Cape Peninsula University of Technology

EFFECTS OF DIETARY *GARCINIA KOLA* SUPPLEMENTATION ON INFLAMMATION AND OXIDATIVE STRESS IN ISOLATED PERFUSED RAT HEARTS

by

Naledi Gape Nyepetsi

Thesis submitted in fulfilment of the requirements for the degree of

Master of Technology: Biomedical Technology

In the Faculty of Health and Wellness Sciences

At the Cape Peninsula University of Technology

Supervisors: Prof. Adriaan J Esterhuyse
Dr Dirk J Bester

Bellville

January 2014

DECLARATIONS

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

N. G. N Nyepetsi **07 March 2014**

Signed **Date**

ABSTRACT

Background: Oxidative stress and chronic inflammation contributes significantly to the pathogenesis of several ischaemic heart diseases, including atherosclerotic plaque rupture and myocardial infarction. It is widely demonstrated that ischaemia, followed by reperfusion, results in alterations of the mitochondrial and endothelial function through uncontrolled cascades of events characterized by free radical release and inflammation. Recent experimental evidence shows that modulation of inflammatory and antioxidant signaling mediators may determine the host outcome following myocardial ischaemia-reperfusion injury.

Investigations from the past decade indicate that food supplements may play an important role in the prevention and management of chronic inflammatory diseases. *Garcinia kola* seeds are flavonoid rich nut from a tropical flowering, non-timber plant of the *Guttiferae* family. This plant is highly valued in several African cultures for its use in herbal medicine. Recently, the majority of experimental research has linked phytochemicals found in *Garcinia kola* nut, to its proposed beneficial effects in treatment and management of oxidative stress related-chronic diseases. Research performed in our laboratory demonstrated that kolaviron, a prominent *Garcinia kola* flavonoid extract, reduces myocardial apoptosis during ischaemia-reperfusion injury. Therefore, the aim of our current study was to determine the effects of *Garcinia kola* supplementation on cardiac inflammatory and antioxidant signaling pathways during ischaemia-reperfusion using a Wistar rat heart model.

Materials and Methods: Male wistar rats were randomly divided into two groups: a control group receiving 2ml/kg corn oil and the experimental group receiving 100mg/kg *Garcinia kola* dissolved in corn oil, daily for 4 weeks. After the feeding period, blood samples were collected and lymphocytic DNA damage was analyzed using the alkaline comet assay. Furthermore, rat hearts were isolated and perfused with Krebs-Henseleit buffer on a working heart perfusion apparatus to measure myocardial functional parameters. Myocardial functional recovery was measured after 15 minutes global ischaemia followed by 25 minutes reperfusion. Hearts were freeze clamped at three different time points for myocardial cytokine concentration determinations using multiplex electrochemilunescent immunoassay. Nuclear factor

kappa beta (NF- kβ), p38 mitogen activated protein kinases (p38 MAPK), protein kinase B/Akt (PKB/Akt), nitro-tyrosine, inducible nitric oxide (iNOS), cyclooxygenase-2 (COX-2), poly (adenosine-di-phosphate) ribose polymerase-1 (PARP-1) and caspase-3 expression and their phosphorylated forms (where applicable) were analyzed using the Western blot technique.

Results: Dietary *Garcinia kola* supplementation significantly improved functional recovery when compared to the control group as reflected by the improved aortic output recovery (68.47 ± 6.16% *versus* 44.96 ± 7.00%; $p<0.05$). Our biochemical results supports the hypothesis that, dietary *Garcinia kola* supplementation modulates different cardiac proteins in terms of expression and activation at different time points when compared to the control group. We show that, before induction of ischaemia, *Garcinia kola* supplementation attenuates expression of inflammatory mediators and pro-apoptotic proteins when compared to the control group. The improved functional recovery was associated with a prompt inflammatory response, activation of PKB/Akt and attenuation of protein nitrosylation after 10 minutes of reperfusion. Modulation of NF-kβ and the p38 MAPK family proteins expression could have also played a significant role in myocardial functional recovery.

Conclusion: We have shown that a 4 week period of dietary *Garcinia kola* supplementation at 100mg/kg daily improves cardiac functional recovery following ischaemia-reperfusion injury. We propose that dietary *Garcinia kola* supplementation protects cardiac myocytes from ischaemia-reperfusion induced oxidative stress through the induction of a prompt inflammatory response and controlled expression and/or activation of the, NF-kβ, PKB/Akt and p38 MAPK protein signaling pathways PARP-1 and caspase. Finally, we demonstrated that dietary *Garcinia kola* supplementation did not induce rat lymphocytic DNA damage when compared to the control group.

DEDICATIONS

To My beloved Family

My mother: Ausi Mpura

My brothers: Rapula, Shampa and Keke

My sisters: Ausi Tamati and Puna

My nephew and niece: Larona and Kaone

In Memory of my grandparents
Rest in peace

ACKNOWLEDGEMENTS

I wish to thank:

- My supervisors Prof AJ Esterhuyse and Dr DJ Bester for their guidance and support through the course of this project
- Mr W Pantsi for his assistance in taking care of my animals when I couldn't, may God be graceful to your giving hand
- Cape Peninsula University of Technology for giving me the opportunity to carry out this project and funding they provided for this study
- My colleagues and friends for their support during this journey
- Prof I Kasvosve for seeing potential and believing in me

LIST OF ABBREVATIONS

AIF	Apoptosis Inducing Factor
AO	Aortic Output
AOR	Aortic Output Recovery
APAF-1	Apoptosis Protease Activator 1
ATP	Adenosine Tri-Phosphate
BCL	B-cell lymphoma
BPM	Beats per minute
Ca^{2+}	Calcium ion
CF	Coronary Flow
c-IAP	Cellular Inhibitor Apoptosis Protein
CID	Chronic Inflammatory Diseases
COX	Cyclooxygenase
CPUT	Cape Peninsula University of Technology
CVD	Cardiovascular Diseases
DAP	Diastolic Aortic Pressure
DNA	Deoxyribose Nucleic Acid
GSH/GSSG	Reduced/oxidized Glutathione
H_20_2	Hydrogen Peroxide
HR	Heart Rate
IFN	Interferon
IHD	Ischaemic Heart Diseases
IKK	Inhibitor Kappa Beta Kinase
Ikβ	Inhibitor Kappa Beta
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase

IPC	Ischaemia Pre-Conditioning
IRI	Ischaemia Reperfusion injury
LDL-C	Low Density Lipoproteins-Cholesterol
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinases
MCP-1	Monocyte Chemotactic Protein-1
MIP	Macrophage Inflammatory Protein
mmHg	Millimeter of Mercury
NF-kβ	Nuclear Factor Kappa Beta
NO	Nitric Oxide
O_2^-	Superoxide radical
^oC	Degrees Celsius
$ONOO^-$	Peroxynitrite
PARP	Poly (Adenosine-di-phosphate) Ribose Polymerase
PKB/Akt	Protein Kinase B also known as Akt
ROS	Reactive Oxygen Species
RPO	Red Palm Oil
S.E.M	Standard Error of the Mean
SAP	Systolic Aortic Pressure
TNF-α	Tumor Necrosis Factor-Alpha
WHO	World Health Organization
α	Alpha
β	Beta

TABLE OF CONTENT

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 4

CHAPTER 1: INTRODUCTION

Ischaemia-reperfusion injury (IRI) is associated with alterations in the function of the mitochondrial membrane through cascades of events, leading to increased free radical production and activation of inflammatory mediators (Eltzschig and Collard, 2004; Grootjans *et al*., 2010). In the last few decades it has become clear that increased release of free radicals are involved in the pathogenesis of several ischaemic cardiovascular diseases (CVD), in particular atherosclerosis and myocardial infarction (Lakshmi *et al.,* 2009; Vogiatzi *et al.,* 2009). The role of free radicals in atherosclerotic plaque formation is well accepted, however, accumulating evidence shows that the inflammatory response plays a major role in all stages of atherosclerosis (Hansson and Libby, 2006). Furthermore, research evidence indicates that acute inflammatory responses during IRI is predominately an innate immune response, mediating tissue injury and possibly repair mechanisms (Linfert *et al.,* 2009; Arslan *et al.,* 2010).

In 2008 an estimated 17 million global deaths were attributed to CVD, representing approximately 48% of global deaths due to non-communicable disease and over 80% of these deaths were reported from low to middle income countries (WHO, 2011a). From these total deaths, 7.3 and 6.2 million were attributed to coronary heart disease and stroke, respectively (WHO, 2011b), with approximately 42% credited to ischaemic heart diseases (WHO, 2011a; 2011b). Mathers and Loncar (2006) projects that by 2030, CVD will remain the single leading cause of deaths world-wide, with people dying from CVD, increasing to approximately 23.3 million. In the Republic of South Africa alone statistics show that approximately 195 people died per day due to CVD between 1997 and 2004 (Maredza *et al*., 2011). With the disease taking its toll, projections indicate a potential increase in death rate of over 40% in the age group between 35-64 yrs by year 2030 in the Republic of South Africa alone (Maredza *et al*., 2011).

Ischaemia generally results from the occlusion of one of the major arteries causing restricted blood supply and limited oxygen supply (Goldhaber and Weiss, 1992). Restoration of blood supply (reperfusion) is critical for salvaging of injured cells and re-establishment of tissue function (Dhalla *et al*., 2000). Though necessary

for cell survival, it has been shown that reperfusion contributes to myocardial damage through a process referred to as reperfusion injury (Ferrari *et al.,* 1991). The main mechanisms hypothesized to explain the pathogenesis of ischaemia-reperfusion injury includes endothelial injury, oxidative stress and inflammatory response (Vajdovich, 2008). Oxidative stress has been linked with cellular damage observed in cardiovascular related diseases (diabetes and hypertension) and other risk factors (smoking and dyslipidaemia). The majority of experimental evidence implicates oxygen and nitrogen derived free radicals, especially superoxide, hydroxyl, nitric oxide (NO) and peroxynitrite ($ONOO^-$) in the pathogenesis of ischaemic CVD (Ferrari *et al.,* 1991; Closa and Folch-Puy, 2004; Zweier and Talukder, 2006; Pacher *et al*., 2007). On interaction with intracellular macromolecules *in vivo*, free radicals have demonstrated detrimental effects facilitating damage to intracellular organelles, cell membranes, leading to cell death and endothelial dysfunction (Vajdovich, 2008).

Upon tissue injury, an inflammatory response may be initiated as a primary immune system response for elimination of pathogens or stimuli, in order to restore the cell to a state of normal homeostasis or remove dead cells (Pan *et al*., 2010). This is a process by which innate immune system mediators enter the injured tissue area and facilitate tissue healing and repair. Inflammatory response induces increased release of mediators such as leukotrienes, prostaglandins and histamine. The binding of these mediators to their respective receptors on the endothelium facilitates vasodilation, increasing blood flow, capillary permeability and infiltration of plasma fluids, proteins and leukocytes to the area of injury (Buckley *et al*., 2001). The initial inflammatory response referred to as acute inflammation, occurs within seconds and may last for several hours to a few days, to complete the process of tissue healing (Feghali and Wright, 1997). Contrary to the homeostatic function, inflammation which last for weeks to months, and in some instances for years, progresses to a chronic disease state resulting in progressive tissue injury (Feghali and Wright, 1997).

Inflammatory mediators may be derived from the circulation as inactive precursors or may be released by cells of the immune system at the site of injury. Cell derived mediators are rapidly secreted during an acute inflammatory response: histamine by mast cells, prostaglandins and cytokines/chemokines by leukocytes. Other proteins

circulating in inactive form e.g. complement system proteins, undergoes proteolytic cleavage to be transformed to their active state. It has been reported that an inflammatory state is maintained until the offending pathogen is eliminated and the tissue repair process is complete (Nian *et al*., 2004). Therefore, chronic inflammation suggests a dysfunction of the negative regulatory mechanisms regulating acute inflammatory response (Inagaki-Ohara *et al*., 2003; Yoshimura *et al.,* 2003). Chronic inflammation may be defined as an inflammatory process characterised by macrophage, lymphocytes and plasma cell infiltration, exerting its cellular side effects mainly through overproduction of free radicals, prolonged expression of inflammatory mediators and depletion of antioxidants (Percival, 1999).

Chronic inflammation and oxidative stress are complicated processes linked with several chronic diseases such as CVD and cancer. Currently, chronic inflammatory diseases (CID) are still proving to be a major health challenge world-wide (WHO 2011a). Several pharmacological agents available, such as celebrex, ibuprofen and aspirin only targets inhibition of activity of the cyclooxygenase enzyme, thereby blocking prostaglandin synthesis hence their efficiency in treating pain and fever. Infiltration of mononuclear cells, predominantly macrophages and neutrophils in inflammatory state primarily serves to eliminate deleterious agents and initiate tissue repair, however, activation of macrophages may be responsible for much of the injury that occur during chronic inflammation (Butterfield *et al.,* 2006). Activation of these cells may lead to increased release of lysosomal enzymes, reactive oxygen and nitrogen species, as well as cytokines and chemokines, which may be toxic to the host cell. With a rapid increase in disease prevalence currently observed in developing countries (Puoane *et al*., 2008), lifestyle changes and healthy natural dietary components have become the current focus on managing CID.

Phytochemicals have previously been shown to have a beneficial role in reducing the incidence and progression of diseases to a chronic inflammatory state (Middleton *et al.,* 2000; Kim *et al.,* 2004; Yoon and Baek, 2005; Lin *et al.,* 2006). These are plant derived chemicals found in many vegetables, fruits and beverages such as wine and tea, popular for their health benefits, including antioxidant and anti-inflammatory activities (Kim *et al.,* 2004; Yoon and Baek, 2005). Research suggests that flavonoids in particular are an important polyphenolic phytochemical group associated with

decreased risk of chronic oxidative stress induced diseases such as cancer and CVD (Middleton *et al.,* 2000; Lin *et al.,* 2006). These are water soluble polyphenolic compounds characterised by two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a heterocyclic pyran (Beecher, 2003). **Table 1-1** shows the sub-classification of flavonoids, names of prominent food flavonoids and their typical food sources (Garcia-Lafuente *et al*., 2009).

Table 1-1: Subclass and prominent food flavonoids and typical food sources

Flavonoid sub-class	Food Flavonoid	Food source
Flavanols	*Catechin, gallocatechin, epicatechin*	Teas, red grapes and red wines
Flavonones	*Naringenin, hesperetin, eriodictyol*	Citrus food
Flavones	*Apigenin, luteolin*	Green leafy spices
Isoflavones	*Daidzein, genistein, glycitein, biochanin A*	Soyabeans, soy food, legumes
Flavonols	*Kaempferol, myricetin, quercetin, isorhamnetin*	Nearly ubiquitous in food
Anthocyanidins	*Cyanidin, delphinidin, pelargonidin*	Red, purple and blue berries

(Adapted from Garcia-Lafuente *et al.,* 2009)

Garcinia kola, a species of a tropical flowering non-timber plant of *Guttiferae* family, is found mainly in the tropical rain forest regions of west and central Africa (Okwu, 2005). It grows as a medium sized tree, up to 14 metres high and is highly valued in west and central Africa for its extensive use in herbal medicine and as a food source (Okwu, 2005). The plant produces reddish, yellowish or orange coloured fruits, each producing 2 to 4 sour tasting brownish seeds (**Figure 1-1**), which when chewed, have a bitter taste, hence the name “bitter kola” nut (Okwu, 2005). The fruit, seeds, nuts and bark of the plant are used in traditional medicine for the prevention of colic pains and headaches (Ayensu, 1978), relieving chest cold and cough (Iwu, 1993) and as an antidote for venomous stings and bites (Aluka, 1985).

Figure 1-1: *Garcinia kola* nuts

In search of natural compounds with pharmacological benefits, ethanolic extract from the *Garcinia kola* nut, commonly referred to as kolaviron, has shown great potential for use in therapeutic medicine against many health threatening chronic diseases of the liver, reproductive system and diabetes. Adegbehingbe and co-workers (2008) demonstrated that *Garcinia kola* seed shows a significant anti-inflammatory effect in knee osteoarthritis patients. Furthermore, Olaleye and colleagues (2000) showed that kolaviron exhibits strong anti-inflammatory activities when compared to acetyl salicylic acid drug. Thus, the aim of our study was to determine the anti-inflammatory effects of dietary *Garcinia kola* nut against IRI in isolated Wistar rat hearts. We hypothesized that dietary *Garcinia kola* supplementation may offer myocardial protection against induced IRI through modulation of myocardial inflammatory mediators and protein signaling.

1.1: Aims of the study

The aims of this study were to:

1. investigate the effect of dietary *Garcinia kola* supplementation on cardiac functional recovery after ischaemia/reperfusion injury in an isolated rat heart model
2. determine the effect of dietary *Garcinia kola* supplementation on cardiac inflammatory cytokine/chemokine and protein expression before and after induction of IRI
3. investigate the effect of dietary *Garcinia kola* supplementation on pro-oxidative stress mediators before and after IRI
4. determine the effect of dietary *Garcinia kola* supplementation on NF-kβ PKB/Akt, p38 MAPK, Caspase 3 and PARP-1 protein signaling and/or phosphorylation (Where applicable)
5. Investigate the effect of dietary *Garcinia kola* supplementation on *in vivo* lymphocytic deoxyribose nucleic acid (DNA) damage using the alkaline comet assay.

CHAPTER 2: LITERATURE REVIEW

2.1: Flavonoids in health

Research from the past three decades indicates that food supplements play an important role in prevention of chronic diseases like cancer, diabetes, coronary heart diseases, hypertension and hypercholesterolemia (Rao, 2003). Evidence shows that nutritional supplements, especially those of plant origin, contain a wide range of non-nutrient phytochemicals (**Table 2-1**), originally used by plants for their own disease protection and for other biological functions (Rao, 2003). Current interest in the possible health benefits of different flavonoids observed in both *in vivo* and *in vitro* experimental models are increasing (Rice-Evans *et al.,* 1996; Shen *et al*., 2007; Prochazkova *et al*., 2011). These natural food products demonstrates different biological properties such as anti-inflammatory (Subarnas and Wagner, 2000; Widlansky *et al.,* 2005) and anti-carcinogenic benefits (Farombi and Owoeye, 2011). Furthermore, these products have shown potential to alleviate induced oxidative stress (Van Acker *et al.,* 1995; Farombi *et al*., 2004a) through direct scavenging of reactive oxygen species (ROS) (Farombi *et al*., 2002; Farombi, 2006; Santo *et al*., 2010), activation of antioxidant enzymes (Nijveldt *et al*., 2001), chelating of metal (Nwokocha *et al.,* 2011) and reduction of oxidized α-tocopheryl radicals (Hirano *et al.,* 2001; Heim *et al.,* 2002; Fujisawa *et al.,* 2006).

Table 2-1: Disease preventing phytochemicals in plant foods

Food groups	phytochemicals	Useful in the prevention of
Whole cereal, grains	Dietary fibre, *tocopherols*	Diabetes, CVD, hypercholesterolemia
Vegetables, fat	*α-linoleic acid, linoleic acid, tocopherols,* sterols	Cancer, diabetes, CVD, hypercholesterolemia
Palm oil	*Tocopherols, tocotrienols, carotenoids*	Cancer, heart diseases, atherosclerosis, cataract, pulmonary diseases, muscle injury
Yellow or green leafy vegetables, yellow fruits	*Carotenes*, ascorbic acid, dietary fibre,*omega-3 fatty acids*	Cancer, heart diseases, atherosclerosis, pectins cataract, pulmonary diseases, muscle injury
Rice bran oil	Sterols, polyunsaturated fatty acids	Hypercholesterolemia, diabetes, CVD
Linseed oil	Omega-3 fatty acid	Hypercholesterolaemia, diabetes, CVD
Spices, fenugreek seeds, turmeric	Plant gum, *curcumin, eugenol, capsicin*	Cancer, CVD, detoxification of drugs and toxins

(Adopted from: Rao, 2003)

Different flavonoids have demonstrated various beneficial health related roles, triggering pro-cell survival response and/or inhibiting pro-cell death signaling pathways following ischaemia-reperfusion injury (IRI). Kim and co-workers (2009) demonstrated that administration of daidzein, an isoflavone found in soya and soyabeans reduce myocardial IRI in rat hearts by inhibiting the nuclear factor kappa beta (NF-kβ) transcription factor activation. Furthermore, epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea and has shown the ability to inhibit cerebral (Park *et al*., 2009), renal (Jang *et al.,* 2006) and cardiac (Townsend *et al.,* 2004; Kim *et al.,* 2010) injury in animal ischaemia-reperfusion models.

Research has also shown that certain flavonoids modulate expression of inflammatory mediators in lipopolysaccharide (LPS)-activated macrophages (Comalada *et al.,* 2006*;* Boots *et al.,* 2008). Comalada and co-workers (2006) demonstrated that quercetin and catechins inhibits expression of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β coupled with enhancement of IL-10 release, in macrophage cells stimulated with LPS for 24 hours. In another study Lee and colleagues (2009) showed that kaempferol, quercetin, fisetin, and chrysin inhibits TNF-α induced IL-8 promoter activation and gene expression in human embryonic

kidney HEK 293 cells. Supporting these observations, Geraets and co-workers (2009) showed that fisetin down regulates gene expression of TNF-α, IL-1β, macrophage inflammatory protein (MIP) -1α, IL-6 and MIP-2 in mouse model of LPS-induced acute pulmonary inflammation. They observed that anti-inflammatory effects of fisetin were pronounced when compared to flavone or tricetin.

It is widely accepted that myocardial IRI triggers sequential release of deleterious mediators responsible for inducing tissue injury and subsequently leading to endothelial dysfunction (Stenvinkel, 2001; Behrendt and Ganz, 2002; Vajdovich, 2008; Zhang, 2008). Evidence suggests that timely regulation of certain mediators during IRI is essential for optimal tissue healing and recovery of the host cells. The efficacy of several phytochemicals in attenuating disease progression has been clearly established (Liu, 2003; Kennedy and Wightman, 2011), however, evidence indicates that different phytochemicals have the ability to protect against chronic inflammatory disease through targeting multiple signaling pathways (Gillespie and Gavins, 2013). Furthermore, it has been demonstrated that their efficacy may be attributed to several reasons, namely: optimal dose, absorption, type of antioxidants available (Tribble, 1999; Salonen *et al*., 2003; Comalada *et al*., 2006) and their ability to target specific redox reactions and different signaling pathways (Van Acker *et al.,* 1995; Rotelli *et al.,* 2003; Bester *et al.*, 2010; Pantsi *et al*., 2011; Katengua-Thamahane *et al.,* 2012). Hence, better understanding is required on the specific properties and potency of individual phytochemicals.

2.2: *Garcinia kola* in health and disease

Therapeutic values of the *Garcinia kola* nut have been attributed to its high content of phytochemicals, vitamins and minerals (**Table 2-2**) (Okwu, 2005; Adegboye *et al*., 2008; Olajide and Adeniyi, 2011; Eleazu *et al.,* 2012). The antioxidant and anti-inflammatory potential of *Garcinia kola* may be linked to its high flavonoid content of kolaviron (Adaramoye *et al.,* 2005). It has been demonstrated that kolaviron plays a significant role in free radical scavenging of superoxide anions (O_2^-), singlet oxygen radicals ($^1O_2$), hydroxyl radicals (OH^-) and lipid-peroxy radicals (LOO^-) in animal experimental models (Farombi *et al.,* 2002; Farombi *et al.,* 2004a)

and therefore potentially limiting the time span during which these radicals can exert their deleterious effects in the tissue (Toth *et al*., 2003).

Table 2-2: Nutritional composition of *Garcinia kola* seed

Phytochemical	Vitamins	Minerals
Flavonoids	Thiamine(vitamin B_1)	Macro Elements:
Saponins	Riboflavin (vitamin B_2)	Magnesium, Calcium, potassium, phosphorus, sodium.
Tannins	Niacin (Nicotinic acid)	Micro Elements:
Alkaloids	Ascorbic acid (vitamin C)	iron, zinc, copper, manganese, cobalt, cadmium
Phenols		

(Compiled from: Okwu, 2005)

Several investigations using kolaviron have shown its potential for use in chemo-prevention and protection against many health threatening diseases of the liver (Farombi *et al*., 2001; 2005), brain (Adaramoye, 2010), male reproductive system (Adaramoye *et al.,* 2012a; 2012b), lymphocytes (Farombi *et al.,* 2004a), and in blood (Adaramoye *et al.,* 2005; Ajani *et al*., 2008). Recently, Olivier and co-workers (2013) demonstrated that dietary kolaviron supplementation reduces cardiac infarct size and improves myocardial functional recovery following ischaemia-reperfusion injury. However, little is known concerning the mechanisms of dietary *Garcinia kola* supplementation especially in the cardiac tissue.

The ability of kolaviron to inhibit Cyclooxygenase-2 (COX-2) and iNOS expression through the down regulation of NF-kβ and activator protein-1 (AP-1) DNA binding activity has been suggested as a potential mechanism for the hepatoprotection in Wistar rats (Farombi *et al.*, 2009). This study revealed that kolaviron abolishes the expression of COX-2 and iNOS proteins in rat liver treated with highly toxic dimethylnitrosamine (Farombi *et al.*, 2009). The majority of reports suggest that kolaviron elicits its protective effect on the liver by acting as a membrane stabilizer, hence preventing the distortion of cellular ionic environment associated with xenobiotic treatment (Farombi, 2000; Farombi *et al.,* 2004a; 2004b; Madubunyi, 2010; Farombi and Owoeye, 2011). Farombi (2000) demonstrated that

kolaviron significantly attenuates the toxic assaults imposed on hepatic enzymes by carbon tetrachloride, and that it protects the rat liver against accumulation of toxic lipid peroxidation products. They proposed that kolaviron protection against carcinogen-induced hepatotoxicity and membrane damage may be through its *in vivo* antioxidant potency. In another study, Farombi and co-workers (2001) demonstrated that kolaviron supplementation attenuates hepatotoxic effects of Aflatoxin B1 in rats. In the light of kolaviron efficacy to reduce some heavy metal accumulation in the liver, Nwokocha and co-workers (2011) demonstrated that kolaviron protects the rat liver against cadmium, mercury and lead poisoning. Beneficial effects of kolaviron supplementation in these studies where attributed to its antioxidant signaling, metal chelation and free radical scavenging (Farombi, 2000; Farombi *et al.,* 2001; 2005; Nwokocha *et al.,* 2011).

In recent years there has been an increasing concern regarding the role of chronic inflammatory conditions associated with male infertility caused by oxidative stress (Aitken and Baker, 2004; Amaral *et al.,* 2008; Badade and Samant, 2011; Bansal and Bilaspuri, 2010). As such, research on natural products with the potential to reduce oxidative stress is currently on the rise. Recent investigations indicate that kolaviron ameliorate testicular toxicity caused by nevirapine (Adaramoye *et al.,* 2012a), gamma irradiation, (Adaramoye *et al.,* 2012b) and di-n-butyl-phthalate (Farombi *et al.,* 2007) in Wistar rats. The protective effect of kolaviron in these studies was linked to the increased activity of antioxidant enzymes and decreased peroxidation of the lipid membrane. Adaramoye (2010) further demonstrated that kolaviron protects the Wistar rat brain form gamma irradiation-induced oxidative stress. In this study the protective effects observed was also associated with the significantly improved antioxidant indices in kolaviron supplemented animals.

Research indicates that pharmacological benefit of kolaviron in streptozotocin (STZ)-induced diabetic rats, including anti-hypercholestrolaemic (Adaramoye *et al*., 2005), antioxidant activities and hypoglycaemic effects (Adaramoye and Adeyemi, 2006). Ajani and co-workers (2008) have demonstrated that administration of amodiaquine and artesunate malaria drugs for prophylactic purposes increases the risk of atherogenic and coronary diseases. In the same study, kolaviron though reputed to be hepatoprotective, failed to reverse the symptoms of cardiotoxicity

associated with these drugs, however, pre-treatment with kolaviron reversed the increased organ weights observed in prophylactic treated animals (Ajani *et al.,* 2008). In another study, Farombi and Nwaokeafor (2005) demonstrated that kolaviron attenuates oxidation of lipoprotein, possibly by mechanisms involving metal chelation and anti-oxidant activity that may be of importance in prevention of atherosclerotic plaque formation.

2.3: Ischaemic heart diseases

Ischaemic heart diseases (IHD) are a group of pathophysiological related conditions characterised by reduced blood supply to the heart, usually caused by narrowing or occlusion of a coronary artery (Mathers *et al*., 2004; Steyn, 2007). As the only serving path for blood supply to the heart muscle, narrowing of the artery lumen results in reduced blood supply to heart muscle. This may results in a condition referred to as angina pectoris, which is characterised by sudden episodes of chest pain. In advanced cases complete occlusion of the coronary artery may result in myocardial infarction, a severe and life threatening form of IHD (Mathers *et al.,* 2004; Steyn, 2007). Research evidence suggest that IHD is characterised by disruption in the normal endothelial function and active inflammation (Davignon and Ganz, 2004; Zhang, 2008).

Endothelial dysfunction and inflammation have been linked with the pathogenesis of CVD, leading to their role in IHD becoming an area of active investigation (Galle *et al.,* 2003; Hadi *et al.,* 2005; Libby, 2006; Tousoulis *et al.,* 2008). Inflammation is characterised by the release of ROS such as O_2^- while the endothelial cells release NO to maintain vascular tone and homeostatic function (Stenvinkel, 2001; Behrendt and Ganz, 2002; Zhang, 2008). The most significant mechanism associated with vascular endothelial dysfunction involves the formation of the peroxynitrite ($ONOO^-$) radical from a reaction involving O_2^- and NO. Therefore, this may lead to reduction of NO bioavailability and an increase in vascular endothelial permeability, which in turn promotes the adhesion of leukocytes and low density lipoprotein cholesterol (LDL-C) adhesion to the endothelium (Behrendt and Ganz, 2002**;** Galle *et al*., 2003). Furthermore, it has been shown that the release of inflammatory mediators facilitate firm attachment of leucocytes and oxidised-LDL cholesterol to the injured

endothelium, therefore contributing significantly to the process of atherosclerotic plaque formation (Libby *et al.,* 2002; Mestas and Ley, 2008; Ridker and Silvertown, 2008; Libby *et al.,* 2010).

2.3.1: Atherosclerosis

Atherosclerosis is a pathological condition underlying several ischaemic heart diseases such as myocardial infarction, stroke and heart failure (Mathers *et al*., 2004; Steyn, 2007). It is characterised by the formation of a plaque within artery walls, initially presenting as a fatty streak in the inner-most layer of the arterial walls (Libby, 2006; Hansson and Libby, 2006). It has been demonstrated that myocardial cell damage caused by IRI is accompanied by increased release of free radicals which contribute to induced oxidative modification of LDL-C (Vogiatzi *et al*., 2009). Following endothelial damage, inflammation is initiated, promoting migration of immune cells such as the monocytes into the sub-endothelial space to facilitate tissue repair and healing. However, upon interaction with oxidised-LDL-C these cells are easily attached to the endothelium and transformed into “foam cells”, forming a fatty streak in the intima (Lakshmi *et al*., 2009; Vogiatzi *et al*., 2009). The fatty streak has demonstrated to be a pure inflammatory lesion, with accumulation of polymophonuclear leukocytes and their activation playing a key role in atherosclerotic plaque formation and rupture (Entman and Smith, 1994). Persistent inflammation is associated with increased accumulation of macrophages and lymphocytes in the area of the lesion, leading to increased secretion of cytokines and chemokines, which in turn might perpetuate the inflammatory process (Spagnoli *et al.,* 2007).

Atherosclerosis is a complex condition characterised by thickening, loss of contractility and hardening of the arterial wall, resulting in cardiac complications associated with decreased coronary flow (Katz, 2011). In addition, chronic inflammation in the region of the atherosclerotic plaque activates platelets and promotes their adhesion to the injured endothelium, sealing the injured area with fibrinogen and resulting in a fibrous cover referred to as an atheroma (Hansson and Libby, 2006; Libby, 2006; Spagnoli *et al.,* 2007). Active inflammation within the area of the atheroma subsequently leads to the rupture of this plaque into small thrombi

with the potential to occlude one or more major arteries and generally causing ischaemia to the entire organs or tissues (Buja and Willerson, 1994; Mathers *et al.,* 2004).

2.3.2: Myocardial infarction

Occlusion of coronary arteries results in total or partial myocardial ischaemia. This may lead to three characterized functional alterations of the myocardium depending on the severity and duration of the ischaemic insult: 1) myocardial stunning defined as an ischaemic insult resulting in a reversible contractile dysfunction 2) hibernation, a chronic impairment of the myocardium secondary to chronic hypo-perfusion, however the myocardium is still viable and 3) myocardial infarction, the most detrimental result of myocardial ischaemia, also referred to as a true infarction (Kloner *et al*., 1998; Eltzschig and Collard, 2004). Myocardial infarction (MI) occurs when the heart muscle is deprived of oxygenated blood for a prolonged period. It is characterised by decreased cellular oxidative phosphorylation and the onset of adenosine tri-phosphate (ATP) depletion, resulting in irreversible myocardial cell death (Eltzschig and Collard, 2004).

Damaged myocardial cells may initiate an inflammatory response or necrosis leading to increased release of enzymes such as creatinine phosphokinase and lactate dehydrogenase into the circulation, which in turn leads to the activation of apoptotic signaling pathways (Hoffman *et al.,* 2004). Furthermore, damaged cells are replaced with scar tissue and not functional myocardial cells (Schwartz and Kornowski, 2003; Krishna *et al.,* 2011) therefore, MI may lead to reduced cardiac function and output characterized by impaired contractile (systolic) and relaxation (diastolic) functions, athythmogenesis, decreased stroke volume and increased left ventricular end diastolic volume (Hamosh and Cohn, 1971; Mulder *et al.,* 2004). Several compensatory mechanisms such as increased heart rate, force of contraction (e.g. pulse pressure and aortic pressure) and the renin–angiotensin system may be activated in an attempt to restore cardiac pumping function and cardiac output. However, these mechanisms may increase oxygen demand and

cardiac workload, hence, worsening the overall cardiovascular function and prognosis (Mann, 1999; Young, 2010; Tavares *et al.,* 2012).

2.4: Pathophysiology of ischaemia and reperfusion injury

Research indicates that myocardial tissue can tolerate a brief period of ischaemia without resultant cell death (Verma *et al*., 2002; Eltzschig and Collard, 2004). However, this will depend on the effects of the ischaemia on the cellular metabolic and ultra-structural changes (**Table 2-3**). Different modalities of transient periods of ischaemia-reperfusion are encountered in clinical situations such as coronary artery by-pass, grafting and cardiac transplant (Verma *et al*., 2002). In such situations, although necessary for survival of the myocardium, reperfusion itself is associated with dramatic and deleterious changes mediated in part by oxidative stress and inflammation, both contributing significantly to the endothelial cell damage (Verma *et al.,* 2002; Garcıa-Dorado, 2004; Hoffman *et al*., 2004).

Table 2-3: Cellular effects of ischaemia

Cellular acidosis
Altered membrane potential
Altered ion distribution (increased intracellular Ca^{2+} /Na^{+} ratio)
Cellular swelling
Cytoskeletal disorganization
Increased hypoxanthine
Decreased adenosine-tri-phosphate (ATP)
Decreased phosphocreatine
Decreased glutathione
Stabilization and nuclear translocation of hypoxia-inducible factor 1 (HIF-1)
Increased leucocyte adhesion molecule expression
Increased nucleotide phosphohydrolysis (via CD39 and CD73) and adenosine signalling

(Reproduced from: Eltzschig and Collard, 2004)

2.4.1: Mitochondrial injury

There is general consensus on the role of mitochondrial injury in all forms of cardiac cell death during ischaemia-reperfusion (Di Lisa *et al.,* 1998; Di Lisa and Bernandi, 2006; Machado *et al.,* 2009). Evidence shows that ischaemic injury is accompanied by a gradual increase in intracellular calcium ions (ca^{2+}), mediating mitochondrial damage through activation of the mitochondrial permeability transition and oxidative stress (Weiss *et al*., 2003; Di Lisa and Bernandi, 2006).

Calcium overload induces dysfunction in mitochondrial bio-energetics (Duchen, 2004). It has been demonstrated that binding of free Ca^{2+} to calmodulin activates myosin light chain kinase (MLCK), which utilises ATP molecules and transfers phosphate ions (pi) directly to myosin for activation (Kamm and Stull, 2001; Krueger *et al.,* 2001; Roux *et al*., 2012). The activated myosin forms cross-bridges with actin filaments, in turn leading to contraction of the smooth muscles. For the relaxation to occur, Ca^{2+} must decrease to the extent that the Ca^{2+} dissociates from the MLCK, dephosphorylating the myosin and thereby “turning off” the contractile mechanism. Baumgartner and co-workers (2009) demonstrated that elevated Ca^{2+} in the mitochondria is a crucial factor in determining whether cells activate apoptotic mechanisms during oxidative stress. Ischaemia is characterized by decreased synthesis and increased hydrolysis of ATP resulting in prolonged opening of the mitochondrial permeability pores (mPTP) (Di Lisa and Bernandi, 2006). This altered mPTP, favours impairment of the ionic homeostasis, especially for Ca^{2+}, leading to increased calcium sequestration in the mitochondria. Increased mitochondrial Ca^{2+} is associated with detrimental effects in cardiac function, including excessive activation of myofilament, initiation of arrhythmias, transient mechanical dysfunctions, microvascular injury, as well as myocardial stunning during reperfusion (Bolli, 1990).

Anaerobic metabolism characterised by inadequate oxygen supply leads to increased intracellular acidity, build-up of phosphate, calcium overload and free radical release, all contributing to increased mitochondrial injury upon reperfusion (Di Lisa *et al.,* 1998; Zorov *et al*., 2000). It is widely thought that cardiac functional recovery and myocardial survival upon reperfusion ultimately depends on the mitochondrial recovery, as such mechanistic links between cardio-protective signaling and mitochondria toxicity is currently an area of active investigation.

2.4.2: Inflammatory response

One of the major challenges following myocardial IRI is the regulation of the inflammatory response, a well-established phenomenon in IRI and homeostasis. Evidence indicates that the acute inflammatory response plays a profound role in clearing of debris, wound healing and immune-regulation (Frangogiannis, 2008; 2012). It is currently well established that myocardial damage leads to the recruitment of cells of the innate immune system (i.e. polymorphonuclear leukocytes and macrophages/monocytes) to the site of injury (Zuidema and Zhang, 2010; Mann, 2011; Timmers *et al.,* 2012). Activation of these cells mediates the release of other inflammatory mediators and growth factors. This may in turn induce the recruitment of more inflammatory cells, clearance of cellular debris, angiogenesis, and proliferation of fibroblasts, eventually resulting in scar formation and wound healing (Brunelli and Rovere-Querini, 2008) (**Figure 2-1**). The acute inflammatory response has many beneficial effects in preserving cell function. However, prolonged expression of pro-inflammatory mediators such as TNF-α, IL-1β, IL-8, IL-6 and iNOS are implicated in the detrimental effects of IRI and in abnormal myocardial remodelling (Frangogiannis *et al.,* 2002; Nian *et al.,* 2004).

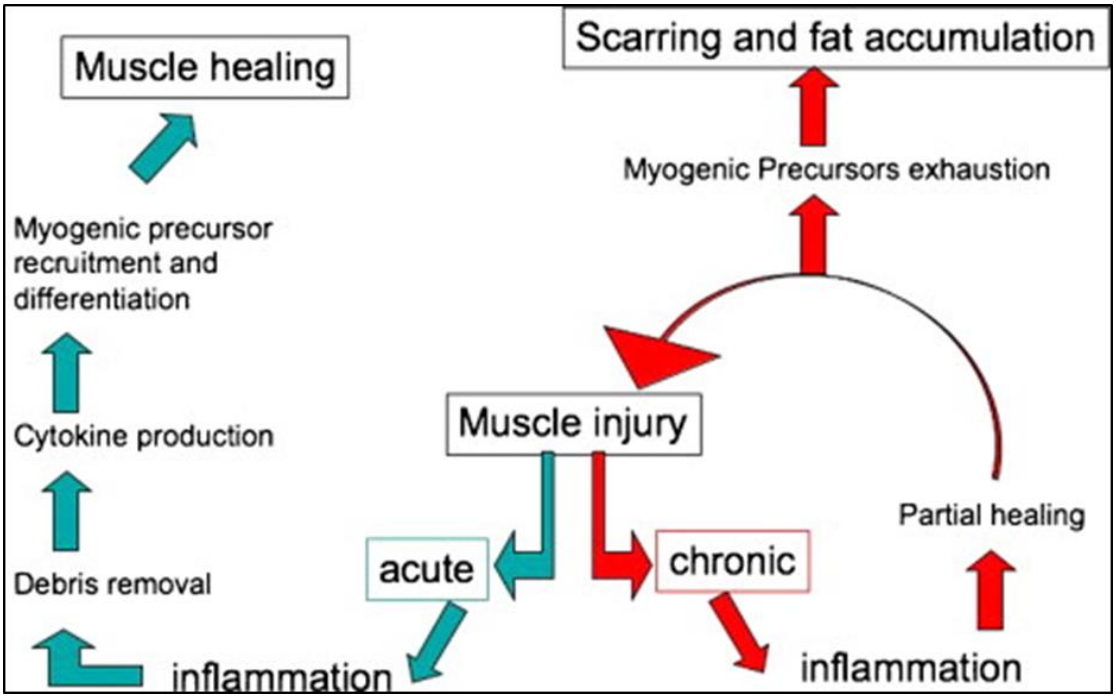


Figure 2-1: Schematic representation of the events occurring following acute (left) or chronic (right) muscle injury. (Adapted from Brunelli and Rovere-Querini, 2008)

Chronic inflammation exerts its deleterious effects mainly through sustained activation of pro-inflammatory mediators and increased free radical release associated with endothelial damage and antioxidant depletion. To regulate the detrimental effects of the inflammatory response, another group of inflammatory mediators are released. The anti-inflammatory cytokine/chemokine exhibits an antagonistic role towards the pro-inflammatory cytokines/chemokines therefore, they may play a significant role in regulating the progression of acute inflammation to chronic inflammation (Hanada and Yoshimura, 2002; Santangelo *et al*., 2007). Anti-inflammatory cytokines/chemokines [IL-4, IL-10, IL-13, IFN-α, and the transforming growth factor (TGF-β)] can directly exert their inhibitory effects on the vascular cells releasing pro-inflammatory mediators and may also modulate different signaling pathways, as such regulating the inflammatory response (Lefer *et al.,* 1990; Perrella *et al.,* 1996; Feghali and Wright, 1997; Hammer *et al*.,2005; Murray, 2006).

2.4.3: Free radical release

Free radicals are defined as molecules that contain one or more unpaired electrons resulting in high affinity to react with other molecules through donating or accepting an electron (Lobo *et al.,* 2010). Evidence shows that O_2^- have a high affinity for NO, forming a more deleterious nitrosative free radical $ONOO^-$ (Turan *et al*., 2006). Upon interaction with macromolecules $ONOO^-$ may trigger cellular responses ranging from loss of enzyme function, altered membrane integrity, DNA mutation, cell necrosis and/or apoptosis (Vinten-Johansen, 2000; Ferdinandy and Schulz, 2001) (**Figure 2-2**). This may lead to endothelial damage and depressed contractile function (Collard and Gelman, 2001; Leifeld *et al*., 2002). Furthermore, ROS have been linked with activation of NF-kβ transcription factor, indirectly stimulating release of pro-inflammatory mediators and the activation of the MAPK family proteins, hence perpetuating myocardial injury and necrosis (Ma *et al*., 2000; Eltzschig and Collard, 2004).

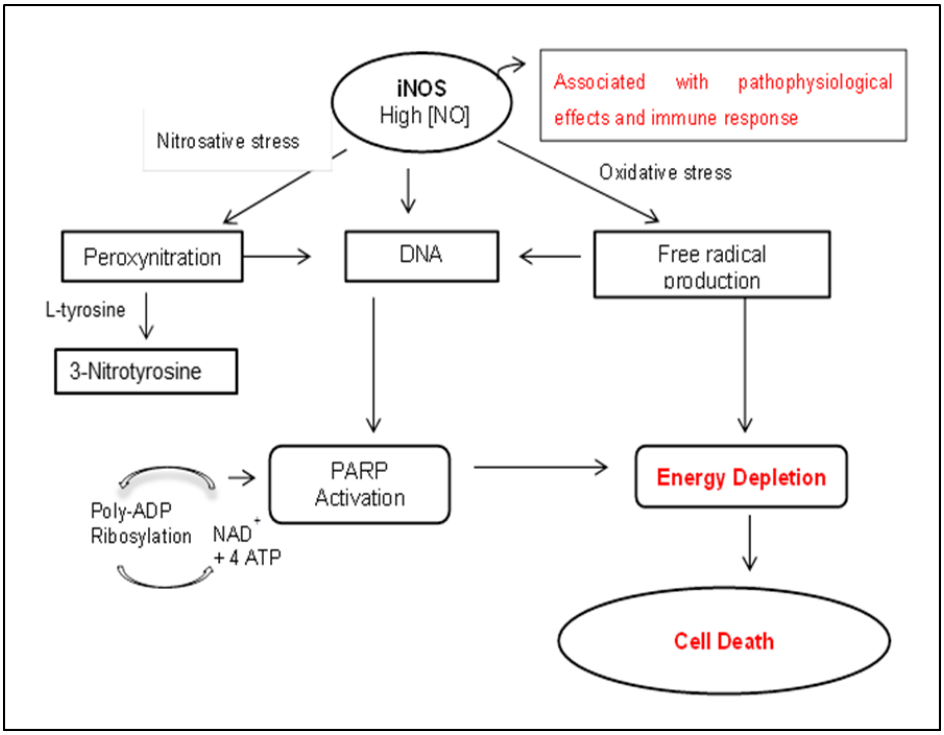


Figure 2-2: Role of nitric oxide and peroxynitrite in myocytes death (Modified from Pacher *et al.*, 2007)

2.5: Role of cytokines/chemokines in myocardial ischaemia and reperfusion

Induced systemic and local expression of inflammatory mediators is generally associated with tissue response to injury. Herskowitz and co-workers (1995) demonstrated that IRI induces local myocardial expression of mRNA for IL-lβ, TNF-α, IFN-γ, IL-2, IL-6 and TGF-1β. Furthermore, they observed an increase in IL-1β and TNF-α proteins and macrophage infiltration during the healing of the ischaemic myocardium using an immuno-staining technique. In another study, increased myocardial expression of IL-6, TNF-α, IL-1β, macrophage inflammatory protein-3α, cytokine-induced neutrophil chemo-attractant 2 and 3 was observed in the myocardium of animals after 8 days of induced ischaemia (Moro *et al.,* 2007). However, expression of IL-4 and IL-10 remained unchanged in this study.

The role of different cytokines/chemokines during myocardial IRI remains speculative. It has been suggested that an adaptive cellular response to myocardial IRI activates the complement system which in turn activates inflammatory cytokines

and leukocytes (**Figure 2-3**) (Sharma and Das, 1997). Several reports suggest that at low dose, expression of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are important mediators during infarct healing and may induce the expression of antioxidant enzymes such as SOD and may trigger the release of heat shock proteins (Hsp) into the extracellular space (Kaur *et al.,* 1989; Ono *et al.,* 1992). Extracellular Hsp such as Hsp27 and Hsp70 has demonstrated anti-inflammatory and immuno-regulatory functions (Pockley, 2003; Luo *et al*., 2008). Luo and co-workers (2008) demonstrated that Hsp70 may inhibit the activation of the MAPK family proteins and nuclear translocation of the NF-kβ transcription factor, thereby regulating the production of pro-inflammatory mediators and tissue injury.

Using a rat model of myocardial infarction, Ono and co-workers (1998) compared gene expression levels of IL-1β, IL-6 and TNF-α in the infarcted area, non-infarcted area and in the myocardium of a sham operated group. They reported that cytokine expression remained significantly high in the non-infarcted area compared to the infarcted zone in the sham operated group. Furthermore, they demonstrated that, the levels of the cytokines in the non-infarcted area correlated well with left ventricular end diastolic diameter and collagen deposition, therefore suggesting a possible involvement of these mediators in the survival process of the myocardium. In addition, Moro and co-workers (2007) proposed that delayed expression of pro-inflammatory cytokines may have contributed to the post-infarct myocardial dysfunction. Furthermore, upregulation of MCP-1 in the infarcted area have been associated with induced recruitment of mononuclear cells which produce the cytokines and growth factors necessary for effective tissue repair and scar formation (Dewald *et al.,* 2005).

Activation of leukocytes are implicated in diminished barrier function of the endothelial cells during IRI. Evidence suggests that these cells have the capacity to induce the release of several mediators such as free radicals, cytokines/chemokines and oxidases (**Figure 2-3**). High levels of IL-1β, TNF-α and IFN-γ in the infarcted rat myocardium have been found to induce iNOS gene expression, hence increasing release of NO (Pinsky *et al.,* 1995). Available evidence suggests that activated leukocytes may increase the release of several mediators during ischaemia-reperfusion that may impair the function of the endothelial barrier, e.g. mast cells

release IL-1, TNF-α and histamine; T-cell releases IL-12 and IFN-γ; macrophages release MIP-1α and IL-6 while neutrophils releases superoxide, hydrogen peroxide radicals and elastase (Granger and Senchenkova, 2010; Rodrigues and Granger, 2010). High levels of these mediators have been linked to events leading to chronic myocardial injury, activation of pro-apoptotic proteins and depletion of ATP with the subsequent induction of apoptosis post-ischaemic injury (Finkel *et al.,* 1992; Roumen *et al.,* 1993; Marra *et al.,* 1998; Takeuchi *et al.,* 2004).

Overall, these studies suggest that control of prompt and regulated expression of pro-inflammatory cytokines/chemokines may be crucial in order to improve myocardial survival post-ischaemia reperfusion injury. Induction of pro-inflammatory cytokines coupled with increased release of anti-inflammatory cytokines such as IL-10 has been associated with mild cardiac dysfunction during IRI (Holzheimer *et al.,* 1999). Furthermore, Yang and co-workers (2000) demonstrated that IL-10 expression inhibits excessive production of TNF-α and suppresses recruitment of neutrophils to the injured area following IRI.

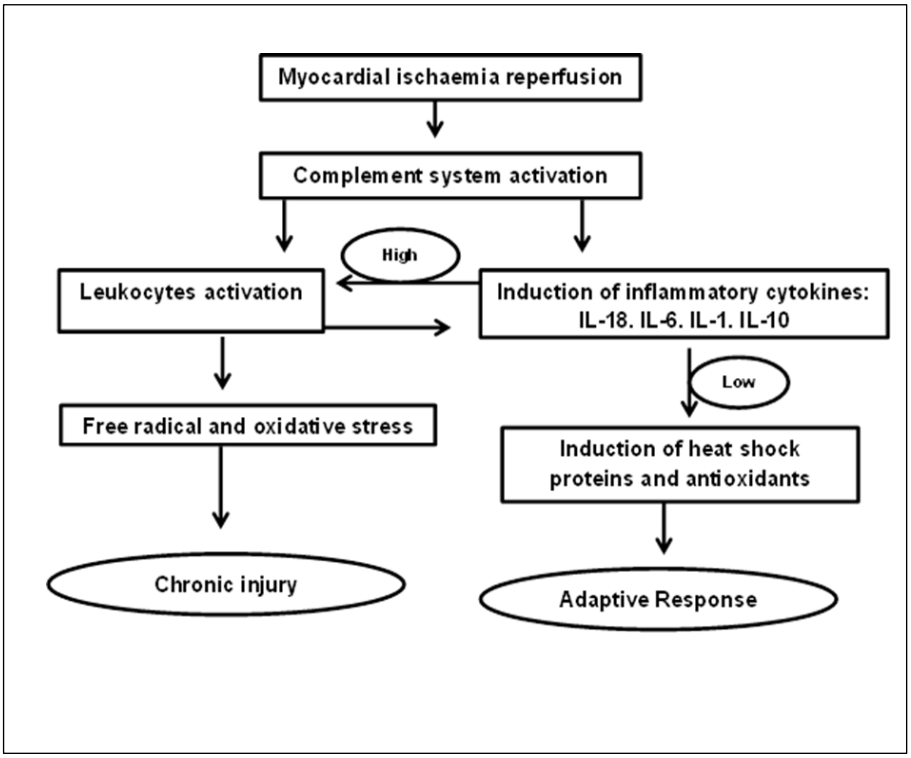


Figure 2-3: A model of the role of cytokines/ chemokines in myocardial ischaemia and reperfusion. (Modified from Sharma and Das, 1997)

2.6: Inflammatory associated intracellular signalling in IRI

Inflammatory mediators may activate several signaling pathways associated with modulating myocardial healing or death following IRI (**Figure 2-4**). The role of the NF-kβ transcription factor is well established in regulating expression of adhesion molecules, inducible enzymes and cytokines involved in inflammatory response (Baichwal and Baeuerle, 1997; Ghosh and Karin, 2002; Karin and Lin, 2002; Sethi *et al*., 2008). Activation of other signaling pathways such as the Protein Kinase B (PKB/Akt) (Mockridge *et al*., 2000) and the p38 MAPK (Herlaar and Brown, 1999) are also linked to the host outcomes following ischaemia and reperfusion.

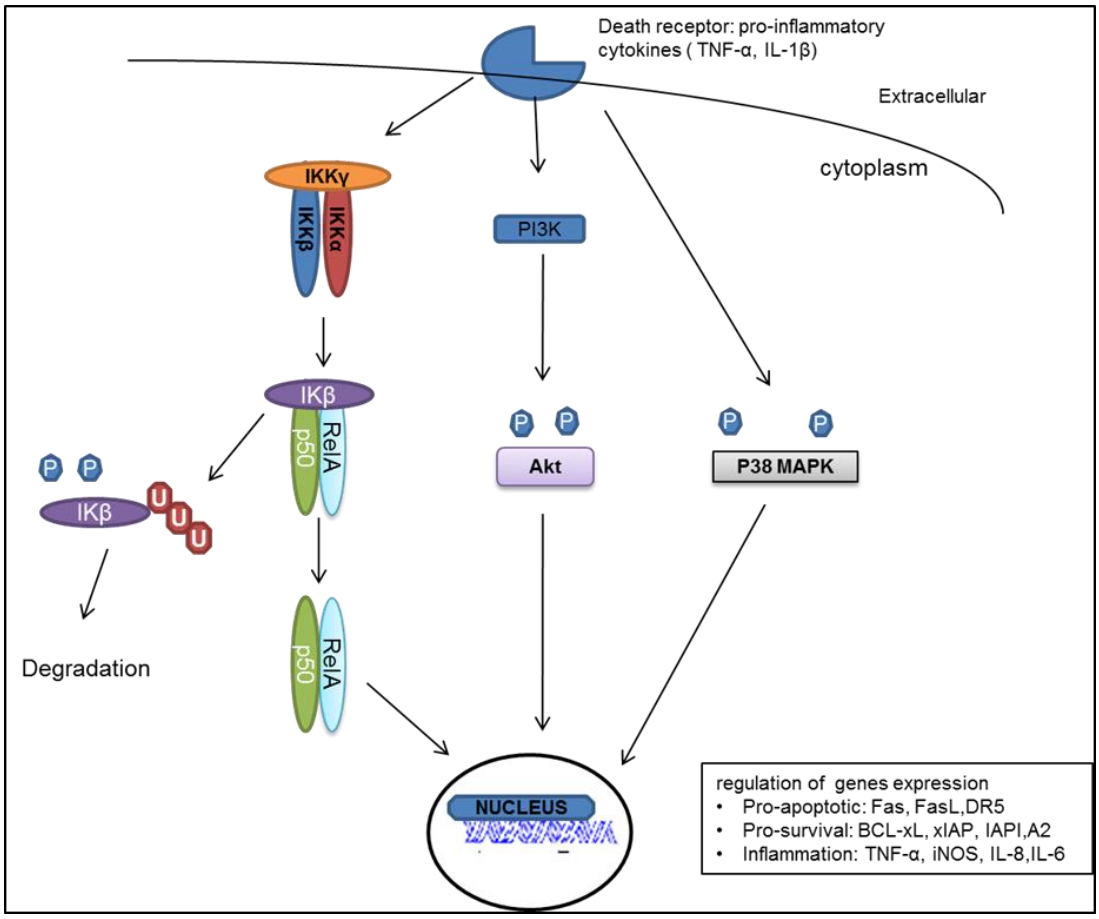


Figure 2-4: Death receptor mediated pathway signaling (Compiled from Katz, 2011)

2.6.1: The NF-kβ pathway

Studies using transgenic animal models showing defective NF-kβ activation or lacking any of the subunits, have demonstrated the importance of the NF-kβ transcription factor in innate immunity, adaptive immunity and inflammation (Sha *et al.,* 1995; Beg and Baltimore, 1996; Franzoso *et al.,* 1998). Beg and Baltimore (1996) demonstrated the potential role of NF-kβ in inhibition of TNF-α induced apoptosis. To date NF-kβ is one of the major transcription factors linked to CVD and health, regulating expression of over 400 genes coding for innate immunity, cell survival and inflammatory response (Sethi *et al*., 2008). The wide variety of genes regulated by this transcription factor includes those encoding for inflammatory mediators such as cytokine (TNF-α, IL-6, IL10), chemokines (IL-8, monocyte chemotactic protein-1), adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1)) and inducible effector enzymes (iNOS, COX-2) (Ghosh and Karin, 2002). Additionally, activation of the NF-kβ proteins induce expression of several anti-apoptotic proteins such as cellular inhibitor of apoptosis-1 (c-IAP-1), c-IAP-2, B cell lymphoma-extra-large (Bcl-xl), and fatty acid synthase ligand (FAS ligand) which are important in preventing TNF-α induced apoptosis (Karin and Lin, 2002).

The family of NF-kβ transcription factors comprises of five members: NF-kβ p65 (RelA), RelB, c-Rel (Rel), NF-kβ p50/105 and NF-kβ p52/100 (Hayden and Ghosh, 2004). These proteins share a highly conserved region of approximately 300 amino acids, which mediates DNA binding, dimerization and is responsible for the interaction with the inhibitor kappa beta (Ikβ) factor. The interaction with the Ikβ assists in retaining the NF- kβ dimers in the cytoplasm in an inactive state (Ghosh and Karin, 2002).

Two signaling pathways are implicated in the activation of the NF-kβ: the canonical (or classical) and the non-canonical (or alternative) pathway (Karin, 1999; Karin and Delhase, 2000; Lawrence, 2009). The two pathways share a common regulatory step of proteolytic degradation of the Ikβ by the inhibitor kappa beta kinase (IKK) complex (Karin, 1999). Proteosomal degradation of the Ikβ through phosphorylation-induced activation of IKK complex results in activation of the NF-kβ dimers with subsequent translocation to the nucleus to induce target gene expression

(Karin, 1999; Maier *et al*., 2012). The IKK complex consists of two catalytic kinase subunits (IKKα and IKKβ) and the regulatory non-enzymatic scaffold protein NEMO (IKKγ) (Karin, 1999; Karin and Lin, 2002). The IKKβ complex in conjunction with the NEMO subunit regulates activation of the NF-kβ dimers comprising of RelA, c-Rel, RelB and p50 in the canonical pathway (Zandi *et al.,* 1997; Karin, 1999; Lawrence, 2009; Maier *et al*., 2012) while the IKKα subunit is required for the instigation of the non-canonical pathway through phosphorylation and processing of the p100 precursor leading to activation of the p52/RelB dimers, independent of the IKKβ and NEMO subunits (Zandi *et al*., 1997; Lawrence, 2009).

2.6.2: PKB/Akt pathway

Activation of Akt has been demonstrated to promote cardiomyocytes survival following myocardial injury through modulation of different proteins and transcription factors (Chaanine and Hajjar, 2011). The B cell lymphoma-2 (Bcl-2) family proteins include both pro-apoptotic molecules (BAD, BIM, BAX) and pro-survival molecules (Bcl-xl, Bcl-w). Evidence shows that phosphorylation of Akt may promote cell survival by inhibiting the binding to and inactivating of the pro-apoptotic Bcl-2 family proteins thus promoting activation of the pro-survival Bcl-2 family proteins (Gustafsson and Gottlieb, 2007). Furthermore, activation of the Akt has been linked to transcriptional regulation of the cyclic adenosine monophosphate response element binding (CREB), foxhead and the NF-kβ transcription factors activity (LoPiccolo *et al.,* 2008; Papanicolaou *et al.,* 2008; Hers *et al*., 2011) (**Figure 2-5**). It has been shown that Akt phosphorylation may promote transcriptional expression of pro-survival genes by activating the NF-kβ and the CREB transcription factors (Romashkova and Makarov, 1999). Additionally, it has been suggested that activation of Akt may alter cellular localisation of the foxhead transcription factor, promoting its cytoplasmic localisation and thereby decreasing transcription of pro-apoptotic genes (Nicholson and Anderson, 2002) (**Figure 2-5**).

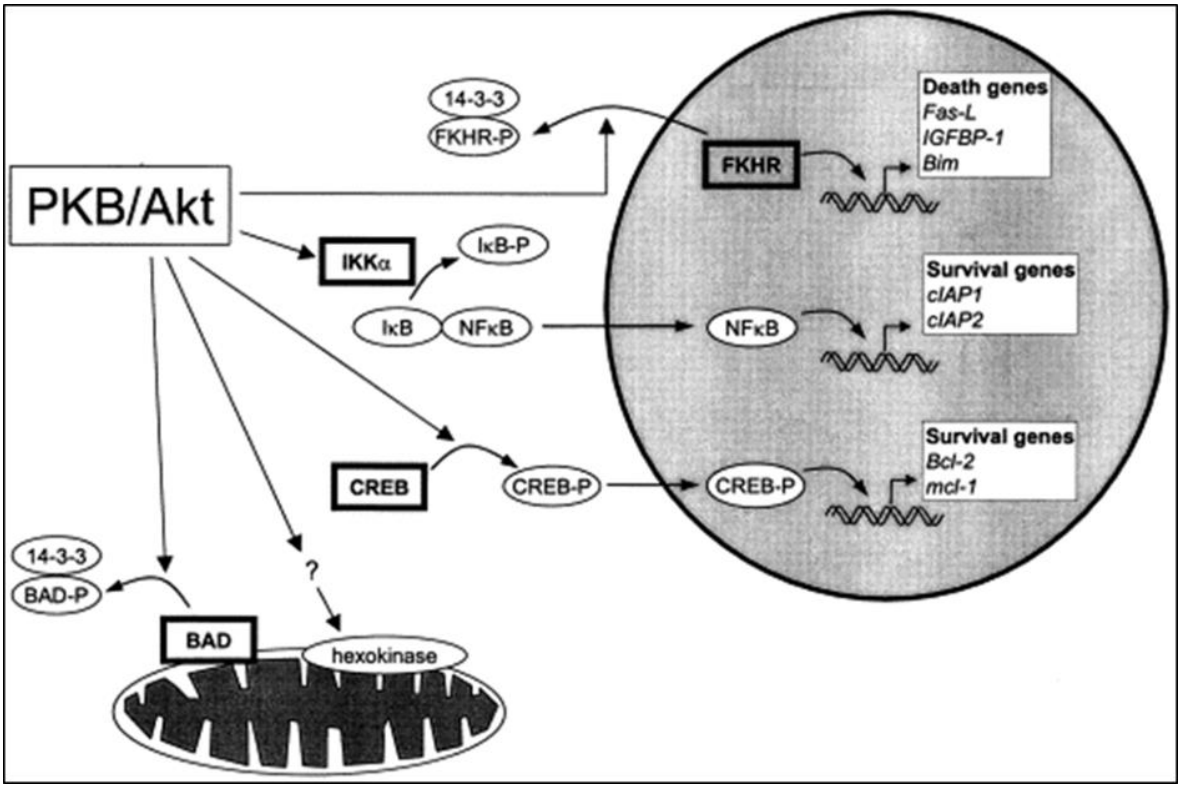


Figure 2-5: Regulation of cell survival by PKB/Akt. (*Adopted from* Nicholson and Anderson*, 2002)*

2.6.3: P38 MAPK pathways

The p38 MAPK is a member of the MAPK family of proteins, which are activated by a variety of environmental stressors and inflammatory cytokines (Cargnello and Roux, 2011). In the heart they are implicated in regulation of cardiac gene transcription, inflammation, myocytes hypertrophy, contractility, proliferation and apoptosis (Bassi *et al.,* 2008). Four isoforms of p38 MAPK has been identified, p38α, p38β, p38γ and p38δ, of which p38α and p38β are reported to be the most abundant within the myocardium (Vassalli *et al.,* 2012).

Activation of p38 MAPK by dual phosphorylation at threonine (Thr^{180}) and tyrosine (Tyr^{185}) during myocardial ischaemia has been reported to cause lethal injury (Bassi *et al*., 2008). However, the role of p38 MAPK activation during cardiac IRI is still controversial, with reports suggesting that mechanisms of activation differ by circumstances and yields either beneficial or detrimental results. Some researchers

have shown that activation of p38 MAPK during IRI causes lethal injury (Barancik *et al*., 2000) and inhibition slows the rate of myocardial cell death (Meldrum *et al*., 1998; Barancik *et al.,* 2000; Gysembergh *et al.,* 2001).

Much of the evidence supporting protective effects of p38 MAPK activation comes from studies in the phenomenon of ischaemic preconditioning (Weinbrenner *et al.,* 1997; Steenbergen, 2002; Bassi *et al.,* 2008). The overall observation shows that transient activation of p38 MAPK may offer myocardial protection during the ischaemic period, however, prolonged activation of p38 MAPK during the reperfusion time is detrimental to the injured myocardium. Furthermore, Mockridge and co-workers (2000) reported that inhibition of p38 MAPK partially blocks activation of PKB/Akt, but, activation of p38 MAPK did not show any direct role in Akt activation. It appears that p38 MAPK modulates certain components of the PKB/Akt pathway indirectly, assisting in sustained Akt activation through mechanisms not yet known.

In an attempt to understand this dual effect of p38 MAPK, studies on the specific isoforms demonstrate some important functional differences (Kaiser *et al.,* 2004). The p38β MAPK isoform in the heart is associated with hypertrophic and anti-oxidant changes during IRI (Kim *et al.,* 2006). Hence, its activation is associated with increased cardiomyocytes survival and ROS suppression during induced stress. However, activation of the p38α MAPK has been demonstrated to be pro-apoptotic and is associated with detrimental effect in the myocardial cells (Saurin *et al.,* 2000; Steenbergen, 2002).

2.7: Role of antioxidant in oxidative stress

2.7.1: Endogenous antioxidants

One of the well-established regulatory mechanisms of IRI involves antioxidant signaling mediators scavenging and neutralizing free radicals, converting these highly reactive molecules into non-toxic water and oxygen molecules (Wendell, 1970; Inal *et al.,* 2001; Townsend *et al.,* 2003; Masella *et al.,* 2005). These mechanisms comprise a series of enzymatic and non-enzymatic components acting cooperatively to neutralize the free radicals. An example of enzymatic mechanism involves the

clearance of highly toxic superoxide ions to a less toxic hydrogen peroxide (H_2O_2) molecule in a reaction catalysed by superoxide dismutase (SOD) (**Equation 1**) (Inal *et al*., 2001). A subsequent reaction catalysed by catalase or peroxidases (**Equation 2**), follows to convert the H_2O_2 molecule produced to a non-toxic water and oxygen molecules (Inal *et al.,* 2001; Masella *et al*., 2005).

$$2O_2^- + 2H^- \xrightarrow{SOD} H_2O_2 \qquad (\textbf{Equation 1}) \qquad \text{Mates } et\ al., 1999$$

$$2H_2O_2 \xrightarrow{Catalase/Gpx} 2H_2O + O_2 \quad (\textbf{Equation 2}) \qquad \text{Mates } et\ al., 1999$$

Another antioxidant mechanism makes use of non-enzymatic antioxidant molecules such as Glutathione (GSH), albumin, uric acid, bilirubin, ascorbic acid (Vitamin C), and α-tocopherol (vitamin E) as electron donors (Masella *et al*., 2005; Lobo *et al.,* 2010). For example, glutathione exist intracellularly in a reduced state (GSH) serving as a source of electrons in the antioxidant reactions (Townsend *et al.,* 2003). In a reaction catalysed by glutathione peroxidase (Gpx), GSH is oxidised to GSSG molecule **(Figure 2-6).** The oxidised GSSG molecule is recycled back to GSH in a reaction catalysed by glutathione reductase (GR) enzyme, utilising nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) from the pentose phosphate pathway (Townsend *et al*., 2003).

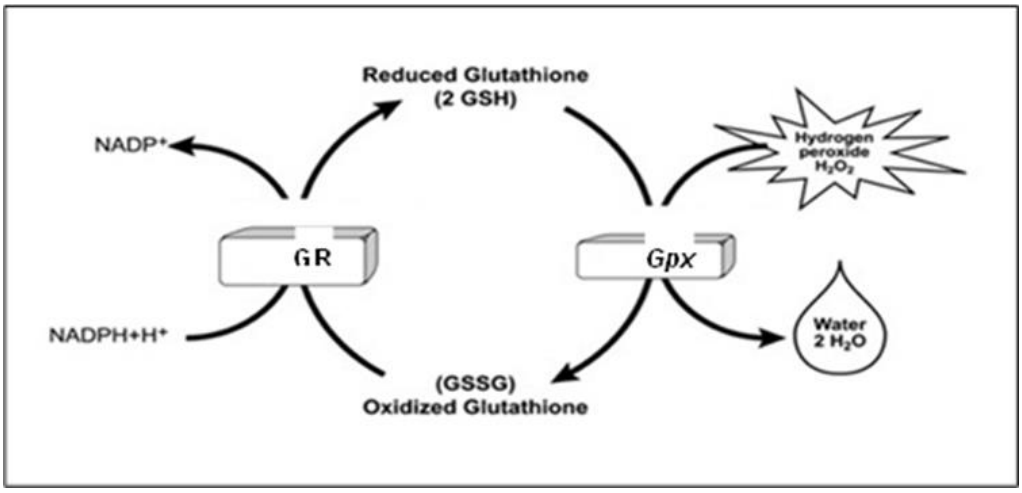


Figure 2-6: Mechanism of H_2O_2 reduction by glutathione enzymes. (Modified from Wendell, 1970)

2.7.2: Exogenous antioxidants

Dietary supplements form an integral part of the body's antioxidant mechanism affecting cellular redox reactions. It is well established that enzymes catalyzing redox reactions require certain minerals acting as co-factors e.g. copper and zinc for SOD, iron for catalase and selenium for Gpx (FAO/WHO, 2002; Shazia *et al*., 2012). Evidence shows that dietary supplements rich in minerals and antioxidants potentially enhance the activity of endogenous antioxidant reactions. A review by Nijveldt and co-workers (2001) suggested that, in addition to enhancing the activity of endogenous antioxidants reactions, flavonoids may directly facilitate scavenging of free radicals through donation of a hydrogen atom to the attacking radical, thus forming a flavonoid-o-free radical molecule which is more stable.

2.8: IRI and myocardial cell death pathways

Ischaemia and reperfusion injury may induce irreversible cell damage, triggering intrinsic cellular suicide mechanisms to remove the damaged cells, a process referred to as programmed cell death or apoptosis (Eefting *et al.,* 2004; Singhal *et al.,* 2010). Reactive oxygen species and inflammatory mediators play a key role in activation of the apoptotic cascade (Hoffman *et al.,* 2004). It has been suggested that inflammatory cytokines may activate caspase proteins directly through binding to their specific death receptors (The extrinsic apoptotic induced pathway). ROS on the other hand, may indirectly activate caspase proteins through increased oxidative DNA damage resulting in activation of p53 protein, which in turn inhibits the release of anti-apoptotic proteins while enhancing the release of pro-apoptotic proteins and cytochrome C (cyto C) (intrinsic apoptotic induced pathway) (**Figure 2-7**) (Eefting *et al.,* 2004).

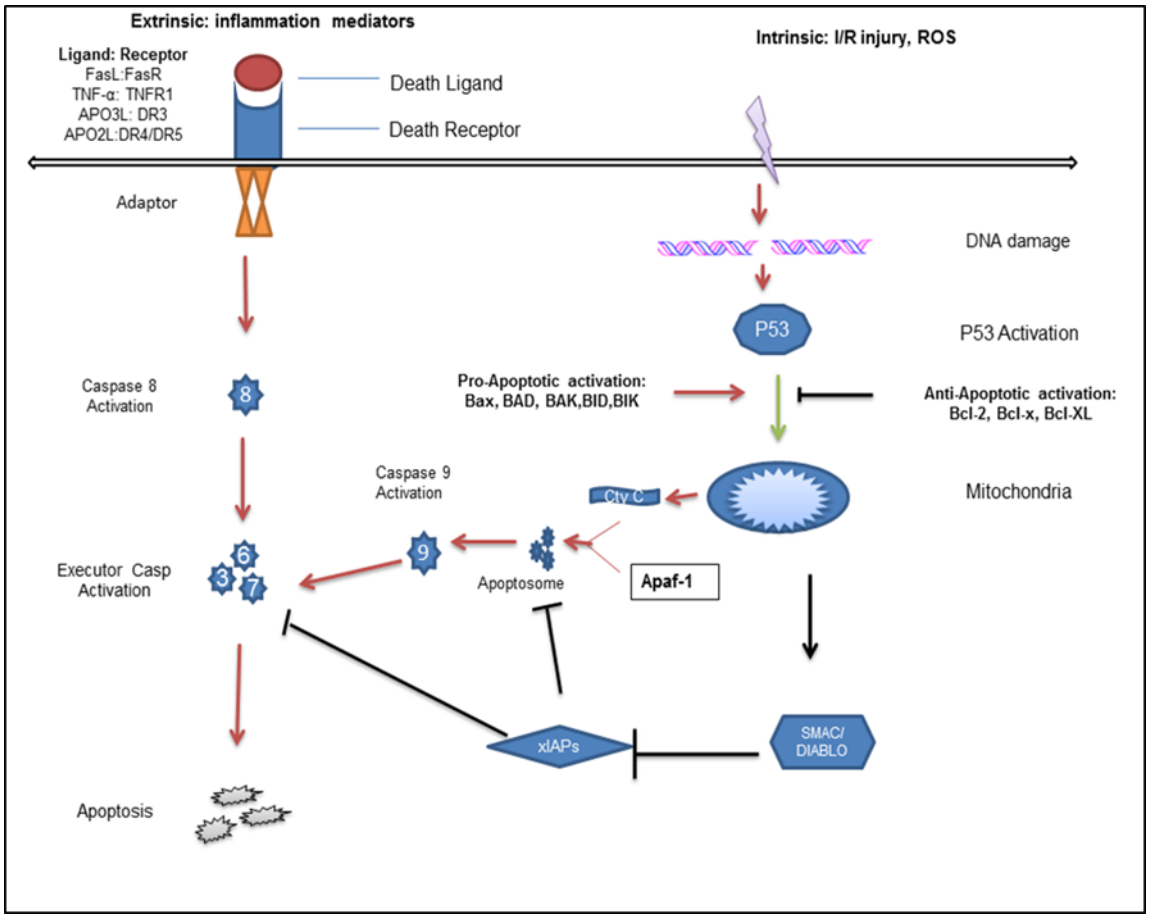


Figure 2-7: Apoptotic pathways in cardiac myocyte. (Modified from Tait and Green, 2010)

2.8.1: Caspase activation

Caspases are cysteine proteases central to execution of apoptosis. They comprise of two distinctive classes: the initiator caspase (e.g. caspase 2, 8, 9, 10) and the executor caspase (e.g. caspase 3, 6, 7) (Zhang *et al*., 2003). Activation of death receptors or increased release of apoptosomes may initiate auto-activation of initiator caspases. The cleaved initiator caspase activates the effector caspase which is responsible for the proteolytic cleavage of cellular targets leading to cell death (Kurokawa and Kornbluth, 2009; Zhang *et al.,* 2003). Caspase 3 is considered the most important death executant, specifically activating endonuclease and caspase activated DNAse (CAD) (Sakahira *et al*., 1998). The CAD is normally bound to its inhibitor (ICAD), forming an inactive ICAD: CAD complex and promoting cell proliferation. However, activation of caspase 3 by the initiator caspases leads to

cleavage of the inhibitor hence releasing the CAD and promoting rapid DNA fragmentation and apoptosis (Sakahira *et al.,* 1998).

2.8.2: Extrinsic pathways

The extrinsic pathway is initiated through binding of death ligands and activation of the death adaptors (TRADD, FADD) to recruit inactive pro-initiator caspases (Katz, 2011). The death inducing signal caspase (DISC) auto-activates and yields active initiator caspase 8 and 10, with subsequent activation of effector caspase 3, 6 and 7 (Katz, 2011). This pathway is generally activated by pro-inflammatory mediators of the TNF-superfamily or the IL-1 superfamily (**Figure 2-7**).

2.8.3: Intrinsic pathway

Mitochondrial DNA damage activates tumor suppressor p53 protein in two phases. Zhang and co-workers (2011) demonstrated that p53 phosphorylation at serine 15 and 20 induces DNA repair while further phosphorylation at serine 46 triggers mitochondrial release of cytochrome C, Smac/DIABLO, Endonuclease G and other apoptogens (**Figure 2-7**).

Myocardial cells naturally demonstrate attenuated levels of pro-apoptotic mediators such as apoptotic peptidase activating factor-1 (Apaf-1) and increased expression of inhibitor apoptosis protein (IAP) (Chiong *et al*., 2011). These cells have controlled mechanisms regulating the intrinsic apoptotic pathway through expression of various members of the Bcl-2 family (Chiong *et al*., 2011). Up-regulation of IAP and c-IAP1/2 in cardiac myocytes directly blocks activation of executioner caspases and indirectly blocks their activation through binding the apoptosomes, allowing the injured cell an attempt at DNA repair before caspase is activated (Crow *et al.,* 2004). However, increased formation of apoptosomes may auto-activate caspase 9, the upstream activator of executioner caspase proteins, which in turn activates the apoptotic cascade.

2.9: Flavonoids and DNA damage

Controversy exists on the dual nature of dietary supplements possessing both antioxidant and pro-oxidant characteristics, with polyphenolic supplements taking the centre stage (Halliwell, 2008; Bouayed and Bohn, 2010; Babich *et al.,* 2011). Studies suggest that dietary antioxidants functioning by donating an electron or hydrogen molecule to reduce a free radical may become a pro-oxidant (Krinsky, 1992; Lobo *et al.,* 2010). It has been shown that Vitamin C donates a hydrogen atom to a free radical molecule such as hydrogen peroxide while becoming an ascorbate radical itself (Erdem *et al.,* 1994). In case of polyphenols Decker (1997) have demonstrated that these molecules can rapidly release complex mixtures of semi-quinones and quinones, all of which are potentially cytotoxic upon interaction with free radicals.

Antioxidant defence mechanisms have proved to be complex and interlocking, functioning to minimize the levels of ROS while allowing other physiological functions of the ROS (Halliwell, 2008). Moller and co-workers (2004a) showed that vitamin C supplementation decreases oxidative DNA damage in monocytes of smokers, however, some researchers claim that consumption of increased amounts of antioxidant vitamin and mineral supplements may induce *in vivo* oxidative stress in supplemented groups (Omenn *et al*., 1996; Bianchini *et al.,* 2000). These variations between experiments make data equivocal and inconclusive. Most reports associate the pro-oxidant effects of polyphenols to their interactions with the transient metal ions and their functional roles, such as metal chelators and reducing characteristics (Decker, 1997; Bohm *et al.,* 1998; Bravo, 1998). However, several other researchers centred their debate on the type, dose and matrix of these supplements as the potential determining factors in the balance between beneficial and deleterious effects (Moller and Loft, 2002; Moller *et al.,* 2004b; Bouayed and Bohn, 2010; Babich *et al.,* 2011; Yordi *et al*., 2012). They argue that consumption of large amounts of these products may not be considered safe until their *in vivo* potential for oxidative stress is evaluated. The question on how much is too much still remains unanswered.

Methods using DNA as the biological target molecule are generally used in antioxidant experimental intervention studies investigating DNA damage caused by polyphenols (Loft and Poulsen, 1999; 2000). The 7-hyroxy-8-oxo-2-deoxyguanosine

(8-oxo-dg) technique utilizes several chromatographic techniques, including high performance liquid chromatographic (HPLC) with electrochemical detection and tandem, mass spectrometry, gas chromatography and thin layer chromatography with ^{32}P post-labeling and antibody based immunoassay (ESCODD, 2000; Moller and Loft, 2007). There is some variation in the different assay results, partly because of artificial generation of 8-oxo-dg, complicated protocols and high cost (ESCODD, 2000; Moller and Loft, 2007), complicating its routine use. In the second method referred to as the comet assay, DNA strand breaks are detected, with the additional option of enzyme digestion allowing detection of oxidatively modified nucleotides bases (Speit and Hartmann, 2005; Moller and Loft, 2007). The method provides an easy protocol and more affordable cost for routine use.

2.10: Detection of DNA damage using alkaline comet assay

The single cell gel electrophoresis (comet) assay is a simple, relatively fast and cheap technique that can be used to detect DNA damage in almost all mammalian cell types (Bowden *et al.,* 2003; Collins, 2004). An expert panel at the international workshop of genotoxicity testing procedures held in Washington, DC (March 25-26, 1999), recommended the alkaline (pH >13) version of comet assay developed by Singh and co-workers (1988) as the optimal version for identifying agents with genotoxic activity (Moller, 2006). It was further recommended that the method be validated scientifically at each laboratory to obtain valid and reproducible results (Tice *et al*., 2000). Protocol validation involves, “scientific confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled,” (ISO/IEC 17025: 2005E 5.4.5.1). The Alkaline comet assay protocol for our laboratory was validated based on the, “NewGeneries comet assay protocols and other useful information, version 2, September 2008 by University of Oslo, Faculty of Medicine, Department of Nutrition, Norway.”

2.10.1: Principle of alkaline comet assay

The cells of interest are isolated and embedded in a thin layer of low melting-point agarose gel, with subsequent cell membranes lysed with high salt concentrated solution at pH 10. The lysis step removes proteins and histones, leaving supercoiled loops of DNA linked to the nuclear matrix (Collins, 2004). The embedded nuclear matrix are then treated in alkaline solution (PH>13) to unwind and denature the DNA. Application of electric current (electrophoresis, approximately 28V) to the gels pulls the DNA loops towards the anode, forming a comet "tail" and "head" (**Figure 2-8**) (Wong *et al.,* 2005; Moller, 2006; Kumaravel *et al.,* 2009). The extent of migration depends on the number of single strand breaks. The migration is microscopically visualized after gel staining using fluorescent dye such as ethidium bromide. Quantification of the amount of DNA in the tail, relative to the head, is achieved by using computerized software, such as the comet assay IV analysis software.

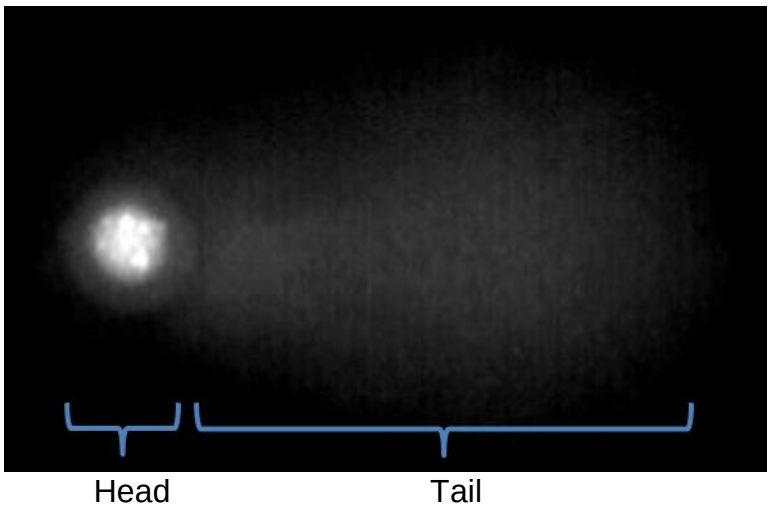


Figure 2-8: Comet cell

2.10.2: Evaluation of cell DNA damage

Visualisation of comet cells are made after gel staining with a fluorescent dye, such as ethidium bromide and using a 40X magnification objective on a fluorescent microscope. The microscope is usually linked to an image software used to quantitate extent of DNA damage per cell**.** Tail parameters are usually computed to

analyse the length plus the amount of DNA migrated from the head and the intensity plus percentage of DNA in the tail. DNA-bound ethidium bromide absorbs green light peak at approximately 530nm and emits orange light peak at approximately 610nm. Quantification of the amount of DNA that migrated out of the nuclei is based on the linear relationship between the DNA bound ethidium bromide and the intensity of emitted light (Vilhar, 2004).

2.10.3: Comet image analysis and end-points data interpretation

Generally analysis of 50 to 100 cells per slide using at least two slides per animal are recommended (Collins, 2004). Primarily tail DNA data, such as tail intensity (pixels), tail length (µm), tail extent moment (µm) and % tail DNA migration are used for the “ENDPOINT” analysis per animal (Bowden *et al.,* 2003). One complication with tail parameter analysis in the comet assay is the presence of zero values which complicates statistical analysis. However, Collins (2004) have suggested that untreated control cells should have a background level of approximately 10% tail DNA migration, which will then alleviates statistical complications.

The “endpoint” analysis describes the individual observation per animal, summarizing DNA damage at individual study subject level. This is followed by computing an “ESTIMATE” analysis per study group for each parameter of interest. The “Estimate” is the mean calculated for each particular “endpoint” parameter per study group. Finally the “EFFECT” of treatment is established comparing the “estimates” of different supplemented or experimental groups to the control group (Moller, 2006).

CHAPTER 3: MATERIALS AND METHODS

3.1: Animal care

Animals received humane care in accordance to South African Medical Research Council and "Guide for the care and use of laboratory animals 8th edition" (2011). The rats were housed at Cape Peninsula University of Technology (CPUT), Bellville Campus, in the experimental animal facility and the room temperature (RT) was set at 25^{o}C, with 12 hours artificial day and night cycle. All animals were allowed water and rat chow *ad libitium* throughout the experimental period.

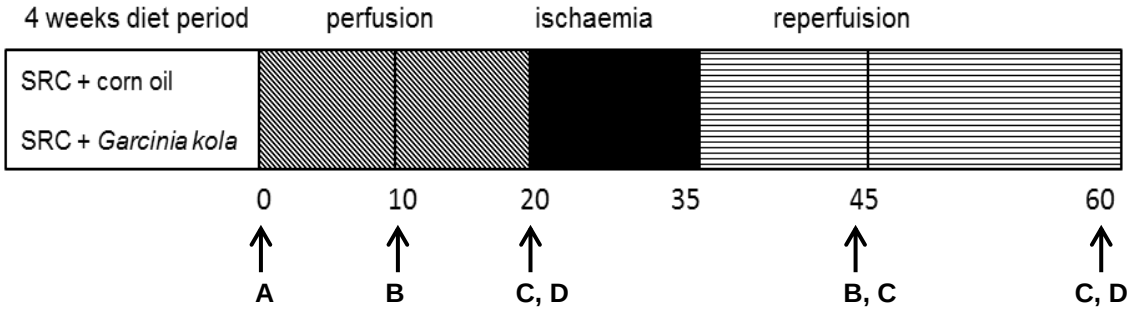
The ethical clearance of the study was granted by Health and Wellness Science Research Ethics Committee at CPUT, Bellville (Ref: CPUT/HW-REC 2012/A03).

3.2: Dietary supplementation

Garcinia kola nuts for this study were supplied by Prof Farombi from the University of Ibadan, Nigeria. Ripe seeds of *Garcinia kola* were air dried and grounded into a uniform powder using an automated plant grinding machine and stored in an air tight opaque bottle in a dry, dust free cupboard. 10g of *Garcinia kola* powder was weighed and mixed with 100ml of corn oil in another opaque brown glass bottle to have a final concentration of 100mg/ml. The daily *Garcinia kola* dose was selected based on the average adult human receiving a minimum of approximately 7g of the *Garcinia kola* nut daily.The *Garcinia kola* supplement was prepared and used within 2 weeks and new supplement was prepared for the remaining feeding period.

3.3: Experimental group

Forty male Wistar rats were randomly divided into two equal groups. The experimental group was fed with 100mg/kg *Garcinia kola* in corn oil daily, while the control group was fed with 2ml/kg of corn oil daily using the oral gavage method for 4 weeks (**Figure 3-1**).



KEY

↑: Time points in minutes

SRC: Standard rat chow

A: Weight recoded and blood sample collected for lymphocyte DNA damage analysis

B: Switch from the Langendorff mode to the working heart mode

C: Hearts were snap frozen for biochemical analysis

D: Functional parameters measured and calculated: Coronary flow, aortic output, heart rate, diastolic, systolic pressure, aortic pressure, pulse pressure.

Figure 3-1: Study design and experimental model

3.4: Heart perfusion protocol

Principle: The working heart perfusion involves the use of two cannulas; one placed in the aorta and the second in the left atrium (Igic, 1996). Prior to the initiation of the working heart perfusion mode, the heart is stabilised in the Langendorff perfusion mode. This involves retrograde perfusion of the heart at a constant flow and hydrostatic pressure through the aorta. At this point the aortic valves are forced shut and the perfusate is directed into the coronary ostia, thus perfusing the entire ventricular mass of the heart and draining the right atrium through the coronary sinus (Depre, 1998). Once the heart is stabilised in the Langendorff mode, the working heart mode is switched on, allowing the perfusate to flow from the left atrium through the pulmonary vein into the left ventricle, which then pumps the perfusate through the aorta, mimicking blood flow *in situ*.

3.4.1: Experimental perfusion protocol

Rats weighing between 250 and 380g were anaesthetized with intraperitoneal injection of 2ml/kg sodium pentobarbital. Once the animal was confirmed to be anesthetized, the heart was excised and immersed in in ice-cold Krebs-Henseleit buffer (118.5 mM NaCl; 4.75 mM KCl; 1.2 mM MgCl 6 H_2O; 1.36 mM $CaCl_2$; 25.0 mM $NaHCO_3$; 1.2 mM KH_2PO_4; 11.0 mM glucose) before it was transferred to the perfusion apparatus.

The heart was mounted on the standard working heart perfusion apparatus and housed in a thermo-controlled organ bath maintained at 37^{o}C. The perfusion was performed with Krebs-Henseleit buffer equilibrated with 95% O_2 and 5% CO_2 at 37^{o}C. Once the aorta was cannulated retrograded perfusion in Langendorff mode was initiated. During this period excess tissue was removed from the heart and the left atrium was cannulated. Following 10 minutes stabilization in the Langendorff mode, the heart was switched to the working heart mode for 10 minutes. Hearts were then subjected to 15 minutes of normothemic global ischaemia followed by reperfusion in the Langendorff mode for 10 minutes and 15 minutes in the working heart mode (**Figure 3-1**). The temperature of both the perfusate and the air surrounding the heart was thermostatically controlled and checked at regular intervals to ensure that the temperature was maintained at 37 °C irrespective of coronary flow. During ischaemia temperature was maintained between 35.9 and 36.5°C.

Coronary flow (CF) (ml/minute), aortic output (AO) (ml/minute), heart rate (HR) (beats/minute), systolic aortic pressure (SAP) (mmHg) and diastolic aortic pressure (DAP) (mmHg) were measured at 5 minutes interval during the working heart perfusion. Hearts were freeze clamped in liquid nitrogen for biochemical analysis after 20 minutes (20min Baseline samples), 45 minutes (10 min reperfusion samples) and 60 minutes (25 min reperfusion samples) perfusion (**Figure 3-1**).

3.4.2: Myocardial functional recovery

Aortic output recovery (AOR) was calculated by dividing the AO after ischaemia by AO before ischaemia and expressing these values as a percentage functional recovery

- $AOR\ \% = \frac{AO\ at\ reperfusion(15\ or\ 20\ or\ 25min)}{AO\ at\ baseline(20\text{min})}$

In addition to the HR and CF rate measured, pulse pressure (*pp*) (mmHg) and aortic pressure (AP) (mmHg) were calculated to determine the cardiac efficiency and contractile functional difference before and after induced ischaemia.

- Pulse pressure was estimated as the difference between SAP and DAP

  $PP = SAP - DAP$ Chemla *et al.,* 2005

- Aortic pressure was estimated as the squire root of the product of SAP and DAP

  $AP = \sqrt{SAP\ X\ DAP}$ Chemla *et al.,* 2005

3.5: Western blot (immunoblotting) protocol

Principle: Western blotting is a standard technique whereby a complex protein mixture is separated by fractionation in polyacrylamide gel according to their molecular size. This is followed by electrophoretic transfer of the protein pattern to a nitrocellulose sheet and detection with specific antibodies (Burnett, 1981). When bound to membranes, proteins are readily accessible for immunological or biochemical analyses, quantitative staining for easy visualisation and identification of proteins of interest. Protein blotting involves four major phases: sample preparation (**3.5.1**), electrophoresis (**3.5.3**), gel-membrane protein transfer (**3.5.4**) and immunological protein detection (**3.5.5**).

The antibodies typically recognize a small portion of the protein, called epitope, which resides within 3D conformation structure. To enable access to this portion, samples are denatured through pre-treatment with laemmli sample buffer and the mixture placed in boiling water for 5 minutes (**3.5.1**). The buffer also contains a detergent such as sodium dodecyl sulfate (SDS) which attaches and imparts a negative charge on the proteins at a constant ratio, therefore giving every protein the same charge-to-mass ratio (Kurien and Scofield, 2006) (**3.5.3**). The transfer step involves moving the proteins from a gel and immobilizing them on a synthetic membrane support (**3.5.4**). Following antigen immobilisation into a membrane, the assay requires the use of protein specific antibodies (primary antibody) for antigen-antibody complex formation, followed by antibody-Antibody complex formation, with a secondary antibody labeled with a tag such as peroxidase or phosphatase. The membrane is then exposed to a substrate that produces a visible signal, indicating the presence of an antigen (**3.5.5**). Colorimetric detection systems are used to provide quantitative results, where signal levels are measured by film/digital imager and the intensity of the signal generated is proportional to the amount of antigen present.

3.5.1: Sample preparation (Lysates)

One hundred mg of ground heart tissue was weighed in 500µl of ice cold radio-Immuno precipitation assay (RIPA) buffer (150mM sodium chloride, 1.0% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris pH 8.0), containing 5µl protease inhibitor cocktail (Sigma Alrich), 2µl phosphatase inhibitor cocktail (Sigma Alrich) and 2µl sodium othovanadate (Sigma Alrich). The tissue was homogenized using tissue grinder at 700g, centrifuged at 4^{o}C, for 10 minutes at 10 000 rpm, and supernatant was collected and stored in -80 ^{o}C freezer till use till use in Bradford protein concentration (**3.5.2**) and SDS-PAGE (**3.5.3**).

3.5.2: Bradford protein concentration

Principle: Bradford protein concentration determination is based on the observation that interactions of proteins with coomassie brilliant blue G250 cause a colour change from brown to blue in proportion to the amount of proteins present in the sample (Bradford, 1976). Protein quantification was made in comparison to assay standard values, prepared as a serial dilution from a known concentration of bovine serum albumin (BSA). Briefly, 5µl of serially diluted five point standard, blank and heart tissue homogenates, were mixed with 250µl of coomassie brilliant blue G250 and allowed to stand in the dark for 10 minutes. This was followed by spectrophotometric determination of optical density (OD) values of each sample at 630nm and protein standard curve plotted. Protein concentration in the heart tissue homogenates were calculated from the standard curve using OD values obtained.

3.5.3: SDS-poly-acrylamide gel electrophoresis (SDS-PAGE)

Tissue lysate (**from 3.5.1**) was diluted in distilled water and laemmli buffer to standardize the amount of protein loaded per sample for each protein of interest (**see Note 3**). The samples were boiled for 10 minutes and allowed to cool prior to loading of 15µl of sample and separated on SDS-PAGE. Gel concentration used was dependent on the protein molecular weight mass of each protein of interest (8-12% acrylamide concentration). Gels were electrophoresed in 10% SDS glycine buffer at a constant voltage of 90V for 10 minutes and the voltage increased to 110V for the rest of the experiment.

3.5.4: Protein Gel-Membrane Transfer

Following SDS-PAGE, the gel and polyvinylidene fluoride membrane (PVDF) were sandwiched between sponge and filter paper, and clamped tightly together after removing air bubbles that might be trapped between the gel and membrane. The sandwich was then submerged in a cold transfer buffer (Tris 25mM, Glycine 192mM, 10% SDS, 10% methanol) to which an electrical field was applied at 300mA for 75 minutes. Transfer of proteins onto the PVDF membrane was checked using ponceau

red. Visual observation of short red bands was regarded as a good transfer before proceeding with the blocking step.

3.5.5: Immunological protein detection

The BCIP/NBT Phosphatase Substrate protein detection kit used in this study detects proteins that have been immobilized on membranes following Western blot transfer. The ready-to-use reagents are based on the hydrolysis of 5-bromo, 4-chloro, 3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) by alkaline phosphatase, resulting in a strong purple reaction product (http://www.kpl.com: catalog/productdetail).

Upon satisfactory transfer, the membrane was washed with Tris-buffed saline (TBS)-tween and incubated with 5% BSA or fat free milk in TBS-tween for 90 minutes depending on manufacturer's recommendations and optimization results for each protein of interest. The blocked membranes were then incubated in primary rat monoclonal antibodies against inducible nitric oxide synthase (iNOS), total p38 mitogen-activated protein kinases (MAPK), phospho-p38 (Thr180/Tyr182) MAPK, P38α MAPK, Nitro-tyrosine, uncleaved and cleaved poly (ADP) ribose polymerase (PARP-1), cyclooxygenase-2, caspase-3, cleaved caspase-3 (Asp175), protein kinase B/Akt (Total-Akt), phospho-Akt (ser473), nuclear factor-kappa beta 65, inhibitor kappa beta kinase IKKα, IKKβ, phospho-IKKα (ser176)/β(ser177) and β-Actin antibodies. The primary antibodies were diluted in 5% fat free milk or BSA and membranes incubated at 4^{o}C for 20 hours, with constant shaking. Antibody dilution factor was used as per manufacturer's recommendation. The membranes were then washed in TBS-tween three times at room temperature (RT=22-25^{o}C) prior to incubation with alkaline phosphatase labeled anti-goat IgG antibody diluted in 5% TBS-tween fat free milk. The membranes were incubated for 2 hours at RT with agitation. This was followed by three times washing with TBS-tween, followed by addition of a BCIP/NBT Phosphatase substrate for 10 minutes.To stop the enzymatic reaction the membranes were submerged in distilled water after a timed and standardized substrate reaction (**Note 3**).

Visualization: The membranes were air dried in a dark and dust free place for 24 hours before scanned into a computer and densitometrically analyzed (UN-SCAN-IT$_{TM}$).

Note 1: *Pre-stained molecular weight marker was loaded with each run to determine the protein size of interest, and β-Actin antibody (cell signaling) was run during the experiment to control sample loading. All blots were normalized to the positive control.*

Note 2: *Normal control and lipopolysaccharide (LPS) treated rat liver and kidney samples (obtained from previous studies performed in our laboratory), were used during optimization and included with each run as a positive control sample. Different control samples were selected depending on the antigen of interest.*

Note 3: *Prior to experimental analysis, optimization for each protein of interest was done to set a standard protein amount loaded, specific blocking solution, and incubation time.*

Note 4: *All antibodies were purchased from Cell Signaling Technology, except for iNOS, which was purchased from Abcam, and other chemicals were obtained from Bio-Rad and Sigma Aldrich.*

Note 5: *detection kit was purchased from KPL, Kirkegaard and Perry Laboratories, Inc.*

3.6: Bio-plex electrochemilunescent immunoassay

Principle: The Bio-plex pro^{TM} assays are built around core elements of the xMAP technology allowing quantification of multiple protein biomarkers in a single well. A multiplex electrochemilunescent immunoassay technology was used for simultaneous quantification of different cytokines and chemokines on the surface of fluorescent-dyed magnetic beads. Beads with distinctive colour coding are covalently coupled with specific antibodies, enabling combination of analytes to be assayed within a single well reaction (http://www.bio-rad.com: Bio-Rad protocols). After analytes from the sample are captured in the beads by different specific antibodies, a biotinylated antibody is introduced, creating a sandwich complex. The final detectable

complex is formed with addition of streptavidin-phycoerythrin conjugate. Phycoerythrin serves as a fluorescent reporter. The complex is then passed through two lasers. The first laser excites the internal dyes and the second laser excites the phycoerythrin (PE) on the reporter molecule. Finally the high speed digital signal processor identifies each individual bead colour and quantifies the fluorescent reporter signal of PE (http://www.bio-rad.com: Bio-Rad protocols).

Sample preparation: See Western blot sample preparation **3.5.1**

Protein concentration assay: See Western blot protein concentration assay **3.5.2**

3.6.1: Bio-plex pro™ rat cytokine assay protocol

Procedure: Rat Cytokine/Chemokine kit consisting of 8-plex array (interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-18, interferon gamma, tumor necrosis factor-alpha and monocyte chemotactic protein-1) antibody-immobilized magnetic beads (Bio-Rad) were assayed using the MAGPIX® system (Reagent kit purchased from Bio-Rad, South Africa). Reagent preparation and protocol for running this assay was based on the manufacturer's instructions. Nine point standard samples, inclusive of a blank sample and experimental samples were run in duplicates per plate.

Briefly, 50µl of prepared magnetic beads were added in each well and pre-washed twice with 100µl assay wash buffer using the automated washer. This was followed by addition of 50µl of each sample, standard and blank to the pre-assigned wells and incubated for one hour at room temperature in the dark, shaking at 300 rpm. This was followed by a washing step and addition of 25µl of detection antibody. After 30 minutes incubation, unbound detection antibody was washed off, followed by addition of 50µl streptavidin-PE and incubation for 10 minutes. A final washing step was performed and followed by re-suspension of the beads in 100µl assay buffer, shaken for 30 seconds at 1100 rpm and analyzed using the MAGPIX® xPONENT software.

Note 6: *All incubations were performed at RT (22-25°C) and shaking at 300RPM with the plate covered with paper foil to create a dark environment.*

Note 7: *The washing step was performed with an automated washer, programmed with 3 cycles of washing with 100µl assay wash buffer.*

Standard concentration values and beads region were pre-assigned in the MAGPIX® xPONENT software and a standard curve for each cytokine/chemokine was plotted allowing determination of cytokine/chemokine concentration in each sample in pico-grams cytokine per milliliter (pg/ml) sample volume in each well. The cytokine/chemokine concentrations obtained for each sample were normalized to the total protein concentration of each sample, hence, expressing cytokine/chemokine concentrations in pg cytokine/ µg total protein.

3.7: Alkaline comet assay

Overview: Single cell gel electrophoresis, commonly known as comet assay, provides a simple and effective way of evaluating DNA damage in single cells. The assay is based on the ability of the negatively charged fragments of DNA to migrate through an agarose gel in response to electric field (Kumaravel *et al*., 2009). The damaged DNA fragments migrate easily out of the nucleoid, forming a "comet" shape, whereas undamaged DNA migrates slower and remains confined within the nucleoid when a current is applied. Evaluation of the comet tail shape and the migration patterns allow for assessment of the level of DNA damage. Two types of comet assays have been reported: neutral comet assay typically used to detect double stranded breaks, and the alkaline comet assay, which is more sensitive and is used to detect smaller amounts of damage including single and double stranded breaks. Blood samples used in this study were collected from the abdominal aorta using needle and syringe into blood collection tube with ethylenediaminetetraacetate (EDTA) anti-coagulant. Samples were processed on the same day of collection.

3.7.1: Validation procedure

Coordination of the validation protocol for continual improvement was archived through use of the Plan-DO-Check-Act (PDCA) cycle model (Moen and Norman, 2006). This is a four step process (**Figure 3-2**) which we followed for the coordination of continual improvement efforts of our alkaline comet assay protocol draft.

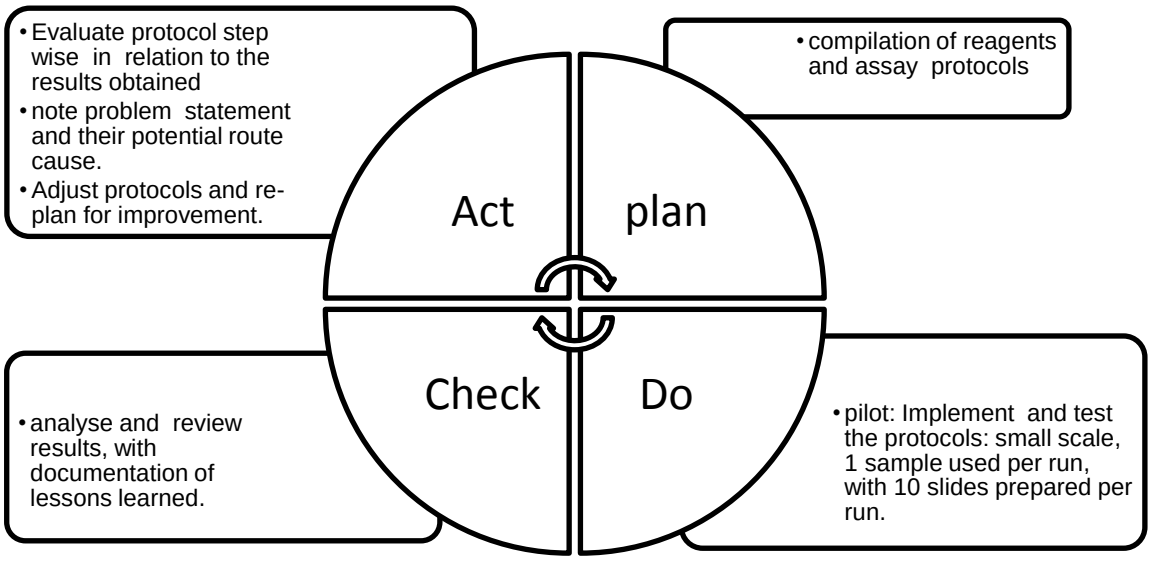


Figure 3-2: Protocol continual improvement using PDCA cycle.

The Alkaline comet assay protocol used here was adopted and modified slightly from NewGeneries comet assay protocols and other useful information, version 2, September 2008 by university of Oslo, Faculty of medicine, Department of Nutrition, Norway.

*Note: All Reagents used in the Alkaline Comet assay were purchased from Sigma-Aldrich (**3.7.2**).*

3.7.2: Alkaline comet assay protocol (Adopted)

Slide preparation: Microscope slides were pre-coated with 1% agarose gel dissolved in double distilled water (ddH_2O). The slides were first wiped with 70%

ethanol, allowed to air dry and dipped into normal melting point agarose gel covering at least two thirds of the slide. The bottom part of the slide was wiped with a tissue to remove excess gel and placed on a clean dust free area to dry overnight (>16 hours). The slides were labeled to identify the gel coated side and stored till required.

Lymphocyte preparation: 30µl of the blood sample was suspended in 1ml of ice cold PBS and incubated on ice for 30 minutes. Using a pipette, 100µl histopaque 1077 (Sigma-Aldrich) was added at the bottom of the sample mixture and centrifuged at 200 x g for 3 min (4°C). Lymphocytes were then retrieved by collecting 100µl from just above boundary between PBS and Histopaque using a pipette. This was re-suspended in 1 ml ice cold PBS mixed and re-spun (200 g, 3 min, 4°C). PBS was removed and the lymphocyte pellet was re-suspended in 100µl ice cold PBS. The lymphocyte preparation was kept on ice at all times.

Embedding cells in LMP agarose: 1% Low melting point (LMP) agarose gel was prepared in PBS and dissolved using a microwave. The LMP gel was kept in liquid form by storing it in a 37 °C water bath. Prepared lymphocytes were re-suspended by tapping the tube with the finger, followed by addition of 140µl of 1% LMP agarose and gently mixed by sucking up and down with a pipette. The mixture was then transferred as two roughly equal drops to the 1% normal agarose gel pre-coated slide, and covered with an 18x18 mm coverslip immediately. The slides were then left at 4 °C in a fridge for 5 min to solidify.

Lysis step: Following removal of the cover slips, the slides were incubated in a lysis buffer containing: 2.5M NaCl, 0.1M EDTA and 10 mM Tris at pH 10 (1%Triton X- and 10%DMSO solution were added to the lysis buffer immediately before use). Lysis was carried out for 1hour 30 minutes at 4 °C.

Alkaline treatment and electrophoresis: Slides were removed from the lysis buffer, placed in the electrophoresis tank and covered with alkaline buffer containing 0.3 M NaOH and 0.001M EDTA at pH 13. The slides were left in a 4ºC walk-in fridge for 40 minutes. After incubation electrophoresis was carried out at the same temperature at a constant voltage of 28V for 40 minutes.

Neutralization: Slides were removed from the alkaline buffer and submerged in PBS, followed by distilled water for 10 minutes each. They were then air dried at room temperature and stored for analysis.

Staining: Comets were visualized by staining the gels with ethidium bromide and viewed on a fluorescence microscope. First 100 comets per slides were randomly scored, measuring the fluorescence intensity over the different parts of the comet using the computer based analysis comet assay IV version 4.3 software.

Data analysis and interpretation: Cells with tail DNA migration less than 10% were excluded from analysis. Mean tail length, % tail DNA migration, tail moment and tail intensity were computed for each sample (as end point analysis). This was followed by an "ESTIMATE" analysis by computing the average endpoints from each animal and finally determining the "EFFECT" of the supplement statistically comparing the mean "ESTIMATE" value for supplemented group to the control group mean values.

3.8: Statistical analysis

GraphPad^{TM} PRISM version 5.0 and Microsoft excel 2010 were used for statistical analysis. Graphs and values are presented as mean ± standard error of mean (S.E.M). Difference between the groups and time points were determined using two tailed unpaired student's *t*-test, with $p\text{-value} < 0.05$ considered statistically significant.

CHAPTER 4: RESULTS

4.1: Effects of dietary *Garcinia kola* supplementation on animal body weight

The weights of the rats after 4 weeks of supplementation were evenly distributed between the two groups, with the rat weights in both the control and *Garcinia kola* supplemented groups ranging between 250 and 380 grams at the point of sacrifice (**Figure 4-1**). There was no significant difference in the mean weights between the two groups.

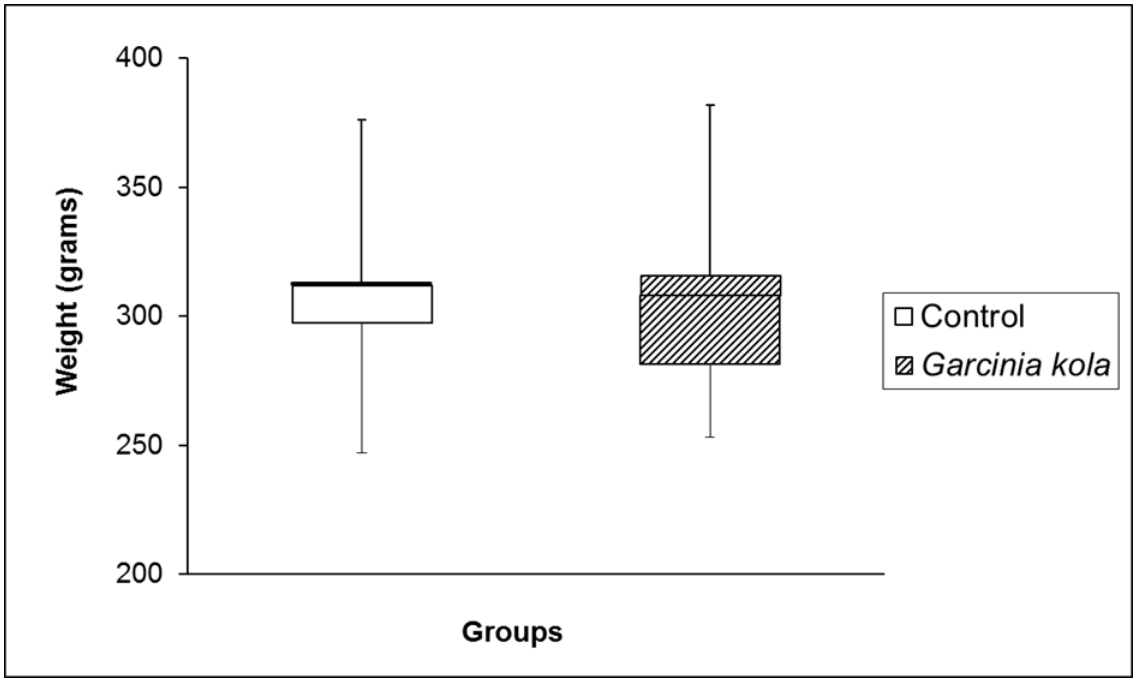


Figure 4-1: Box-whiskers showing distribution of rats weight in the different groups at point of sacrifice, (n=20 per group)

4.2: Effects of dietary *Garcinia kola* supplementation on functional parameters

There was no significant difference in aortic pressure (mmHg), pulse pressure (mmHg), coronary flow (ml/min) and heart rate (BPM) between the two groups and between the baseline and reperfusion time points (**Table 4-1)**.

Table 4-1: Functional parameters measured in perfused rat hearts at baseline and 25 minutes reperfusion time point. (Data presented as mean ± standard error of the mean (S.E.M), number of rats (n) =6 per group)

Function parameters	Groups	Baseline	25minutes Reperfusion
Aortic pressure(mmHg)	Control	100.70 ± 1.85	91.57 ± 1.73
	Garcinia kola	118.67 ± 1.42	107.30 ± 3.21
Pulse Pressure(mmHg)	Control	79.55 ± 5.47	61.50 ± 6.56
	Garcinia kola	82.91 ± 2.42	72.04 ± 2.51
Coronary flow (ml/min)	Control	18.18 ± 1.09	17.52 ± 1.24
	Garcinia kola	20.10 ± 1.40	16.80 ± 1.65
Heart Rate (BPM)	Control	267.00 ± 8.80	205.70 ± 24.81
	Garcinia kola	261.6 0 ± 11.85	240.80 ± 7.06

4.3: Effects of dietary *Garcinia kola* supplementation on Aortic output recovery

Percentage aortic output recovery (AOR) was higher in supplemented group when compared to the control group at 15, 20 and 25 minutes reperfusion time point with a significant difference observed at 20 minutes reperfusion (*p=0.0366*) and 25 minutes reperfusion (*p=0.0324*) (**Figure 4-2**).

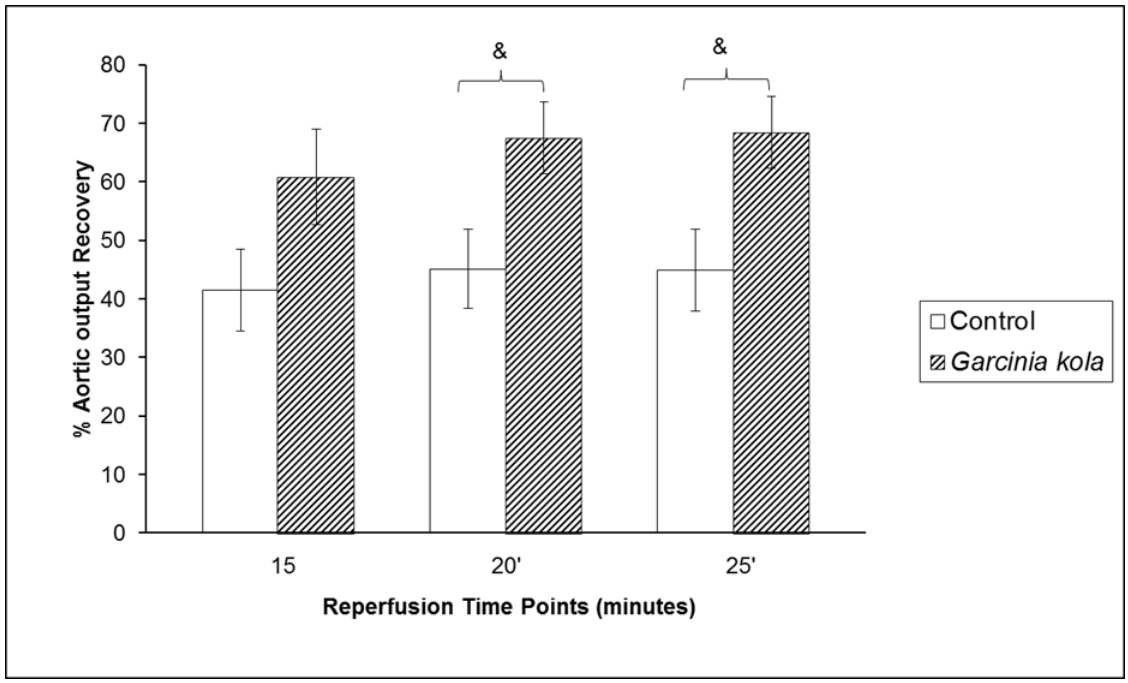


Figure 4-2: %AOR in isolated rat hearts using working heart perfusion apparatus. Results are expressed as %mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group)

4.4: Effects of dietary *Garcinia kola* supplementation on cardiac pro-inflammatory cytokine/chemokine expression

Garcinia kola supplemented group showed a significantly low expression of pro-inflammatory cytokines when compared to the control group at baseline: interleukin (IL)-1β (8.89 ± 0.56 *versus* 11.55 ± 0.27, *p=0.0056*), tumor necrosis factor alpha (TNF-α) (4.67 ± 0.37 *versus* 7.47 ± 0.560, *p=0.0058*), IL-6 (5.54 ± 0.47 *versus* 8.47 ± 0.64, *p=0.0072*) and IL-18 (8.90 ± 1.03 *versus* 13.48 ± 0.82, *p=0.0090*) (**Figure 4-3a, b, c, d**). However, there was no statistical difference between the two groups in the expression of interferon gamma (IFN-γ) and monocyte chemotactic protein-1 (MCP-1) at baseline (**Figure 4-3e, f**).

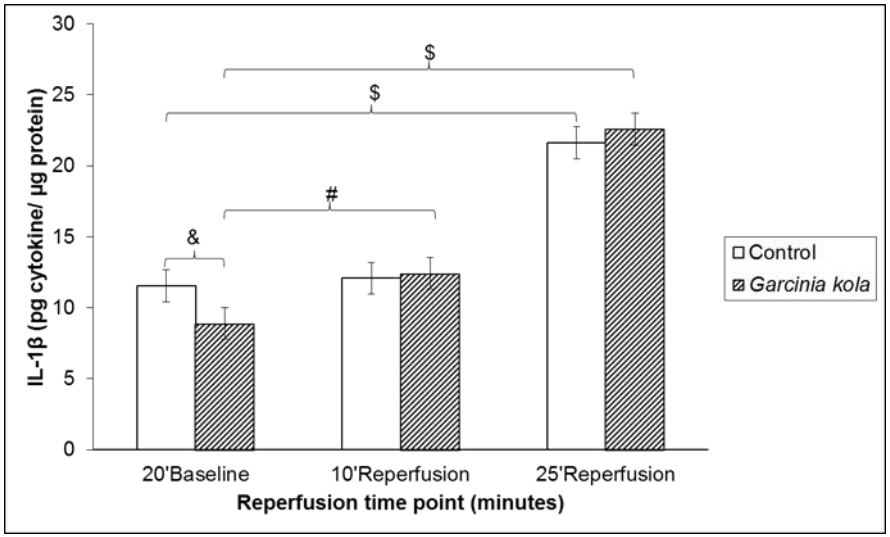
Control rat hearts did not show significant change in the concentration of the above mentioned cytokines/ chemokine after 10 minutes of reperfusion when compared to the baseline expression level. Dietary *Garcinia kola* supplemented group showed a significantly increased concentration of IL-1β (12.43 ± 0.937 *versus* 8.89 ± 0.56, *p=0.0012*) and IL-6 (8.90 ± 0.63 *versus* 5.54 ± 0.47, *p=0.0021*) at 10 minutes reperfusion when compared to the baseline expression levels. However,

there was no significant change observed in the other cytokines between baseline and 10 minutes reperfusion in the supplemented group (**Figure 4-3**).

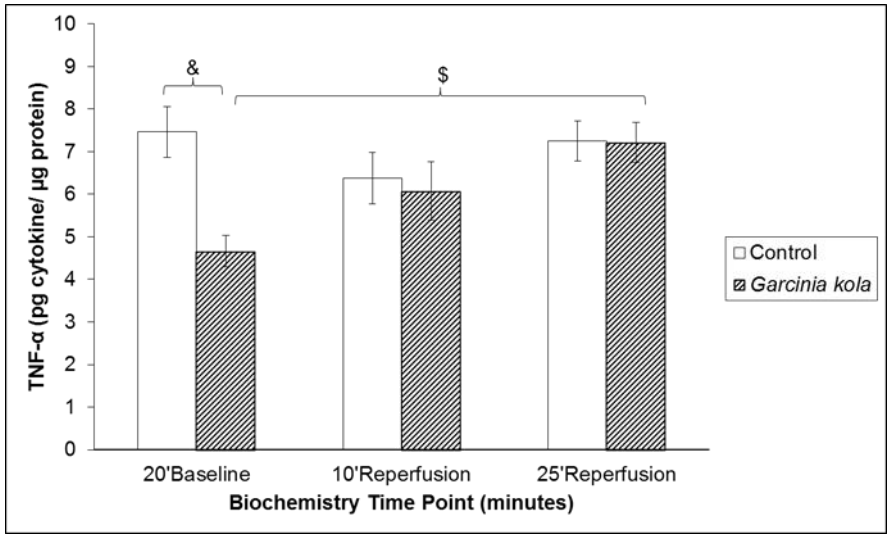
Comparing the baseline and the 25 minutes reperfusion time points, all 8 measured cytokines demonstrated a significant increase in the *Garcinia kola* supplemented group while the control group only showed a significant increase in expression of IL-1β, IL-6 and MCP-1 (**Table 4-2**).

Table 4-2: Pro-inflammatory cytokines expression: comparison of the baseline and 25 minutes reperfusion time points *per* group expression levels. Results expressed as mean ± S.E.M, *$p < 0.05$.

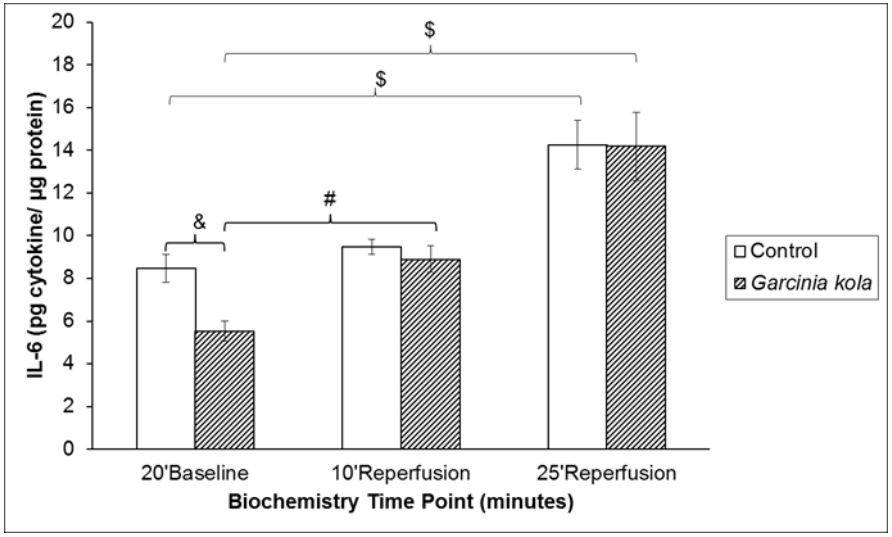
Cytokines	Groups	Baseline	25 minutes Reperfusion	*p-value*
IL-1β	Control	11.55 ± 0.27	21.62 ± 1.11	***<0.0001****
	Garcinia kola	8.89 ± 0.56	22.58 ± 2.09	***0.0002****
TNF-α	Control	7.47 ± 0.60	7.25 ± 0.47	*0.7817*
	Garcinia kola	4.67 ± 0.37	7.21 ± 0.47	***0.0014****
IL-6	Control	8.47± 0.64	14.26 ± 1.14	***0.0012****
	Garcinia kola	5.54 ± 0.47	14.18 ± 1.59	***0.0008****
IL-18	Control	13.48 ± 0.82	12.01 ± 0.65	*0.1954*
	Garcinia kola	8.90 ± 1.03	12.49 ± 0.71	***0.0217****
IFN-γ	Control	2.91 ± 0.09	3.21 ± 0.15	*0.1257*
	Garcinia kola	2.59 ± 0.12	3.12 ± 0.165	***0.0256****
MCP-1	Control	2.96 ± 0.18	4.90 ± 0.40	***0.0005****
	Garcinia kola	2.47±0.17	5.18 ± 0.47	***0.0004****



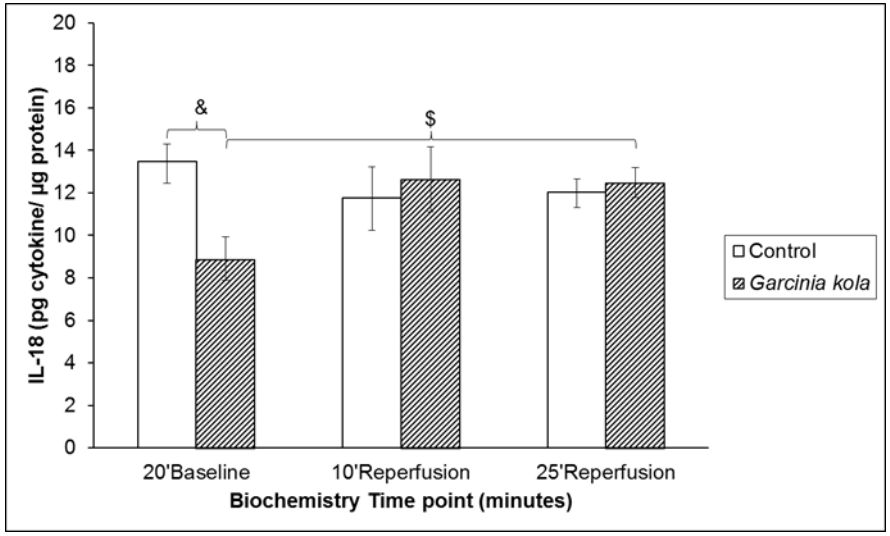
4-3a) IL-1β concentration (pg/µg protein)



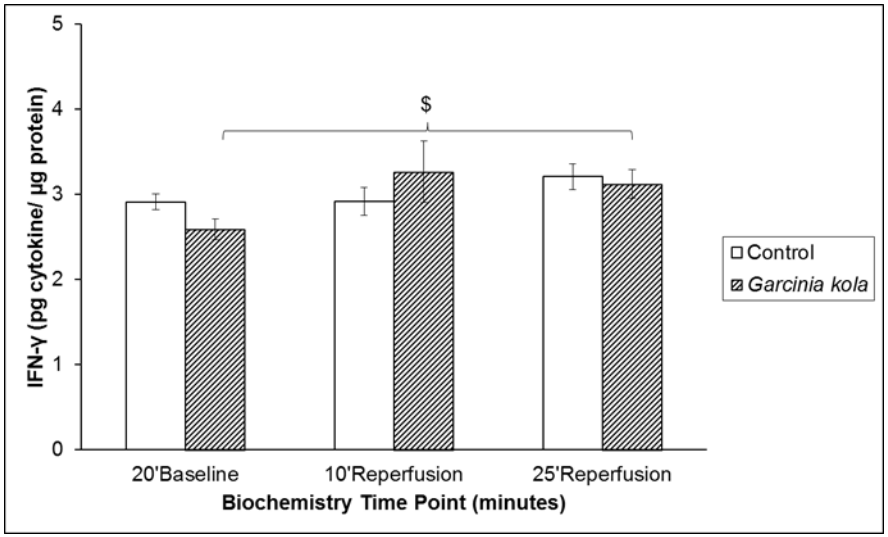
4-3b) TNF-α Concentration(pg/µg protein)



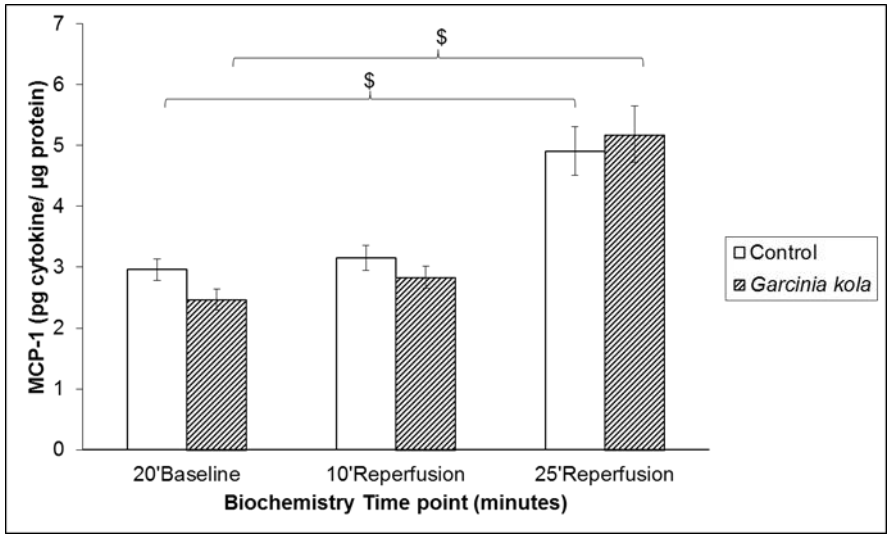
4-3c) IL-6 Concentration(pg/µg protein)



4-3d) IL-18 Concentration(pg/µg protein)



4-3e) INF-γ Concentration(pg/µg protein)



4-3 f) MCP-1 Concentration(pg/µg protein)

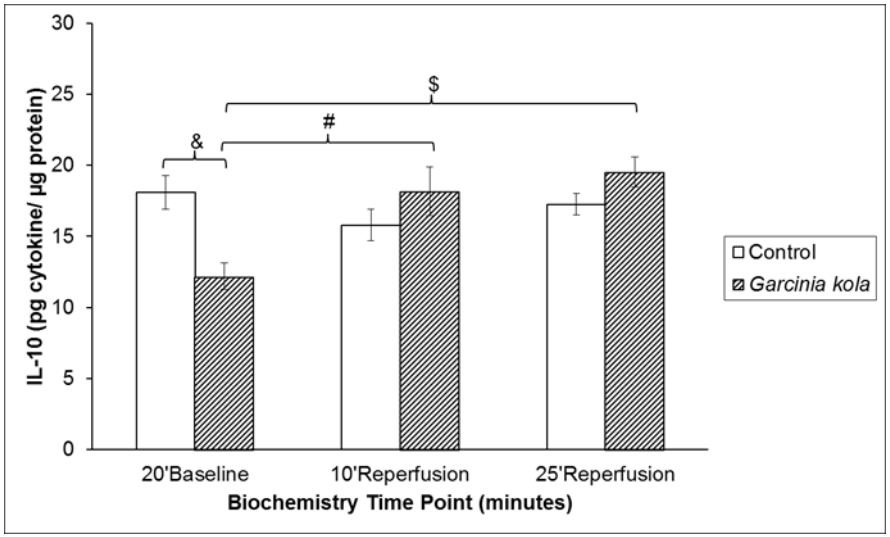
Figure 4-3: Effects of dietary *Garcinia kola* supplementation on: a) IL-1β, b) TNF-α, c) IL-6, d) IL-18, e) IFN-γ and f) MCP-1 expression. Results are expressed as mean ± S.E.M, (n=6-8). &: p<0.05 control versus *Garcinia kola* supplemented group at same time point; #: *p<0.05* baseline *versus* 10 minutes reperfusion for the same group; $: *p<0.05* baseline versus 25 minutes reperfusion for the same group.

4.5: Effects of dietary *Garcinia kola* supplementation on cardiac anti-inflammatory cytokines expression

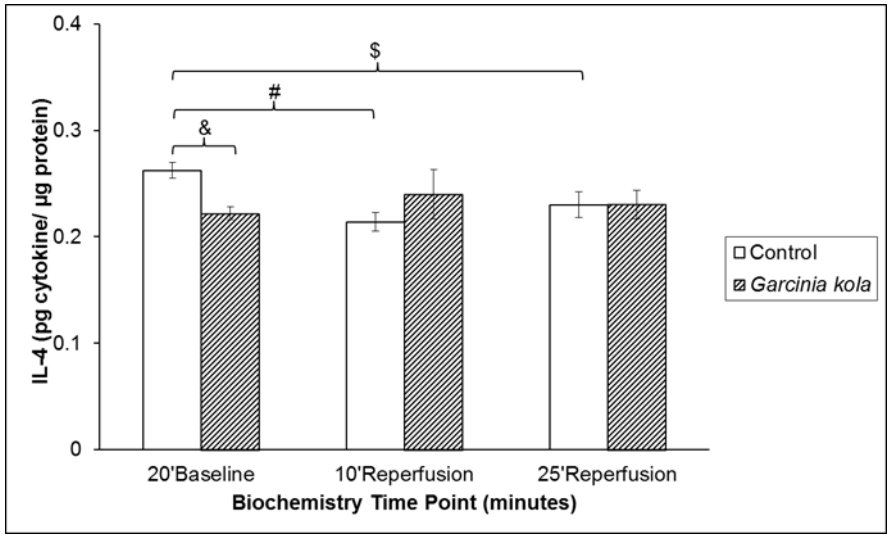
Expression of IL-10 and IL-4 were significantly lower in *Garcinia kola* supplemented group when compared to the control group at baseline: IL-10 (12.18 ± 0.94 *versus* 18.10 ± 1.18, *p=0.0049*) and IL-4 (0.22 ± 0.006 *versus* 0.262 ± 0.007, *p=0.0031*) (**Figure 4-4**).

Garcinia kola supplemented group showed a significant increase in expression of IL-10 at 10 minute reperfusion (12.18 ± 0.94 *versus* 18.18 ± 0.936, *p=0.016*) and 25 minutes reperfusion (12.18 ± 0.94 *versus* 19.52 ± 1.05, *p=0.0003)* when compared to the baseline expression level (**Figure 4-4a**).There was no significant change in expression of IL-4 at both reperfusion time points when compared to the baseline expression level in *Garcinia kola* supplemented group (**Figure 4-4b**).

In the control group there was no significant change observed in IL-10 expression at the two reperfusion time points when compared to baseline expression level (**Figure 4-4a**). Furthermore, the control group showed a significantly decreased IL-4 expression at 10 minutes reperfusion (0.26 ± 0.01 *versus* 0.21 ± 0.01, *p=0.0032*) and 25 minutes reperfusion (0.26 ± 0.01 *versus* 0.23 ± 0.01, *p=0.043*) when compared to the baseline expression level (**Figure 4-4b**).



4-4a) IL-10 concentration (pg/µg protein)



4-4b) IL-4 concentration (pg/µg protein)

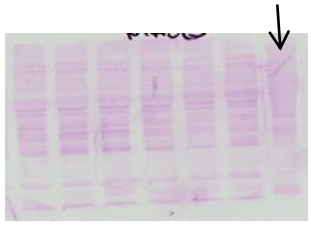
Figure 4-4: Effects of dietary *Garcinia kola* supplementation on IL-10 and IL-4 expression. Results are expressed as mean concentration (pg cytokine/µg protein) ± S.E.M, (n=6-8). &: *p<0.05 for* control versus *Garcinia kola* supplemented group at same time point: #: *p<0.05 for* baseline *versus* 10 minutes reperfusion results the same group: $: *p<0.05 for* baseline versus 25 minutes reperfusion results of the same group.

4.6: Effects of dietary *Garcinia kola* supplementation on cardiac oxidative stress mediator

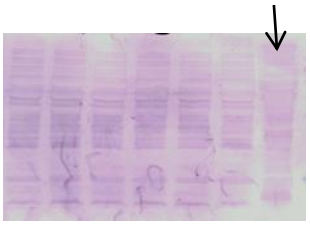
There was no significant difference in expression of nitro-tyrosine before ischaemia and at 25 minutes reperfusion between the control and supplemented groups, however, at 10 minutes reperfusion a significantly low expression of nitro-tyrosine was observed in the *Garcinia kola* supplemented group when compared to the control group (28.86 ± 1.25 *versus* 52.66 ± 3.03, *p=0.0002*) (**Figure 4-5**).

Myocardial expression of nitro-tyrosine protein in the control group showed a significant increase at 10 minutes reperfusion when compared to the baseline expression level (35.59 ± 1.25 *versus* 52.66 ± 3.03, *p=0.0020*), while a significant decrease was observed in the *Garcinia kola* supplemented group between the same time points (28.86 ± 1.25 *versus* 36.18 ± 1.30, *p=0.0023*) (**Figure 4-5**).

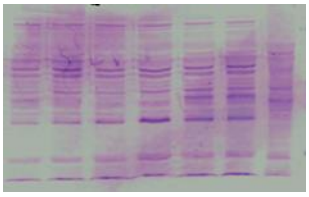
Key: ↓ **liver sample lane.**



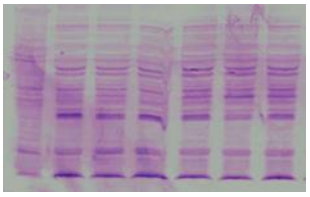
Control baseline (CB)



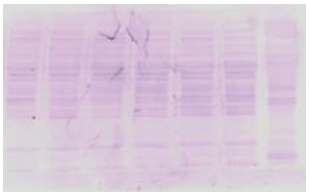
Garcinia kola baseline (GKB)



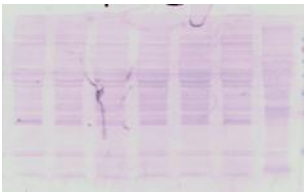
Control 10 min reperfusion (C10)



Garcinia kola 10 min reperfusion (GK10)



Control 25 min reperfusion (C25)



Garcinia kola25 min reperfusion (GK 25)

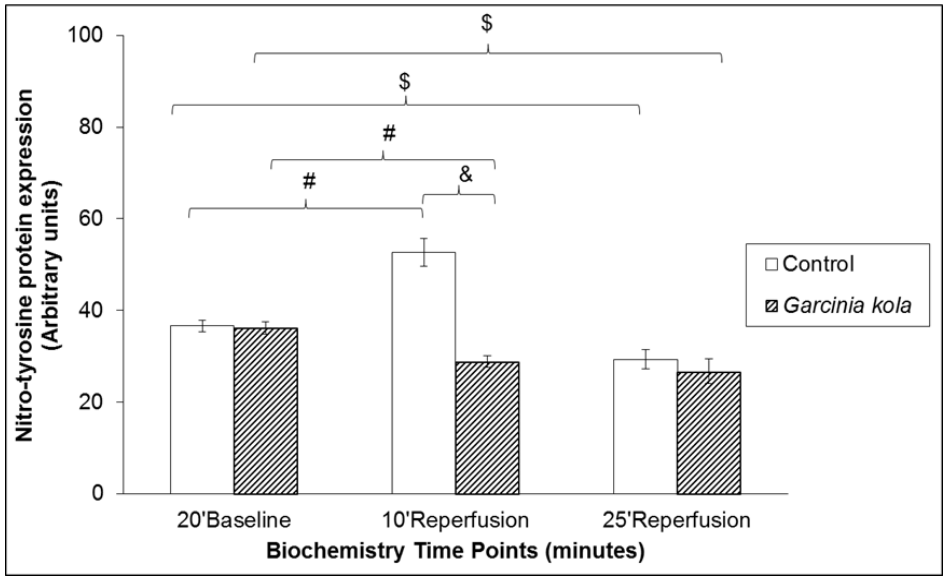


Figure 4-5: Effects of dietary *Garcinia kola* supplementation on cardiac nitro-tyrosine expression using western blot technique. Results are expressed as mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus* 10 minutes reperfusion for the same group; $: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group.

4.7: Effects of dietary *Garcinia kola* supplementation on iNOS and COX-2 protein expression

4.7.1: Inducible nitric oxide synthase (iNOS) protein expression

There was no significant difference in iNOS protein expression between the two groups and across the time points (**Figure 4-6**).

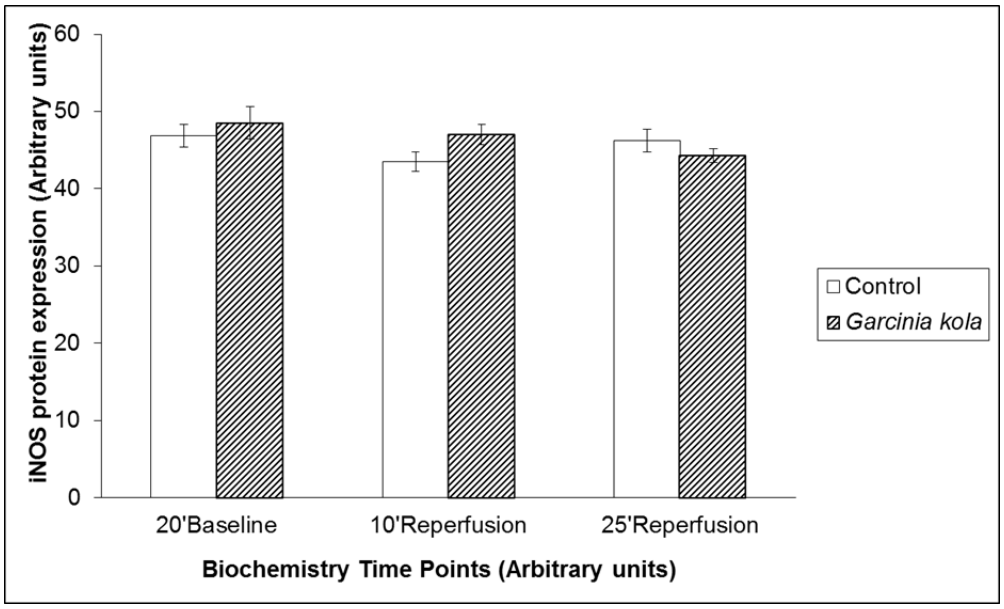


Figure 4-6: Effects of dietary *Garcinia kola* supplementation on iNOS using western blot. Results are expressed as mean ± S.E.M, n=6 per group.

4.7.2: Cyclooxygenase (COX)-2 protein expression

There were no detectable bands in our study samples for both the control and *Garcinia kola* supplemented groups at all-time points (sampled membranes shown). **Figure 4-7** show Western blots pictures `for COX-2 protein expression in, control and

garciniakola supplemented rat hearts at 10 minutes reperfusion, rat kidney and hearts treated with 1µg/ml LPS for 2 hours. The LPS kidney and heart lysate were usedas in-house control to confirm that our method and COX-2 primary antibody are working optimally.

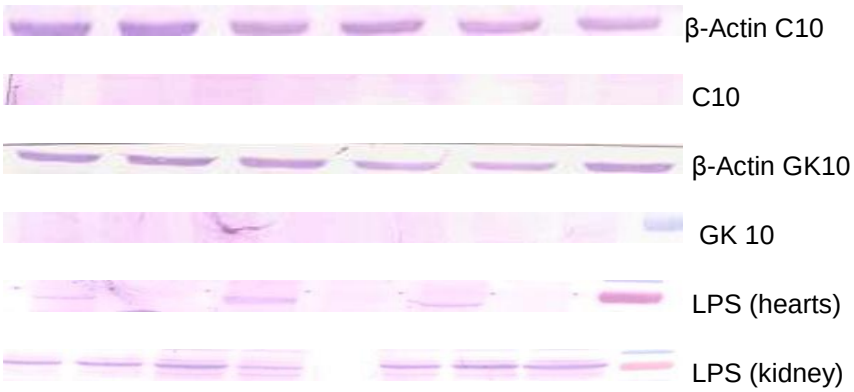


Figure 4-7: Western blot picture for COX-2 protein expression

4.8: Effects of dietary *Garcinia kola* supplementation on cardiac nuclear factor kappa beta (NF-kβ) protein expressions

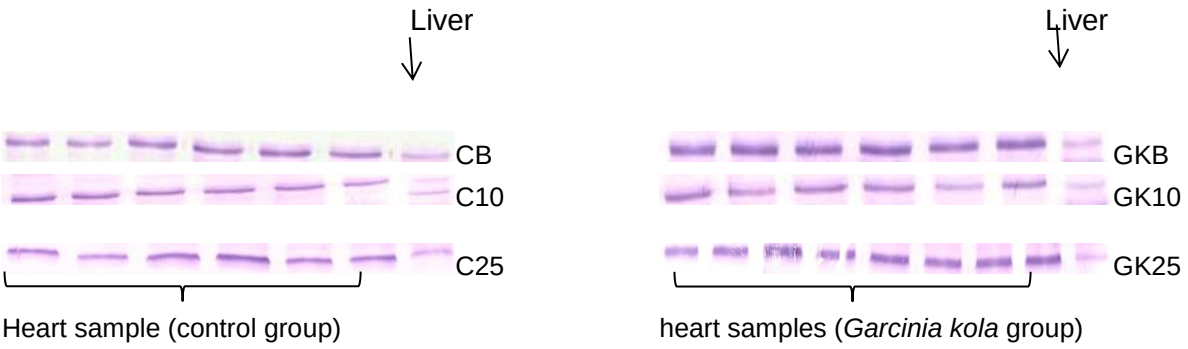
4.8.1: NF-kβ65 protein expression

Garcinia kola supplemented group showed a significantly high NF-kβ65 protein expression at baseline (60.42 ± 1.14 *versus* 45.81 ± 1.59, *p<0.0001)* and at 25 minutes reperfusion (49.14 ± 1.79 *versus* 41.42 ± 1.62, *p=0.0066*) when compared to the control group (**Figure 4-8**). There was no significant difference at 10 minutes reperfusion between the two groups.

Furthermore, when comparing the baseline expression level with reperfusion time point expression levels, a significant decrease was observed in *Garcinia kola* supplemented group at 10 minutes reperfusion (60.42 ± 1.14 *versus* 50.20 ± 1.69, *p=0.0008*) and 25 minutes reperfusion (60.42 ± 1.14 *versus* 49.14 ± 1.79, *p=0.0002*). A significant increase was observed in the control group at 10 minutes reperfusion when compared to the baseline time point (45.81 ± 1.59 *versus* 55.47 ± 2.11,

p=0.0045). There was no significant difference between the baseline and 25 minutes reperfusion expression levels in the control group (Figure 4-8).

Note: Sample 3 and 4 were excluded from analysis in the GK25 group due to bubble formation during gel preparation Figure 4-8 blots picture.



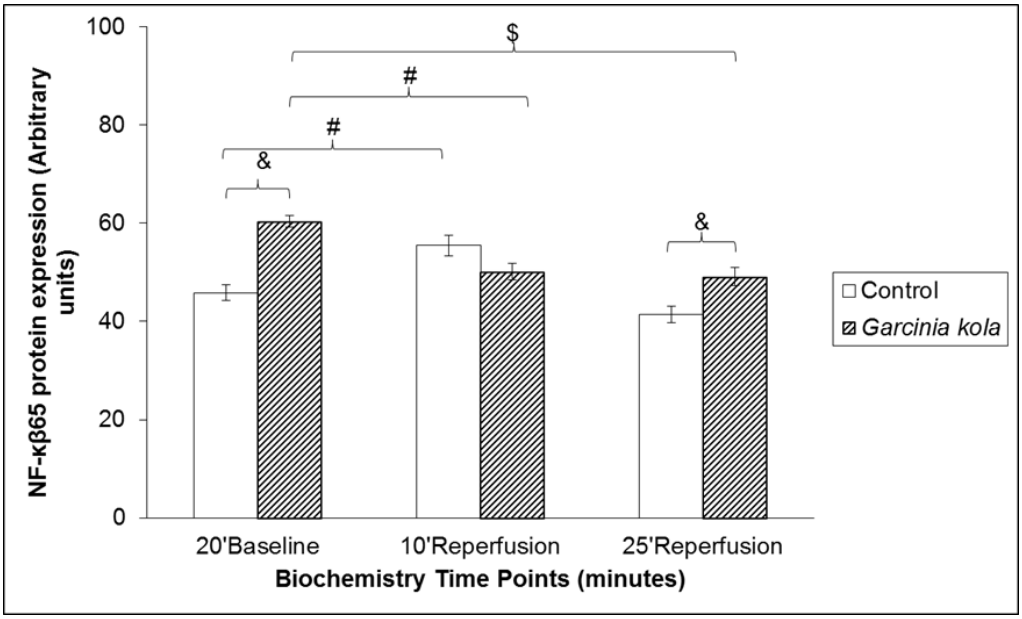


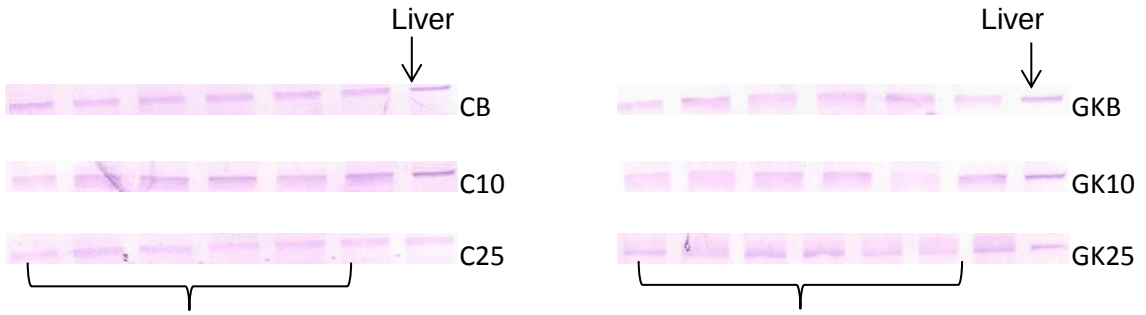
Figure 4-8: Effects of dietary *Garcinia kola* supplementation on NF-kβ65 expression using western blot. Results are expressed as mean ± S.E.M, n=6 per group (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus 10 minutes reperfusion for the same group; $*: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group).

4.8.2: Inhibitor kappa beta kinase beta (IKKβ) protein expression

IKKβ protein expression was significantly decreased in the *Garcinia kola* supplemented group at baseline (35.48 ± 0.91 *versus* 42.68 ± 0.45, *p=0.0002)* and 10 minutes reperfusion (36.14 ± 0.65 *versus* 45.53 ± 1.28, *p=0.0002*) when

compared to the control group (**Figure 4-9**). However, there was no significant difference between the two groups at 25 minutes reperfusion.

A significant decrease was observed in the control group at 25 minutes reperfusion when compared to the baseline expression level (38.78 ± 0.81 *versus* 42.68 ± 0.45, *p<0.0001*), while a significant increase was observed in the *Garcinia kola* supplemented group between the same time points (35.48 ± 0.91 *versus* 38.78 ± 0.81, *p=0.0187*) (**Figure 4-9**). No significant change was observed between baseline and 10 minutes reperfusion in both groups.



Heart sample (control group) heart samples (*Garcinia kola* group)

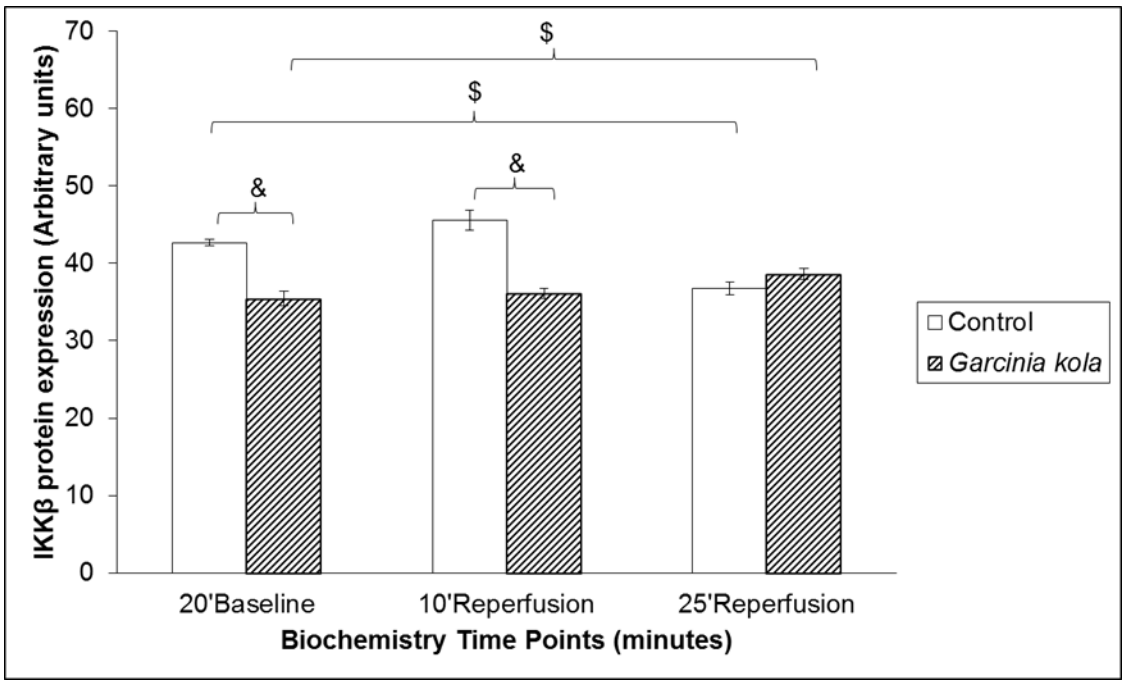
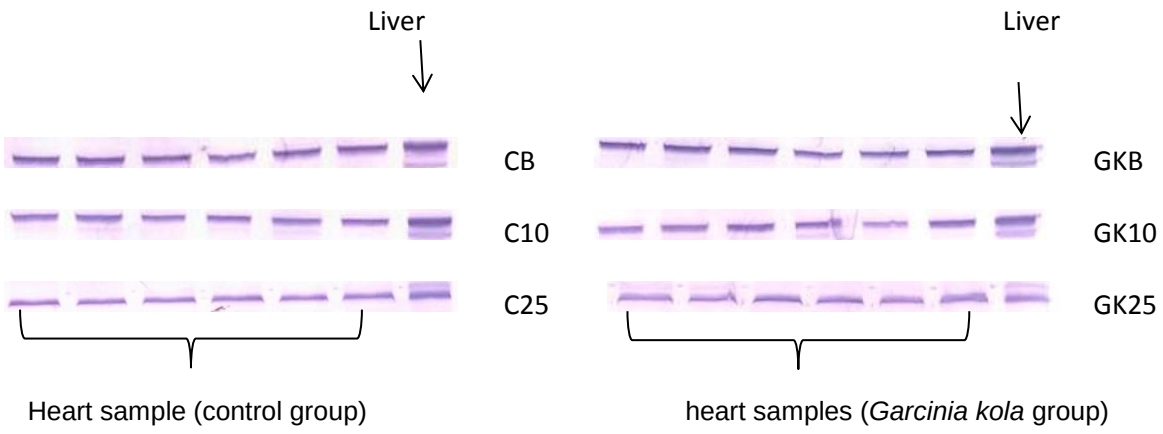


Figure 4-9: Effects of dietary *Garcinia kola* supplementation on IKKβ expression using western blot. Results are expressed as mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus 10 minutes reperfusion for the same group; $*: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group.

4.8.3: Inhibitor kappa beta kinase alpha (IKKα) protein expression

The *Garcinia kola* supplemented group showed a significantly increased IKKα expression at 25 minutes reperfusion when compared to the control group at the same time point (95.54 ± 1.50 *versus* 85.77 ± 1.82, *p=0.0011*) (**Figure 4-10**). However, there was no significant difference at baseline and 10 minutes reperfusion between the two groups.

Comparing the expression levels in the control group at baseline and reperfusion, there was a significant decrease in IKKα expression at 10 minutes (73.19 ± 1.61 *versus* 64.17 ± 1.11, *p=0.0013*) and a significant increase at 25 minutes (73.19 ± 1.61 *versus* 85.77 ± 1.82, *p=0.0002*) when compared to the baseline expression level. However, the *Garcinia kola* supplemented group showed a significant increase in IKKα protein expression at 25 minutes reperfusion when compared to the baseline expression level of the same group (95.54 ± 1.50 *versus* 69.94 ± 1.24, *p<0.0001*) (**Figure 4-10**).There was no significant difference between the baseline and 10 minutes reperfusion time points in the supplemented group.



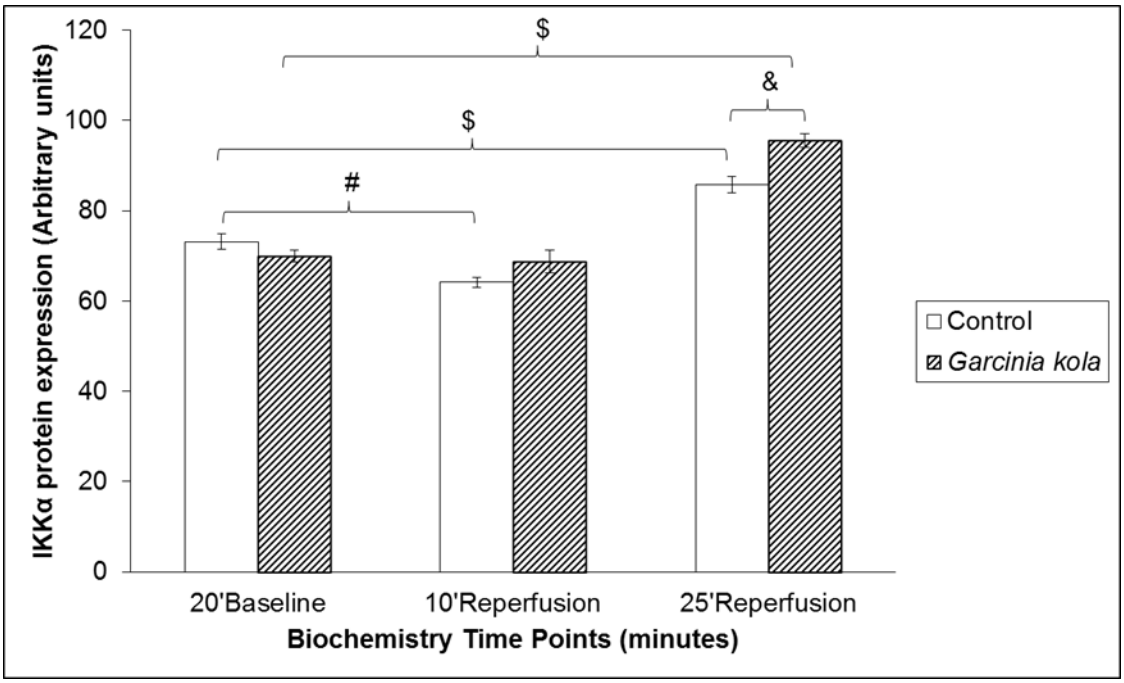


Figure 4-10: Effects of dietary *Garcinia kola* supplementation on IKKα expression using western blot. Results are expressed as mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus 10 minutes reperfusion for the same group; $*: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group.

4.8.4: Phosphorylation of IKKα/β (Ser176/180)

No bands were detected for both the control and *Garcinia kola* supplemented group for phospho-IKKα/β (Ser176/180) rabbit monoclonal antibody detecting IKKα only when phosphorylated at Ser176/180, and IKKβ only when phosphorylated at Ser177/181(**Figure 4-11**).The membrane did not show any band between the 75 and

100kDa marker, however, beta actin was used as a loading control. Rat kidney and heart samples treated with 2µg/ml LPS for 2 hours were analysed, however, the samples did not show any expression of phospho-IKKα/β also (membranes not shown).

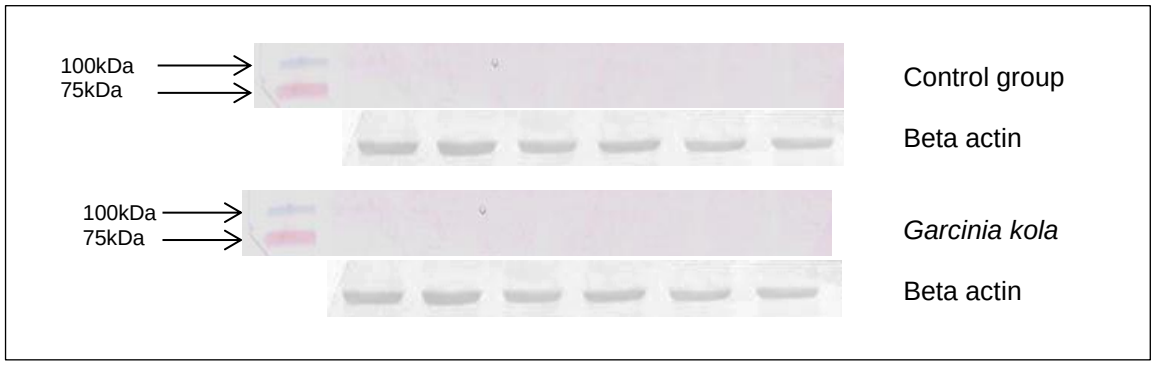
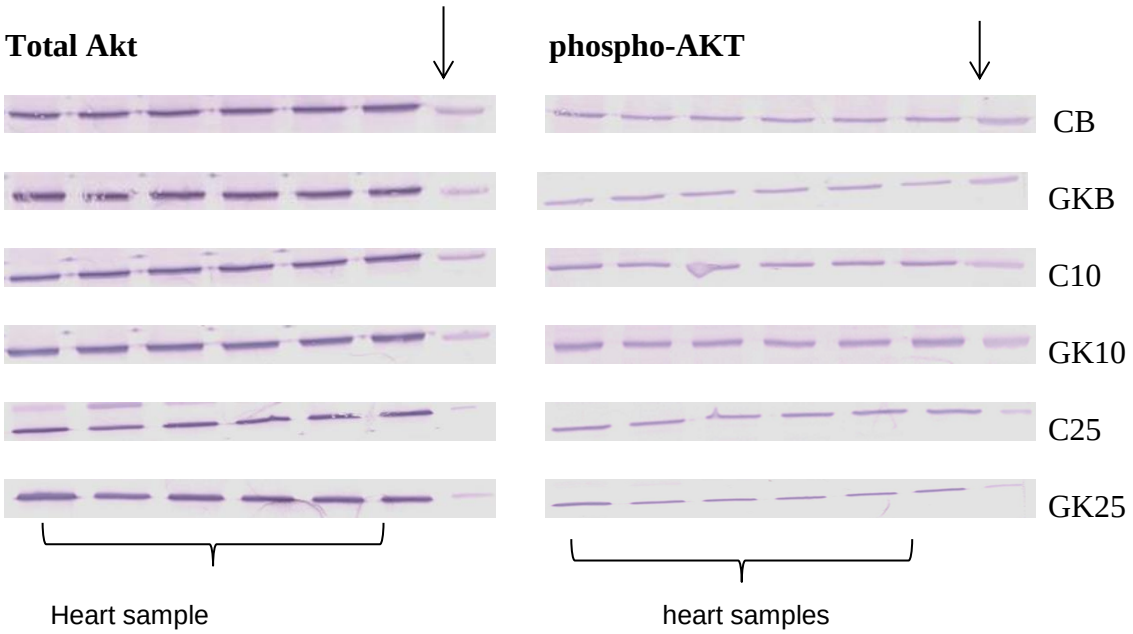


Figure 4-11: Western blots pictures of *phospho*-IKKα/IKKβ protein expression

4.9: Effects of dietary *Garcinia kola* supplementation on protein kinase B/Akt protein

The *Garcinia kola* supplemented group showed a significantly high ratio of phosphorylated Akt to total Akt at 10 minutes reperfusion when compared to the control group at the same time point (0.58 ± 0.02 *versus* 0.49 ± 0.02, *p= 0.0028*) (**Figure 4-12**). However, there was no significant difference between the two groups at baseline and 25 minutes reperfusion.

Furthermore, a significant increase was observed in the *Garcinia kola* supplemented group at both 10 minutes reperfusion (0.50 ± 0.03 *versus* 0.58 ± 0.02, *p=0.0187)* and 25 minutes reperfusion (0.50 ± 0.03 *versus* 0.71 ± 0.0251, *p=0.0026*) when compared to baseline time point, while the control group only showed a significant increase in phosphorylated Akt ratio at 25 minutes reperfused when compared to the baseline ratio (0.71 ± 0.0270 *versus* 0.50 ± 0.01*, p<0.0001*) (**Figure 4-12**).



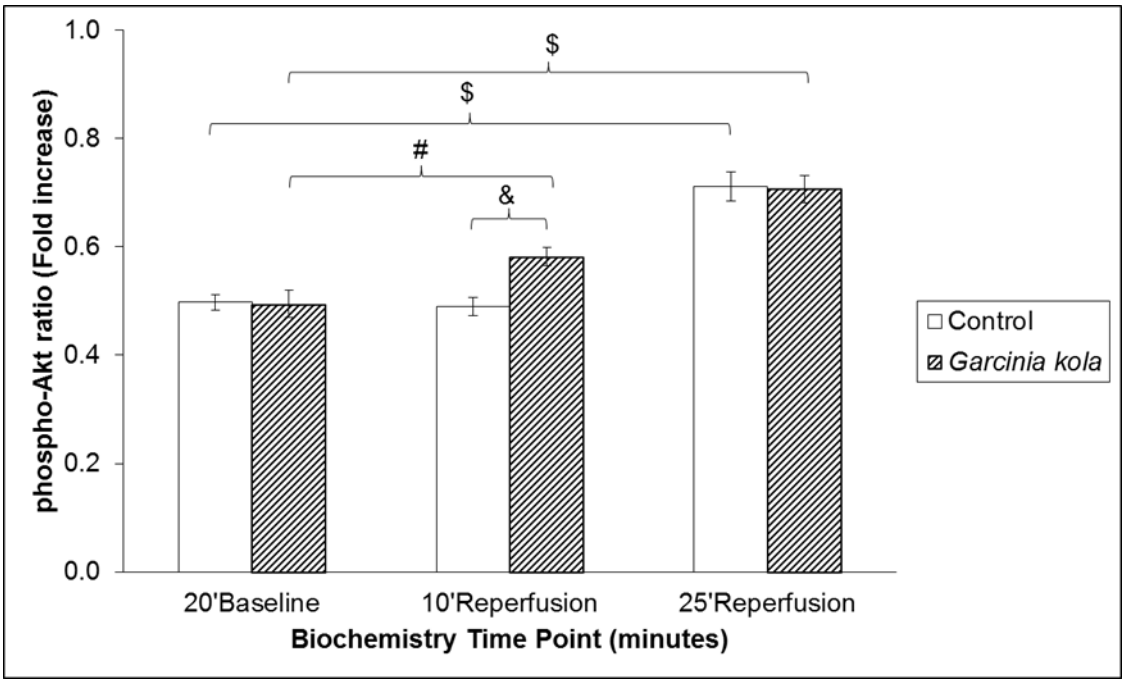


Figure 4-12: Effects of dietary *Garcinia kola* supplementation on Akt phosphorylation using western blot. Results are expressed as mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus 10 minutes reperfusion for the same group; $*: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group.

4.10: Effects of dietary *Garcinia kola* supplementation on p38 mitogen activated protein kinase (MAPK) proteins

4.10.1: Total p38 MAPK protein expression

The *Garcinia kola* supplemented group showed a significantly increased expression of total p38 MAPK at baseline (59.55 ± 1.14 *versus* 55.80 ± 0.78, *p=0.0243*) and 25 minutes reperfusion (73.31 ± 3.96 *versus* 51.17 ± 0.94, *p=0.0182*) when compared to the control group at the same time points. However, at 10 minutes reperfusion control rat hearts showed a significantly increased expression of total p38 MAPK when compared to the *Garcinia kola* supplemented group (66.56 ± 1.60 *versus* 60.58 ± 1.36, *p=0.0006*) (**Figure 4-13a**).

Control rat hearts showed a significant increase in total p38 MAPK expression at 10 minutes reperfusion (66.56 ± 1.60 *versus* 55.80 ± 0.78, *p=0.0005*) and a significant decrease at 25 minutes reperfusion (51.17 ± 0.94 *versus* 55.80 ± 0.78, *p=0.0026*) when compared to baseline time point (**Figure 4-13a**). There was no significant change in total p38 MAPK expression between baseline and 10 minutes reperfusion time point however, a significant increase was observed between the baseline and 25 minutes expression (73.31 ± 3.96 *versus* 59.55 ± 1.14, *p=0.0099)* (**Figure 4-13a**).

4.10.2: Ratio of phosphorylated total p38 MAPK

The *Garcinia kola* supplemented rat hearts showed a significantly low ratio of phosphorylated tototal p38 MAPK when compared to the control group at baseline (0.62 ± 0.01 *versus* 0.67 ± 0.01; *p=0.0175*) and at 25 minutes reperfusion (0.72 ± 0.05 *versus* 0.95 ± 0.02; *p=0.0016*). However, a significantly high ratio was observed in the supplemented group at 10 minutes reperfusion when compared to the control group ratio (0.65 ± 0.01 *versus* 0.52 ± 0.02, *p=0.0020*) (**Figure 4-13b**).

There was no significant difference in phospho- p38 MAPK ratio at reperfusion time points when compared to baseline time point in supplemented group. However, the control group showed a significantly decreased phospho-p38 MAPK ratio at 10 minutes reperfusion (0.52 ± 0.02 *versus* 0.67 ± 0.01, *p0.0009)* and a significantly

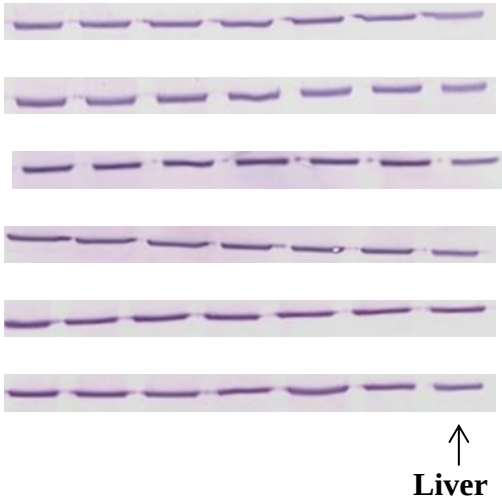
increased phospho-p38 MAPK ratio at 25 minutes reperfusion when compared to the baseline time point (0.95 ± 0.02 *versus* 0.67 ± 0.01, *p<0.0001*) **(Figure 4-13b)**.

4.10.3: Ratio of p38α MAPK isoform

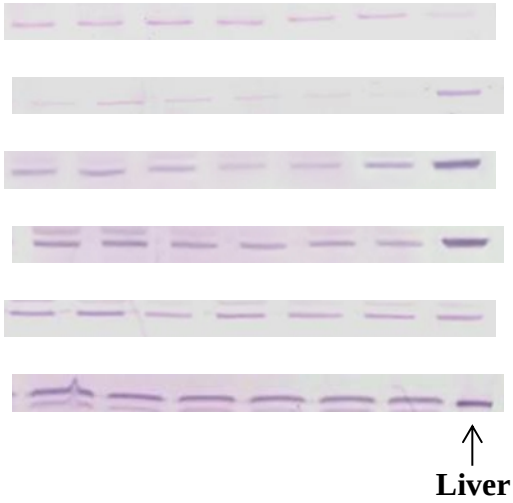
The ratio of p38α MAPK was significantly lower in the supplemented group at baseline (0.82 ± 0.02 *versus* 0.93 ± 0.02, *p=0.0029*) and 25 minutes reperfusion (0.62 ± 0.05 *versus* 0.84 ± 0.02, *p=0.0043*) when compared to the control group. However, at 10 minutes reperfusion control rat hearts showed a significantly lower ratio of p38α MAPK when compared to the *Garcinia kola* supplemented group (0.73 ± 0.02 *versus* 0.85 ± 0.02, *p=0.0016*) (**Figure 4-13c**).

There was no significant difference in p38α MAPK ratio between baseline and 10 minutes reperfusion in the supplemented group however a significant decrease in p38α MAPK ratio was observed in the *Garcinia kola* supplemented group at 25 minutes reperfusion when compared to the baseline time point (0.62 ± 0.05 *versus* 0.82 ± 0.02, *p=0.0078).* The control group showed a significant decrease in p38α MAPK ratio at 10 minutes reperfusion (0.73 ± 0.02 *versus* 0.93 ± 0.02, *p<0.0001*) and at 25 minutes reperfusion (0.84 ± 0.02 *versus* 0.93 ± 0.02, *p=0.0150*) (**Figure 4-13c)**.

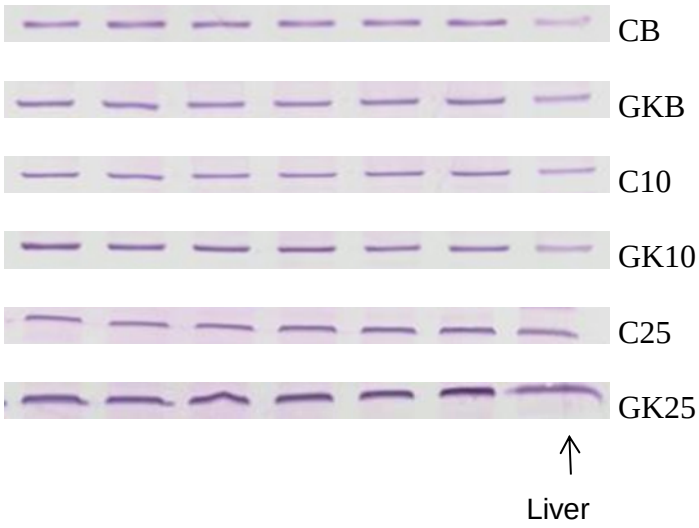
Total p38-MAPK

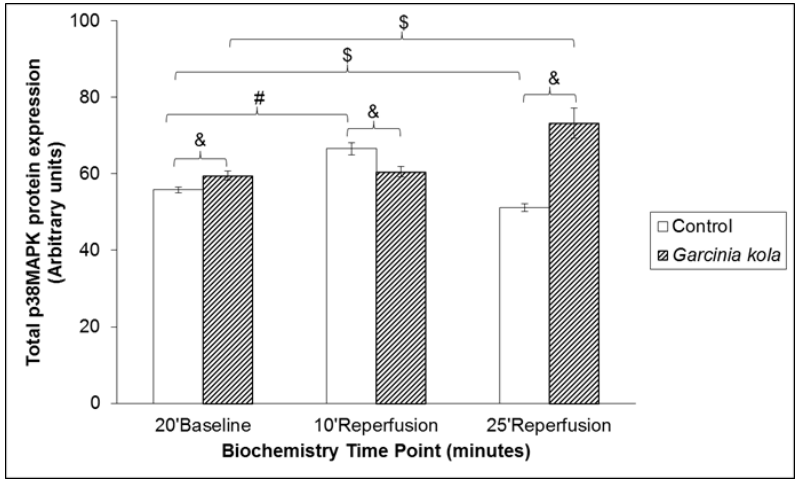


phospho p38-MAPK

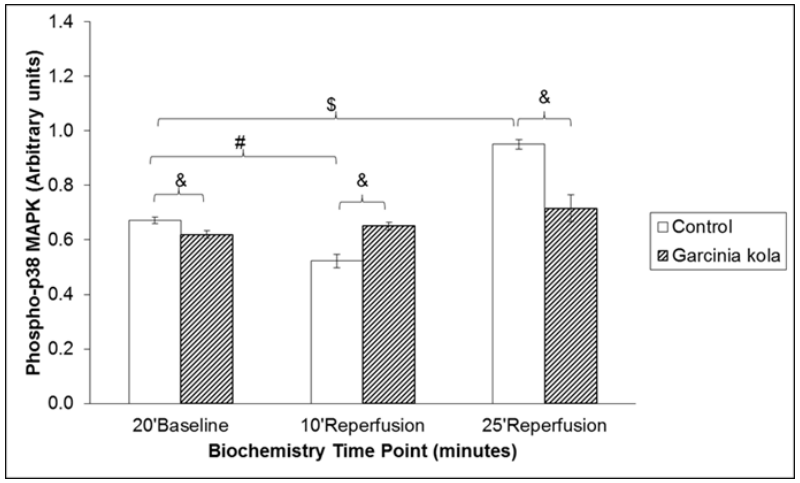


p38α-MAPK

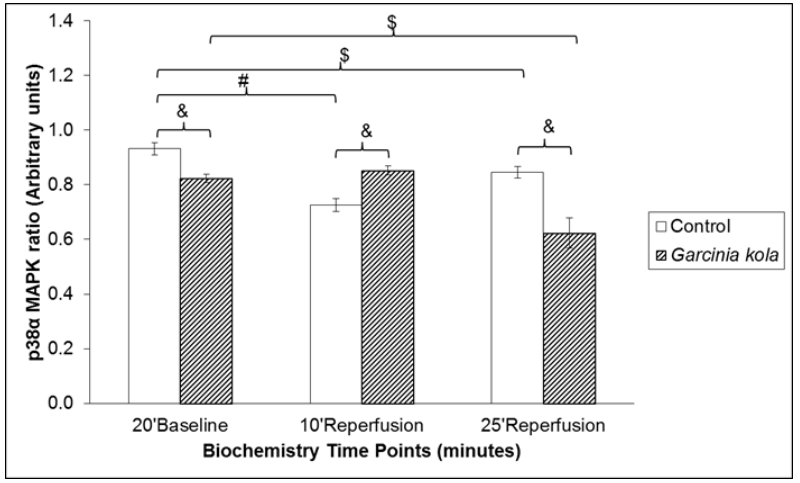




a)



b)



c)

Figure 4-13: Effects of dietary *Garcinia kola supplementation on a) total* p38 MAPK, b)ratio of phospho- p38 MAPK and c) ratio of p38α MAPK expression using western blot. Results are expressed as mean ± S.E.M, n= 6-8 *per group per time point.* (&: *p<0.05 for* control versus *Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus* 10 minutes reperfusion results the same group, $: *p<0.05* baseline *versus* 25 minutes reperfusion results of the same group).

4.11: Effects of dietary *Garcinia kola* supplementation on poly (ADP) ribose polymerase (PARP-1) protein expression

Uncleaved PARP-1 protein was significantly increased in the *Garcinia kola* supplemented group when compared to the control group at 10 minutes reperfusion (37.13 ± 1.02 *versus* 32.53 ± 0.71; *p=0.0049*) and 25 minutes reperfusion (32.66 ± 0.56 *versus* 30.05 ± 0.26; *p*=0.0017) however, there was no significant difference in cleaved PARP-1 expression between the two groups at baseline and reperfusion time points (**Table 4-3**).

Uncleaved PARP-1 showed a significant increase in the control group 32.53 ± 0.71 *versus* 29.30 ± 0.43; *p=0.0042*) and in the *Garcinia kola* supplemented group (37.13 ± 1.02 *versus* 31.19 ± 1.07; *p*=0.0025) at 10 minutes reperfusion when compared to the baseline time point (**Table 4-3**).

Cleaved PARP-1 expression showed a significantly increase in the control group at 10 minutes reperfusion (153.76 ± 3.27 versus 114.22 ± 2.77, *p<0.0001*) and at 25 minutes reperfusion (175.37 ± 4.15 versus 114.22 ± 2.77, *p<0.0001*) when compared to the baseline expression level, respectively. *Garcinia kola* supplemented group also demonstrated significant increase in cleaved PARP-1 at 10 minutes reperfusion (183.34 ± 2.88 versus 110.99 ± 2.10, *p<0.0001*) and at 25 minutes reperfusion (154.61 ± 2.77 versus 110.99± 2.10, *p<0.0001*) when compared to baseline expression level, respectively (**Table 4-3**).

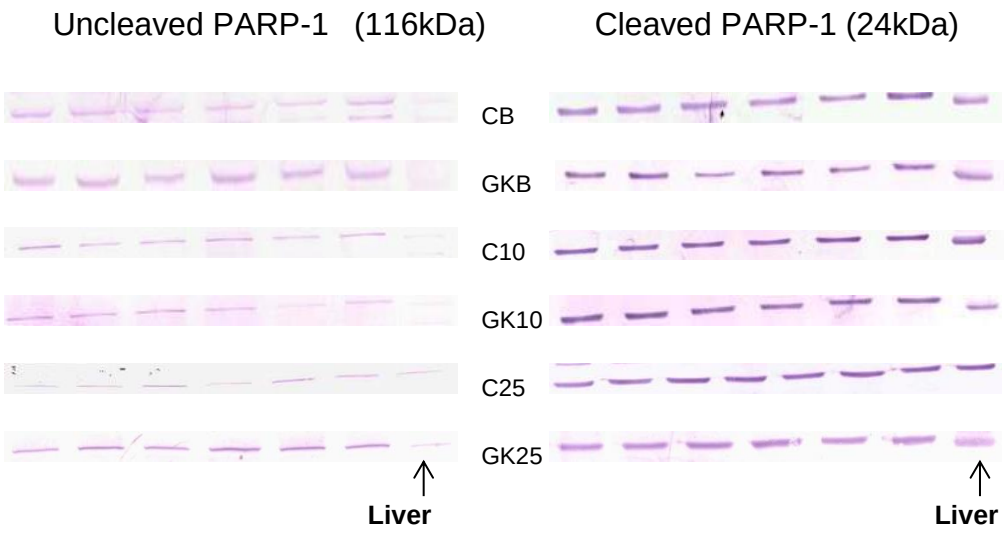


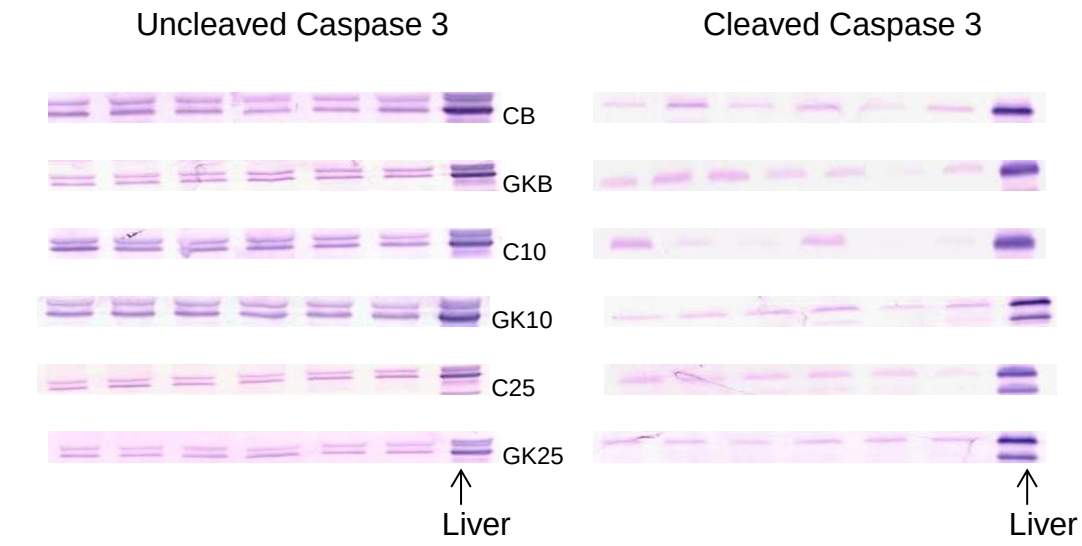
Table 4-3: Effect of dietary *Garcinia kola* supplementation on PARP-1 protein expression using western blot (Results are expressed as mean ± S.E.M, n=6-8 per group)

	Uncleaved PARP-1		Cleaved PARP-1	
Time points	**Control**	***Garcinia kola***	**Control**	***Garcinia kola***
20' Baseline	29.30 ± 0.43$^{\#}$	31.19 ± 1.07$^{\#}$	114.22 ± 2.77 $^{\#,\$}$	110.99 ± 2.10$^{\#,\$}$
10' Reperfusion	32.53 ± 0.71$^{\&}$	37.13 ± 1.02	175.37 ± 4.15	183.34 ± 2.88
25' Reperfusion	30.05 ± 0.26$^{\&}$	32.66 ± 0.56	153.76 ± 3.27	154.61 ± 2.77

&: *p<0.05 for* control versus *Garcinia kola* supplemented group at same time point
#: *p<0.05 for* 20 minutes Bseline *versus* 10 minutes reperfusion results the same group
$: *p<0.05 for* 20 minutes Baseline versus 25 minutes reperfusion results of the same group

4.12: Effects of dietary *Garcinia kola* supplementation on Caspase-3 expression

The *Garcinia kola* supplemented group showed a significantly decreased cleaved caspase-3 ratio at 10 minutes reperfusion when compared to the control group at the same time point (0.32 ± 0.01 *versus* 0.38 ± 0.01; *p<0.0027*). Furthermore, there was a significant decrease in cleaved caspase-3 at 10 minutes reperfusion when compared to baseline ratio in the supplemented group (0.47 ± 0.03 *versus* 0.32 ± 0.01 *p=0.0030*) (**Figure 4-14**). No significant change was observed at 25 minutes reperfusion and in the control group (**Figure 4-14**).



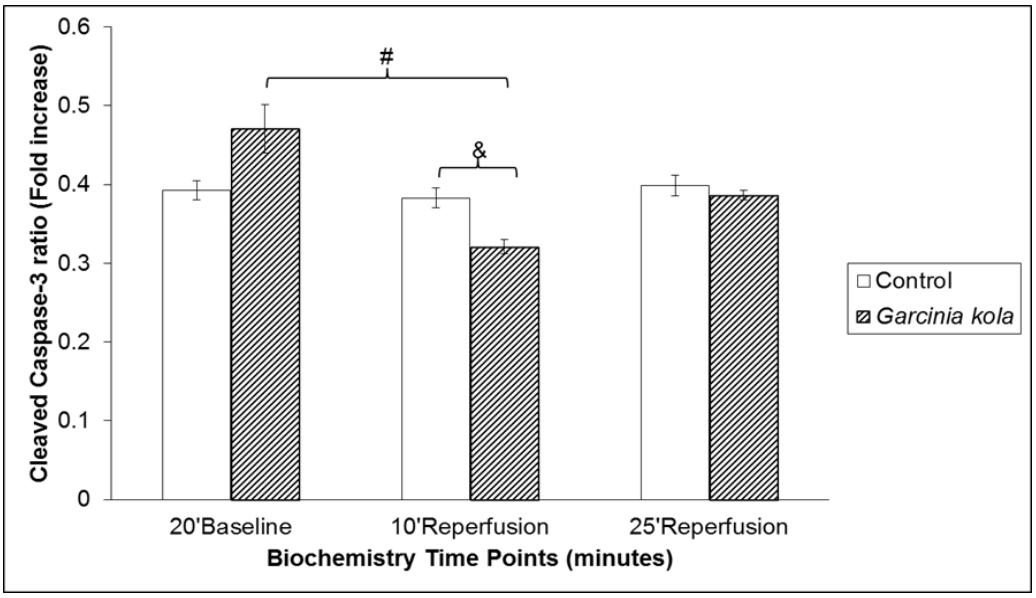


Figure 4-14: **Effects of dietary *Garcinia kola* supplementation on caspase-3 protein expression using western blot. Results are expressed as mean ± S.E.M, n=6 per group. (&: *p<0.05* control *versus Garcinia kola* supplemented group at same time point, #: *p<0.05* baseline *versus 10 minutes reperfusion for the same group.***

4.13: Effects of dietary *Garcinia kola* supplementation on rat lymphocytes DNA

Figure 4-15 shows appearance of ethidium bromide stained lymphocytic cells using fluorescence microscope.

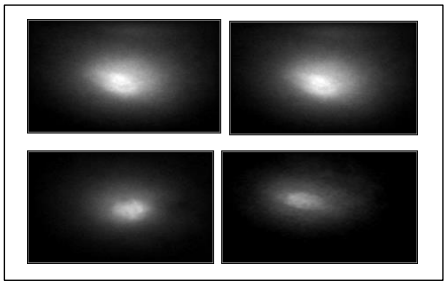


Figure 4-15: photographs of comet cells

There was no significant difference in tail length, tail moment, tail intensity and % tail DNA migration between the two groups (**Figure 4-16**).

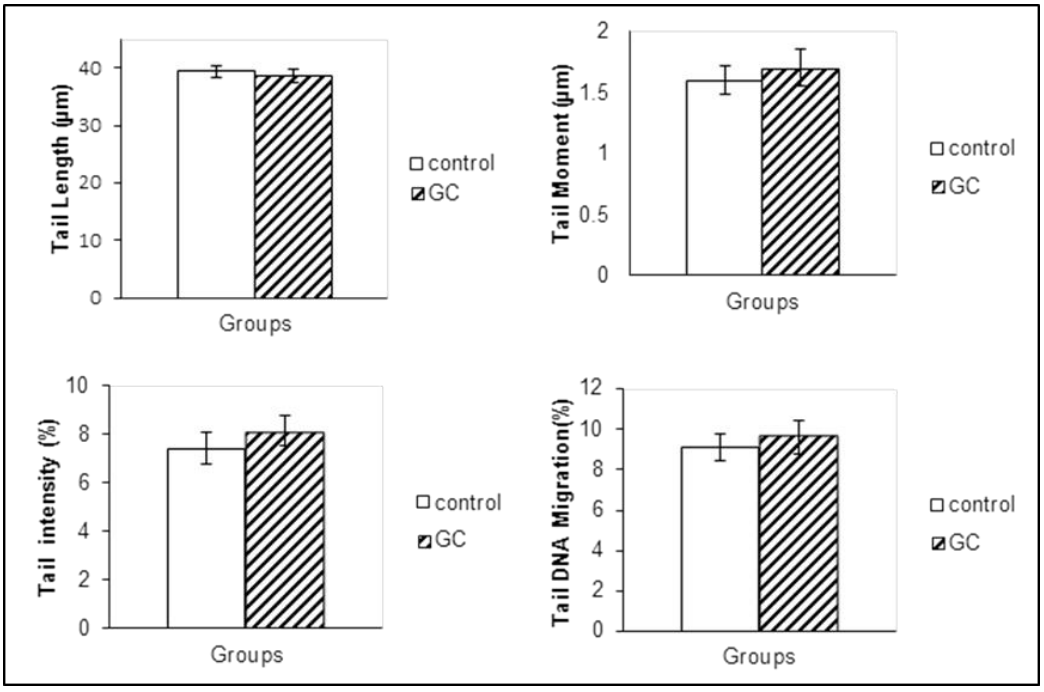


Figure 4-16: Effects of dietary *Garcinia kola* supplementation on Tail length, tail moment, percentage intensity and percentage DNA migration on rat lymphocytes using alkaline comet assay technique. Results are expressed as mean ± S.E.M (n= 6 per group).

CHAPTER 5: DISCUSSION

Inflammation and oxidative stress are well accepted to play a critical role in cardiovascular diseases (CVD). Gradually more studies show that modulation of signaling pathways during cardiac ischaemia-reperfusion injury (IRI) by phytochemicals may play a significant role in prevention and alleviation of ischaemic heart diseases (IHD). Therefore, the physiological role of different polyphenolic antioxidants and their anti-inflammatory properties in IHD are currently being pursued, with new interactions and benefits being recognized.

Our study follows on previous work with Red Palm Oil (RPO) and Rooibos (*Aspalathus linearis*) herbal tea in our laboratory. Improved functional recovery, reduced infarct size and myocardial tolerance against oxidative risk induced diet in RPO supplemented group was associated with RPO rich oleic acid (42.7–43.9%), beta-carotene and vitamin E (*tocopherols and tocotrienols*) content (Esterhuyse *et al.,* 2005; Esterhuyse *et al*., 2006; Bester *et al*., 2010; Katengua-Thamahane *et al.,* 2012). In another study the unique *aspalathin* flavonoid content of Rooibos tea was associated with antioxidant signaling in isolated Wistar rat hearts (Pantsi *et al.,* 2011).

In the current study we aimed to determine the effects and mechanisms of cardio-protection of dietary *Garcinia kola* supplementation using the isolated Wistar rat heart model. For the first time we demonstrated the potential effects of dietary *Garcinia kola* supplementation on the antioxidant and anti-inflammatory signaling pathways during ischaemia and reperfusion in the Wistar rat heart model.

5.1: Effects of dietary *Garcinia kola* supplementation on cardiac functional recovery

Body weight is well documented as a cardiovascular risk factor, with change in body weight reported to affect cardiac contractility and heart rate (Karason *et al.,* 1999; McMillen *et al.,* 2013). Our results did not show any statistical difference in body weight between the two groups at point of sacrifice (**Figure 4-1**). Furthermore, there was no statistical difference in cardiac contractile functional parameter (**Table**

4-1) before and after induce IRI between the control and the supplemented group. However, we did observe a significantly improved aortic output recovery in dietary *Garcinia kola* supplemented group when compared to the control group after 25 minute reperfusion (**Figure 4-2**). Cardiac output is defined as the amount of blood the heart pump per minute and it has been demonstrated to be dependent on several factors including the heart rate, contractility and end systolic stress (Vincent, 2008). Experimental and clinical observation reviewed by Singh (2003) provides convincing evidence which implicates increased heart rate as a risk factor for ischaemic heart diseases. These studies highlighted the importance of pharmacological agents that improves cardiac output without increasing the heart rate and myocardial contractility. Our results indicates that dietary *Garcinia kola* supplementation seem to offer cardiac protection linked to improved functional recovery through mechanisms of acute inflammatory response, antioxidant signaling and prompt activation of the PKB/Akt protein (**Figure 4-12**) earlier into reperfusion when compared to the control group.

Similar to dietary *Garcinia kola* supplementation, several other researchers have demonstrated that dietary antioxidant rich foods, including polyphenols improves cardiac functional recovery in reperfused isolated rat hearts (Pataki *et al.,* 2002; Esterhuyse *et al.,* 2005; Wang *et al.,* 2009; Pantsi *et al.,* 2010; Testai *et al.,* 2013). Sun and co-workers (2012) reported that *luteolin* improves cardiac functional recovery after 30 minutes of ischaemia and 3 hours of reperfusion in diabetic rats. The improved functional recovery was associated with up-regulation of anti-apoptotic proteins, activating PI3K/Akt pathway, increased BAD protein phosphorylation and decreased ratio of Bax to Bcl-2 protein.

5.2: Effects of dietary *Garcinia kola* supplementation on inflammatory cytokines/chemokines before IRI

Our data shows that dietary *Garcinia kola* supplementation attenuates myocardial expression of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-18, IL-10 and IL-4 when compared to the control group expression levels before induced ischaemia (**Figure 4-3 and 4-4**). The role of inflammatory mediators in atherogenesis have been demonstrated in several experimental studies using genetically modified laboratory animals (Mallat *et al*., 2001; Whitman *et al.,* 2002; Elhage *et al.,* 2003; Kirii

et al., 2003; Branen *et al.,* 2004; Ohta *et al.,* 2005). Supporting our data, Kirii and co-workers (2003) have demonstrated that mice lacking IL-1β (apoE-/-/IL-1 β -/-) significantly decreases the size of atherosclerotic lesion formation by approximately 30% when compared to control apoE-/-/IL-1 β +/+ mice. In other studies a significant decrease in atherosclerotic lesion formation was also observed in TNF-α, IL-18, IFN-γ gene deleted mice when compared to the wild type group (Whitman *et al.,* 2002; Elhage *et al.,* 2003; Branen *et al.,* 2004; Ohta *et al.,* 2005). Mallat and co-workers (2001) further demonstrated that inhibition of IL-18 may prevent fatty streak development in the thoracic aorta of apoE knockout mice and may slows progression of advanced atherosclerotic plaques in the aortic sinus. More importantly, they demonstrates that transfection with the IL-18 binding protein plasmid induces profound changes in plaque composition with resultant stable plaque phenotype.

Garcinia kola supplementation was able to down regulate both the pro- (IL-1β, TNF-α, IL-6, IL-18) and anti-inflammatory (IL-10 and IL-4) cytokines at baseline (**Figure 4-3 and 4-4**). This attenuation of cytokines/chemokines may be due to alteration of homeostatic control by *Garcinia kola*. We propose that *Garcinia kola* supplementation may offer cardiac protection (in part) through down regulation of chronic expression of circulating inflammatory cytokines/chemokines, as such potentially minimizing long term deleterious effects associated with these mediators.

5.3: Effects of dietary *Garcinia kola* supplementation on inflammatory cytokine/chemokines after induced IRI

We observed that *Garcinia kola* supplemented rat hearts showed a significantly increased expression of IL-1β and IL-6 at 10 minutes reperfusion when compared to the baseline time point, while the control group only demonstrated a significant change from baseline expression at 25 minutes reperfusion (**Figure 4-3a and 4-3c**). The supplemented group showed a significant increase in TNF-α, IL-18, IFN-γ and MCP-1 in addition to IL-1β and IL-6 at 25 minutes reperfusion when compared to the baseline time point (**Figure 4-3**).

In line with our observation, Gurantz and co-workers (2005) supports the concept that early expression of IL-1β initiates the stimulus for Angiotensin II (Ang II) type

1 (AT_1) receptor density, playing an important role in the post myocardial infarction. Furthermore, they demonstrated that subsequent increase in TNF-α was associated with sustained and high levels of AT_1 density, enhancing pro-fibrotic effects of Ang II post-myocardial infarction (Gurantz *et al.,* 1999; 2005). Rapid angiogenesis provide blood supply to the remaining cardiomyocytes, as such facilitating infarct healing. IL-6 and TNF-α are also implicated in promotion of pro-survival signaling pathway, termed, the SAFE (survivor activating factor enhancement) pathway, which protects against myocardial infarction (Lecour *et al.,* 2002; Lecour, 2009a, b). In this pathway cardio-protective effects of TNF-α were associated with dose level and type of receptors activated. Furthermore, IL-6 and TNF-α activation are linked to the protective effect of preconditioning, demonstrated by the reversed protective effect of preconditioning in mice lacking either IL-6 or TNF-α (Lecour *et al*., 2002).

Though crucial, pro-inflammatory cytokines/chemokines can exert detrimental effects to the myocardial cells if not regulated. It has been demonstrated that optimal myocardium healing requires timely initiation and regulation of inflammation response, mediating resolution of the inflammatory infiltrates. Importantly, expression of pro-inflammatory cytokines/chemokines in dietary *Garcinia kola* supplemented rat hearts in our study was accompanied by an increase in anti-inflammatory IL-10 (**Figure 4-4a**), while the control group showed a significant decrease in expression of IL-4 (**Figure 4-4b**) and no change in IL-10 when compared to baseline time point expression levels. It is widely accepted that optimal myocardial healing requires timely resolution of inflammatory response, therefore, expression of anti-inflammatory cytokines/chemokines may play a significant role in regulating the acute inflammatory response and the extracellular matrix metabolism. Mechanisms utilized by IL-10 in suppression and regulation of inflammatory response have been thoroughly investigated in human and murine cells of myeloid origin in response to LPS treatment (Berlato *et al.,* 2002; Murray, 2006; Prele *et al.,* 2007; Rossato *et al.,* 2007). In these studies it was demonstrated that anti-inflammatory effects of IL-10 is mediated through signal transducers and activators of transcription-3 (STAT-3), a downstream activator of IL-10 receptor. It has been suggested that IL-10 may be responsible for several inflammatory repressor effects attributed to STAT-3 activation, tempering with the output of pro-inflammatory mediators and inhibiting pro-inflammatory response from activated macrophages (Murray, 2006).

Furthermore, Hammer and co-workers (2005) demonstrated that IL-10, or its downstream mediators, may interfere with both NF-kβ and p38 MAPK signaling pathways. They showed that induced expression of dual-specificity phosphatases (DUSP-1) in IL-10-treated macrophages was associated with inhibition of pro-inflammatory cytokine production and down-regulation of p38 MAPK activation (Hammer *et al.,* 2005).

To this point the majority of research demonstrated the beneficial role of flavonoids in chronic inflammation using LPS induced cell line (Literat *et al.,* 2001; Xagorari *et al.,* 2001; Hougee *et al.,* 2005; Comalada *et al.,* 2006). For the first time we have demonstrated the potential role of *Garcinia kola* in acute inflammatory response during ischaemia and reperfusion in the isolated Wistar rat heart model.

5.4: Effects of dietary *Garcinia kola* supplementation on cardiac oxidative stress mediator before and after IRI

Nitro-tyrosine is regarded as a marker of protein tyrosine nitration, associated with post translational oxidative modification of nitric oxide (NO) and its products (Eiserich *et al*., 1998). This marker has also been described as a hallmark of inflammatory tissue damage, often associated with release of superoxide, nitric oxide and myeloperoxidase (Guzik and Harrison 2006). It is widely accepted that measuring the levels of nitro-tyrosine protein expression may serve as a marker of nitrosative and oxidative stress. In the current study we observed that dietary *Garcinia kola* supplemented rat hearts expressed reduced levels of nitro-tyrosine after 10 minutes reperfusion when compared to the control group (**Figure 4-5**). Although antioxidant mediated protection could have also played a significant role scavenging the released free radicals, we associated the attenuated expression of nitro-tyrosine in supplemented rat hearts with prompt inflammation response observed in this group. It is important to note that the control group also showed attenuated levels of nitro-tyrosine at 25 minutes reperfusion, the same time point that we observed an increase in IL-1β and IL-6 expression when compared to baseline expression level (**Figures 4-3a and 4-3c**).

Ability of several flavonoids (quercetin, rutin, baicalein, baicalin, apigenin, puerarin, (+)-catechin) to protect the rat heart from oxidative induced injury using nitrite-glucose-glucose oxidase system *in vitro,* has been reported previously (Lu *et al.,* 2009). In these studies tyrosine protein nitration was initiated by applying nitrite and glucose oxidase into the heart homogenate without exogenous hemoproteins. The study reported the effectiveness of several flavonoids on protein nitration, with apigenin reported as the most effective of them all, followed by quercetin, rutin, baicalin and puerarin, and least of the group was baicalein and (+)-catechin. It was reported that the protective effects of these flavonoids on protein nitration were closely related to their antioxidant activities, whereas their protective effectiveness on protein oxidation was almost proportional to their antioxidant activities.

5.5: Effects of dietary *Garcinia kola* supplementation on inducible enzymes

Cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) enzymes are implicated in mechanisms by which IRI-induced inflammation catalyse the rapid and increased production of prostaglandin and NO molecules respectively (Kim *et al.,* 2005). Both molecules at high concentrations are associated with risks of pro-atherosclerosis and further inflammation activation contributing to further tissue damage. In our study we did not observe any significant difference in iNOS expression between the two groups. We also didn't observe a significant change after reperfusion (**Figure 4-6**). We attempted to detect the expression of COX-2 using Western blot method (**Figure 4-7**), however, our samples appeared to express COX-2 levels below detectable limits for our method of choice.

Our results are in agreement with several other COX-2 expression profiles previously described in other tissues including the heart (Shinmura *et al.,* 2000), kidney (Slimane *et al.,* 2002) and skeletal muscle (Dupouy *et al.,* 2006). In these studies the levels of COX-2 mRNA was undetectable or faintly detectable, but protein expression was not detectable. In addition, statistically detectable levels of COX-2 mRNA were only evident 6 to 10 hours post-IRI. Similar to COX-2 expression mechanisms, Hangai and co-workers (1996) demonstrated that iNOS mRNA expression doesn't show significant change immediately after IRI, however,

expression rapidly increases after 6 hours reaching peak 12 hours after reperfusion injury in rat retina.

5.6: Effects of dietary *Garcinia kola* supplementation on nuclear factor kappa beta (NF-kβ65) proteins expression

Major mechanisms underlying inflammatory response are thought to revolve around control of the NF-kβ transcription factor. Our results show that RelA (NF-kβ65) protein expression was significantly higher in supplemented rat hearts at baseline and 25 minutes reperfusion when compared to the control group. No significant difference was observed at 10 minutes reperfusion between the two groups (**Figure 4-8**). Beg and Baltimore (1996) demonstrated that NF-kβ65 transcription factor is important for lymphocytes response to TNF-α induced gene expression. They showed that treatment of RelA transgenic (RelA+/+, RelA +/- and RelA-/-) embryonic fibroblast and macrophages with TNF-α resulted in a significant reduction in cell viability in RelA (-/-), followed by RelA (+/-), whereas RelA (+/+) cells were unaffected. To validate this observation, reintroduction of RelA into RelA (-/-) fibroblasts resulted in enhanced cell survival, demonstrating that the presence of RelA is required for protection from TNF-α induced cell death. We also observed that *Garcinia kola* supplemented rat hearts expressed lower levels of NF-kβ65 at the two reperfusion time points when compared to the baseline expression. This decrease may be due to transcriptional activation and subsequent modification, hence we recommend further investigation on NF-kβ65 transcriptional activation.

Activation of the NF-kβ transcription factors by inhibitor kappa beta kinase (IKK) subunits have demonstrated opposing, but complementary roles, controlling inflammatory response and innate immunity (Lawrence *et al*., 2005). Recently evidence indicates that IKKβ protein represents a potential target for strategies aiming at regulation of NF-kβ activation and expression of pro-inflammatory genes. In our study we demonstrated that dietary *Garcinia kola* supplementation attenuated expression of IKKβ in isolated rat hearts when compared to control at baseline and 10 minutes of reperfusion (**Figure 4-9**). Further, expression did not change significantly in the supplemented group after reperfusion when compared to the baseline time point. To support our data Moss and co-workers (2007) demonstrated

that inhibition of IKKβ provides both acute and delayed cardio-protection in C57BL/6 mice following acute IRI. In this study they showed that inhibition of IKKβ significantly reduced left ventricular infarct size, preserved cardiac function and decreased NF-kβ associated protein expression.

It has been demonstrated that IKKα respond to TNF-α stimuli and translocate to the nucleus where it binds with creb binding protein (CBP) at ser1382 and ser1386 to phosphorylate histone H3 at ser10 (Yamamoto *et al.,* 2003), hence switching the binding preference of CBP from p53 to NF-kβ, thus enhancing expression of NF-kβ mediated gene and suppressing p53-mediated gene expression (Huang *et al.,* 2007). Evidence supports that NF-kβ pathway promotes resistance to apoptosis (Beg and Baltimore, 1996), whereas P53 pathway is associated with promotion of apoptosis (Amaral *et al.,* 2010). In addition, IKKα has been demonstrated to contribute in control of inflammatory mediated gene transcription through phosphorylation of RelA at ser536, as such facilitating the turnover of RelA and c-Rel in response to TNF-α stimulation (Lawrence *et al.,* 2005). Expression of IKKα was significantly higher in supplemented rat hearts at 25 minutes reperfusion when compared to control group at the same time point (**Figure 4-10**). Both groups showed a significant increase between baseline time point and 25 minutes reperfusion time points.

Attempts to detect the expression of phosphorylated IKKα/β using Western blot method (**Figure 4-11**) were unsuccessful. Detection of phosphorylated IKK subunits is complicated by the expression time of the proteins following activation. Kupfer and Scheinman (2002) used the kinetic assay to observe the kinetics of T-cell receptor (TCR) mediated activation of IKK subunits in primary rat T-cells. The study demonstrated that TCR litigation induces maximal IKK activity after 5 minutes of stimulation and this activity rapidly diminishes to background levels. These data suggest that IKK is closely linked to the rapid phosphorylation and dephosphorylation.

5.7: Effects of dietary *Garcinia kola supplementation* on protein kinase B/Akt signaling

Activation of Akt, a downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway, is well known to promote cell survival following cellular injury (Fujio

et al., 2000; Mockridge *et al.,* 2000; Matsui *et al.,* 2001). Our results show that dietary *Garcinia kola* supplementation promptly increases Akt phosphorylation following IRI when compared to the control group (**Figure 4-12**). It has been demonstrated that activation of Akt at ser473 and Thr308 mediates activation and expression of several cytosolic and nucleic proteins involved in regulation of cardiac growth, contractile function and angiogenesis (Liao and Hung, 2010). In one study Katengua-Thamahane and colleagues (2012) demonstrated that addition of A6730 inhibitor (Akt phosphorylation inhibitor) to perfusate fluid, partially attenuates functional recovery in control and RPO supplemented rat hearts during IRI when compared to rat groups perfused in the absence of the inhibitor. Therefore, these results support that increased activation of Akt may play a significant role in cardio- protection and functional recovery during induced-IRI.

5.8: Effects of dietary *Garcinia kola supplementation* on p38 mitogen activated protein kinase (MAPK) signaling

In cardiomyocytes the p38 MAPK is thought to be primarily activated in response to both G-protein-coupled receptor activation and stress stimulation, therefore increased phosphorylated p38 MAPK is associated with induced stress. Furthermore, the p38α MAPK isoform in the heart have been linked to induced apoptosis, therefore attenuated levels are associated with potential pro-survival mechanisms. To support this observation Ma and co-workers (1999) have demonstrated that phosphorylation of p38 MAPK plays a vital role in the signal transduction pathway mediating post-ischaemic myocardial apoptosis and that inhibition of p38 MAPK activation attenuates IRI. Additional evidence of the opposing effects of p38α and p38β MAPK was demonstrated by Kim and co-workers (2006). They reported that inhibiting activation of the alpha isoform significantly prevents cell death while dominant negative p38β-MAPK significantly reverses the survival effects of myocytes following injury. Other studies also support that attenuation of the alpha isoform limits myocardial injury during reperfusion (Ren *et al.,* 2005; Clark *et al.,* 2007).

Our results show that the ratio of phosphorylated p38 MAPK and p38α MAPK were attenuated significantly, in supplemented rat hearts at baseline and at 25 minutes reperfusion when compared to the control group (**Figure 4-13b and c**).

Furthermore, the ratio of p38α MAPK only demonstrated a significant decrease between baseline expression level and 25 minutes reperfusion level in the supplemented group, with no significant difference observed between the baseline and 10 minutes reperfusion of the same group. However, in the control group, ratio of p38α MAPK significantly decreased at 10 minutes reperfusion and increased at 25 minutes reperfusion when compared to the baseline expression level (**Figure 4-13c)**. The change in the ratio of p38α MAPK showed a direct relation to the change in total p38 MAPK expression in both groups. An increase in total p38 MAPK resulted in a decrease in p38α MAPK in both groups, therefore this results suggest that the increased p38 MAPK could have been mainly due to expression of the other isoforms increasing levels of total p38 MAPK, hence resulting in a decreased ratio of the alpha isoform (**Figure 4-13a and 4-13c**). Stimulation ofl eukocytes by pro-inflammatory cytokines is known to result in the activation of the MAPK p38. Furtheremore, p38 MAPK has been demonstrated to play a central role in the regulation of a variety of inflammatory responses such as expression of pro-inflammatory mediators, leukocyte adhesion, migration and effector functions such as oxidative burst and degranulation (Herlaar and Brown, 1999).We proposed that the observed increase in total p38 MAPK in the control group observed at 10 minutes reperfusion could have resulted from the rat adaptive immune response to tissue injury.

While inhibition of p38 MAPK activation is widely thought to be cardio-protective, several studies have reported beneficial effects of transient activation of the p38 MAPK during ischaemia in a mechanism termed ischaemia preconditioning (IPC) (Maulik *et al.,* 1996). Weinbrenner and co-workers (1997) demonstrated that transient activation of the P38 MAPK correlates with myocardial survival in ischaemia preconditioned rabbit hearts. To validate their observation, they showed that p38 MAPK inhibitor SB203580 completely abolishes the protective mechanism of IPC, whereas anisomycin, a p38 MAPK activator, mimicked IPC protection in non-IPC rabbit hearts. This data may support our observation at 10 minutes reperfusion between the two groups. We argue that the sustained activated p38 MAPK in the supplemented rat heart group between baseline and 10 reperfusion time points may have contributed to the enhanced functional recovery, supported by the decreased –cleaved caspase-3 ratio at 10 minutes reperfusion (**Figure 4-14**).

In addition, Mockridge and colleagues (2000) reported that activation of p38 MAPK appears to be involved in maintaining Akt activation during injury. They showed that blocking p38 MAPK activation using SB203580 inhibitor partially inhibited Akt activation. This data supports that control and balancing of signaling proteins may play a vital role in cardiomyocytes survival post IRI. We argue that though ratio of phosphorylated p38 MAPK was significantly lower at 10 minutes reperfusion in the control group. The sustained activation of p38 MAPK in the supplemented rat hearts at 10 minutes reperfusion followed by a decrease observed at 25 minutes reperfusion (**Figure 4-13c**), may contribute significantly to myocardial protection, potentially using mechanisms similar to those of IPC and sustained Akt activation upto the 25 minutes reperfusion time point.

5.9: Effects of dietary *Garcinia kola supplementation* on PARP-1 and caspase-3 proteins

In physiological conditions PARP-1 is well established as a nuclear chromatin-bound DNA repair enzyme, catalysing transfer of ADP-ribose moieties from NAD^+ to acceptor DNA binding proteins, however, under pathophysiological conditions such as IRI and inflammation, increased PARP-1 activation is associated with increased energy consumption, mitochondrial dysfunction and ultimately necrotic cell death (Virag and Szabo, 2002; Toth-Zsamboki *et al.,* 2006). Our data shows a significant increase in PARP-1 expression (uncleaved) after reperfusion when compared to the control group for both 10 and 25 minutes reperfusion (**Table 4-3**), however, cleaved PARP-1 expression did not differ significantly between the two groups before and after IRI (**Table 4-3**).

PARP-1 and caspase-3 activation are widely associated with apoptotic cell death and increased infarct size after IRI (Condorelli *et al.,* 2002). Our results show that ratio of activated caspase-3 was significantly decreased in supplemented rat hearts after 10 minutes reperfusion when compared to the control group (**Figure 4-14**). Yaoita and colleagues (1998) demonstrated that inhibition of caspase activation reduces myocardial reperfusion injury.

5.10: Effects of dietary *Garcinia kola supplementation* on DNA damage to rat lymphocytes

Evidence indicates that certain polyphenolic flavonoids generally considered as antioxidants and anti-carcinogens, also possess pro-oxidant and pro-carcinogenic properties, suggesting their possible dual role in mutagenesis and carcinogenesis (Babich *et al.,* 2011). It has been reported that quercetin and kaempferol induces single strand DNA breaks in isolated liver nuclei (Sahu and Washington, 1991; Sahu and Gray, 1994). Few studies have reported on the potential genotoxicity and cytotoxicity of dietary flavonoids in *in vivo* experimental models. In this study we have shown that supplementation with *Garcinia kola* did not induce significant changes in the lymphocytes DNA tail parameters lymphocytes (**Figure 4-15**) after four weeks of supplementation when compared to the control group. These results suggest that our daily dose of dietary *Garcinia kola* supplementation did not induce *in vivo* lymphocytic DNA damage.

CHAPTER 6: CONCLUSION

In support of our results, several clinical studies have linked decreased expression levels of inflammatory cytokines in plasma (Testa *et al.,* 1996; Torre-Amione *et al.,* 1996; Tsutamoto *et al.,* 1998) and myocardium (Devaux *et al.,* 1997) with reduced risks of atherosclerotic plaque formation and instability. Furthermore, chronic expression of inflammatory mediators is emerging as one of the important tools used in the prognosis of myocardial remodeling and heart failure (Testa *et al.,* 1996; Torre-Amione *et al.,* 1996; Ono *et al.,* 1998 Tsutamoto *et al.,* 1998). Increased circulating levels are associated with poor prognosis, influencing heart contractility by inducing hypertrophy and promoting apoptosis/ fibrosis post-myocardial injury (Torre-Amione *et al.,* 1996; Tsutamoto *et al.,* 2000; Li *et al.*, 2006; Sun *et al.,* 2012).

Our results demonstrated that *Garcinia kola* supplemented rat hearts promptly respond to ischaemia and reperfusion injury through different mechanisms, involving acute inflammatory response and decreased protein nitrosylation. Our observation of improved cardiac functional recovery following ischaemia and reperfusion injury (IRI) was associated with the anti-inflammatory and antioxidant potency of *Garcinia kola*. We have shown for the first time that dietary *Garcinia kola* supplementation modulates cardiac protein signaling before and after induced IRI.

In summary, we have shown that dietary *Garcinia kola* supplementation improves cardiac functional recovery and may offer cardiac protection during ischaemia and reperfusion injury through acute inflammation response, Akt and p38 MAPK pathways protein signaling. We propose that dietary *Garcinia kola* supplementation offer cardiac protection during ischaemia and reperfusion through prompt activation of the anti-apoptotic Akt protein and attenuated/delayed expression of pro-apoptotic p38 MAPK proteins. Furthermore, our results show that dietary *Garcinia kola* supplementation modulates expression of NF-kβ pathway proteins, hence we suggest that dietary *Garcinia kola* supplementation limit the release of free

radicals and/or promptly scavenge the free radicals produced, limiting their time span and deleterious effects in the myocardium.

6.1: Study Limitation and Recommendations

This study raised several interesting observations that may need further investigations. We could only speculate to the level of protein expression in the NF-kβ pathways and the possible effects of *Garcinia kola* in atherosclerotic plaque formation. We propose further investigation to elucidate on the effects of *Garcinia kola* on transcriptional activation and also in the atherosclerotic experimental model. The findings of this study also speculated on the mechanism of acute inflammation and its potential regulation points. Further investigations are needed to validate our speculations using chronic inflammatory models.

References

Adaramoye OA, Nwaneri VO, Anyanwu KC, Farombi EO, Emerole GO. Possible anti-atherogenic effect of kolaviron (a *Garcinia kola* seed extract) in hypercholestrolaemic rats. *Clin Exp Pharmacol Physiol*. 2005; 32:40-6.

Adaramoye OA. Protective effect of kolaviron, a biflavonoid from *Garcinia kola* seeds, in brain of Wistar albino rats exposed to gamma-radiation. *Biol Pharm Bull.* 2010; 33(2):260-6.

Adaramoye OA, Adeyemi EO. Hypoglycaemic and hypolipidaemic effects of fractions from kolaviron, a biflavonoid complex from *Garcinia Kola* in streptozotocin-induced diabetes mellitus rats. *J Pharm Pharmacol.* 2006; 58(1):121-8.

Adaramoye OA, Akanni OO, Farombi EO. Nevirapine induces testicular toxicity in Wistar rats: reversal effect of kolaviron (biflavonoid from *Garcinia kola* seeds). *J Basic Clin Physiol Pharmacol.* 2012a; 8:1-8.

Adaramoye OA, Adedara IA, Farombi EO. Possible ameliorative effects of kolaviron against reproductive toxicity in sub-lethally whole body gamma-irradiated rats. *Exp Toxicol Pathol.* 2012b; 64(4):379-85.

Adegbehingbe OO, Adesanya SA, Idowu TO, Okimi OC, Oyelami OA, Iwalewa EO. Clinical effects of *Garcinia kola* in knee Osteoarthritis. *J Orthop Surg*. 2008;3:34

Adegboye MF, Akinpelu DA, Okoh AI. The bioactive and phytochemical properties of *Garcinia kola* (Heckel) seed extract on some pathogens. *Afr J Biotechnol.* 2008; 7 (21):3934-38.

Aitken RJ, Baker MA. Oxidative stress and male reproductive biology. *Reprod Fertil Dev*. 2004; 16(5):581-8.

Ajani EO, Shallie PD, Adegbesan BO, Salau BA, Adesanya M. Protective effect of *Garcinia kola* (kolaviron) extract on predisposition of rats to cardiovascular diseases following separate administration of amodiaquine and artesunate. *Afr J Tradit Complement Altern Med*. 2008; 5(2):180-6.

Aluka. In: Entry for Garcinia kola Heckel (Guttiferae), the useful plants of West Tropical Africa. Burkill, HM. 1985; Vol 2.

Amaral JD, Xavier JM, Steer CJ, Rodrigues CM. The role of p53 in apoptosis. *Discov Med*. 2010; 9(45):145-52.

Amaral S, Oliveira PJ, Ramalho-Santos J. Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. *Curr Diabetes Rev*. 2008; 4(1):46-54.

Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation.* 2010; 121(1):80-90.

Ayensu ES. Medicinal plants of West Africa. Algonac: Reference Publications Inc.; 1978 pp162.

Babich H, Schuck GA, Weisburg JH, Zuckerbraun HL. “Research Strategies in the Study of the Pro-Oxidant Nature of Polyphenol Nutraceuticals.” *J Toxicol.* 2011; Article ID 467305:12.

Badade ZG, Samant PM. Role of Oxidative Stress in Male Infertility.*J Biomed Sci and Res.* 2011; 3 (2):385-91.

Baichwal VR, Baeuerle PA. Activate NF-kappa B or die? *Curr Biol*. 1997; 7(2):R94-6.

Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. *Vet Med Int*. 2010; 2010 (Pii): 686137.

Barancik M, Htun P, Strohm C, Kilian S, Schaper W. Inhibition of the cardiac p38-MAPK pathway by SB203580 delays ischemic cell death. *J Cardiovasc Pharmacol.* 2000; 35(3):474-83.

Bassi R, Heads R, Marber MS, Clark JE. Targeting p38-MAPK in the ischaemic heart: kill or cure? *Curr Opin Pharmacol.* 2008; (2):141-6.

Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV. Calcium elevation in mitochondria is the main Ca2+ requirement for mitochondrial permeability transition pore (mPTP) opening. *J Biol Chem*. 2009; 284(31):20796-803.

Beecher GR. Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake. *J. Nutr*. 2003; 133(10): 3248S-3254S.

Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science*. 1996; 274(5288):782-4.

Behrendt D, Ganz P. Endothelial function. From vascular biology to clinical applications. *Am J Cardiol.* 2002; 90(10C):40L-48L.

Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. *J Immunol.* 2002; 168(12):6404-11.

Bester D, Esterhuyse AJ, Truter EJ, van Rooyen J. Cardiovascular effects of edible oils: a comparison between four popular edible oils. *Nutr Res Rev.* 2010; 23(2):334-48.

Bianchini F, Elmstahl S, Martinez-Garcia C, van Kappel AL, Douki T, Cadet J, Ohshima H, Riboli E, Kaaks R. Oxidative DNA damage in human lymphocytes: correlations with plasma levels of alpha-tocopherol and carotenoids. *Carcinogenesis*. 2000; 21(2):321-4.

Bohm H, Boeing H, Hempel J, Raab B, Kroke A. *Flavonols*, *flavone* and *anthocyanins* as natural antioxidants of food and their possible role in the prevention of chronic diseases. *Z Ernahrungswiss*. 1998; 37(2):147-63.

Bolli R. Mechanism of myocardial "stunning".*Circulation*. 1990; 82(3):723-38.

Boots AW, Guido Haenen RMM, Aalt Bast. Health effects of quercetin: From antioxidant to nutraceutical. *Eur J Pharmacol.* 2008; 585: 325-37.

Bouayed J, Bohn T. Exogenous antioxidants - Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses: *Oxid Med Cell Longev.* 2010; 3(4):228-237.

Bowden RD, Buckwalter MR, McBride JF, Johnson DA, Murray BK, O'Neill KL. Tail profile: a more accurate system for analyzing DNA damage using the Comet assay. *Mutat Res.* 2003; 537(1):1-9.

Bradford, MM. A dye binding assay for protein. *Anal. Biochem.*1976; 72:248-54.

Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol*. 2004; 24(11):2137-42.

Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev.* 1998; 56(11):317-33.

Brunelli S, Rovere-Querini P. The immune system and the repair of skeletal muscle. *Pharmacol Res*. 2008; 58(2):117-21

Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol.* 2001; 22(4):199-204.

Buja LM, Willerson JT. Role of inflammation in coronary plaque disruption. *Circulation*. 1994; 89(1):503-5.

Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem.* 1981; 112(2):195-203.

Butterfield TA, Best TM, Merrick MA. The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. *J Athl Train.* 2006; 41(4):457-65.

Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev.* 2011; 75(1):50-83.

Chaanine AH, Hajjar RJ. AKT signaling in the failing heart. *Eur J Heart Fail.* 2011; 13(8):825-9.

Chemla D, Antony I, Zamani K, Nitenberg A. Mean aortic pressure is the geometric mean of systolic and diastolic aortic pressure in resting humans. *J Appl Physiol.* 2005; 99(6):2278-84.

Chiong M, Wang ZV, Pedrozo Z, Cao DJ, Troncoso R, Ibacache M, Criollo A, Nemchenko A, Hill JA, Lavandero S. Cardiomyocyte death: mechanisms and translational implications*. Cell Death Dis.* 2011; 2:e244.

Clark JE, Sarafraz N, Marber MS. Potential of p38-MAPK inhibitors in the treatment of ischaemic heart disease. *Pharmacol Ther*. 2007; 116(2):192-206.

Closa D, Folch-Puy E. Oxygen free radicals and the systemic inflammatory response. *IUBMB Life.* 2004; 56(4):185-91.

Collard C, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia reperfusion injury. *Anesthesiology* 2001;94:1133– 8.

Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol.* 2004; 26(3):249-61.

Comalada M, Ballester I, Bailon E, Sierra S, Xaus J, Galvez J, Sanchez de Medina F, Zarzuelo A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure–activity relationship*. Biochem Pharmacol.* 2006; 72:1010-21.

Condorelli G, Morisco C, Latronico MV, Claudio PP, Dent P, Tsichlis P, Condorelli G, Frati G, Drusco A, Croce CM, Napoli C. TNF-alpha signal transduction in rat neonatal cardiac myocytes: definition of pathways generating from the TNF-alpha receptor. *FASEB J*. 2002; 16(13):1732-7.

Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res.* 2004; 95(10):957-70.

Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004; 109(23 Suppl 1):III27-32.

Decker EA. Phenolics: prooxidants or antioxidants? *Nutr Rev*. 1997; 55(11 Pt 1):396-8.

Depre C. Isolated working heart: description of models relevant to radioisotopic and pharmacological assessments. *Nucl Med Biol.* 1998; 25(8):711-3.

Devaux B, Scholz D, Hirche A, Klövekorn WP, Schaper J. Upregulation of cell adhesion molecules and the presence of low grade inflammation in human chronic heart failure. *Eur Heart J*. 1997; 18(3):470-9.

Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, Michael LH, Rollins BJ, Entman ML, Frangogiannis NG. CCL2/Monocyte Chemoattractant Protein-1 regulates inflammatory responses critical to healing myocardial infarcts. *Circ Res.* 2005; 96(8):881-9.

Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc Res*. 2000; 47(3):446-56.

Di Lisa F, Menabo R, Canton M, Petronilli V. The role of mitochondria in the salvage and the injury of the ischemic myocardium. *Biochim Biophys Acta*. 1998; 1366(1-2):69-78.

Di Lisa F, Bernardi P. Mitochondria and ischemia-reperfusion injury of the heart: fixing a hole. *Cardiovasc Res*. 2006; 70(2):191-9.

Duchen MR. Roles of mitochondria in health and disease. *Diabetes.* 2004; 53(1):S96-102.

Dupouy VM, Ferre PJ, Uro-Coste E, Lefebvre HP. Time course of COX-1 and COX-2 expression during ischemia-reperfusion in rat skeletal muscle. *J Appl Physiol.* 2006; 100(1):233-9.

Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Jan Cramer M, Lips DJ, Doevendans PA. Role of apoptosis in reperfusion injury. *Cardiovasc Res*. 2004; 61(3)414-26.

Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature*. 1998; 391(6665):393-7.

Eleazu CO, Eleazu KC, Awa E, Chukwuma SC. Comparative study of the phytochemical composition of the leaves of five Nigerian medicinal plants. *JBPR.* 2012; 3(2):42-46.

Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res.* 2003; 59(1):234-40.

Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. *Br Med Bull.* 2004; 70:71-86.

Entman ML, Smith CW. Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. *Cardiovasc Res.* 1994; 28(9):1301-11.

Erdem G, Oner C, Onal AM, Kisakurek D, Ogus AY. Free radical mediated interaction of ascorbic acid and ascorbate/Cu (II) with viral and plasmid DNAs. *J Biosci*.1994;19(1): 9-17.

ESCODD (European Standards Committee on Oxidative DNA Damage). Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. *Free Radic Res.* 2000; 32:333–41.

Esterhuyse AJ, Toit ED, Rooyen JV. Dietary red palm oil supplementation protects against the consequences of global ischemia in the isolated perfused rat heart. *Asia Pac J Clin Nutr*. 2005; 14(4):340-7.

Esterhuyse JS, van Rooyen J, Strijdom H, Bester D, du Toit EF. Proposed mechanisms for red palm oil induced cardioprotection in a model of hyperlipidaemia in the rat. *Prostaglandins Leukot Essent Fatty Acids*. 2006; 75(6):375-84.

FAO /WHO: Human Vitamin and Mineral Requirements. Report of a Joint FAO/WHO Expert Consultation, Bangkok. WHO/FAO, Rome, 2002.

Farombi EO, Tahnteng JG, Agboola O, Nwankwo JO, Emerole GO. Chemoprevention of 2-acetyl aminofluorene- induced hepatotoxicity and lipid peroxidation in rats by kolaviron-A *Garcinia kola* seed extract. *Food Chem Toxicol.* 2000a; 38: 535–41.

Farombi EO. Mechanisms for the hepatoprotective action of kolaviron: studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbontetrachloride-treated rats. *Pharmacol Res*. 2000b; 42(1):75-80.

Farombi EO, Adepoju BF, Ola-Davies OE, Emerole, G.O. Inhibition of Aflatoxin B1-induced clastogenicity and hepatocarcinogenicity by kolaviron (*Garcinia biflavanones*) in rats. *Mutat Res.* 2001*;* 483:S106.

Farombi EO, Akanni O, Emerole GO. Antioxidant and scavenging activities of kolaviron in vitro. *Pharm Biol.* 2002; 40:107–16.

Farombi EO, Moller P, Dragsted LO. *Ex vivo* and *in vitro* protective effects of kolaviron against oxygen-derived radical-induced DNA damage and oxidative stress in human lymphocytes and rat liver cells. *Cell Biol Toxicol.* 2004a; 20: 1315–22.

Farombi EO, Hansen M, Rav-Haren G, Moller P, Dragsted LO. Commonly consumed and naturally occurring dietary substances affect biomarkers of oxidative stress and DNA damage in healthy rats. *Food Chem Toxicol.* 2004b; 42(8):315-22.

Farombi EO, Adepoju BF, Ola-Davie OE, Emerole GO. Chemoprevention of aflatoxinb1- induced genotoxicity and hepatic oxidative damage in rats by kolaviron, a natural bioflavonoidof *Garcinia kola* seed. *Eur J Cancer Prev*. 2005a;14:207-14.

Farombi EO, Nwaokeafor IA. Anti-oxidant mechanisms of kolaviron: studies on serum lipoprotein oxidation, metal chelation and oxidative membrane damage in rats. *Clin Exp Pharmacol Physiol.* 2005; 32: 667-74.

Farombi EO. Genotoxicity of chloroquine in rat liver cells: protective role of free radical scavengers. *Cell Biol Toxicol.* 2006; 22:159-67.

Farombi EO, Abarikwu SO, Adedara IA, Oyeyemi MO. Curcumin and Kolaviron Ameliorate Di-n-Butylphthalate-Induced Testicular Damage in Rats. *Basic Clin Pharmacol Toxicol.* 2007; 100: 43–48.

Farombi EO, Shrotriya S, Surth YJ. Kolaviron inhibits dimethyl nitrosamine-induced liver injury by suppressing COX-2 and iNOS expression via NF-κβ and AP-1. *Life Science.* 2009; 84:149-55.

Farombi EO, Owoeye O. Antioxidative and Chemopreventive Properties of *Vernonia amygdalina* and *Garcinia biflavonoid*. *Int. J. Environ Res Public Health.* 2011; *8*: 2533-55.

Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. *Front Biosci.* 1997; 2:d12-26.

Ferdinandy P, Schulz R. Peroxynitrite: toxic or protective in the heart? *Circ Res*. 2001; 88 (2):E12-3.

Ferrari R, Ceconi C, Curello S, Cargnoni A, Pasini E, De Giuli F, Albertini A. Role of oxygen free radicals in ischemic and reperfused myocardium. *Am J Clin Nutr*. 1991; 53(1 Suppl):215S-22S.

Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science.* 1992;257(5068):387-9.

Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002; 53(1):31-47.

Frangogiannis NG. The immune system and cardiac repair. *Pharmacol Res*. 2008; 58(2):88-111.

Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. *Circ Res.* 2012; 110(1):159-73.

Franzoso G, Carlson L, Poljak L, Shores EW, Epstein S, Leonardi A, Grinberg A, Tran T, Scharton-Kersten T, Anver M, Love P, Brown K, Siebenlist U. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J Exp Med*. 1998; 187(2):147-59.

Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation.* 2000; 101(6):660-7.

Fujisawa S, Ishihara M, Atsumi T, Kadoma Y. A quantitative approach to the free radical interaction between alpha-tocopherol or ascorbate and flavonoids. *In Vivo*. 2006; 20(4):445-52.

Galle J, Quaschning T, Seibold S, Wanner C. Endothelial dysfunction and inflammation: what is the link? *Kidney Int Suppl*. 2003; (84):S45-9.

Garcia-Dorado D. Myocardial reperfusion injury: a new view. *Cardiovasc Res*. 2004; 61(3):363-4.

Garcia-Lafuente A, Guillamon E, Villares A, Rostagno MA, Martinez JA. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res*. 2009; 58(9):537-52.

Geraets L, Haegens A, Brauers K, Haydock JA, Vernooy JH, Wouters EF, Bast A, Hageman GJ. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. *Biochem Biophys Res Commun*. 2009; 382(3):598-603.

Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. 2002; 109 Suppl: S81-96.

Gillespie S, Gavins FN. Phytochemicals: countering risk factors and pathological responses associated with ischaemia reperfusion injury. *Pharmacol Ther*. 2013; 138(1):38-45.

Goldhaber JI, Weiss JN. Oxygen free radicals and cardiac reperfusion abnormalities. *Hypertension*. 1992; 20:118–27.

Granger DN, Senchenkova E. Inflammation and the Microcirculation. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Chapter 10, Endothelial Barrier Dysfunction. *Available from:http://www.ncbi.nlm.nih.gov/books/NBK53374/*

Grootjans J , Lenaerts K, Derikx JPM, Matthijsen RA, de Bruine AP, van Bijnen AA, Ronald M. van Dam RM, Dejong CHC, Buurman WA . Human Intestinal Ischemia-Reperfusion–Induced Inflammation Characterized, Experiences from a New Translational Model*. Am J Pathol*. 2010; 176(5):2283–91.

Guide for the Care and Use of Laboratory Animals, 8th edition. National Research Council. Washington (DC): National Academies Press (US); 2011.

Gurantz D, Cowling RT, Villarreal FJ, Greenberg BH. Tumor necrosis factor-alpha upregulates angiotensin II type 1 receptors on cardiac fibroblasts. *Circ Res*. 1999; 85(3):272-9.

Gurantz D, Cowling RT, Varki N, Frikovsky E, Moore CD, Greenberg BH. IL-1beta and TNF-alpha upregulate angiotensin II type 1 (AT1) receptors on cardiac fibroblasts and are associated with increased AT1 density in the post-MI heart. *J Mol Cell Cardiol*. 2005; 38(3):505-15.

Gustafsson AB, Gottlieb RA. Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol.* 2007; 292(1):C45-51.

Guzik TJ, Harrison DG. Vascular NADPH oxidases as drug targets for novel antioxidant strategies. *Drug Discov Today*. 2006; 11(11-12):524-33.

Gysembergh A, Simkhovich BZ, Kloner RA, Przyklenk K. p38 MAPK activity is not increased early during sustained coronary artery occlusion in preconditioned versus control rabbit heart. *J Mol Cell Cardiol.* 2001; 33(4):681-90.

Hadi HA, Carr CS, Al Suwaidi J. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc Health Risk Manag.* 2005; 1(3):183-98.

Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Arch Biochem Biophys.* 2008; 476(2):107-12.

Hammer M, Mages J, Dietrich H, Schmitz F, Striebel F, Murray PJ, Wagner H, Lang R. Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *Eur J Immunol.* 2005; 35(10):2991-3001.

Hamosh P, Cohn JN. Left ventricular function in acute myocardial infarction. *J Clin Invest*. 1971; 50(3):523-33.

Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev*. 2002; 13(4-5):413-21.

Hangai M, Yoshimura N, Hiroi K, Mandai M, Honda Y. Inducible nitric oxide synthase in retinal ischemia-reperfusion injury. *Exp Eye Res*. 1996; 63(5):501-9.

Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immuno*l. 2006; 6(7):508-19.

Hayden MS, Ghosh S. Signaling to NF-kappa B. *Genes Dev.* 2004; 18(18):2195-224.

Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *J Nutr Biochem.* 2002; 13:572–84.

Herlaar E, Brown Z. p38 MAPK signaling cascades in inflammatory disease. *Mol Med Today*. 1999; 5(10):439-47.

Hers I, Vincent EE, Tavare JM. Akt signaling in health and disease. *Cell Signal.* 2011; 23(10):1515-27.

Herskowitz A, Choi S, Ansari AA, Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. *Am J Pathol.* 1995;146(2):419-28.

Hirano R, Sasamoto W, Matsumoto A, Itakura H, Igarashi O, Kondo K. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. *J Nutr Sci Vitaminol*. 2001; 47(5):357-62.

Hoffman JW Jr, Gilbert TB, Poston RS, Silldorff EP. Myocardial reperfusion injury: etiology, mechanisms, and therapies. *J Extra Corpor Technol*. 2004; 36(4):391-411.

Holzheimer RG, Gross J, Schein M. Pro- and anti-inflammatory cytokine-response in abdominal aortic aneurysm repair: a clinical model of ischemia-reperfusion. *Shock.* 1999; 11(5):305-10.

Hougee S, Sanders A, Faber J, Graus YM, van den Berg WB, Garssen J, Smit HF, Hoijer MA. Decreased pro-inflammatory cytokine production by LPS-stimulated PBMC upon in vitro incubation with the flavonoids apigenin, luteolin or chrysin, due to selective elimination of monocytes/macrophages. *Biochem Pharmacol.* 2005; 69(2):241-8.

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110004F.pdf Accessed on 28102013 at 2300 hrs

http://www.kpl.com/catalog/productdetail.cfm?Catalog_ID=17&Category_ID=420&Product_ID=573. Accesse 05032014 at 2146hrs

Huang WC, Ju TK, Hung CM, Chen CC. Phosphorylation of CBP by IKKα promotes cell growth by switching the binding preference of CBP from p53 to NF-kB. *Mol Cell*. 2007; 26:75-87.

Igic R. The isolated perfused "working" rat heart: a new method*. J Pharmacol Toxicol Methods.* 1996; 35(2):63-7.

Inagaki-Ohara K, Hanada T, Yoshimura A. Negative regulation of cytokine signaling and inflammatory diseases. *Curr Opin Pharmaco*l. 2003; 3(4):435-42.

Inal ME, Kanbak G, Sunal E. Antioxidant enzyme activities and malondialdehyde levels related to aging. *Clin Chim Acta.* 2001; 305(1-2):75-80.

ISO/IEC 17025: 2005(E). General requirements for the competence of testing and calibration laboratories.

Iwu MM. Handbook of African Medical plants. Boca Raton, FL: CRC press; 1993. pp183

Jang YH, Lee YC, Park NH, Shin HY, Mun KC, Choi MS. Polyphenol (-)-epigallocatechin gallate protection from ischemia/reperfusion-induced renal injury in normotensive and hypertensive rats. *Transplant Proc.* 2006; 38:2190–94

Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF, Molkentin JD. Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion *in vivo*. *J Biol Chem*. 2004; 279(15):15524-30.

Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. *J Biol Chem.* 2001; 276(7):4527-30.

Karason K, Molgaard H, Wikstrand J, Sjostrom L. Heart rate variability in obesity and the effect of weight loss. *Am J Cardiol.* 1999; 83(8):1242-7.

Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene*. 1999; 18(49):6867-74.

Karin M, Delhase M. The Inhibitor kappa B kinase (IKK) and NF-kappa B: key elements of pro-inflammatory signaling. *Semin Immunol*. 2000; 12(1):85-98.

Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol.* 2002; 3(3):221-7.

Katengua-Thamahane E, Engelbrencht A, Esterhuyse AJ, Van Rooyen J. Inhibition of Akt attenuates RPO-induced cardioprotection. *Cardiol Res Pract*. 2012; Vol 2012, Article ID 392457, 9 pages.

Katz AM. Physiology of the Heart, 5th Edition. Philadelphia, USA: Wolters Kluwer/Lippincott Williams & Wilkins. 2011; 177-266

Kaur P, Welch WJ, Saklatvala J. Interleukin 1 and tumour necrosis factor increase phosphorylation of the small heat shock protein. Effects in fibroblasts, Hep G2 and U937 cells. *FEBS Lett*. 1989; 258(2):269-73.

Kennedy DO, Wightman EL. Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Adv Nutr*. 2011; 2(1):32-50.

Kim CJ, Kim JM, Lee SR, Jang YH, Kim JH, Chun KJ. Polyphenol (-)-epigallocatechin gallate targeting myocardial reperfusion limits infarct size and improves cardiac function. *Korean J Anesthesiol*. 2010; 58(2):169-75.

Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J Pharmacol Sci.* 2004; 96(3):229-245

Kim JK, Pedram A, Razandi M, Levin ER. Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isoforms. *J Biol Chem*. 2006; 281(10):6760-7.

Kim JW, Jin YC, Kim YM, Rhie S, Kim HJ, Seo HG, Lee JH, Ha YL, Chang KC. Daidzein administration in vivo reduces myocardial injury in a rat ischemia/reperfusion model by inhibiting NF-kappaB activation. *Life Sci.* 2009; 84(7-8): 227-34.

Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science*. 2005; 310(5756):1966-70.

Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol.* 2003; 23(4):656-60.

Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E. Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop. *Circulation*. 1998; 97(18):1848-67.

Krinsky NI. Mechanism of action of biological antioxidants. *Proc Soc Exp Biol Med.* 1992; 200(2): 248-54.

Krishna KA, Krishna KS, Berrocal R, Rao KS, Rao KRSS. Myocardial infarction and stem cells. *J Pharm Bioallied Sci*. 2011; 3(2):182–8.

Krueger JK, Gallagher SC, Zhi G, Geguchadze R, Persechini A, Stull JT, Trewhella J. Activation of myosin light chain kinase requires translocation of bound calmodulin. *J Biol Chem*. 2001; 276(7):4535-8.

Kumaravel TS, Vilhar B, Faux SP, Jha AN. Comet Assay measurements: a perspective. *Cell Biol Toxicol*. 2009; 25:53–64.

Kupfer R, Scheinman RI. Measurement of IKK activity in primary rat T cells: rapid activation and inactivation. *J Immunol Methods*. 2002; 266(1-2):155-64.

Kurien BT, Scofield RH. Western blotting. *Methods.* 2006; 38(4):283-93.

Kurokawa M, Kornbluth S. Caspases and kinases in a death grip. *Cell*. 2009; 138(5):838-54.

Lakshmi SV, Padmaja G, Kuppusamy P, Kutala VK. Oxidative stress in cardiovascular disease. *Indian J Biochem Biophys*. 2009; 46(6):421-40.

Lawrence T, Bebien M, Liu GY, Nizet V, Karin M. IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. *Nature.* 2005; 434(7037):1138-43.

Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol.* 2009; 1(6).

Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. *J Mol Cell Cardiol*. 2002; 34(5):509-18.

Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? *J Mol Cell Cardiol*. 2009a; 47(1):32-40.

Lecour S. Multiple protective pathways against reperfusion injury: a SAFE path without aktion? *J Mol Cell Cardiol.* 2009b; 46:607-9.

Lee S, Kim YJ, Kwon S, Lee Y, Choi SY, Park J, Kwon HJ. Inhibitory effects of flavonoids on TNF-alpha-induced IL-8 gene expression in HEK 293 cells. *BMB Rep*. 2009; 42(5):265-70.

Lefer AM, Tsao P, Aoki N, Palladino MA Jr. Mediation of cardioprotection by transforming growth factor-beta. *Science*. 1990; 249(4964):61-4.

Leifeld L, Fielenbach M, Dumoulin FL, Speidel N, Sauerbruch T, Spengler U. Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expression in fulminant hepatic failure. *J Hepatol*. 2002; 37(5):613-9.

Li Y, Takemura G, Okada H, Miyata S, Maruyama R, Li L, Higuchi M, Minatoguchi S, Fujiwara T, Fujiwara H. Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure. *Cardiovasc Res.* 2006; 71(4):684-94.

Liao Y, Hung MC. Physiological regulation of Akt activity and stability. *Am J Transl Res*. 2010; 2(1):19-42.

Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002; 105:1135-43.

Libby P. Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr*. 2006; 83(2):456S-460S.

Libby P, Okamoto Y, Rocha VZ, Folco E. Inflammation in atherosclerosis: transition from theory to practice. *Circ J*. 2010; 74(2):213-20.

Lin J, Zhang SM, Wu K, Willett WC, Fuchs CS, Giovannucci E. Flavonoid intake and colorectal cancer risk in men and women. *Am J Epidemiol.* 2006; 164(7):644-51.

Linfert D, Chowdhry T, Rabb H. Lymphocytes and ischemia-reperfusion injury. *Transplant Rev (Orlando)*. 2009; 23(1):1-10.

Literat A, Su F, Norwicki M, Durand M, Ramanathan R, Jones CA, Minoo P, Kwong KY. Regulation of pro-inflammatory cytokine expression by curcumin in hyaline membrane disease (HMD). *Life Sci.* 2001; 70(3):253-67.

Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr*. 2003; 78(3 Suppl):517S-520S.

Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev.* 2010; 4(8):118-26.

Loft S, Poulsen HE. Antioxidant intervention studies related to DNA damage, DNA repair and gene expression. *Free Radic Res.* 2000; 33:S67–83.

Loft S, Poulsen HE. Markers of oxidative damage to DNA: antioxidants and molecular damage. *Methods Enzymol*. 1999; 300:166–84.

LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Up dat*. 2008; 11(1-2):32-50.

Lu N, Zhang Y, Gao Z. Nitrite-glucose-glucose oxidase system directly induces rat heart homogenate oxidation and tyrosine nitration: effects of some flavonoids. *Toxicol In Vitro.* 2009; 23(4):627-33.

Luo X, Zuo X, Zhou Y, Zhang B, Shi Y, Liu M, Wang K, McMillian DR, Xiao X. Extracellular heat shock protein 70 inhibits tumour necrosis factor-alpha induced proinflammatory mediator production in fibroblast-like synoviocytes. Arthritis Res Ther. 2008;10(2):R41.

Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue TL. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation.* 1999; 99(13):1685-91.

Ma XL, Gao F, Lopez BL, Christopher TA, Vinten-Johansen J. Peroxynitrite, a two-edged sword in post-ischemic myocardial injury-dichotomy of action in crystalloid- versus blood-perfused hearts*. J Pharmacol Exp Ther*. 2000; 292(3):912-20.

Machado NG, Alves MG, Carvalho RA, Oliveira PJ. Mitochondrial involvement in cardiac apoptosis during ischemia and reperfusion: can we close the box? *Cardiovasc Toxicol.* 2009; 9(4):211-27.

Madubunyi II. "Mechanism of the Hepatoprotective Activities of the *Garcinia* Hydroxybiflavanonol, GB1." *JCIM*. 2010; 7.1

Maier HJ, Schips TG, Wietelmann A, Kruger M, Brunner C, Sauter M, Klingel K, Bottger T, Braun T, Wirth T. Cardiomyocyte-specific IκB kinase (IKK)/NF-κB activation induces reversible inflammatory cardiomyopathy and heart failure. *Proc Natl Acad Sci U S A*. 2012; 109(29):11794-9.

Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res*. 2001; 89(7):E41-5.

Mann DL. Mechanisms and models in heart failure: A combinatorial approach. *Circulation*. 1999; 100(9):999-1008.

Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. *Circ Res*. 2011; 108(9):1133-45

Maredza M, Tollman S, Hofman K. A hidden menace: Cardiovascular disease in South Africa and the costs of an inadequate policy response, *SA Heart*. 2011; 8(1): 48-55.

Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, Romanelli RG, Laffi G, Gentilini P. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am J Pathol.* 1998; 152(2):423-30.

Masella R, Di Benedetto R, Varì R, Filesi C, Giovannini C Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J Nutr Biochem*. 2005; 16(10):577-86.

Mates JM, Perez-Gomez C, Nunez de Castro I. Antioxidant enzymes and human diseases. *Clin Biochem.* 1999; 32(8):595-603.

Mathers CD, Truelson T, Begg S, Satoh T: Global burden of ischemic heart disease in the year 2000. In *Global Burden of Disease 2000*. Geneva: World Health Organization; 2004.

Mathers CD, Loncar D. "Projections of global mortality and burden of disease from 2002 to 2030". *PLoS Medicine* 3. 2006; no. 11:e442.

Matsui T, Tao J, Del Monte F, Lee KH, Li L, Picard M, Force TL, Franke TF, Hajjar A, Rosenzweig A. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia *in vivo*. *Circulation*. 2001; 104:330 –335.

Maulik N, Watanabe M, Zu YL, Huang CK, Cordis GA, Schley JA, Das DK. Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. *FEBS Lett.* 1996; 396(2-3):233-7.

McMillen TS, Minami E, Leboeuf RC. Atherosclerosis and cardiac function assessment in low-density lipoprotein receptor-deficient mice undergoing body weight cycling. *Nutr Diabetes*. 2013; 3:e79.

Meldrum DR, Dinarello CA, Cleveland JC Jr, Cain BS, Shames BD, Meng X, Harken AH. Hydrogen peroxide induces tumor necrosis factor alpha-mediated cardiac injury by a P38 mitogen-activated protein kinase-dependent mechanism. *Surgery*. 1998; 124(2):291-6.

Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*. 2008; 18(6):228-32.

Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*. 2000; 52(4):673-751.

Mockridge JW, Marber MS, and Heads RJ. Activation of Akt during simulated ischemia/reperfusion in cardiac myocytes. *Biochem Biophys Res Commun.* 2000; 270:947–52.

Moen R, Norman C. Evolution of the PDSA cycle. Retrieved on 04102013: 2030; from *http://pkpinc.com/files/NA01MoenNormanFullpaper.pdf;* 2006

Moller P, Loft S. Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *Am J Clin Nutr*. 2002; 76(2):303-10.

Moller P, Viscovich M, Lykkesfeldt J, Loft S, Jensen A, Poulsen HE. Vitamin C supplementation decreases oxidative DNA damage in mononuclear blood cells of smokers. *Eur J Nutr*. 2004a; 43(5):267-74.

Moller P, Loft S, Alfthan G, Freese R. Oxidative DNA damage in circulating mononuclear blood cells after ingestion of blackcurrant juice or anthocyanin-rich drink. *Mutat Res.* 2004b; 551(1-2):119-26.

Moller P. The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic Clin Pharmacol Toxicol.* 2006; 98(4):336-45.

Moller P, Loft S. "The Role of Antioxidants in the Prevention of Oxidative Damage to Nucleic Acids." Oxidative Damage to Nucleic Acids. Springer New York, 2007. 207-23.

Moro C, Jouan MG, Rakotovao A, Toufektsian MC, Ormezzano O, Nagy N, Tosaki A, de Leiris J, Boucher F. Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. *Am J Physiol Heart Circ Physiol*. 2007; 293(5):H3014-9.

Moss NC, Stansfield WE, Willis MS, Tang RH, Selzman CH. IKKbeta inhibition attenuates myocardial injury and dysfunction following acute ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2007; 293(4):H2248-53.

Mulder P, Barbier S, Chagraoui A, Richard V, Henry JP, Lallemand F, Renet S, Lerebours G, Mahlberg-Gaudin F, Thuillez C. Long-term heart rate reduction induced by the selective I(f) current inhibitor ivabradine improves left ventricular function and intrinsic myocardial structure in congestive heart failure. *Circulation.* 2004; 109(13):1674-9

Murray PJ. Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. *Curr Opin Pharmacol.* 2006; 6(4):379-86.

Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. *Circ Res*. 2004; 94(12):1543-53.

Nicholson KM, Anderson NG. The protein kinase B/Akt signaling pathway in human malignancy. *Cell Signal*. 2002; 14(5):381-95.

Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr.* 2001; 74:418-25.

Nwokocha CR, Nwangwa EK, Udekweleze DC. The detoxification of lead poisoning by G*arcinia kola* in albino wistar rats. *C J Biol Sci.* 2011; 1:32-36.

Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, Saito K, Sekikawa K, Seishima M. Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis*. 2005; 180(1):11-7.

Okwu DE. Phytochemicals, Vitamins and Mineral Contents of Two Nigerian medicinal plants, *Int J Mol Med Adv Sci*. 2005; 1:375-81.

Olajide OJ, Adeniyi PA. Studies on effects of aqueous *Garcinia kola* extract on the lateral geniculate body and rostral colliculus of adult Wistar rats, *Med. Pract, Rev.* 2011*; 2(2):23-28.*

Olaleye SB, Farombi EO, Adewoye EO, Onasanwo SO, Owoyele BV, Elegbe RA. Analgesic and anti-inflammatory effects of kolaviron (A *Garcinia kola* seed extract). *Afr J Biomedical Res*. 2000; 3:163–166.

Olivier TT, Esterhuyse AJ, Truter E, Bester DJ. Dietary kolaviron supplementation increases aortic output recovery and decreases myocardial infarct size in an isolated perfused rat heart model. *Brit J Biomed Technol.* 2013; (submitted).

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med*. 1996; 334(18):1150-5.

Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Cytokine gene expression after myocardial infarction in rat hearts: possible implication in left ventricular remodeling. *Circulation*. 1998; 98(2):149-56.

Ono M, Kohda H, Kawaguchi T, Ohhira M, Sekiya C, Namiki M, Takeyasu A, Taniguchi N. Induction of Mn-superoxide dismutase by tumor necrosis factor, interleukin-1 and interleukin-6 in human hepatoma cells. *Biochem Biophys Res Commun.* 1992; 182(3):1100-7.

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease*. Physiol Rev.* 2007; 87(1):315-424.

Pan MH, Lai CS, Ho CT. Anti-inflammatory activity of natural dietary flavonoids. *Food Funct*. 2010; 1(1):15-31

Pantsi WG, Marnewick JL, Esterhuyse AJ, Rautenbach F, van Rooyen J. Rooibos (*Aspalathus linearis*) offers cardiac protection against ischaemia/reperfusion in the isolated perfused rat heart. *Phytomedicine*. 2011; 18(14):1220-8.

Papanicolaou KN, Izumiya Y, Walsh K. Forkhead transcription factors and cardiovascular biology. *Circ Res.* 2008; 102(1):16-31.

Park JW, Jang YH, Kim JM, Lee H, Park WK, Lim MB. Green tea polyphenol (-)-epigallocatechin gallate reduces neuronal cell damage and up-regulation of MMP-9 activity in hippocampal CA1 and CA2 areas following transient global cerebral ischemia. *J Neurosci Res*. 2009; 87:567–75

Pataki T, Bak I, Kovacs P, Bagchi D, Das DK, Tosaki A. Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. *Am J Clin Nutr.* 2002; 75(5):894-9.

Percival M. Understanding the natural management of pain and inflammation. *Clinical Nutrition Insights.* 1999; 4-99.

Perrella MA, Patterson C, Tan L, Yet SF, Hsieh CM, Yoshizumi M, Lee ME. Suppression of interleukin-1beta-induced nitric-oxide synthase promoter/enhancer activity by transforming growth factor-beta1 in vascular smooth muscle cells. Evidence for mechanisms other than NF-kappaB. *J Biol Chem.* 1996; 271(23):13776-80.

Pinsky DJ, Cai B, Yang X, Rodriguez C, Sciacca RR, Cannon PJ. The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor beta. *J Clin Invest.* 1995; 95(2):677-85.

Pockley AG. Heat shock proteins as regulators of the immune response. Lancet. 2003; 362(9382):469-76.

Prele CM, Keith-Magee AL, Murcha M, Hart PH. Activated signal transducer and activator of transcription-3 (STAT3) is a poor regulator of tumour necrosis factor-alpha production by human monocytes. *Clin Exp Immunol*. 2007; 147(3):564-72.

Prochazkova D, Bousova I, Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia.* 2011; 82:513–23.

Puoane T, Tsolekile L, Sanders D, Parker W. Chronic non-communicable diseases P Barron, J Roma-Reardon (Eds.). South African Health Review 2008, Health Systems Trust, Durban. 2008, 73–87.

Rao N. Bioactive phytochemicals in Indian foods and their potential in health promotion and disease prevention. *Asia Pacific J Clin Nutr* 2003: 12(1): 9-22.

Ren J, Zhang S, Kovacs A, Wang Y, Muslin AJ. Role of p38alpha MAPK in cardiac apoptosis and remodeling after myocardial infarction. *J Mol Cell Cardiol.* 2005; 38: 617–23.

Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* 1996; 20:933–56.

Ridker PM, Silvertown JD. Inflammation, C-reactive protein, and atherothrombosis. *J Periodontol.* 2008; 79(8 Suppl):1544-51.

Rodrigues SF, Granger DN. Role of blood cells in ischaemia-reperfusion induced endothelial barrier failure. *Cardiovasc Res*. 2010; 87(2):291-9.

Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature.* 1999; 401(6748):86-90.

Rossato M, Cencig S, Gasperini S, Cassatella MA, Bazzoni F. IL-10 modulates cytokine gene transcription by protein synthesis-independent and dependent mechanisms in lipopolysaccharide-treated neutrophils. *Eur J Immunol.* 2007; 37(11):3176-89.

Rotelli AE, Guardia T, Juárez AO, de la Rocha NE, Pelzer LE. Comparative study of flavonoids in experimental models of inflammation. *Pharmacol Res*. 2003; 48(6):601-6.

Roumen RM, Hendriks T, van der Ven-Jongekrijg J, Nieuwenhuijzen GA, Sauerwein RW, van der Meer JW, Goris RJ. Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. *Ann Surg.* 1993; 218(6):769-76.

Roux E, Mbikou P, Fajmut A. Role of Protein Kinase Network in Excitation-Contraction Coupling in Smooth Muscle Cell, Protein Kinases, Dr. Gabriela Da Silva Xavier (Ed.), 2012; Available from: *http://www.intechopen.com/books/protein-kinases/role-of-protein-kinase-network-in-excitation-contraction-coupling-in-smooth-muscle-cell*

Sahu SC, Gray GC. Kaempferol-induced nuclear DNA damage and lipid peroxidation. *Cancer Lett.* 1994; 85(2):159-64.

Sahu SC, Washington MC. Effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation. *Cancer Lett.* 1991; 60(3):259-64.

Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*. 1998; 391(6662):96-9.

Salonen RM, Nyyssönen K, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Rissanen TH, Tuomainen TP, Valkonen VP, Ristonmaa U, Lakka HM, Vanharanta M,Salonen JT, Poulsen HE. Antioxidant Supplementation in Atherosclerosis Prevention Study. Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression: the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study. *Circulation.* 2003; 107(7):947-53.

Santangelo C, Vari R, Scazzocchio B, Di Benedetto R, Filesi C, Masella R. Polyphenols, intracellular signaling and inflammation. *Ann Ist Super Sanita.* 2007; 43(4):394-405.

Santos C, Treitas M, Ribero D, Gomes A, Silvia A, Cavaleiro J, Fernandes E. 2, 3-Diarylxanthones as strong scavengers of reactive oxygen and nitrogen species: A structure–activity relationship study. *Bioorg Med Chem.* 2010; 18:6776–84.

Saurin AT, Martin JL, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber MS. The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *FASEB J.* 2000; 14(14):2237-46.

Schwartz Y, Kornowski R. Progenitor and embryonic stem cell transplantation for myocardial angiogenesis and functional restoration. *Eur Heart J*. 2003; 24(5):404-11.

Sethi G, Sung B, Aggarwal BB. Nuclear factor-kappaB activation: from bench to bedside. *Exp Biol Med (Maywood).* 2008; 233(1):21-31.

Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell.* 1995; 80(2):321-30.

Sharma HS, Das DK. Role of cytokines in myocardial ischemia and reperfusion. *Mediators Inflamm*. 1997; 6(3):175-83.

Shazia Q, Mohammad ZH, Rahman T, Shekhar HU. Correlation of oxidative stress with serum trace element levels and antioxidant enzyme status in Beta thalassemia major patients: a review of the literature. *Anemia.* 2012; 2012:270923.

Shen L, Ji H, Zhang H. How to understand the dichotomy of antioxidants. *Biochem. Biophys. Res. Commun.* 2007; 362:543–45.

Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci U S A*. 2000; 97(18):10197-202.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for the quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988; 175:184–91.

Singh BN. Increased heart rate as a risk factor for cardiovascular disease. *Eur. Heart J. Suppl.* 2003; 5: G3-G9.

Singhal M, Raina V, Gupta R, Das P. Role of Endothelial Cells in Myocardial Ischemia-Reperfusion Injury*. VDP.* 2010; *7,* 1-14.

Slimane MA, Ferlicot S, Conti M, Droupy S, Cosson C, Paradis V, Loric S, Bedossa P, Gauthier F, Legrand A, Benoit G, Eschwege P .Expression of cyclooxygenase 2 and prostaglandin E synthase after renal ischemia-reperfusion. *Transplant Proc.* 2002; 34(7):2841-2.

Spagnoli LG, Bonanno E, Sangiorgi G, Mauriello A Role of inflammation in atherosclerosis*. J Nucl Med*. 2007; 48(11).

Speit G, Hartmann A. The comet assay: a sensitive genotoxicity test for the detection of DNA damage. *Methods Mol Biol.* 2005; 291:85-95.

Steenbergen C. The role of p38 mitogen-activated protein kinase in myocardial ischemia/reperfusion injury; relationship to ischemic preconditioning. *Basic Res Cardiol*. 2002; 97(4):276-85.

Stenvinkel P. Endothelial dysfunction and inflammation-is there a link? *Nephrol Dial Transplant.* 2001; 16(10):1968-71.

Steyn K. Heart Disease in South Africa, Heart and Stroke foundation South Africa. *JM Fourie*, Editor. 2007; 2-27.

Subarnas A, Wagner H. Analgesic and anti-inflammatory activity of the proanthocyanidins shellegueain A from Polypodium feei. *Phytomedicine.* 2000; 7:401–5.

Sun D,Huang J,Zhang Z,Gao H,Li J,Shen M,Cao F,Wang H. Luteolin limits infarct size and improves cardiac function after myocardium ischemia/reperfusion injury in diabetic rats**.** *PLoS ONE*. 2012; 7(3):1-10.

Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology. 2010;* 11,621-32.

Takeuchi D, Yoshidome H, Kato A, Ito H, Kimura F, Shimizu H, Ohtsuka M, Morita Y, Miyazaki M. Interleukin 18 causes hepatic ischemia/reperfusion injury by suppressing anti-inflammatory cytokine expression in mice. *Hepatology*. 2004; 39(3):699-710.

Tavares AM, da Rosa Araujo AS, Llesuy S, Khaper N, Rohde LE, Clausell N, Bello-Klein A. Early loss of cardiac function in acute myocardial infarction is associated with redox imbalance. *Exp Clin Cardiol.* 2012; 17(4):263-7.

Testa M, Yeh M, Lee P, Fanelli R, Loperfido F, Berman JW, LeJemtel TH. Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. *J Am Coll Cardiol.* 1996; 28(4):964-71.

Testai L, Martelli A, Cristofaro M, Breschi MC, Calderone V. Cardioprotective effects of different flavonoids against myocardial ischaemia/reperfusion injury in Langendorff-perfused rat hearts. *J Pharm Pharmacol.* 2013; 65(5):750-6.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen.* 2000; 35(3):206-21.

Timmers L, Pasterkamp G, de Hoog VC, Arslan F, Appelman Y, de Kleijn DP. The innate immune response in reperfused myocardium. *Cardiovasc Res.* 2012; 94(2):276-83

Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB, Mann DL. Pro-inflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). *J Am Coll Cardiol.* 1996; 27(5):1201-6.

Toth A, Halmosi R, Kovacs K, Derea P, Kalai T, Hideg K, Toth K, Sumegi B. Akt activation induced by an antioxidant compound during ischemia-reperfusion. *Free Radic Biol Med.* 2003; 35:1051-63.

Toth-Zsamboki E, Horvath E, Vargova K, Pankotai E, Murthy K, Zsengeller Z, Barany T, Pek T, Fekete K, Kiss RG, Preda I, Lacza Z, Gero D, Szabo C. Activation of poly(ADP-ribose) polymerase by myocardial ischemia and coronary reperfusion in human circulating leukocytes. *Mol Med*. 2006; 12(9-10):221-8.

Tousoulis D, Charakida M, Stefanadis C. Endothelial function and inflammation in coronary artery disease. *Postgrad Med J*. 2008; 84(993):368-71.

Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother*. 2003; 57(3-4):145-55.

Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, Suzuki H. Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. *FASEB J*. 2004; 18:1621–3.

Tribble DL. AHA Science Advisory. Antioxidant consumption and risk of coronary heart disease: emphasison vitamin C, vitamin E, and beta-carotene: A statement for healthcare professionals from the American Heart Association. *Circulation*. 1999; 99(4):591-5.

Tsutamoto T, Hisanaga T, Wada A, Maeda K, Ohnishi M, Fukai D, Mabuchi N, Sawaki M, Kinoshita M. Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure. *J Am Coll Cardiol*. 1998; 31(2):391-8.

Tsutamoto T, Wada A, Maeda K, Mabuchi N, Hayashi M, Tsutsui T, Ohnishi M, Sawaki M, Fujii M, Matsumoto T, Kinoshita M. Angiotensin II type 1 receptor antagonist decreases plasma levels of tumor necrosis factor alpha, interleukin-6 and soluble adhesion molecules in patients with chronic heart failure. *J Am Coll Cardiol.* 2000; 35(3):714-21.

Turan N, Csonka C, Csont T, Giricz Z, Fodor G, Bencsik P, Gyongyosi M, Cakici I, Ferdinandy P. The role of peroxynitrite in chemical preconditioning with 3-nitropropionic acid in rat hearts. *Cardiovasc Res*. 2006; 70(2):384-90.

Vajdovich P. Free radicals and antioxidants in inflammatory processes and ischemia-reperfusion injury. *Vet Clin North Am Small Anim Pract.* 2008; 38(1):31-123.

Van Acker SA, Tromp MN, Haenen GR, van der Vijgh WJ, Bast A. Flavonoids as scavengers of nitric oxide radical. *Biochem Biophys Res Commun.* 1995; 214(3):755-9.

Vassalli G, Milano G, Moccetti T. Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation. *J Transplant*. 2012; 2012:928954.

Verma S, Fedak PW, Weisel RD, Butany J, Rao V, Maitland A, Li RK, Dhillon B, Yau TM. Fundamentals of reperfusion injury for the clinical cardiologist. *Circulation*. 2002; 105(20):2332-6.

Vilhar B. Help! There is a comet in my computer! A dummy's guide to image analysis used in the comet assay. 2004; available at *http://www.botanika.biologija.org/exp/comet/* Comet-principles.pdf (accessed 28092013: 1830)

Vincent JL. Understanding cardiac output. *Crit Care*. 2008; 12(4):174-6.

Vinten-Johansen J. Physiological effects of peroxynitrite: potential products of the environment. *Circ Res*. 2000; 87(3):170-2.

Virag L, Szabo C. The therapeutic potential of poly (ADP-ribose) polymerase inhibitors. *Pharmacol Rev*. 2002; 54(3):375-429.

Vogiatzi G, Tousoulis D, Stefanadis C. The role of oxidative stress in atherosclerosis. *Hellenic J Cardiol.* 2009; 50(5):402-9.

Wang S, Thomas CJ, Dusting GJ, Woodman OL, May CN. 3', 4'-Dihydroxyflavonol improves post-ischaemic coronary endothelial function following 7days reperfusion in sheep. *Eur J Pharmacol.* 2009; 624(1-3):31-7.

Weinbrenner C, Liu GS, Cohen MV, Downey JM. Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J Mol Cell Cardiol.* 1997; 29(9):2383-91.

Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ Res*. 2003; 93(4):292-301.

Wendell PL., Measurement of oxidized glutathione and total glutathione in the perfused rat. *Heart Biochem J*. 1970; 117(4):661–665.

Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E (-/-) mice through release of interferon-gamma. *Circ Res*. 2002; 90(2):E34-8.

WHO(a). Global status report on noncommunicable diseases 2010. Geneva, World Health Organization, 2011a.

WHO(b). Global atlas on cardiovascular disease prevention and control. Geneva, World Health Organization, 2011b.

Widlansky ME, Duffy SJ, Hamburg NM, Gokce N, Warden BA, Wiseman S, Keaney JF Jr, Frei B, Vita JA. Effects of black tea consumption on plasma catechins and markers of oxidative stress and inflammation in patients with coronary artery disease. *Free Radic Biol Med*. 2005; 38,499–506.

Wong VWC, Szeto YT, Collins AR, Benzie IFF. The comet assay: a biomonitoring tool for nutraceutical research. *Curr Top Nutraceutical Res.* 2005; 3(1) 31–14.

Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, Roussos C. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and pro-inflammatory cytokine production in macrophages. *J Pharmacol Exp Ther*. 2001; 296(1):181-7.

Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature*. 2003; 423(6940):655-9.

Yang Z, Zingarelli B, Szabo C. Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. *Circulation.* 2000; 101(9):1019-26.

Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation*. 1998; 97(3):276-81.

Yoon JH, Baek SJ. Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med J.* 2005; 46(5):585-96.

Yordi E, Molina Perez E, Matos MJ, Uriarte Villares E.. Antioxidant and pro-oxidant effects of polyphenolic compounds and structure-activity relationship evidence. In: Bouayed, J., Bohn, T. (Eds.), Nutrition, Well-Being and Health. *InTech*. 2012:24–48.

Yoshimura A, Mori H, Ohishi M, Aki D, Hanada T .Negative regulation of cytokine signaling influences inflammation. *Curr Opin Immunol*. 2003; 15(6):704-8.

Young DB. Control of Cardiac Output. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Chapter 6, Analysis of Cardiac Output Control in Response to Challenges. Available at: *http://www.ncbi.nlm.nih.gov/books/NBK54474/* (Accessed on 29092013: 1423)

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell.* 1997; 91(2):243-52.

Zhang C. The role of inflammatory cytokines in endothelial dysfunction. *Basic Res Cardiol*. 2008; 103(5):398-406.

Zhang JH, Zhang Y, Herman B. Caspases, apoptosis and aging. *Ageing Res Rev.* 2003; 2(4):357-66.

Zhang XP, Liu F, Wang W. Two-phase dynamics of p53 in the DNA damage response. *Proc Natl Acad Sci USA*. 2011; 108(22):8990-5.

Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med.* 2000; 192(7):1001-14.

Zuidema MY, Zhang C. Ischemia/reperfusion injury: The role of immune cells. *World J Cardiol*. 2010; 2(10):325-32.

Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res*. 2006; 70(2):181-90.