRESSION TO ATHEROSCLEROSIS

High oxidative stress is found in hypertension. This provides a compelling convergence of mechanisms for the major risk factors for atherosclerosis (Scott, 2004). Oxidative stress is associated with several cardiovascular diseases including atherosclerosis, hypertension, congestive heart failure and diabetes. Oxidation of LDL plays a key role in pathogenesis of atherosclerosis. Oxidative stress plays a key role in endothelial dysfunction associated in these diseases, as superoxide (O_2) inactivates nitric oxide (NO) causing endothelial dysfunction. Endothelial dysfunction is associated with and may contribute to an increased risk of cardiovascular events (Heitzer *et al.*, 2001).

As oxidative stress has been observed in several diseases of macro vascular and micro vascular origin, oxidised low density lipoprotein (oxLDL) plays a central pathogenic role in the arterial wall (Stocker and Keaney, 2004). Various known risk factors for atherosclerosis lead to a state of oxidative stress characterized by excessive production of ROS that is not neutralized by the body's antioxidant defense (Bonomini *et al.*, 2008). Now it is well known that oxLDL induced pro -oxidant state is present in all stages of atherosclerosis from the beginning to the acute thrombotic event. oxLDL inhibits the expression of the constitutive endothelial enzyme nitric oxide synthase (eNOS), which inhibits a number of steps involved in atherogenesis (Aviram *et al.*, 2000). In addition, it results in the generation of ROS from endothelial cells, vascular smooth muscle cells and macrophages. Release of ROS also includes expression of adhesion molecules on endothelial cells, proliferation of macrophages, stimulates collagen formation in fibroblasts, leads to proliferation and migration of vascular smooth muscle cells and activation of platelets (Aviram *et al.*, 2000).

2.9 HIGH FRUCTOSE DIET

A high intake of sugars has been linked to diet-induced health problems. Sugars are important sources of energy in our diet, but a high intake of sugars has increasingly been identified as a considerable cause of major diet-induced health problems (Kolderup and Svihus, 2015). Recent findings support that the high consumption of fructose may be an important contributor to the metabolic syndrome, resulting in hyperinsulinemia, insulin resistance, hypertension, hypertriacylglycerolaemia and atherosclerosis (Suwannaphet *et al.*, 2010; Khitan and Kim, 2013). These abnormalities enhance cardiac and endothelial problems (Edwards, 2009; Akar *et al.*, 2012). Studies have shown that high fructose diet-fed rats display hepatic oxidative damage and altered lipid metabolism due to hepatic stress as a result of the burden of fructose metabolism (Suwannaphet *et al.*, 2010).

High-fructose corn syrup (HFCS) has several industrial advantages such as freshness, texture, not masking original taste, and, especially, low price (Saygin *et al.*, 2016). Fructose is found in a variety of foods. In table sugar, it is bound to glucose to form the disaccharide sucrose, whereas in honey it occurs in monosaccharide form. In fruit, berries, and vegetables, fructose occurs in both monosaccharide and disaccharide forms (Kolderup and Svihus, 2015).
Consumption of sucrose and more recently of HFCS has increased dramatically through processed foods such as beverages, jams, jellies, baked goods and dairy products. An increase in high fructose corn syrup, as well as total fructose consumption over the past years has been linked to a rise in obesity and metabolic disorders (Rizkalla, 2010).

De Novo Lipogenesis (DNL) is the metabolic pathway that transforms excess non-fat energy into fat by synthesis of fatty acids from acetyl-CoA (Hellerstein *et al.*, 1996). The liver is the primary site of DNL. Higher percentage of fructose compared to glucose can be converted to fat in the liver via DNL (Parks *et al.*, 2008). The greater potential of fructose to stimulate DNL is the main reason why fructose has been showed as particularly harmful. Excessive intake of fructose, and hence increased DNL, may increase the risk of disease, because it may potentially cause both increased cholesterol levels in the blood and accumulation of fat in the liver (Lê *et al.*, 2009)

Lipid metabolism is a central element in metabolic syndrome and often in type 2 diabetes as well as cardiovascular diseases. High fructose intake may induce hypertriglyceridemia and lipogenesis , which in addition may contribute to weight gain and obesity resulting in increasing circulating triglycerides levels in the blood (Rizkalla, 2010). Fructose intake has been found to

increase the small dense LDLs, the type of LDLs that may in particular be linked to cardiovascular risk (Aeberli *et al.*, 2011). Fructose has been shown to be the risk of atherosclerosis on the effect of blood uric level (Feig *et al.*, 2008; Rodrigues *et al.*, 2010). Increased uric level has been associated with an increase in atherosclerosis and, fructose appears to increase uric levels in the blood (Feig *et al.*, 2008; Segal *et al.*, 2007). An increased blood level of uric acid can theoretically lead to elevated blood pressure because uric acid inhibits an enzyme in the endothelial cells of the arteries called endothelial nitric oxide synthase (eNOS). Activated eNOS leads to increased production of nitric oxide (NO), an important vasodilator, thus inhibition of eNOS may lead to vasoconstriction (Kolderup and Svihus, 2015).

High fructose feeding promotes insulin resistance by the alteration of enzymes that regulate hepatic carbohydrates metabolism, including decreased glucokinase and increased glucose-6-phosphatase activities (Thorburn, *et al.*, 1983; Higashura, *et al.*, 2000; Abdula, *et al.*, 2010). High fructose-induced insulin resistance generates oxidative stress in response to an overproduction of superoxide anions by the activation of NADPH oxidase (Sandrine *et al.*, 2004). There is evidence that treatment with antioxidants improves insulin resistance in fructose fed rats (Faure *et al.*, 1997). The oxidative damage caused by dietary fructose was due to a defect in the antioxidant defense mechanism and increased free radical production (Busserolles *et al.*, 2002). Fructose containing diet increase serum tumour necrosis factor alpha and depleted hepatic glutathione levels (Kitagawa *et al.*, 2012).

2.10 OLEANOLIC ACID

Triterpenoids are a diverse family of secondary plant metabolites that are minor but ubiquitous components of the human diet and are considered relatively non-toxic (Liu, 1995; Fukushima *et al.*, 2011; Xu *et al.*, 2014). Oleanolic acid is a pentacyclic triterpenoid that can be found in the form of free acid or aglycones in various medicinal plants and fruits (Phillips *et al.*, 2006). Oleanane triterpenoids are a wide group that is present in nature and includes oleanolic acid (3β-hydroxy-olean-12-en28-oic acid), maslinic acid (2α, 3β-dihydroxyolean-12-en-28-oic acid), morolic acid (3βhydroxyolean-18-en-28-oic acid), and moronic acid (3-oxoolean-18-en-28-oic acid) (Liu, 1995; Fai and Tao, 2009; Fukushima *et al.*, 2011). Oleanolic acid existed for the past two decades and it has attracted interest because of its pharmacological and biochemical effects including anti-inflammatory (Liu, 2005; Ikeda *et al.*, 2008), anti-tumour (Tokuda *et al.*, 1986; Liu, 1995; Young *et al.*, 1995), hepatoprotective (Liu, 1995; Kim *et al.*, 2004; Saravanan *et al.*,

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2006), anti-hyperlipidemic (Somova *et al.*, 2003), anti-bacterial (Liu, 1995; Fontanay *et al.*, 2008) and anti-diabetic (Teodoro *et al.*, 2008; Jang *et al.*, 2009) properties.

Oleanolic acid treatment in high-fat fed animals decreased the levels of serum glucose, insulin, cholesterol and triglycerides and the accumulation of visceral fat (Sato *et al.*, 2007; Demelo *et al.*, 2010). Oleanolic acid has the capacity to modulate the immune system (Liu, *et al.*, 1995) and has been found to reduce TNF-α expression in human endothelial cells (Takada *et al.*, 2010) and inhibit IL-6 (Saaby *et al.*, 2011). Oleanolic acid derivatives potentially stimulate glucose transport in 3T3-L1 adipocyte via multitargeted mechanisms indicating its antidiabetic application (Onat, 2010). Oleanolic acid (pentacyclictriterpenoid) can protect cells from oxidative stress by improving antioxidant defence mechanisms (Zhang *et al.*, 2012).

Oleanolic acid has been shown to play a significant role in protecting hepatocytes from carbon tetrachloride (a hepatotoxicant that causes oxidative stress) (Liu *et al.*, 1998; Reisman *et al.*, 2009). It is a known activator of the NRF-2 pathway, providing a mechanism for antioxidant cell protection (Reisman *et al.*, 2009). The high antioxidant activity of oleanolic acid has effective functions in inhibiting cancer cell proliferation, decreasing lipid oxidation, oxidative stress and lowering cholesterol, which has confirmed its role in potentially reducing the risk of chronic diseases and metabolic syndrome (Boyer and Liu, 2004). Recent studies in humans and animals suggest that intake of oleanolic acid can positively affect lipid metabolism, insulin resistance associated with metabolic disorders (Ravn-Haren, *et al.*, 2013; Nagasako-Akazome *et al.*, 2007), weight management (De Oliveira *et al.*, 2003) and vascular function (Gasper *et al.*, 2014).

CHAPTER 3

3.0 METHODOLOGY

3.1 ANIMAL

The study was conducted by obtaining Animal Ethical Committee clearance from the University of Witwatersrand, Johannesburg and North-West University, Mafikeng campus. The pups used for this study were nursed and raised under necessary conditions; room temperature controlled at 22-25°C, humidity of 45%-55%, 15-20 air changes per hour and on a 12-hour light / dark cycle.

3.2 STUDY DESIGN

The Sprague Dawley male rats (Pups) were divided into four groups, each consisting of six rats (n=6)

Group A: (Normal control): Rats were given normal diet (breast milk).

Group B: (Oleanolic Acid): Rats were given breast milk and oleanolic acid (60mg/kg).

Group C: Rats were given breast milk and high fructose diet only (25% w/v).

Group D: Rats were given breast milk, oleanolic acid and high fructose diet (60 mg/kg).

3.4 PREPARATION OF SAMPLES

Pups were given the dietary treatments between postnatal day 7 to day 14. On postnatal day 14, the pups were weighed and then killed. The heart was dissected out, snap frozen in liquid nitrogen, and then stored at -80°C in a cryovial tubes for further experiments.

3.5 CATALASE ASSAY

The heart samples were done by preparing a homogenate in (10% w/v) phosphate buffer, centrifuged in 10,000 x g (4°C) for 10min. The supernatant was separated out and preserved at -80°C for enzyme analysis. Catalase activity was determined according to the method described by (Sinha, 1972). In this method, dichromate in acetic acid is reduced to chromic acetate when

heated in the presence of hydrogen peroxide(H_2O_2 65 mM) in 50 mmol/L sodium, potassium phosphate buffer(pH 7.4), with the formation of perchromic acid as an unstable intermediate (Hadwan, 2016). The procedure was performed using 1.5 ml tubes adding 50 µl of sample and 250 µl of hydrogen peroxide to tube labelled test, control test consisting of 50 µl of heart tissue and 250 µl of distilled water, standard consisting of 50 µl of distilled water and 250 µl of hydrogen peroxide and lastly, blank consisting of 300 µl of distilled water. The tubes containing the reagents were mixed by vortexing and incubated at 37°C for 3 minutes after that, 500 µl of dichromate/acetic acid (prepared by mixing 50 ml of a 5% aqueous solution of potassium dichromate with 150 ml of glacial acetic acid) was added to all the four test tubes. The tubes were then kept at 100°C for 10 minutes and allowed to cool with tap water and centrifuged to remove precipitated protein. 200 micro litre (µl) of the supernatant was then taken to 96 well plate and the absorbance was read at 570 nm against reagent blank.

3.6 GENE EXPRESSION OF THE ANTIOXIDANT ENZYME

3.6.1 RNA EXTRACTION FROM THE HEART

The tissue was immersed in liquid nitrogen until the procedure was completed. Upon completion of the harvest procedure, the heart tissue was emptied to an empty falcon tube and stored on dry ice. The heart tissue was then kept frozen until homogenization procedure was ready to be performed. Thereafter, 1 ml of triazole was added to the sample and it was then homogenized while on ice. An amount of 200 µl chloroform was then added to the homogenate and it was vortexed vigorously, and then incubated for 15 minutes on ice. The homogenate was then centrifuged at 12000 xg for 15 minutes at 4°C for phase separation; the aqueous phase was transferred to a fresh tube. Thereafter, the RNA was precipitated by mixing it with 0.5 ml of isopropanol and incubated on ice for 10 minutes; it was then centrifuged for 10 minutes at 12000 xg at 4°C and the supernatant was then removed. The pellet was then washed with 1 ml 70% ethanol and thereafter, centrifuged at 7500 xg for 10 minutes at 4°C and the supernatant was removed. The remains were air dried and RNA pellet was dissolved in an appropriate volume of RNase free water.

3.6.2 AGAROSE GEL PREPARATION

1.5 gram (g) of agarose was weighed and added to150 ml TAE (buffer solution containing mixture of Tris base, acetic acid and EDTA, it is used in separation of nucleic acids such as DNA and RNA). And mixed well. The mixture was heated for 60 s in the microwave to dissolve

the agarose and was allowed to cool to 50°C; 2 µl of ethidium bromide was added and mixed. The mixture was then poured into a gel tray and allowed to solidify. After the gel solidified, 4 µl of RNA + 6 µl of bromophenol loading dye was poured inside the gel wells. The gel was ran at 100 V for 60 mins and after the run the gel was place in a UV box to observe the bands.

3.6.3 DNA SYNTHESIS

The cDNA synthesis was performed using Applied Biosystems Superscript VILO cDNA Synthesis Kit Product information sheet. Amount of 4µl (5X VILO reaction mix), 2µl (10X SuperScript enzyme mix), 7.39 µl RNA and 6.61 µl RNase free water were gently mixed in a tube and incubated at 25°C for 10 minutes. Thereafter, the tube contents were further incubated at 42°C for 60 minutes and reaction was terminated at 85°C for 5 minutes.

3.6.4 REAL-TIME PCR ASSAY

The PCR gene expression analysis assay was performed using Applied Biosystems Thermo Fisher Scientific protocol. Firstly, a PCR reaction mixture was prepared, which consisted of the following: 5 µl PowerUp™ SYBR Green Master Mix (2X), 1µl forward primer and 1 µl reverse primer, 2 µl cDNA template + 1 µl RNase-free water and the total reaction equated to 10 µl. The components were mixed thoroughly and centrifuged to spin down the contents to eliminate any air bubbles. An appropriate volume of each reaction made was transferred to each well of an optical plate and the plate was sealed with an optical adhesive cover. The reaction plate was then placed in the PCR instrument and the thermal cycling conditions were set using the default PCR thermal-cycling conditions.

3.7 ANTIOXIDANT CAPACITY ASSAYS

3.7.1 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

The FRAP assay was done using the method described by Benzie and Strain (1996). Briefly, 100 µl sample was mixed with 200µl FRAP reagent. The FRAP reagent consisted of 30 ml acetate buffer (pH 3.4), 3 ml tripyridyl triazine (TPTZ), 3ml of 0.033M $FeCl_3$ and 6.6 ml dH_2O. The mixture was allowed to incubate for 30 minutes at room temperature and the absorbance of the mixture was read at the wavelength of 539 nm using a Multiskan™GO microplate spectrophotometer. Ascorbic acid (AA) was used as the standard and the results were expressed as µmole AAE/g sample.

3.7.2 TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC)

The TEAC assay was carried out using the principle of 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity according to a method described by (Ou *et al*, 2001). The ABTS was prepared by mixing 88 µl of 0.039M K2S2O8 and 5 ml of 0.043 ABTS, and the mixture was left overnight before it was used the following day. An amount of 25 µl of the sample was mixed with 200 µl ABTS solution and the mixture was left for incubation for 30 minutes at room temperature. The absorbance of the mixture was read at 734 nm using spectrophotometer. Trolox was used as the standard and the results were expressed as µmol TE/g.

3.8 OXIDATIVE STRESS BIOMARKERS

3.8.1 LIPID PEROXIDATION

Malondialdehyde (MDA) is a part of thiobarbituric acid reacting substance (TBARS) often used as an indicator of lipid peroxidation (Seljeskog *et al.*, 2006; Buege and Aust, 1978). The Thiobarbituric acid (TBARS) was performed according to a method described by (Buege and Aust, 1978). The levels of MDA were measured by preparing stock solution of 4 µm MDA from 4 mm MDA solution. 50 micro litre (µl) of 40 µm MDA stock solution prepared was added to clean tubes and 50µl of samples was added to clean empty tubes, after that 100 µl of 15% (w/v) of trichroloacetic acid (TCA) and 0.375% of thiobabituric acid (TBA) was added to all the stock solution and sample tubes. The reagent mixtures were then heated at 100°C for 15 minutes and allowed to cool at room temperature and centrifuged at 3000g for 5 minutes. The results were read at 535nm using microplate reader.

3.8.3 GLUTATHIONE LEVEL

The assay was performed by preparing the stock standard solution (0.001M prepared by dissolving 0.03 g of GSH in a final volume of 100 ml of 0.2 M EDTA solution. The dilutions were made in EDTA solution to volumes of 0, 25, 50, 100, 150 and 250 µl. The reaction mixture of 50 µl of phosphate buffer, 10 µl sodium azide (NaN3), 20 µl GSH, 1 µl H_2O_2, 50 µl sample and distilled water was incubated at 37°C for 3 minutes. After which, 50 µl of TCA was added and thereafter centrifuged at 3000rpm for 5 minutes at 4°C. Supernatant was collected and 50 µl of supernatant was then used for determination of GSH as follows; 100ul of phosphate buffer and 50 µl of DTNB was added and immediately the absorbance was read at 412nm.

3.9 LEVELS OF INFLAMMATORY BIOMARKERS

The levels of inflammatory biomarkers in the heart samples were measured using Bio-Plex Pro magnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-Plex platform (Bio-Rad). Following previous optimization, samples were evaluated undiluted in a blinded manner. Samples were reacted with a mixture of fluorescent polystyrene beads bound with specific anticytokine primary antibodies, resulting in binding of the cytokines to the beads with the corresponding antibody. The biotinylated anticytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. All analytes levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all analyte levels ranged from 3 to 12000 pg/mL. Bio-Plex Manager software, version 6.0, was used for bead acquisition and analysis.

3.10 STATISTICAL ANALYSIS

Statistical analysis was done by one-way analysis of variance using Graphpad prism version 6.0. and data expressed as mean ± standard deviation The differences were considered significant at $p<0.05$.

CHAPTER 4

RESULTS

4.1 ANTIOXIDANT CAPACITY

In Figure 1, the levels of TEAC were not significantly different in all the groups. The values for the treatment groups were control group (53.43 ± 3.90 µmol/ml), OA (47.90 ± 1.55 µmol/ml), HFD (52.82 ± 5.08 µmol/ml) and HFD+OA (45.98 ± 7.32 µmol/ml). No significant differences were observed in the TEAC values in all treatment groups. Figure 2 shows the level of FRAP in the control group (7.0 ± 1.39 µmol/ml), OA (5.92 ± 0.70 µmol/ml), HFD (8.51 ± 1.20 µmol/ml) and HFD+OA (8.77 ± 2.37 µmol/ml). The results showed no significant difference in the FRAP values in normal control when compared with OA, HFD and HFD + OA groups.

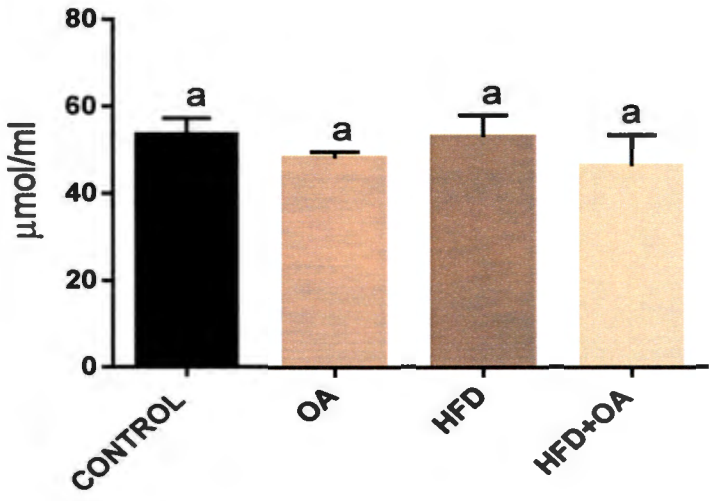

Figure 1: Effect of treatment with oleanolic acid on trolox equivalent antioxidant capacity (TEAC) in heart tissue of Sprague dawley rats.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

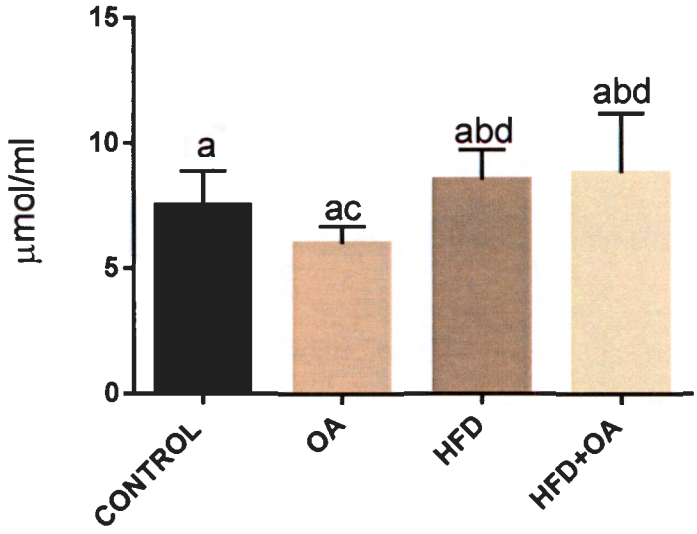

Figure 2: Effect of treatment with oleanolic acid on Ferric reducing antioxidant power (frap) in heart tissue of Sprague dawley rats.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

4.2 INDICATORS OF OXIDATIVE STRESS

As shown in Figure 3, there were no significant differences in the levels of lipid peroxidation in all the groups. The values for different treatment groups were control group (21.66±6.89ng/MDA/ngProtein), OA (30±7.35 ng/MDA/ngprotein), HFD (28.45±9.19 ng/MDA/ngprotein) and HFD+OA (27.16±7.37 ng/MDA/ngprotein). Figure 4 shows a decrease in glutathione level in HFD group though not significant when compared with normal control and treatment with OA increased the level of glutathione. The values for different groups were normal control (237.43 ± 65.30µM/mgProtein), OA (213.75 ±13.20 µM/mgProtein), HFD (149.51±37.60 µM/mgProtein) and HFD+OA (182.60 ± 48.79 µM/mgProtein).

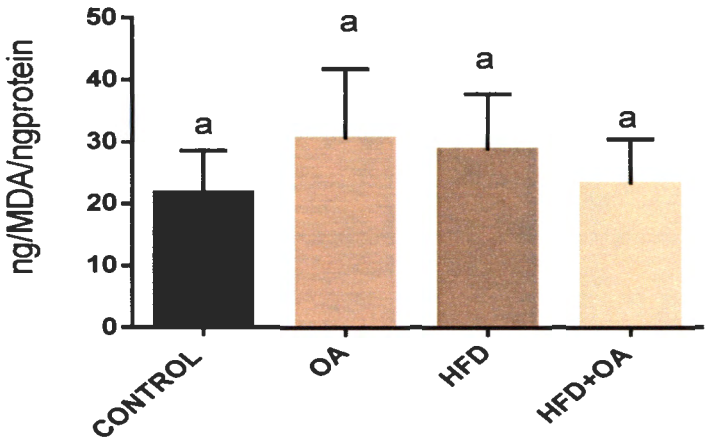

Figure 3: Effect of treatment with oleanolic acid on the level of lipid peroxidation in heart tissue of Sprague dawley rats.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

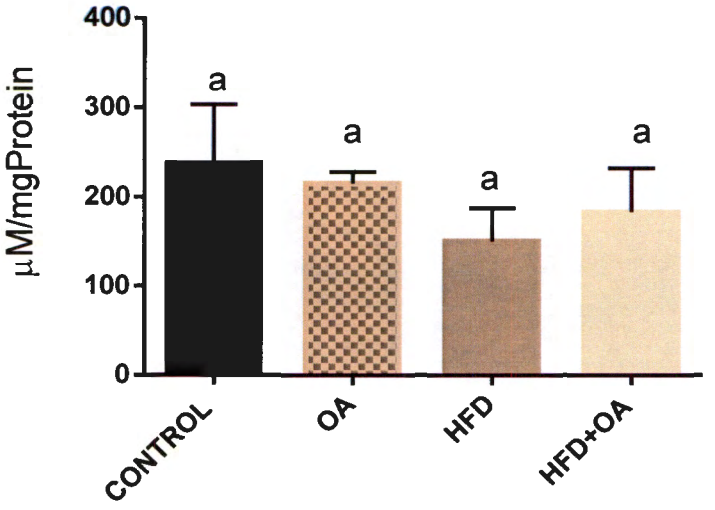

Figure 4: Effect of treatment with oleanolic acid on the total glutathione in heart tissue of Sprague dawley rats.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant difference ($p<0.05$).

As shown in Figure 5, though not significant, the results showed reduction in the activity of catalase in HFD (6.670 ± 0.988Ku). Catalase activity was increased in HFD+OA (6.07±0.660Ku) when compared with normal control following treatment. The OA (4 ± 2.49Ku) group also showed no significant difference when to compared to normal control, HFD and HFD+OA. The gene expression of catalase also showed no significant difference across all the treatment groups (figure 5b).

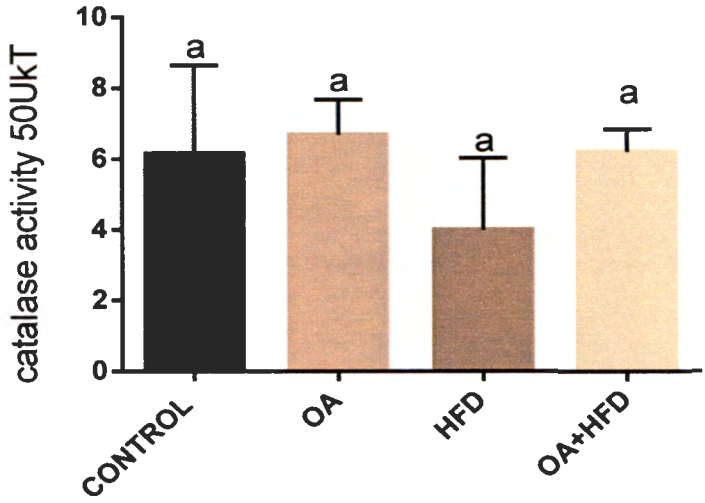

Figure 5a: Effect of treatment with oleanolic acid on the activity of catalase in heart tissue of Sprague dawley rats.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

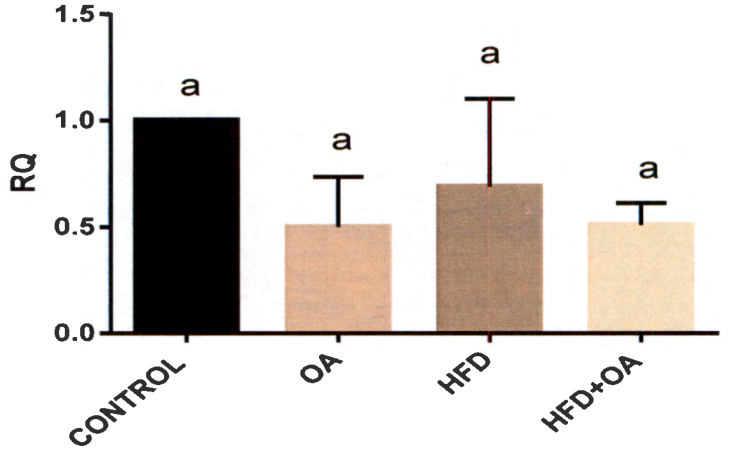

Figure 5b: The gene expression of catalase in treatment groups.

Oleanolic Acid (OA), High Fructose Diet (HFD) and High fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

4.3 INFLAMMATORY BIOMARKERS

4.3.1 PRO-INFLAMMATORY BIOMARKERS

As shown in Figure 7-12, the levels of pro-inflammatory IL-1b, IL-6, IL-12, IFN-g, TNF-alpha and MCP-1 were consistently elevated following administration with HFD, but were not significantly different when compared to the normal controls. In a similar vein, treatments with OA consistently decreased the levels of pro-inflammatory cytokines to levels that are almost the same as normal controls.

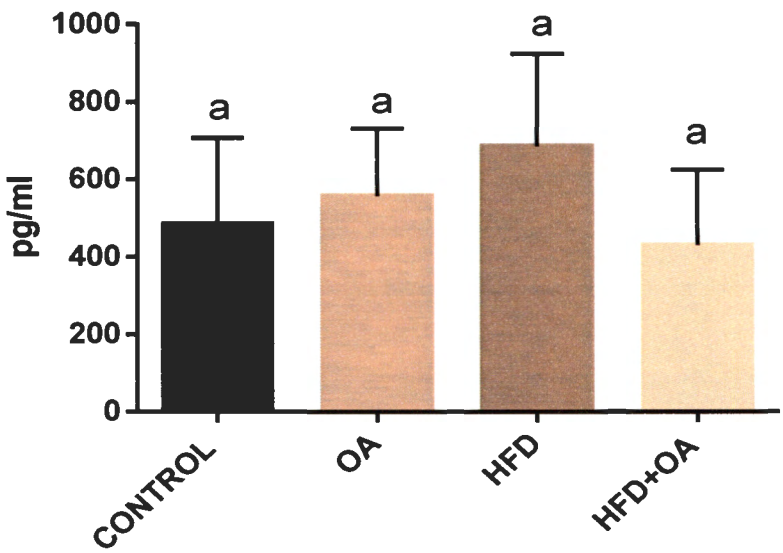

Figure 7: The level of IL-1b in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

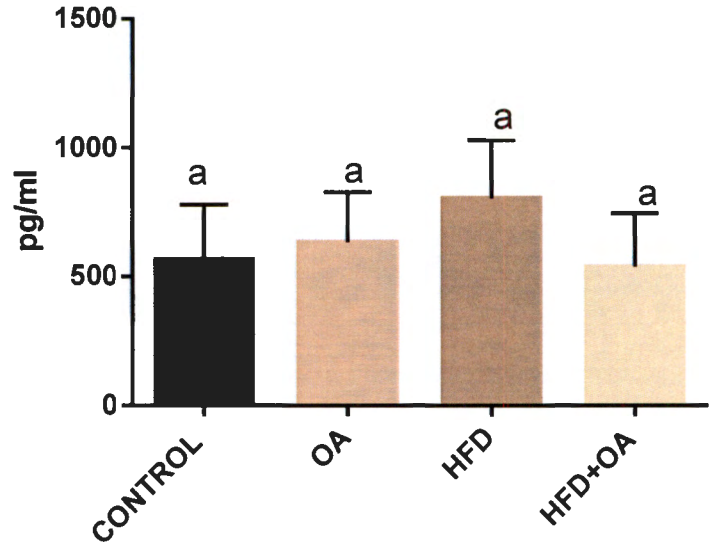

Figure 8: The level of IL-6 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

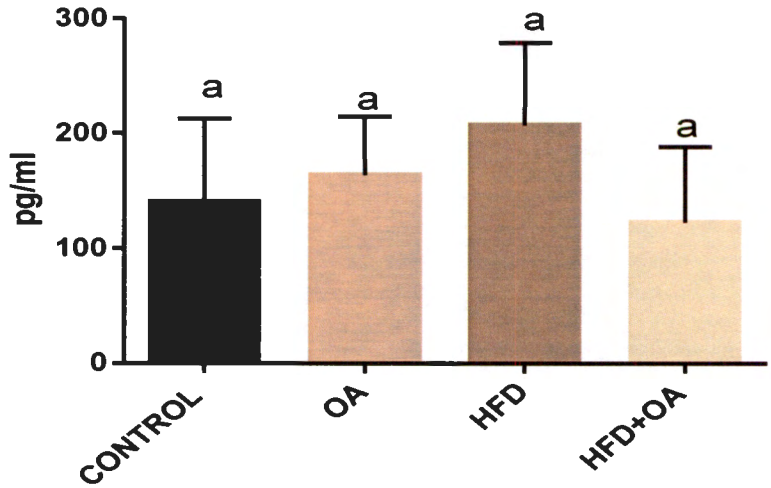

Figure 9: The level of IL-12 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

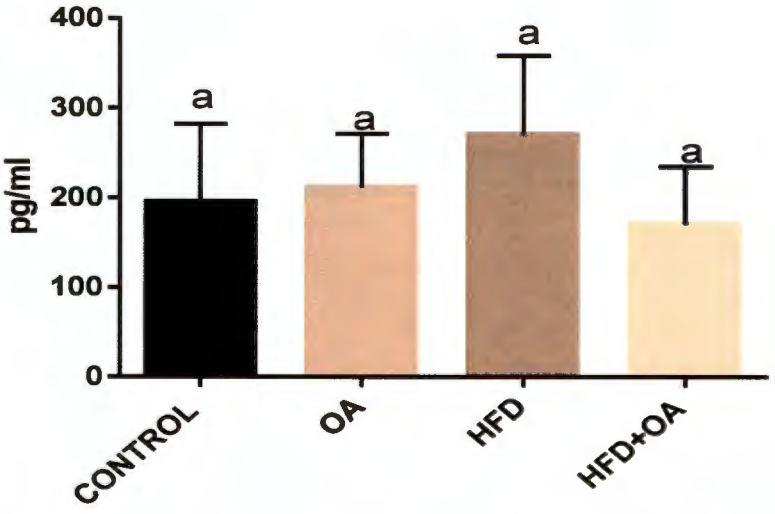

Figure 10: The level of IFN-g in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

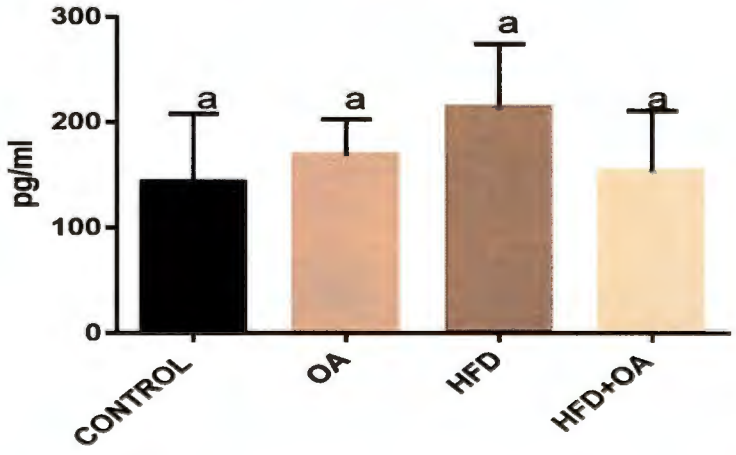

Figure 11: The level of TNF-alpha in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

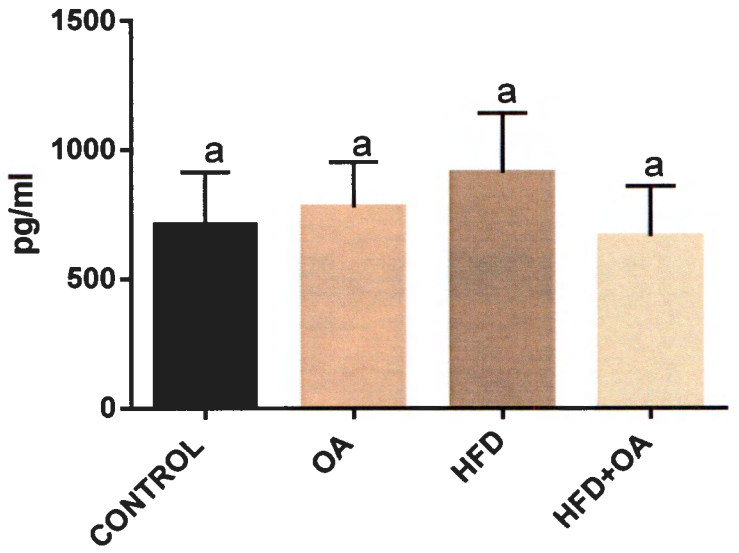

Figure 12: The level of MCP-1 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

4.4.2 ANTI-INFLAMMATRY BIOMARKERS

As shown in Figures 13-15, the level of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 were increased in HFD when compared with normal controls, though not significant. There were non-significant decreases in the levels of the cytokines in HFD+OA when compared with normal control and HFD groups.

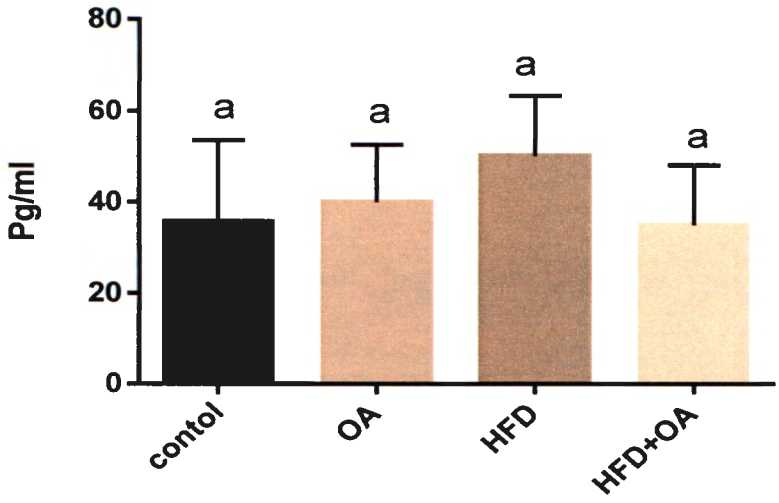

Figure 13: The level of IL-4 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

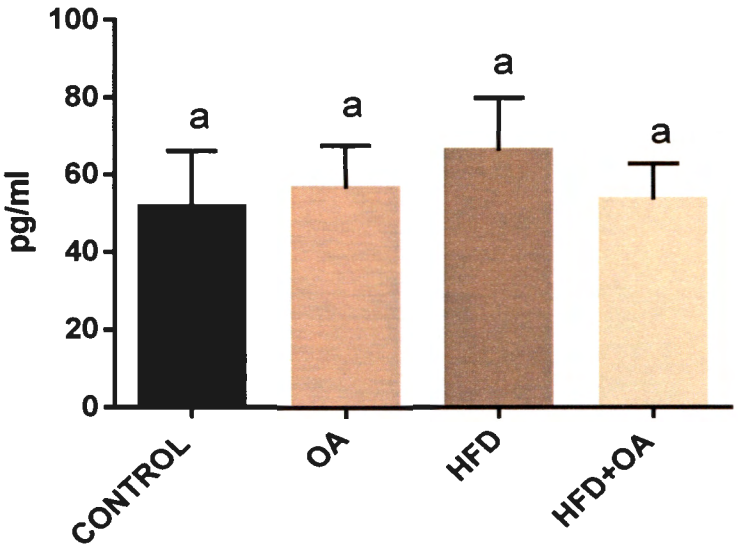

Figure 14: The level of IL-5 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

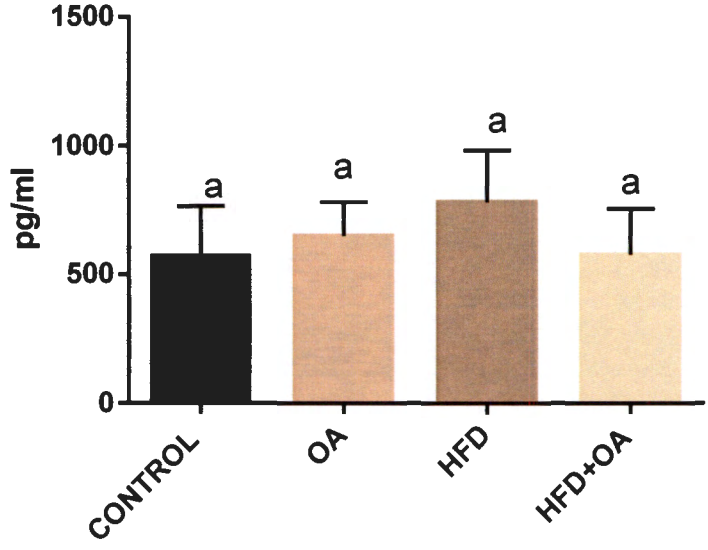

Figure 15: The level of IL-10 in the treatment groups

Oleanolic Acid (OA), High Fructose Diet (HFD) and High Fructose Diet + Oleanolic Acid (HFD + OA).

Same bars across the groups showed no significant differences ($p>0.05$) while different bars across the groups showed significant differences ($p<0.05$).

CHAPTER 5

DISCUSSION

Reactive oxygen species (ROS) are produced as a consequence of cellular metabolism, however under certain conditions excessive amounts produced result in increased oxidative stress (Sharma *et al.*, 2012). Excessive ROS can activate numerous pathways leading to increased expression of pro-inflammatory cytokine mediators (Lee and Yang, 2012). High fructose diet can interfere with the antioxidant defence system which can lead to oxidative stress and inflammation. The reducing capacity of a compound could be used as an important indicator of its possible antioxidant activity (Hazra *et al.*, 2008). The trolox equivalent antioxidant capacity (TEAC) assay or 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay is based on scavenging of the $ABTS^{\bullet+}$ radical cation by the antioxidants present in test sample (Zulueta *et al.*, 2009). It evaluates the relative ability of antioxidant to scavenge the $ABTS^{+}$ generated in aqueous and organic solvent systems (Matsinkou *et al.*, 2012). The results showed that oleanolic acid did not alter the heart TEAC status and no significant difference in the TEAC among the treatment groups of rats was observed. Ferric reducing antioxidant Power (FRAP) assay is commonly used to assess the total antioxidant capacity of biological sample by measuring the sample's ability to reduce the intense blue ferric tripyri-dyltriazine complex to its ferrous form (Benzie and Strain, 1996). There were no significant differences ($p>0.05$) in the antioxidant capacity assessed by FRAP assay in all the treatment groups.

Antioxidant enzymes, i.e. catalase scavenges superoxide radical by accelerating its conversion to hydrogen peroxide while glutathione peroxidase which uses reduced glutathione as a substrate detoxifies hydrogen peroxides to water and oxygen. Catalase (CAT) breaks down hydrogen peroxide (H_2O_2) to water and molecular oxygen (Bailly, 2004) It has been reported that low catalase activity in the heart is responsible for the high sensitivity of this organ to oxidative stress(Zhou and Kang, 2000; El Hassan *et al.*, 2003). In this study, there was decrease in the activity of CAT in the high fructose fed rat and CAT activity was increased, though with no significant difference following treatment with OA.

The release of free radicals in oxidative stress causes considerable damage to biological systems(Finkel and Holbrook, 2000). The study also showed the ability of OA to protect against lipid peroxidation by reducing the levels of malonaldehyde (MDA), a product of lipid peroxidation in the OA + HFD group though not significantly. In this study, there was a slight decrease in the glutathione level of the HFD group and the elevated level of glutathione level in the HFD + OA treatment group is an indication of beneficial effects of OA.

Inflammation occurring because of oxidative stress is the biological manifestation of excess ROS/RNS (Roberts *et al.*, 2010), resulting from oxidative stress is the cause of much human disease (Martinon, 2010). Cytokines are low-molecular-weight regulatory proteins that are produced during all phases of an immune response(Duque and Descoteaux, 2014) . Interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) are the major mediators of the early inflammatory response (Saini *et al.*, 2005). An increase in inflammatory cytokines IL-6 (Interleukin-6), IL-18 (Interleukin -18), IL-1 (Interleukin -1) and TNF-α (Tumour necrosis factor alpha) has been reported in oxidative stress associated with heart diseases (Esposito *et al.*, 2002). Increase in oxidative stress can increase the production of inflammatory proteins and vice-versa (Esposito *et al.*, 2002).

Interactions between oxidative stress and inflammatory signals play a major role in disease progression and tissue damage (Elmarakby and Sullivan, 2012; Vadla and Vellaichamy, 2013). In this study, the levels of pro-inflammatory IL-1b, IL-6, IL-12, IFN-g, TNF-alpha and MCP-1 were consistently elevated following administration with HFD but not when compared to the normal controls but not significantly different. Oleanolic acid consistently decreased the levels of pro-inflammatory cytokines in high fructose-fed rats. The anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 were increased in HFD group when compared with normal controls though not significant ($p>0.05$). There were non-significant decreases in the levels of the cytokines in HFD+OA when compared with normal control and HFD groups.

CONCLUSION

Oleanolic acid, a plant phytochemical, has been reported to have potential therapeutic effects against many diseases. This study explored the effect of oleanolic acid on oxidative stress and inflammation in the pups heart tissue through improvement of antioxidant defence. The study revealed that intake of high fructose has the tendency to reduce antioxidant defence and increase pro-inflammatory biomarkers. Though not significantly, the treatment of high fructose toxicity in the rats with oleanolic acid showed the ability to alleviate the effects of oxidative stress and inflammation at the early stages of life.

STUDY LIMITATIONS

While this study indicated that oleanolic acid was effective at improving specific markers of Oxidative stress and inflammation on high fructose fed rats, further studies should focus On the possible effects of oleanolic acid on specific pathways which have been implicated in increased generation of oxidants and inflammation to elucidate further specific targets in the oxidative stress and inflammation which leads to progression of diseases.

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