IMPROVED MODULATION OF THE ENDOGENOUS ANTIOXIDANT SYSTEM AND INFLAMMATORY RESPONSES IN MALE WISTAR RATS BY ROOIBOS (*Aspalathus linearis*) AND RED PALM OIL (*Elaeis guineensis*)

By

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DECLARATION

I, Olawale Razaq Ajuwon, 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.

Signed **Date**

ABSTRACT

Oxidative stress and chronic (low-grade) inflammation are inseparably interconnected and both have been implicated in the aetiology of many disease states. The use of plant extracts for disease prevention and therapeutic purposes, is gaining more attention because of the health benefits of the bioactive phytochemicals in these extracts. This thesis reports on the antioxidant status, the oxidative stress modulation and anti-inflammatory properties of fermented rooibos (*Aspalathus linearis*) and red palm oil (RPO) from the oil palm plant (*Elaeis guineensis*) using a long-term and two short-term *in vivo* models.

In the first (long-term) study, the effect of chronic feeding of rooibos, RPO or their combination on the endogenous antioxidant system was investigated. Data from this study provided:

- The first scientific evidence that chronic feeding of rooibos, RPO or their combination for 22 weeks did not adversely affect the liver or kidney function parameters.
- The first scientific evidence that chronic feeding of rooibos alone, or together with RPO for 22 weeks modulated the endogenous antioxidant system by inhibiting MDA formation and augmenting the reduced glutathione status.
- The first scientific evidence of an additive or synergistic interaction in the ability of rooibos and RPO to modulate the endogenous antioxidant system.

The second (short-term) study investigated the protective effects of rooibos, RPO or their combination on tert-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatotoxicity, and results from this study provided:

- The first evidence of the ability of the two extracts, either alone or in combination to protect against *t*-BHP induced hepatotoxicity. Supplementation of rooibos, RPO or their combination for eight weeks reversed the hepatic damage induced by *t*-BHP. The changes induced by *t*-BHP in the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were reversed by these plant extracts. The extracts, supplemented alone or combined prevented lipid peroxidation as shown by the decreased levels of conjugated dienes

(CD) and malondialdehyde (MDA) and reversed the *t*-BHP.-induced impairment of the glutathione redox status.

The third (short-term) study provided the first scientific evidence of the *in vivo* anti-inflammatory properties of rooibos and RPO. The properties were demonstrated using a lipopolysaccharide (LPS)-induced hepatic endotoxemic model by:

- Providing the first evidence of an additive or synergistic interaction in the ability of the combined rooibos extract and RPO supplementation to reverse LPS-induced hepatic damage.
- Providing the first evidence for the modulation of the inflammatory responses by rooibos extract, RPO or their combination. Supplementation of rooibos extract, RPO or their combination for four weeks, modulated LPS-induced inflammatory responses by inhibiting the synthesis of pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6).
- Providing evidence that the observed anti-inflammatory effects of rooibos and RPO may be related to their ability to modulate oxidative stress. Supplementation of rooibos extract, RPO or their combination for four weeks, prevented hepatic lipid peroxidation induced by LPS by decreasing CD and MDA formation in the liver. Changes induced in the activities of antioxidant enzymes were reversed and the glutathione redox status was augmented.

Based on these study results, it is proposed that the ability of rooibos and RPO to protect the liver, modulate endogenous antioxidant system and inhibit inflammatory responses may be associated with the unique combination of antioxidant phytochemicals in both plant extracts. The study suggested possible mechanism(s) for the observed health effects, and the development of rooibos and RPO as nutraceuticals, which may be beneficial in the prophylactic management of oxidant-induced liver injury.

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DEDICATION

This thesis is dedicated to the memory of my late sister FOLASADE. You encouraged me to go for it, but you were not alive to witness its completion. We will continue to miss you.

PREFACE

This thesis is submitted to fulfil the requirements for the degree of Doctor of Technology in the discipline of Biomedical Technology. In chapter one, a brief introduction is given to the study and the aims and approach are stated. A literature review in chapter two discusses concepts, which are key in understanding why the study was done and to help interpret the results obtained. Three articles, which will be submitted for publication, form chapters three, four and five, and gives the result of the three study phases. These three chapters contain separate introductions, results and discussions. A general discussion and conclusions chapter ends the thesis and is followed by addendums, which contain additional material and results relevant to the study. Since the thesis is written in article format, each chapter has separate numbering systems and references listed according to the respective journals and university requirements.

GLOSSARY

Antioxidant	A molecule that reduces or prevents oxidation of other molecules by being oxidized.
Apoptosis	A genetically controlled mechanism of cell suicide involved in the regulation of tissue homeostasis.
Autophagy	A catabolic process involving the digestion of cell's own components through the lysosomal machinery.
Biomarker	A measurable biochemical indicator of a biological state, such as severity of presence of disease.
Chemoprevention	The use of naturally occurring or synthetic substances to prevent or slow down the development of a disease.
Chemokines	Low molecular weight cytokines that is able to induce directed chemotaxis in nearby responsive cells.
Chronic disease	A disease of long duration and slow progression that can be controlled but not cured.
Cytokines	Cell signalling protein molecules made by cells, which modulate immune response.
Cytosolic	Relating to the cytoplasm of a cell, excluding the organelles and other intracellular structures.
Dismutation	A chemical reaction in which two identical molecules are converted to two different molecules.
Endogenous	Originating within an organism, tissue or cell.
Endotoxemia	Presence of endotoxin in the blood
Ex vivo	Experimentation done in/on a living tissue outside of the organism, with the minimum alteration of the natural conditions.
Exogenous	Originating from outside of an organism.
Fenton reaction	Non-enzymatic reaction of hydrogen peroxide with ferrous iron to produce hydroxyl radicals and ferric iron.
Flavonoids	Plant secondary metabolites that are subclass of polyphenols.
Free radical	Highly reactive molecule with one or more unpaired electrons.
Inflammation	A protective tissue response to harmful stimuli, such as pathogens, damaged cells or irritants.

Interleukins	Family of cytokines produced by white blood cells to modulate inflammation and immune response.
In vitro	Experimentation on isolated cell component e.g. in a test tube.
In vivo	Experimentation in/on intact or whole organism.
Macrophages	White blood cells produced by differentiation of monocytes in tissues.
Mutagenic	having ability to induce genetic mutations.
Oxidation	Loss of electron from a molecule.
Oxidative stress	Cellular condition resulting from imbalance between free radical formation and antioxidant defence mechanisms in favour of the former.
Phytochemical	A chemical compound that occur naturally in plants
Polyphenol	Compound containing one or more phenol ring.
Pro-oxidant	A species or chemical that causes or promote oxidation.
Reactive oxygen and nitrogen species	Molecules produced by incomplete reduction of oxygen and nitrogen.
Reduction	The gain of electron from a molecule.
Synergistic interaction	Interaction between two compounds such that the total effect is greater than the sum of individual effects.

ABBREVIATIONS

4-HNE	4-Hydroxyl-2-nonenal
8-OHdG	8-Hydroxyl-2'-deoxyguanosine
AA	Ascorbic acid
AAPH	2,2'-Azo-bis(2-methylpropionamidine)dihydrochloride
ABTS	2,2'-Azino-bis(3-ethylbenzothiozoline-6-sulphonic acid)
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAT	Catalase
CCL2	Chemokine (C-C) motif ligand 2
CD	Conjugated dienes
CNCDs	Chronic non-communicable diseases
CPO	Crude palm oil
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL2	Chemokine (C-X-C) motif ligand 2
CXCL8	Chemokine (C-X-C) motif ligand 8
DETAPAC	Diethylenetriaminepentaacetic acid
DMACA	4-(Dimethylamino)-cinnamaldehyde
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FRAP	Ferric reducing ability of plasma
GC	Gas chromatography
GC-MS	Gas chromatography tandem mass spectrometry
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1α
HPLC	High performance liquid chromatography
ICAM 1	Inter-cell adhesion molecule 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
M2VP	1-Methyl-2-vinylpyridinium triflate
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
mRONS	Mitochondrial reactive oxygen and nitrogen species
NADPH	β-Nicotinamide adenine dinucleotide phosphate reduced
$NO^{\bullet}$	Nitric oxide radical
NO^{-}	Nitroxyl anion
$^{\bullet}NO_2$	Nitrosonium cation

nNOS	neuronal nitric oxide synthase
O_2	Molecular oxygen
$^1O_2^{\bullet}$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
$ONO\text{-}OCO_2^-$	Nitroso-peroxocarbonate
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PDK1	Pyruvate dehydrogenase kinase 1
PPARs	Peroxisome proliferators-activated receptors
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RPO	Red palm oil
SAA	Serum amyloid A
SOD	Superoxide dismutase
sGC	Soluble guanylate cyclase
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric reacting substances
TEAC	Trolox equivalent antioxidant capacity
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-α
TNFRs	Tumour necrosis factor receptors
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1

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CHAPTER 1

Introduction

1 Introduction

Scientific evidence has revealed that oxidative stress, inflammation and apoptosis are central factors that have been implicated in the pathophysiology of many chronic diseases (Koenig and Khuseyinova, 2006; Braunwald, 2008), and using biomarkers of oxidative stress, chronic inflammation and antioxidant status to identify persons at risk of chronic diseases is a rational first step towards addressing modifiable risk factors and preventing the development of serious, disabling and costly diseases. Oxidative stress refers to the cytopathological consequences of an imbalance between production of free radicals and the ability of the cell to defend against them (Franco and Panayiotidis, 2009). Free radicals are highly reactive compounds, with an unpaired electron which make them capable of causing oxidative damage to major macromolecules in cells, including lipids, proteins and nucleic acids (Franco and Panayiotidis, 2009). Major families of free radicals include the reactive oxygen species (ROS) and reactive nitrogen species (RNS). As a result of oxidative attack on proteins, protein carbonyls are formed, often with the loss of functionality of the parent protein (Zoccali *et al.,* 2000). Biological membranes are particularly prone to the ROS effect. Oxidative lipid injuries, referred to as lipid peroxidation, produce a progressive loss of membrane fluidity, reduced membrane potential and increased its permeability to ions such as Ca^{2+} (Halliwell, 1987; Simonian and Coyle 1996). In humans, oxidative stress is involved in many diseases, such as neurodegenerative disorders including Parkinson's and Alzheimer's disease, cardiovascular diseases, diabetes, cancers and ischemic cascade due to oxygen reperfusion injury followed by hypoxia (Johansen *et al*., 2005; Dalle-Donne *et al*., 2006; Blum, 2009). Evidence also suggests that reactive oxygen and nitrogen species (RONS) participate in the normal aging process as well as in age related diseases (Finkel, 2003).

Inflammation is a biological process initiated by the immune system in response to tissue injury caused by microbial infection and other noxious stimuli. Acute inflammatory response is characterized by vasodilation, leakage of the vasculature, and infiltration of leukocytes into the site of infection to destroy invading pathogens and is followed by a rapid resolution phase and repair of the damaged tissue (Kundu and Surh, 2012). Although acute inflammation plays a beneficial role against infection and injury, when there is inadequate resolution of such inflammatory responses, chronic, low-grade inflammation may result (Schottenfeld and Beebe-Dimmer, 2006; Aggarwal *et al*., 2006; Kundu and Surh, 2008). Several studies have shown a strong relationship between low-grade systemic (chronic) inflammation and disease conditions including insulin resistance, diabetes, cardiovascular diseases, metabolic syndrome, Alzheimer's disease and cancers (Achike *et al*., 2011; Callewaert, 2012; Kundu and Surh, 2012). Substantial evidence suggests that chronic inflammation is associated with

enhanced production of reactive oxygen and nitrogen species (RONS) resulting in deregulated inflammatory responses including, but not limited to pro-inflammatory cytokine production (Reuter *et al*., 2010; Bulua *et al*., 2011; Naik and Dixit, 2011). Oxidative stress and inflammation have been shown to be involved in a self-perpetuating cycle, since by activating NF-kappa β and activator protein-1, oxidative stress stimulates production of chemokines, cytokines, and adhesion molecules as well as activation and proliferation of lymphocytes and these events, in turn, result in immune cell activation, adhesion, and infiltration. Conversely, inflammation causes oxidative stress since production of the ROS is an inherent property of activated immune cells (Vaziri, 2008).

Under normal circumstances, the body has evolved several endogenous protective mechanisms to limit generation of free radicals and the damage caused by them. Several antioxidant defense systems, including the action of some enzymes are available to prevent oxidative damage (Valko *et al*., 2006). These include catalase (CAT) for hydrogen peroxide, superoxide dismutase (SOD) for superoxide and glutathione peroxidase (GPX) for hydrogen peroxide and lipid peroxide. Moreover, other endogenous antioxidants such as reduced glutathione (GSH), thioredoxin and peroxiredoxin systems are also available to prevent tissue damage induced by free radicals (Rahman, 2007). However, since this endogenous protection may not be complete when the formation of free radicals is excessive, especially during chronic disease conditions, additional protective mechanisms by dietary antioxidants are of great importance. Evidence from epidemiological studies has led to the conclusion that healthy diet is a key factor and a potential tool for the prevention/control of chronic diseases (Darnton-Hill *et al*., 2004; WHO, 2003; Daar *et al*., 2007; Mirmiran *et al*., 2008). More specifically, fruits, vegetables and teas have been shown to exert protective effects (Joshipura *et al.,* 1999; Cox *et al.,* 2000; Azuma *et al.,* 2007). The main factor that is probably responsible for these protective effects by fruits, vegetables and teas is the high content of polyphenolic antioxidants, antioxidant vitamins and trace elements which they contain (Cao *et al.,* 1996; Dreosti, 1996; Trevisanato and Kim, 2000).

Rooibos (*Aspalathus linearis*) and red palm oil, from the fruit of the oil palm tree (*Elaeis guineensis*) are two plant extracts exhibiting high antioxidant qualities. Rooibos is a shrubby legume that is indigenous to the mountainous area of Clanwilliam in the Western Cape Province of South Africa, and is used to make a herbal beverage that is naturally caffeine-free, low in tannin and rich in polyphenol antioxidants (Cheney and Scholtz, 1963; Morton, 1983; Joubert *et al*., 2008). The polyphenolic constituents identified in rooibos include aspalathin (major polyphenol and unique to rooibos), nothofagin, quercetin, rutin, isoquercitrin, orientin, luteolin, vitexin and crysoeriol (Rabe *et al.,* 1994; Joubert, 1996;

Bramati *et al.,* 2002). Rooibos, due to its rich content of different compounds with antioxidant, antimutagenic and antitumourigenic properties, is gaining a lot of attention because of its potential use for clinical purposes (Hesseling and Joubert, 1982; Inanami *et al*., 1995; Nakano *et al*., 1997a, b; Bramati *et al*., 2002, 2003; Ulicna *et al*., 2003; Marnewick *et al*., 2000, 2003, 2005; Marnewick, 2009). Several *in-vitro, in vivo* and human studies have demonstrated the health promoting effects of rooibos. Rooibos has been shown to be antimutagenic (Marnewick *et al*., 2000, 2004), cancer modulating (Marnewick *et al*., 2005, 2009; Sissing *et al*., 2011), anti-inflammatory (Baba *et al*., 2009), antidiabetic (Kawano *et al*., 2009), cardiac-protective (Pantsi *et al*., 2011) as well as modulating oxidative stress (Ulicna *et al*., 2006; Nikolova *et al*., 2007; Marnewick *et al*., 2011; Awoniyi *et al*., 2012).

Red palm oil is a lipid extract obtained from the fleshy orange-red mesocarp of the fruits of the oil palm tree. It is a unique oil with equal amount of saturated and unsaturated fatty acids. Red palm oil is an excellent source of fat soluble antioxidants such as the tocopherols, tocotrienols and carotenoids (Sambanthamurthi *et al.,* 2000; Edem, 2002; Engelbrecht *et al.,* 2009). It is the richest natural food source of carotenoids containing altogether about 500 ppm of which 90% are made up of α-carotene (37%) and β-carotene (47%), with other minor carotenoids such as lycopene and cis-α-carotene making up the difference (Van Rooyen *et al*., 2008). About 70% of the vitamin E content of red palm oil is in the form of tocotrienols (mainly as α-, β- and γ-tocotrienols) while tocopherols account for the remaining 30% (Al Saqer *et al.,* 2004). Recent evidence have shown that the oil palm fruit contain several phenolic compounds including gallic, chlorogenic, gentisic, coumaric and caffeic acids, as well as catechins, hesperidin, narirutin, and 4- hydroxyl benzoate, all of which have appreciable radical scavenging and antioxidant ability (Tan *et al.,* 2001; Loganathan *et al*., 2010; Atawodi *et al*., 2011). Studies using various animal models have demonstrated the beneficial role of red palm oil. Its ability to reduce oxidative stress and protect serum lipids has been highlighted (Ebong *et al*., 1999; Oluba *et al*., 2008; Budin *et al*., 2009). Previous reports have shown the protective effect of RPO against oxidative stress and its association with improved recovery and protection of the heart subjected to ischaemia/reperfusion (Esterhuyse *et al.,* 2005, 2006; Bester *et al.,* 2006; Engelbrecht *et al.,* 2009; Van Rooyen *et al.,* 2008). Another study showed that RPO supplementation reduces caspase-3 activity, thereby inhibiting apoptosis caused in rat sperm by the *in vivo* induction of hydroperoxides (Aboua *et al.,* 2009).

Though, several studies have investigated the health potential of rooibos and red palm oil individually, to the best of our knowledge, there is no report of a comparative study on these two herbal extracts. It is against this background that this study investigated whether rooibos

in a commonly used concentration as consumed by humans and red palm oil will have a synergistically positive effect on biomarkers of oxidative stress and inflammation in experimental rat models. The study also compared the effects of each extract on the aforementioned biomarkers.

2 Aims and approach of this study

The general objective of this study was to investigate the potential health benefits of rooibos and RPO either alone or in combination using biomarkers of oxidative stress and inflammation in different rat models. The study was divided into three phases and the effect of the supplementation of both antioxidant-rich extracts were studied on each group of biomarkers (i.e. oxidative stress and inflammation) in each phase using long- and short-term models. Prior to supplementation of the plant extracts in each phase, the antioxidant capacity, polyphenol and flavonoids contents and concentration of major individual components in the rooibos extract, as well as phytonutrients composition of RPO were determined to discuss their possible role(s)/contribution to the bioactivities measured.

2.1 Long-term (chronic) feeding study

In the first *phase,* the modulation by the two plants extracts, following chronic feeding of rats for 22 weeks on the antioxidant and oxidative stress status was investigated. As a secondary goal, markers of liver and kidney function were monitored to serve as surrogate indicator of safety of the two extracts, since no previous study has reported on this. A possible additive or synergistic interaction in the effects shown by this extracts was also investigated in this phase. Rats were supplemented with rooibos and/or RPO in the water and/or diet for 22 weeks. At the end of the experimental period, the rats were sacrificed and tissue samples were collected. Markers of liver and kidney functions [alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total protein (TP), creatinine (CREA), blood urea nitrogen (BUN) and uric acid] were assessed in the serum to evaluate any possible deleterious effects by the extracts. Oxidative stress and antioxidant status markers including total antioxidant capacity, lipid peroxidation indicators [such as malondialdehyde (MDA) and conjugated dienes (CDs)], GSH/GSSG ratio and activities of various antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR)] were determined in various tissue samples collected.

2.2 Oxidative stress study

The effects of rooibos and RPO supplementation on biomarkers of oxidative stress were investigated. Rats were supplemented with rooibos and/or RPO in the water and/or diet for

eight weeks and challenged daily with *tert*-butyl hydroperoxide (30 µmol/100 g body weight, i.p.) during the last two weeks of the study. The rats were sacrificed and various biological samples collected. Oxidative stress and antioxidant status markers including total antioxidant capacity, lipid peroxidation indicators [such as malondialdehyde (MDA) and conjugated dienes (CDs)], GSH/GSSG ratio and activities of various antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR)] were determined in various tissue samples collected. Figure 2 presents a schematic diagram of the oxidative stress phase of the study.

2.3 Inflammation study

The effects of rooibos and RPO supplementation on biomarkers of acute inflammatory response were studied. Rats were fed rooibos and/or RPO in the diet and/or in water for 27 days and were then challenged with a lipopolysaccharide (*Escherichia coli*, serotype 0111:B) (0.5 mg/kg body weight, i.p.) on the 28th day. Rats were sacrificed 16 hours later, tissue samples were collected and analysed for cytokines (TNF-α, IL-1β, IL-6 and IL-10) as markers of the inflammatory response. All the oxidative stress and antioxidant status markers determined in the oxidative stress phase were also measured in these tissue samples. Figure 3 presents a schematic diagram of the inflammation phase of the study.

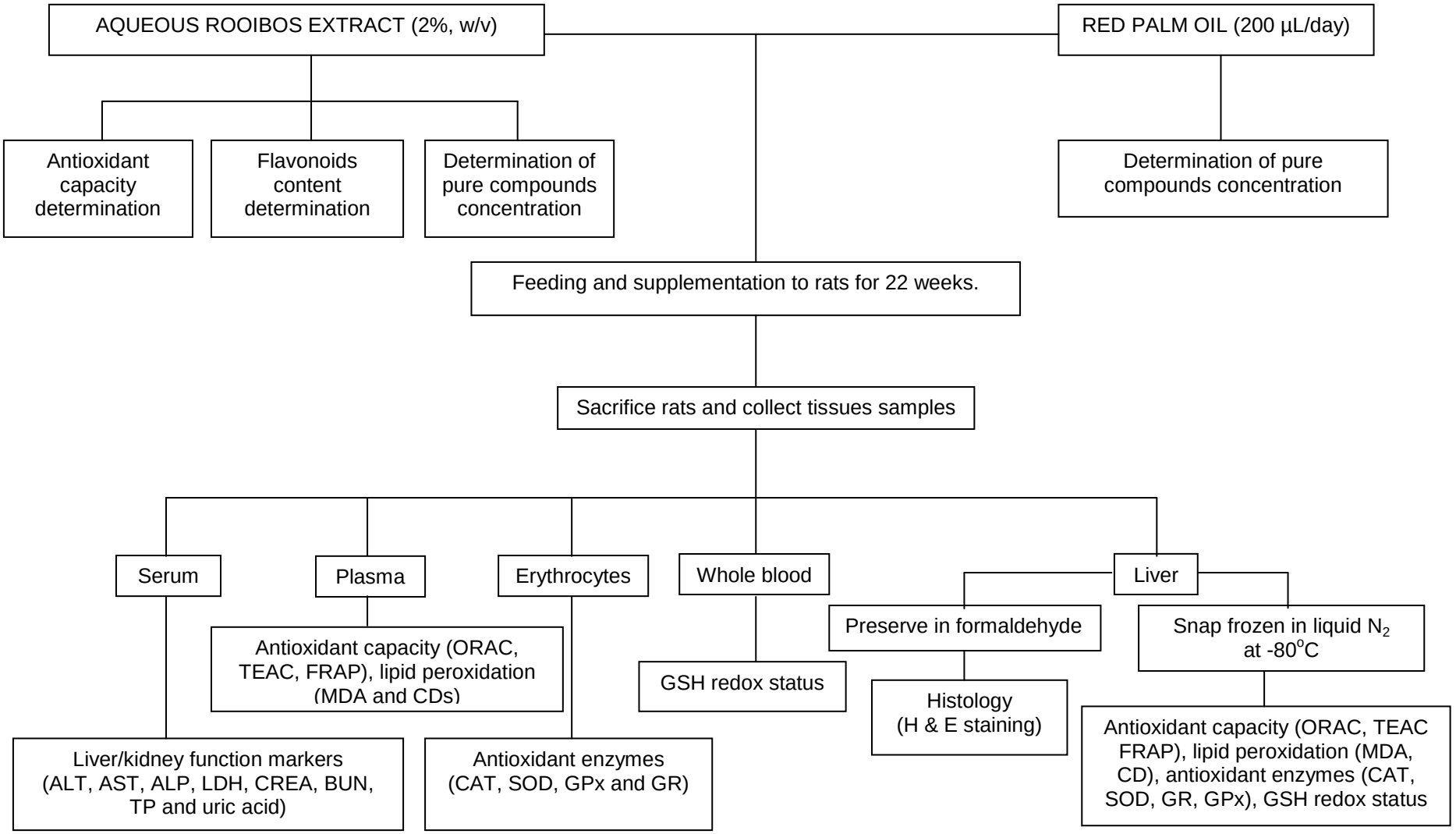

Figure 1: Schematic diagram showing the approach followed in the chronic study phase

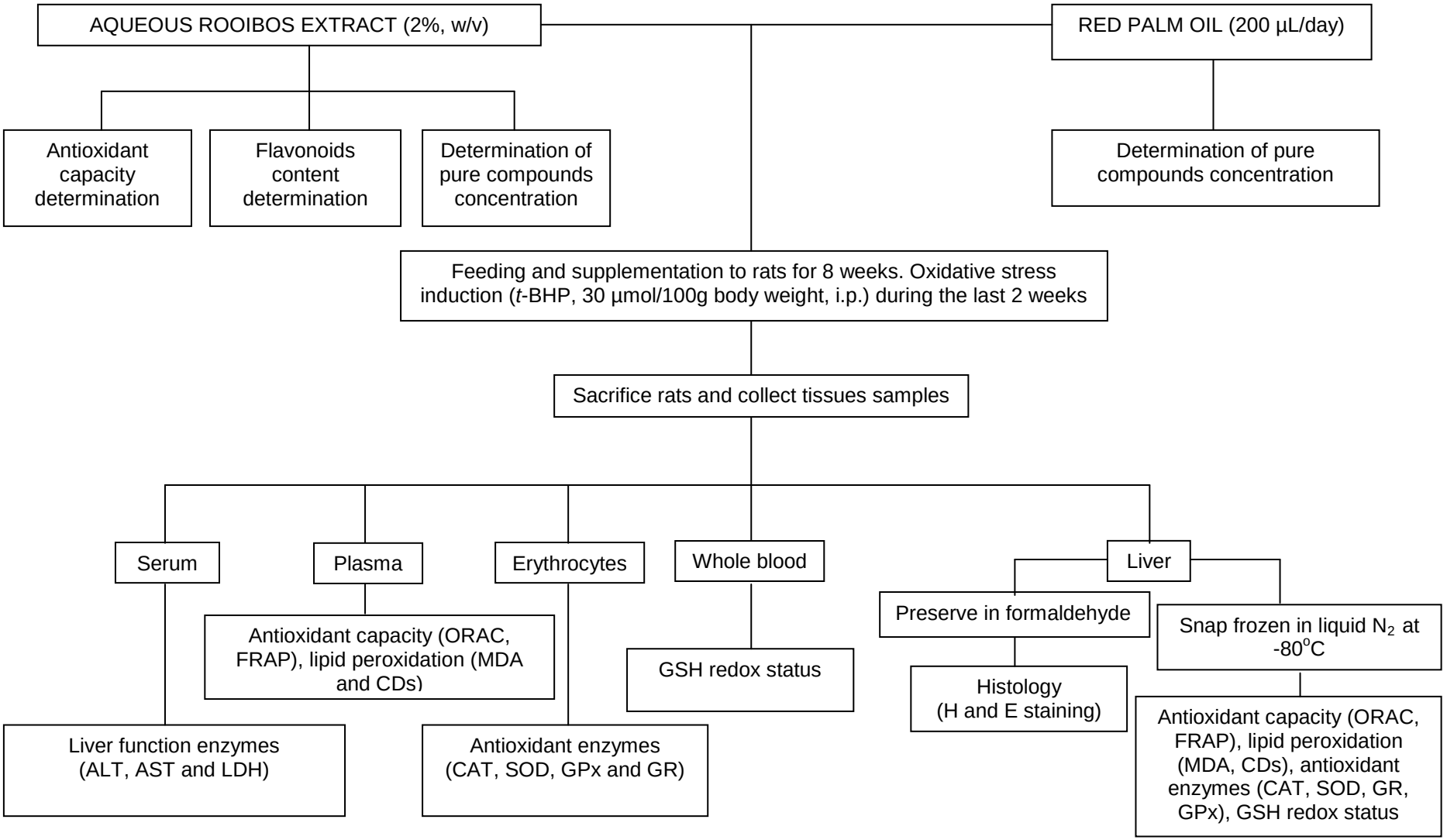

Figure 2: Schematic diagram showing the approach followed in the oxidative stress phase

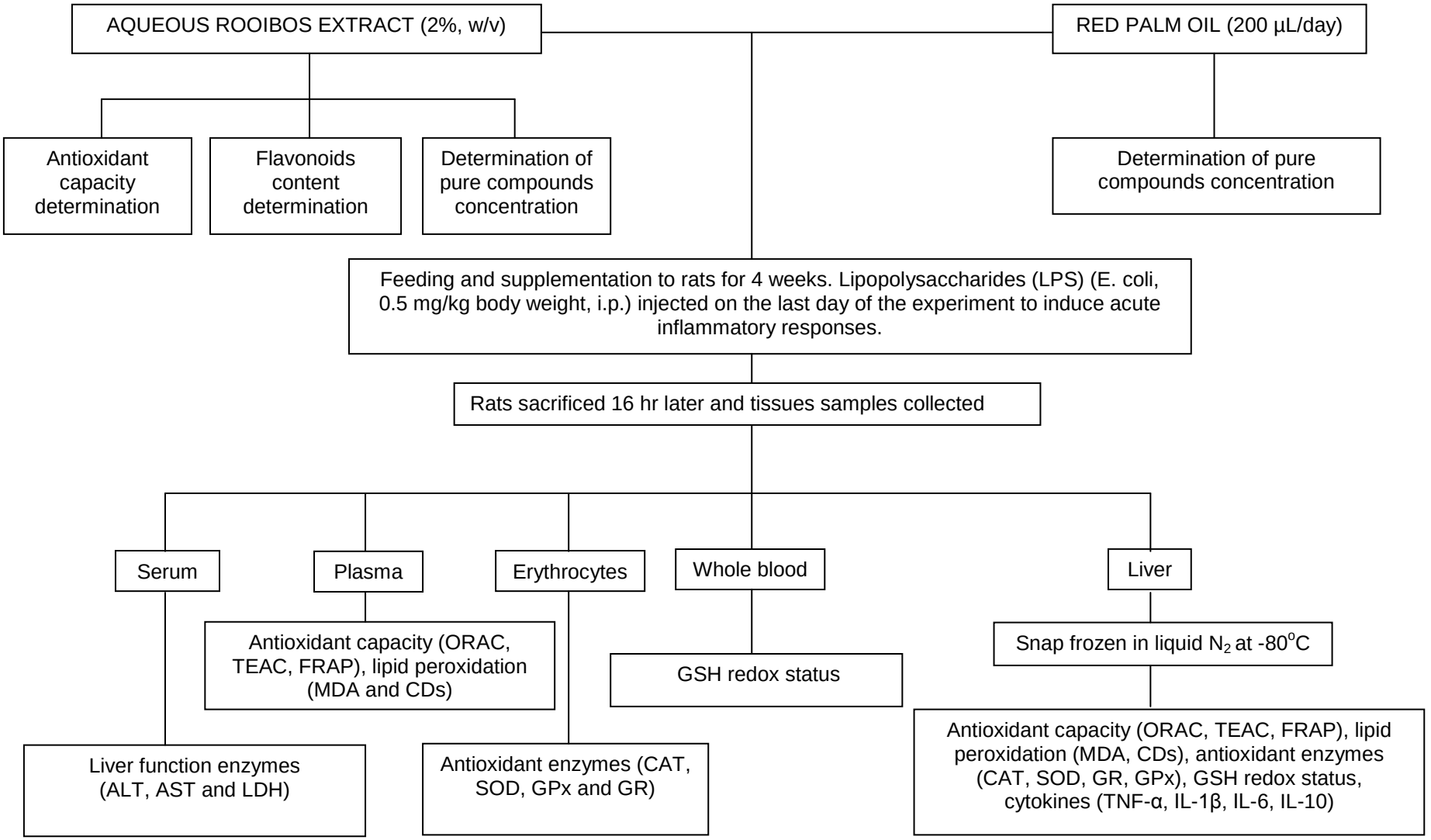

Figure 3: Schematic diagram showing the approach followed in the inflammation phase

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CHAPTER 2

Literature Review

1. Chronic diseases, prevalence and burden

Chronic diseases (CDs) affect people of all ages, nationalities and classes and are reaching epidemic proportions worldwide (WHO, 2005; Egger, 2012). These conditions cause the greatest global share of death and disability, accounting for 63% of all deaths and 44% of premature deaths worldwide (WHO, 2011). Accordingly, four diseases [cardiovascular diseases, cancers, chronic obstructive pulmonary diseases (COPD) and diabetes] have been identified as the leading cause of mortality in the world in 2008, and they account respectively for 17, 7.6, 4.2 and 1.3 million deaths (WHO, 2011). It has been forecasted that by 2020 the global death due to chronic diseases will worsen by 15-20%, and that the four major non communicable diseases would account for 75% of deaths worldwide by 2030 (Terzic and Waldman, 2011; WHO, 2011). With nearly 80% of these deaths occurring in low- and middle income countries, chronic non-communicable diseases (CNCDs) remain the most frequent cause of death in the Americas, the Eastern Mediterranean, Europe, South-East Asia and the Western Pacific (WHO, 2011). The European Union (EU) has identified chronic diseases as the greatest challenge to the goal that it has set for itself of contributing to the achievement of an increase of 2 years in the number of years spent in good health by the EU population, by 2020 (EU, 2012). Although, the average EU death rate from many chronic diseases, including cardiovascular and respiratory diseases, have declined over the last decade. However, the number of people actually suffering from chronic diseases such as diabetes, depression, Parkinson disease, Alzheimer's disease, musculoskeletal disorders and some cancers is rising, leading to increases in long term disability and reductions in the average number of years spent in good health in many parts of the European Union (EU, 2012).

In Africa and other developing nations, the situation is grimmer, with reports indicating that chronic disease conditions are already a burden and are increasing rapidly (Cooke, 2009) with stroke, hypertension, diabetes and cancers causing a greater number of adult medical admissions and deaths compared to communicable diseases such as HIV/AIDS or tuberculosis (Aikins *et al*., 2010). It has been estimated that CNCDs in Africa will cause almost three-quarter as many deaths as infectious, maternal, prenatal and nutritional diseases by 2020, and far exceed them as the most common causes of death by 2030 (WHO, 2011). The economic costs of CNCDs are enormous as it impact negatively by depriving individuals of their health and productivity potentials and invariably challenge the individuals or household incomes. On the country, it reduces life expectancy, depleting the quality and quantity of labour force and ultimately lower economic productivity (Abegunde and Stanciole, 2006).

1.1 Risk factors for increasing burden of chronic diseases

Risk factors for the growing burden of chronic diseases can be modifiable or non-modifiable. The non-modifiable risk factors include age, sex and genetic susceptibility (WHO, 2011). Several modifiable risk factors have been implicated in the increasing burden of chronic diseases, including behavioural or lifestyle factors (e.g. diet, physical inactivity, tobacco use and excessive alcohol consumption), biological factors (e.g. dyslipidemia, hypertension, overweight and hyperinsulinemia) and finally societal factors, which include a complex mixture of interacting socioeconomic, cultural and other environmental parameters (Steyn and Damasceno, 2006; Maher and Sekajugo, 2011; WHO, 2011). Scientific evidence has revealed that diet is an important modifiable risk factor for chronic diseases and that change in diet in the developed world and more recently in the developing countries from largely plant based diets to increased consumption of animal fat products, with consequent increase in fat content and hence high energy density, is a major reason for the increasing burden of chronic diseases (Darnton-Hill *et al*., 2004; Givens, 2005). Therefore, using the identified modifiable risk factors to inform public policy and preventing development of serious disabling and costly diseases, should be the goal of preventive medicine.

1.2 Central pathophysiological conditions linking chronic diseases

The pathophysiology of many chronic disease conditions has been linked to the three central factors of oxidative stress, inflammation and apoptosis. Oxidative stress has been recognized to be an important feature of many acute and chronic diseases including cancer, cardiovascular disease, neurodegenerative disease, lung disease and even the normal aging process (Montuschi *et al*., 2004). Oxidative stress is a consequence of an imbalance between free radical generation and antioxidant capacity of the cell and increasing evidence has emerged pointing to a causal link between oxidative stress and several chronic disease states (**Figure 1**). Several reports have shown that oxidative stress is closely linked with cardiovascular diseases (CVD) and coronary disease risk factors (such as endothelial dysfunction, myocardial ischaemic injury, atherosclerosis, and hypertension) (Moreno and Fuster, 2004; Dzau *et al*., 2006; Vaziri, 2008; Blum, 2009; Shahbaz *et al*., 2010; Pashkow, 2011), chronic kidney disease (Gosmanova and Le, 2011; Small *et al*., 2012; Tanaka *et al*., 2012; Tang *et al*., 2012), cancers (Valko *et al*., 2006, 2007; Shah *et al*., 2009; Acharya *et al*., 2010; Gupta-Elera *et al*., 2011) and diabetes (Abou-Seif and Youssef, 2004; Giacco and Brownlee, 2010; Matough *et al*., 2012). Oxidative stress causes a complex dysregulation of cell metabolism and cell-cell homeostasis and the emerging concept based on the study of several diseases, is that it is the final converging pathway through which risk factors of several diseases exert their deleterious effects (Pitocco *et al*., 2010).

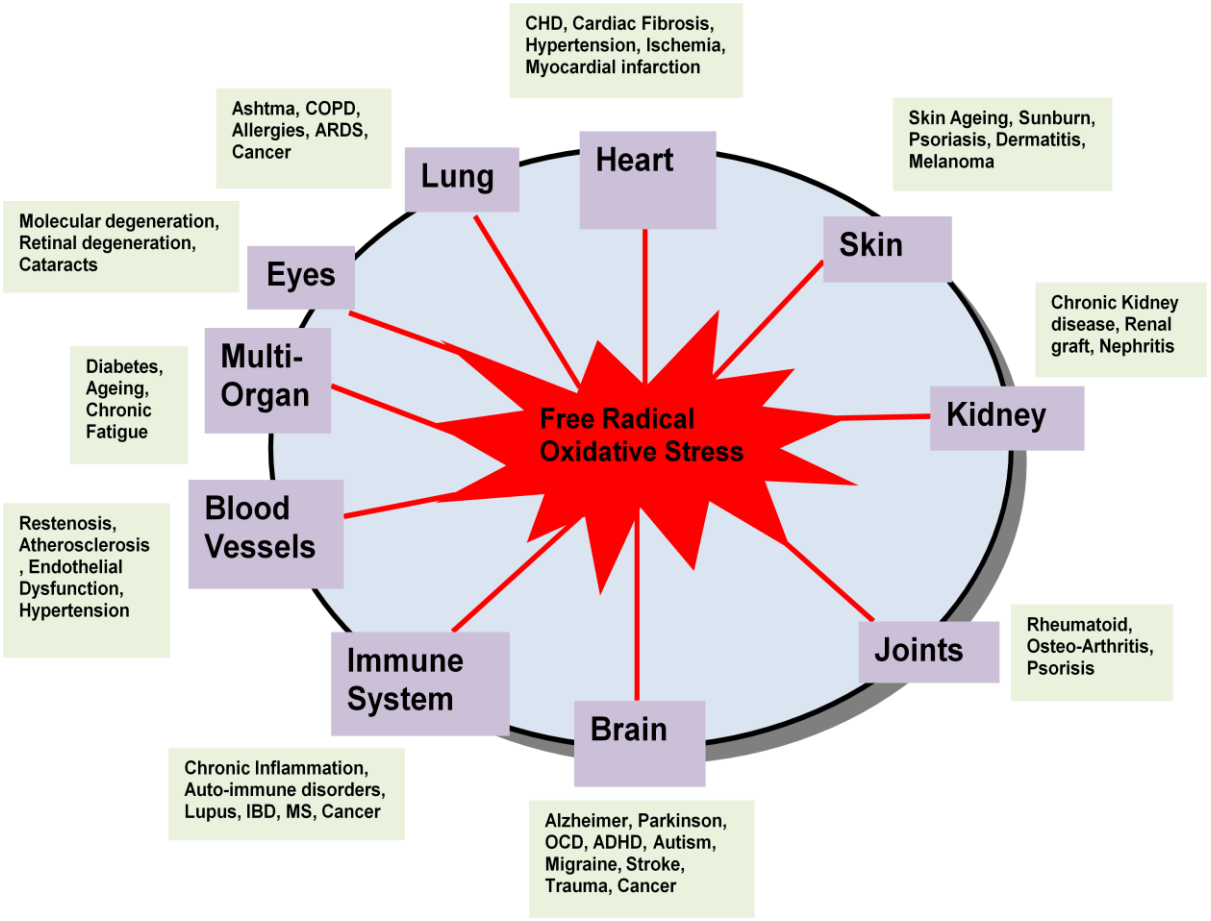

Figure 1: Some examples of diseases whose pathogenesis have been linked to oxidative stress and chronic inflammation. (Source: http://qvhealthwealthprosper.com/2012/04/18/39-deadly -diseases-linked-to-oxidative-stress/ Retrieved on 13/07/2012).

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

2. Oxidative stress

Oxidative stress may be defined as a disturbance in regular cellular and molecular function caused by an imbalance between production of free radicals (reactive species) and the natural antioxidant ability of cells resulting in oxidative damage to macromolecules (Small *et al*., 2012). A free radical is any chemical species (molecule, ion or atom) that contains an unpaired or odd electron in the outer orbit of its molecule. As a result of the presence of unpaired electrons, free radicals are usually very reactive and highly unstable (Freeman and Campo, 1982; Halliwell, 2005). Free radicals target macromolecules in their proximity for their electrons, thereby oxidising them and generating other free radicals. If the macromolecules targeted are important parts of the cellular structure such as nucleic acid, proteins and lipids, considerable oxidative injury can occur (Halliwell and Gutteridge, 2007). Free radicals are classified into two groups; reactive oxygen species (ROS) and reactive nitrogen species (RNS) and both often act together to create a state of oxidative stress (Small *et al*., 2012). Examples of reactive oxygen species are superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and thiyl ($RS^{\bullet}$) while examples of reactive nitrogen species include nitric oxide (NO) and nitrogen dioxide (NO_2). Apart from the reactive free radical species, there are also non-radical reactive species which though, do not contain unpaired electrons, are either oxidising agents or are easily converted to free radicals. Examples are hydrogen peroxide (H_2O_2), organic peroxides such as lipid hydroperoxides (ROOH), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{\bullet}$) (Droge, 2002; Valko *et al*., 2007; Small *et al*., 2012). ROS and RNS often act together to create a state of oxidative stress, either as a result of depletion of cellular antioxidant defense molecules or over-production of the reactive species.

2.1 Generation and interactions of reactive oxygen and nitrogen species (RONS)

Reactive oxygen species can be generated from many sources which can either be endogenous or exogenous. Most ROS are generated via endogenous sources as by-products of normal physiological and metabolic reactions such as energy generation through the mitochondrial electron transport chain reaction and protein assemblage by the endoplasmic reticulum (Valko *et al.,* 2006; 2007). The various endogenous and exogenous sources of ROS generation are shown in **Table 1**.

Table 1: Endogenous and exogenous sources of reactive species

Endogenous Sources	Exogenous Sources
Mitochondrial catalysed electron transport reaction	Radiation (UV light, X-ray and γ-radiation
Neutrophils and macrophages during inflammation	Environmental pollutant and toxin
Xanthine oxidoreductase, NADPH oxidase	Cigarette smoke, excessive alcohol , high calorie diet
Protein assemblage by endoplasmic reticulum	Hypoxia
Peroxisome	Infectious agent
Endosome and lysosome degradation	Glucotoxicity
Angiotensin II	Strenuous exercise
Fibroblasts	Heavy metals

The superoxide radical ($O_2^{\bullet-}$) is considered as the primary ROS and it can further interact with other molecules either directly or prevalently through enzyme- or metal-catalysed processes to generate secondary ROS (Valko *et al*., 2004; 2007). The superoxide anion is formed mostly within the mitochondria of a cell during the electron transport chain reactions. The electron transport chain is made up of five multi-enzyme complexes which are responsible for ATP generation and maintenance of mitochondrial membrane potential. The major site of ROS production is thought to be complex I and III (Murphy, 2009), however, other electron complexes, as well as other mitochondrial enzymes can also generate ROS (Finkel, 2012). During energy transduction, there is a premature leakage of electrons to molecular oxygen at complex I and III forming superoxide anion radical (Liu *et al*., 2002; Valko *et al*., 2007; Small *et al*., 2012). The superoxide anion radical produced as a result of one electron reduction of molecular oxygen, is a relatively stable intermediate and its dismutation by the enzyme manganese superoxide dismutase (MnSOD) will result in the formation of H_2O_2 (Ott *et al*., 2007). Protein assemblage by the endoplasmic reticulum and fatty acid degradation by peroxisomes, also account for $O_2^{\bullet-}$ which is dismutated to H_2O_2 by copper/zinc superoxide dismutase (Cu/ZnSOD). The H_2O_2 produced is a non radical molecule and it is normally neutralized by H_2O_2-removing enzymes, catalase and the glutaredoxin enzyme system (consisting of glutathione peroxidase, glutathione reductase and GSH) to water and molecular oxygen (Michiels *et al*., 1994). Glutathione peroxidase use reduced GSH as a substrate and it is converted to the oxidized form (GSSG). Reduced

glutathione is regenerated in a reaction catalysed by glutathione reductase with subsequent oxidation of NADPH. However, during a period of iron overload (e.g. conditions of haemochromatosis, haemolytic anaemia and haemodialysis), in the presence of high amounts of H_2O_2, heavy metals such as iron (Fe) or copper (Cu) are freed from iron-containing molecules and there is a subsequent interaction of $O_2^{\bullet-}$ and H_2O_2 in a Haber-Weiss reaction or Fe^{2+}- (Cu^{2+})-driven cleavage of H_2O_2 in a Fenton reaction to generate the highly reactive hydroxyl radical ($^{\bullet}OH$) (**Figure 2)** (Ott *et al*., 2007; Jomova *et al*., 2011; Small *et al*., 2012). The $^{\bullet}OH$ radical is highly reactive because of its very short half-life (< 1 ns), thus *in vivo*, it can react with macromolecules such as lipids, proteins and nucleic acids in the vicinity of its site of formation, resulting in oxidative damage to these important cellular components (Jomova *et al*., 2011). Other oxygen-derived free radicals that can be formed *in vivo* are the peroxyl radicals ($ROO^{\bullet}$) (**Figure 2**) which are involved in DNA damage and protein backbone modification and have also been found to synergistically enhance the induction of DNA damage by superoxide (Valko *et al*., 2006).

Reactive nitrogen species are nitrogen centred free radicals and include the nitric oxide radical ($NO^{\bullet}$), nitrosonium cation ($^{\bullet}NO_2$), nitroxyl anion (NO^-), nitrous oxide radical ($NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$). The $NO^{\bullet}$ radical is the precursor of other RNS and is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine in a reaction catalysed by nitric oxide synthase (NOS) (Palmer *et al*., 1988; Bryan *et al*., 2009). The NOS family includes endothelial NOS (eNOS) and neuronal NOS (nNOS), both constitutively expressed isoforms, as well as the transcriptionally regulated isoform, inducible NOS (iNOS) which has been shown to play an important role in host defence (Nathan, 1992; Geller and Billiar, 1998; White *et al*., 2010). Overproduction of the RNS above the ability of the cell to neutralise them, is known as nitrosative stress and may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function (Valko *et al*., 2006). Oxidative burst during inflammatory processes trigger the formation of both superoxide and nitric oxide radical from immune cells and they react together to produce the highly reactive peroxynitrite anion radical which is a potent oxidising agent that can cause DNA fragmentation (Carr *et al*., 2000; Valko *et al*., 2006; 2007). Other nitrating agents include the nitrosonium cation formed in a myeloperoxidase-catalysed reaction involving nitrite (NO_2^-) and hydrogen peroxide (Eiserich *et al*., 1998; Sampson *et al*., 1998) and nitroso-peroxocarbonate ($ONO\text{-}OCO_2^-$) formed via the reaction of CO_2 with $ONOO^-$ (Denicola *et al*., 1996; Lang *et al*., 2000). A recent study has also shown that lipid peroxyl radicals are able to promote tyrosine nitration by inducing tyrosine oxidation and also by reacting with $NO_2^{\bullet}$ to produce nitrosonium cation ($^{\bullet}NO_2$) (Bartesaghi *et al*., 2010).

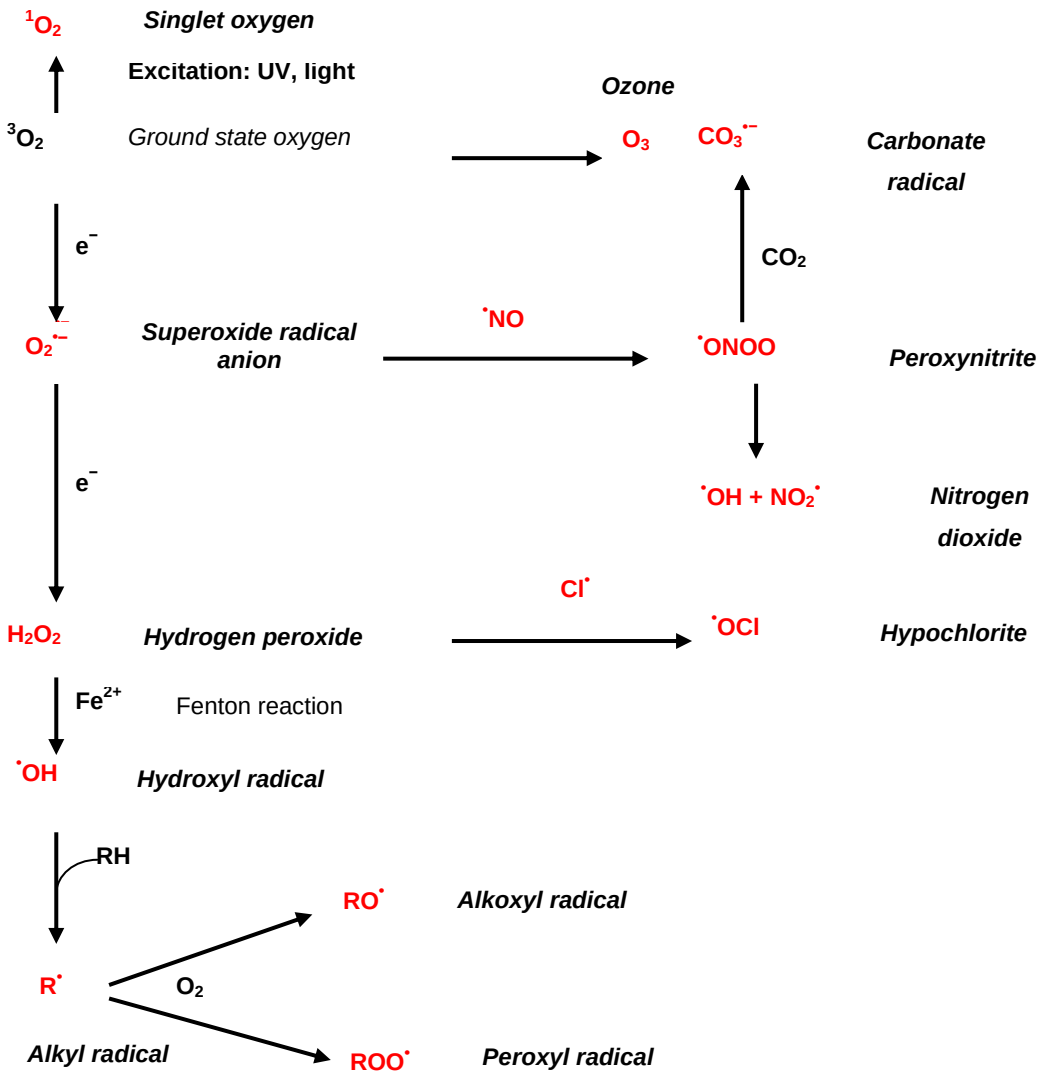

Figure 2: Major reactive oxygen and nitrogen species (red colour). RH: organic molecule (Source: Bartosz, 2009).

2.1.1 Physiological importance of reactive oxygen and nitrogen species (RONS)

Reactive oxygen and nitrogen species have generally been considered as being highly reactive and cytotoxic molecules, however accumulating evidence has shown that beside their noxious effects, RONS participates in physiological processes in a well controlled manner. Physiological sources of RONS in the cell are many, but in a number of cells RONS generated by the NADPH oxidase enzyme are those implicated in important physiological responses, such as defence against environmental pathogens or in cell signalling (Pourova *et al*., 2010). Reactive oxygen and nitrogen species are able to influence physiological processes because of their ability to modify the activity of key protein molecules containing domains sensitive to redox conditions. Reversible oxidation of the target domain results in the initiation of coupled events, such as modification of the activity of downstream enzymes and/or transcription factors. Recent evidence has shown that at moderate concentrations, nitric oxide (NO), superoxide anion, and related reactive species play an important role as regulatory mediators in cell signaling processes and that many of the ROS-mediated responses actually protect the cells against oxidative stress and re-establish redox balance (Droge, 2002). Reactive species have been implicated as signaling molecules in physiological processes, such as regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes (Droge, 2002).

Accumulating evidence suggests that mitochondrial oxidants may be important regulators of the cellular response under hypoxic conditions (oxygen concentrations between 1 and 5%), and that mitochondria might actually increase their release of ROS (Finkel, 2012). Although the molecular basis for this is still obscure, nonetheless, evidence suggests that the release of ROS under these conditions functions as an important physiological regulator of hypoxia-inducible factor 1α (HIF-1α), which under low oxygen conditions, respond to a rise in ROS and then feedback and inhibit the production of ROS levels (Brunelle *et al*., 2005; Guyz *et al*., 2005; Mansfield *et al*., 2005; Finkel, 2012). Other HIF-1α activities include the transcriptional regulation of metabolic enzymes such as lactate dehydrogenase A and pyruvate dehydrogenase kinase 1 (PDK1), which control the flow of carbon substrates into the mitochondria (Semenza *et al*., 1996; Kim *et al*., 2006). Other studies have also shown that HIF-1α regulate the expression of certain cytochrome components directly, and also regulate specific microRNAs that in turn regulate the expression of components of the electron transport chain (Fukuda *et al*., 2007; Chan *et al*., 2009; Finkel, 2012).

RONS mediate inflammatory responses in a number of ways. In an inflammatory environment, activated neutrophils and macrophages produce large quantities of superoxide

radicals and other ROS via the phagocytic isoform of NADPH oxidase for the destruction of intracellular microbes (Keisari *et al*., 1983; Valko *et al*., 2007). Recent evidence has shown that mitochondrial ROS also contribute to the phagocytic response of the innate immune system as a result of the activation of a subset of Toll-like receptors (TLR1, TLR2 and TLR4) (West *et al*., 2011). Mitochondrial RONS (mRONS) have also been suggested to be involved in the activation of the inflammasome, thus acting as important signaling molecules to regulate inflammatory responses. Specifically, mRONS have been implicated in the activation of NLRP3 (NOD-like receptor, pyrin domain-containing 3) receptor (an intracellular receptor that senses a wide range of damage signals, including microorganisms, endogenous danger signals such as ATP and uric acid, and environmental irritants), which when activated, forms a high molecular weight intracellular complex with a number of other protein partners to regulate the maturation and secretion of pro-inflammatory cytokines such as IL-1β (Nakahira *et al*., 2011; Zhou *et al*., 2011; Bulua *et al*., 2011; Finkel, 2012).

Another physiological role of RONS is the regulation of vascular tone by cGMP. Soluble guanylate cyclase (sGC) is a heterodimeric protein which catalyses the formation of cGMP. Soluble guanylate cyclase can be activated by both $NO^{\bullet}$ and H_2O_2, with NO• activating sGC by binding to its Fe^{2+}-haem groups resulting in a conformational change at Fe^{2+} that activates the enzyme. The cGMP formed is used as an intracellular amplifier and second messenger to modulate the function of protein kinases, ion channels, and other physiologically important targets, especially regulation of smooth muscle tone and the inhibition of platelet adhesion (Valko *et al*., 2007). Reactive oxygen and nitrogen species have been implicated in the regulation of authophagy and apoptosis, while mRONS have been recognised as important signalling molecules in the regulation of autophagy through direct regulation of Atg4 (autophagy-related gene 4) activity (Scherz-Shouval *et al*., 2007; 2011). Mitochondria-generated ROS also play an important role in the release of cytochrome c and other pro-apoptotic proteins, which can trigger caspase activation and apoptosis, therefore small molecules like ROS, can impact on the complex networks of proteins mediating the induction and execution of cell death (Ott *et al*., 2007).

2.2 Consequences of oxidative stress

Oxidative attack by RONS is manifested as damage to nucleic acid bases, lipids, and proteins, which can severely compromise cell health and viability or induce a variety of cellular responses through generation of secondary reactive species, ultimately leading to cell death by necrosis or apoptosis (Dalle-Donne *et al*., 2006). Macromolecular damage via oxidative and/or nitrosative stress, if unchecked, can theoretically contribute to disease development and an increasing amount of evidence suggests that oxidative and/or

nitrosative stress is linked to the pathophysiologic mechanisms of a myriad of human diseases (Dalle-Donne *et al*., 2003; 2006; Halliwell and Gutteridge, 2007; Klaunig *et al*., 2010; Small *et al*., 2012).

2.2.1 Oxidative lipid damage (lipid peroxidation)

Lipid peroxidation refers to the oxidative degradation of lipids that occur when RONS attack lipids and extract a hydrogen atom from a methylene carbon in the side chain, and is one of the major outcomes of free radical-mediated injury. The cell membrane is one of the most susceptible sites to RONS damage because the polyunsaturated fatty acid (PUFA) residues of phospholipids are very sensitive to oxidation. RONS such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), alkylperoxyl radical ($RCOO^{\bullet}$) and peroxynitrite ($ONOO^{\bullet}$) are all potent initiators of lipid peroxidation.

The process of lipid peroxidation occurs in three stages: initiation, propagation and termination (Valko *et al*., 2006). During initiation, the fatty acid containing the double bonded carbon chains is oxidised by a free radical to produce a lipid radical which stabilizes itself by reacting with oxygen to produce a new radical, the peroxyl radical which then can oxidize a neighbouring fatty acid to become a stable hydroperoxide. The oxidized fatty acid neighbour becomes a lipid radical, which propagate the oxidation process across the lipid membrane (Valko et al., 2006; Jomova et al., 2011) The lipid hydroperoxides formed may then be converted into conjugated dienes which are unstable and generate a variety of relatively stable end products, mainly aldehydic by-products, such as malondialdehyde, (MDA) and more reactive α, β-unsaturated aldehydes, such as trans-4-hydroxy-2-nonenal (4-HNE) and 2- propenal (acrolein) (Uchida, 2003; Carini *et al*., 2004; Dalle-Donne *et al*., 2006; Mangialasche *et al*., 2009; Vignini, 2011). Isoprostanes and neuroprostanes and more recently neurofurans are other products that have been derived from the endocyclization of lipid hyroperoxyl radicals (Roberts and Morrow, 2002; Cracowski *et al*., 2002; Song *et al*., 2008; Halliwell and Lee, 2010; Niki, 2010).

Oxidative stress-induced lipid peroxidation is a very damaging, self perpetuating process which can alter the biological properties of the membrane (such as the degree of fluidity), inactivate membrane-bound receptors or enzymes and consequently impair normal cellular function and increase tissue permeability (Dalle-Donne *et al.,* 2006). Moreover, lipid peroxidation may cause and amplify cellular damage resulting from generation of highly reactive oxidized products, which bind and covalently modify important macromolecules, as seen in reaction of MDA with guanine, adenine and cytosine to form MDA-DNA adducts (Jomova *et al*., 2011). Evaluation of these end products has been used as an index to

determine the extent of oxidative damage in cells. One of the most popular assays for the assessment of lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) assay. The TBARS assay measures the level of MDA, since MDA form a stable MDA-TBA adduct when it reacts with thiobarbituric acid (TBA), which can be quantified spectrophotometrically or by high performance liquid chromatography (HPLC). The widely used spectrophotometric assay for TBARS to assess the extent of oxidative stress *in vivo* has been criticised for some obvious limitations, including lack of specificity, sensitivity and reproducibility (Halliwell, 2009; Mangalasche *et al*., 2009). It has been established that (i) aldehydes other than MDA may react with TBA to produce a compound that absorbs in the same range as MDA, (ii) during the assay, lipid peroxides may be degraded and this may mask the actual amount of MDA in the sample, (iii) the presence or absence of metal ions or other undefined radicals affect the rate of this decomposition, making reliability a problem, and (iv) most TBA-reactive materials, including MDA, *in vivo* are not specific products of lipid peroxidation and may produce false-positive results (Halliwell and Whiteman 2004; Halliwell, 2009; Halliwell, 2011). As a result of these obvious limitations, the simple spectrophotometric TBARS assay is regarded as obsolete and unsuitable. However, despite these shortcomings, the spectrophotometric TBARS assay is still widely used because it is a relatively simple and rapid assay which can be performed in most laboratories. The direct assessment of MDA or MDA-TBA adducts by HPLC is more specific and reliability and reproducibility of the results are better, but reports have shown that this approach does not deduce all the limitations of this biomarker (Halliwell and Whiteman 2004, Halliwell and Gutteridge, 2007; Halliwell, 2009, Halliwell, 2011). Some authors are of the opinion that since unsaturated aldehydes such as 4-hydroxynonenal and acrolein may cause considerably more cytotoxicity *in vivo* than MDA, it is perhaps more logical to measure these products (Uchida, 1999; 2003).

The isoprostanes, of which F_2-isoprostanes (F_2-IsoPs) have been extensively studied, are considered for now, the best biomarker of oxidative stress and lipid peroxidation because of their chemical stability *in vivo* and *ex vivo*, and minimal metabolism *in situ* (Roberts and Morrow, 2002; Basu, 2004; Halliwell and Whiteman, 2004; Montuschi *et al*., 2004; Morrow, 2005; Spickett *et al*., 2010). F_2-isoprostanes are made up of 64 different prostaglandin (PG)-like compounds with the F-type prostane ring. They are generated *in vivo* by the non-enzymatic free radical-mediated peroxidation of esterified arachidonic acid (AA), cleaved by phospholipases or platelet-activating factor (PAF) acetylhydrolases, released into the circulation and excreted in urine as free isoprostanes (Morrow, 2005; Mangalasche *et al*., 2009; Spickett *et al*., 2010). Plasma and urine are the sample types of choice for analysis, because, they are the most convenient and the least invasive to obtain. Isoprostanes have also been found in measurable quantities in most of the biological fluids analysed, including

synovial fluid, bronchoalveolar fluid, bile, lymph, microdialysis fluid from various organs, and amniotic, pericardial, and seminal fluid (Dalle-Donne *et al*., 2006). Recent evidence has shown that F_2-IsoPs are elevated in animal models of oxidant injury and in human diseases associated with oxidative stress (Spickett *et al*., 2010; Billing *et al*., 2011; Il'yasova *et al*., 2012; Chang *et al*., 2012). Current techniques for measuring F_2-isoprostanes includes gas chromatography-mass spectrometry (GC-MS), GC-tandem MS (GC-MS/MS), liquid chromatography (LC)-MS, LC-MS/MS. Various enzyme immunoassay and radioimmunoassay methods are also used but their precision, accuracy and reliability is still being investigated further (Dalle-Donne *et al*., 2006; Mangalasche *et al*., 2009).

2.2.2 Oxidative protein damage

Proteins are primarily responsible for most functional processes within cells and are thus highly abundant in biological systems, making them important targets of RONS attack The attack of RONS on the polypeptide backbone is initiated by an •OH-dependent abstraction of the α-hydrogen atom from an amino acid residue to form a carbon-centered radical, which under aerobic conditions readily reacts with molecular oxygen to form peroxyl radicals, which reacted with the protonated form of superoxide ($HO_2^{\bullet}$) and are converted to the alkyl peroxides (Stadtman, 1992; Valko *et al*., 2007). Attack by RONS on proteins may lead to the oxidation of amino acid residue side chains, as well as oxidation of the protein backbone and formation of protein-protein cross-linkages. Consequently, protein fragmentation and generation of many protein oxidation products which can cause damage to other biomolecules may occur (Mangalasche *et al*., 2009, Pandey and Rizvi, 2010; 2011). All amino acids within proteins may be attacked by RONS; however sulphur containing and aromatic amino acids are the most susceptible (Stadtman and Levine, 2003). Carbonyl derivatives are usually formed when amino acids are oxidized and tyrosine groups of proteins undergo nitration by $ONOO^{\bullet}$ to form 3-nitotyrosine, while dityrosine may also be formed (Mangialasche et al., 2009). Every level of protein structure, from primary to quaternary (if multimeric proteins), may be altered when proteins are exposed to oxidative attack by RONS, causing major physical changes in protein structure (Dalle-Donne et al., 2006).

Because most protein damage is irreparable, oxidative changes to the structure of protein can have a wide range of functional consequences including affecting the function of receptors, enzymes, and transport proteins as well as generating new antigens that can provoke immune responses (Halliwell and Whiteman, 2004). Secondary damage to other biomolecules can also result in inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA (Wiseman and Halliwell, 1996; Halliwell and

Whiteman, 2004). Oxidized proteins are usually degraded by proteosomal and lysosomal pathways, and since degradation of damaged proteins are not completely efficient, functionally inactive proteins that are poorly degraded may form high molecular mass aggregates which accumulate with age in separate compartments within cells or in the extracellular environment (Dunlop *et al*., 2009; Seifert *et al*., 2010; Avery, 2011). It is also possible that the proteolytic systems responsible for removal of oxidized proteins may be impaired by oxidative stress itself, thereby accelerating the accumulation of damaged aggregating proteins (Cecarini *et al*., 2007). Autophagic destruction of protein aggregates which is the last line of defence against these toxic species, may not be wholly efficient (Pan *et al*., 2008; Madeo *et al*., 2009). Protein aggregates can be highly cytotoxic (Campioni *et al*., 2010) and increased levels of carbonylated aggregates have been observed in patients with age-related disorders such as Parkinson disease, Alzheimer's disease and cancer (Avery, 2011).

There are several advantages in measuring protein oxidation as compared to lipid peroxidation or oxidative DNA damage as marker of oxidative and/or nitrosative stress because (i) proteins play a key role in maintaining cellular structure and functions, therefore, alterations of the protein structure due to oxidative and/or nitrosative stress may be reflected at functional level, and the change of activity can be assayed, (ii) the products of oxidative/nitrosative modifications of proteins are relatively stable, and (iii) there are sensitive techniques available to detect and measure them (Chakravarti and Chakravarti, 2007). Protein bound carbonyls has been extensively assayed as a biomarker of oxidative damage to proteins. A number of methods have been developed for the detection of protein carbonyl including the use of radiolabel incorporation from reductants, derivatization by 2,4-dinitrophenylhydrazine (DNPH) and quantification by spectrophotometry, spectrophotometric DNPH derivatization coupled to protein fractionation by HPLC, enzyme linked immunosorbent assay (ELISA) and one- or two-dimensional electrophoresis followed by western blot immunoassay (Winterbourn and Buss, 1999; Levine *et al*., 1994; Levine *et al*., 2000; Catalgol *et al*., 2011). 3-Nitrotyrosine (3-NT) is another biomarker that can be used to quantify the extent of oxidative protein damage *in vivo*. Several methods of analyses are available to assess 3-NT, including chemical analysis using HPLC and GC couple to a mass spectrometer and immunochemical detection using specific antibodies (Castegna *et al*., 2003; Dalle-Donne *et al*., 2006). It can also be assess using specific ELISA kits (Korolainen and Pirtilla, 2009). Another analogue of 3-NT, dityrosine (DT) can be analysed by mass spectrometry coupled with electrochemical detection (Hensley *et al*., 1998; Di Domenico *et al*., 2011). Protein glutathionylation may occur under oxidative stress conditions, but it is also important under normal condition especially for regeneration of several thiol peroxidases.

Considering its importance, several methods are available for its detection. Visualization and quantitation of protein glutathionylation using radiolabeled GSH detected by fluorography (Costa *et al*., 2003) and analysis with immunohistochemical methods exploiting monoclonal antibody anti-GSH bounded to the proteins (Gao *et al*., 2009) are two available methods.

2.2.3 Oxidative DNA damage

Reactive oxygen species, especially $OH^{\bullet}$ radical generated during oxidative stress can react with and cause modifications in all the components of the DNA molecule (the purine and pyrimidine bases and deoxyribose sugar backbone), causing damage such as base or sugar lesions, single strand breaks, double strand breaks, abasic site formation and DNA-DNA or DNA-protein cross-links (Dizdaroglu *et al*., 2002; Halliwell and Gutteridge, 2007; Mangialasche *et al*., 2009). Reports have indicated that reactive oxygen and nitrogen species alone generate several kinds of single-strand breaks and more than 70 oxidative base and sugar products in DNA (Sander *et al*., 2005; Hoeijmakers, 2009). It has been estimated that the frequency of oxidative DNA damage in human cells is 10^4 lesions /cell/day (Fraga *et al*., 1990; Lu *et al*., 2001). Although hydrogen peroxide is less reactive, it's more readily diffusible and thus more likely to be involved in the formation of oxidized bases through Fenton and Haber-Weiss reactions (Guyton and Kensler 1993; Barber and Harris 1994; Klaunig *et al*., 2010). Oxidative damage not repaired prior to DNA replication, may cause cell death, DNA mutation, replication errors and genomic instability (Marnett, 2000; Cooke, 2003; Klaunig and Kamendulis, 2004; Valko *et al*., 2006). Reports by Halliwell (2002) and Valko *et al*. (2004) indicated that oxidative DNA damage may be related to increased risk of cancer development later in life. DNA can also undergo nitrative damage as a result of attack of RNS on DNA bases to form 8-nitroguanine (Kawanishi and Hiraku, 2006). Experimental evidence has shown that 8-nitroguanine is a mutagenic DNA lesion which can preferentially lead to G-T transversions and its formation has been observed in human samples (Kawanishi and Hiraku, 2006). Attack on DNA by aldehydic products of lipid peroxidation such as acrolein and 4-HNE, can lead to the formation of bulky exocyclic adducts, which can promote DNA–DNA and DNA–protein cross linking and impair transcription factors binding (Kozekov *et al*., 2003; Liu *et al*., 2006; Lovell and Markesbery, 2007).

The most commonly used biomarker of DNA damage is the concentration of the nucleoside, 8-hydroxy-2′-deoxyguanosine (8-OHdG) which has been found to be mutagenic in bacterial and in mammalian cells (Guetens *et al*., 2002; Halliwell and Gutteridge, 2007; Mateos and Bravo, 2007; Klaunig *et al*., 2010). Numerous reports have shown that urinary levels of 8-OHdG was elevated in humans with various malignancies (Kuo *et al*., 2007; Rasanen *et al*.,

2007; Chang *et al*., 2008; Yang *et al*., 2009; Valavanidis *et al*., 2009; Agha-Hosseini *et al*., 2012; Gonenc *et al*., 2012) and also in experimental animal models of tumors (Gottschling *et al*., 2001; Muguruma *et al*., 2007; Iqbal *et al*., 2009; Kumar *et al*., 2012; Sintara *et al*., 2012). The common techniques used in determining the level of 8-OHdG includes HPLC coupled with electrochemical detection (HPLC-ECD), GC-MS, HPLC-MS/MS, various antibody based techniques, aldehyde reactive probe (ARP, biotinylated alkoxyamine) assay in combination with an ELISA-like assay and comet assay (Kow and Dare, 2000; Guetens *et al*., 2002; Cadet *et al*., 2003; Halliwell and Whiteman, 2004; Mateos and Bravo, 2007).

2.3 Antioxidant defence system

Under normal circumstances, eukaryotic cells have evolved a defence mechanism to limit free radicals and the damage caused by them. These include systems based on the presence of antioxidant molecules, repair of injured molecules and removal of damaged molecules. The antioxidant defence system can be endogenous and/or exogenous. The endogenous system is made up of a network of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the glutaredoxin and peroxiredoxin system as well as low molecular weight antioxidant molecules such as vitamin E (major membrane bound antioxidant), vitamin C (ascorbic acid, AA) (major aqueous phase antioxidant), uric acid, glutathione and ceruloplasmin (Davies, 2000). Superoxide dismutase catalyses the dismutation of superoxide anion, converting it to molecular oxygen and H_2O_2. There are three isoforms of the SOD family and they all utilise a transition metal at their active site. There is a CuZnSOD form in the cytosol and the intermembrane mitochondrial compartment (Okado-Matsumoto and Fridovich, 2001), a MnSOD in the mitochondrial matrix and another form in the extracellular compartment (e.g. blood) (Pamplona and Costantini, 2011).

Though, less reactive than the $O_2^{\bullet-}$ anion, the H_2O_2 must still be rapidly removed and this can be accomplished by the enzymes CAT and GPx working co-ordinately. Catalase remove H_2O_2 at a high rate but show low affinity for the peroxide, thus it should be most useful during the peak of H_2O_2 production or accumulation (Pamplona and Costantini, 2011). Evidence has shown that this peak should occur *in vivo* since acatalasemia (i.e. disorder caused by lack of catalase) increases oxidative stress and induce certain pathologies in human (Goth, 2000). Glutathione peroxidase may be present in the selenium- and non-selenium-dependent forms and it has been shown that there are at least five selenium-containing GPxs in humans (Brigelius-Flohe and Kipp, 2009) which activities may be manipulated by changing dietary selenium levels (Arthur, 2000). The isoforms are the ubiquitously expressed cytosolic GPx (GPx-1) and phospholipid hydroperoxide GPx (GPx-4), epithelium-specific gastrointestinal

GPx (GPx-2), secreted plasma GPX (GPx-3) and GPx-6 found in the olifactory epithelium and embryonic tissue (Papp *et al*., 2007; Steinbrenner and Sies, 2009). All the GPx isoforms (GPx 1-4 and GPx-6) can metabolise H_2O_2 and soluble fatty acid hydroperoxides, however, only GPx-4 can metabolise complex phospholipid hydroperoxides (Brigelius-Flohe and Kipp, 2009). The phospholipid hydroperoxide GPx-4 also differs from other isoforms in that it is a monomer while others are tetrameric proteins (Arthur, 2000). Glutathione peroxidases utilize the reducing power of GSH (and other thiols, such as thioredoxin) to decompose H_2O_2 and it is the sulfhydryl moiety of the cysteine residue that supplies the reducing equivalent for GPx activity. Two molecules of GSH are oxidized for every one molecule of H_2O_2 decomposed, resulting in the formation of GSSG which can be re-reduced back to two molecules of GSH by glutathione reductase (Davies, 2000).

Various low molecular weight endogenous non-enzymatic antioxidants are found in animal and human tissues. These are usually depleted when they react with RONS, but are actually recycled back to the antioxidant form due to reduction by other molecules. Because of their low molecular weight, they are able to eliminate RONS at sites that much larger enzymes cannot access (Pamplona and Costantini, 2011). Reduced glutathione (GSH), thioredoxin and ascorbate are the main low molecular weight hydrophilic non-enzymatic antioxidant molecules in the cell (Pamplona and Costantini, 2011). Glutathione (L-γ-glutamyl-L-cysteinylglycine) is the predominant intracellular non-protein thiol in eukaryotic cells. It possesses strong antioxidative properties and consequently plays a crucial role in intracellular protection against compounds such as RONS and other free radicals (Anderson, 1996; Anderson and Luo, 1998; Nordberg and Arner, 2001). It functions as a nucleophile to form conjugates with many xenobiotics and/or their metabolites and can also serve as a reductant in the metabolism of hydrogen peroxides and other organic hydroperoxides (Anderson, 1996; Anderson and Luo, 1998; Rahman *et al*., 1999; Deneke, 2000; Suntres, 2002). The thioredoxin system is another major intracellular antioxidant system and comprises of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH. It is an oxidoreductase with a redox-active disulfide/dithiol at the active site (Hashemy, 2011; Pamplona and Costantini, 2011). Report has indicated that Trx is cytoprotective against oxidative stress in cooperation with peroxiredoxin/thioredoxin-dependent peroxidases (Mitsui *et al*., 2002, Nakamura, 2005).

The other most abundant reduced non-enzymatic antioxidant in cells is ascorbate. It is endogenously synthesized and maintained at high levels in tissues (about 1 mM in rats), but in primates (including human), guinea pigs, fruit-eating bats and many bird species, it is obtained through diet (Barja, 1996). Ascorbate can scavenge RONS and other free radicals

by readily donating an electron to potentially damaging radicals such as hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($LOO^{\bullet}$), thiol radical ($GS^{\bullet}$) and tocopheroxyl radicals ($TO^{\bullet}$) becoming oxidized to the ascorbate radical ($Asc^{\bullet-}$) during the process (Buettner, 1993; Du *et al*., 2012). Ascorbate is regenerated from the $Asc^{\bullet-}$ by the action of (i) NADH- and NADPH-dependent dehydroascorbate reductase (Rose and Bode, 1993; Navas *et al*., 1994; Linster and Schaftingen, 2007), (ii) GSH-dependent dehydroascorbate reductase (Maellaro *et al*., 1994; Wells and Xu, 1994) or (iii) pH dependent disproportionation reaction which result in the formation of ascorbate and dehydroascorbate (Bielski *et al*., 1981; Corti and Casini, 2010). Reports have shown that GSH and ascorbate interact cooperatively *in vivo* to cope with RONS (Meister, 1994) and that ascorbate serves as a co-antioxidant with vitamin E *in vivo* to protect LDL from detectable oxidative damage induced by aqueous peroxyl radicals (Frei *et al*., 1989).

Vitamin E and carotenoids are the most important antioxidants in the lipophilic environment of the cell. There are 8 known isoforms of the vitamin E family, comprising of α-, β-, γ- and δ-tocopherol and the corresponding tocotrienols (Wang and Quinn, 1999; Bron and Asmis, 2001; Surai, 2002). Because of the presence of an –OH group on the chromanol ring of vitamin E, it is able to function as an antioxidant and reduces lipid peroxyl groups to hydroperoxides, thus terminating the propagation of lipid peroxidation (Esterbauer et al., 1991; Pamplona and Costantini, 2011). Empirical evidence for carotenoids being important antioxidants *in vivo* is weak (Pamplona and Costantini, 2011, Halliwell, 2012). Recent reports in birds showed that carotenoids have a very low contribution to protection against oxidative damage under stressful conditions (Costantini and Moller, 2008; Costantini *et al*., 2008; Isaksson and Anderson, 2008; Horak *et al*., 2010; Larcombe *et al*., 2010). Many human trials where carotenoids and other dietary antioxidants (vitamin C and E) were administered singly or together, did not show any positive effect, and even contributed to mortality among study subjects (Heinonen and Albanes, 1994; Albanes *et al*., 1996; Lee *et al*., 1999; Muntwyler *et al*., 2002).

2.4 Pro-oxidant activity of flavonoids

Pro-oxidant activities of dietary antioxidants such as carotenoids or polyphenols have also been reported (Halliwell, 2008; Pamplona and Costantini, 2011). It has been suggested that the antioxidant/pro-oxidant properties of flavonoids could be important in determining the fate of a cell, with the biological response, beneficial or deleterious, depending on the prevailing oxidative status in the cell (Klaunig and Kamendulis, 2004; Joubert *et al*., 2005). Evidence has shown that the antioxidant/pro-oxidant activity of flavonoids depend on certain factors, including metal-reducing potential, chelating ability, pH and solubility characteristics (Decker,

1997; Sakihama *et al*., 2002). Polyphenols are oxidized to produce superoxide anion radicals, hydrogen peroxide and a complex mixture of semiquinones and quinones, which are all potentially cytotoxic (Awad *et al*., 2001; Lambert *et al*., 2007; Halliwell, 2008). Some reports have suggested that a pro-oxidant effect of flavonoids can be beneficial, because imposing a mild oxidative stress, might induce an upregulation of antioxidant defenses and xenobiotic metabolising enzymes, resulting in overall cytoprotection (Fahey and Kensler, 2007; Halliwell, 2008; Halliwell, 2009; Tang and Halliwell, 2010) Also the pro-oxidant effect of flavonoids has been proposed as one of the mechanisms for the anti-cancer function of most flavonoids, including EGCG, quercetin and luteolin (Sang *et al*., 2005; Lin *et al*., 2008; Zhang *et al*., 2012). The ROS and oxidative stress generated by these flavonoids have been reported to play important role in inhibition of tumour cell growth and induction of apoptosis, which eliminates cancer cells (Salganik, 2001, Lambert and Elias, 2010).

3. Inflammation

Inflammation is a complex, vascular and cellular reaction by the immune system to either external or internal injurious agents such as pathogens, irritants or other noxious stimuli characterised by pain, redness, swelling and sometimes loss of function (Aggarwal *et al*., 2006; Shanmugam *et al*., 2012). Inflammation may be local or systemic, and it can be acute or chronic. Acute inflammation is a short term non-specific response to harmful stimuli initiated by several chemical mediators that promote vascular and cellular changes characterized by vasodilatation, leakage of vasculature, and the flow of plasma and leukocytes to the site of injury, followed by rapid resolution phase and repair of the damaged tissue (Kundu and Surh, 2012). Therefore, acute inflammation is fundamentally a protective response with ultimate goal to eliminate the injury-inducing stimuli, prevent tissue damage and/or initiate repair processes (Allam *et al*., 2008). However, chronic low-grade inflammation may result when there is inadequate resolution of acute inflammation which can prove harmful and may lead to disease development (Callewaert, 2012). Chronic inflammatory states are associated with the homeostatic imbalance of one of several physiological systems that are not directly functionally related to host defence or tissue repair (Medhizhitov, 2008). Several laboratory and population-based studies have suggested a strong association between chronic low-grade inflammation and several disease conditions including insulin resistance, diabetes, metabolic syndrome, cardiovascular disease, cancer, neurodegenerative diseases (Parkinson's and Alzheimer's disease) and even aging (Dandona *et al*., 2004; Achike *et al*., 2011; *Rossi et al*., 2011; Callewaert, 2012; Emmanuela *et al*., 2012; Kundu and Surh, 2012).

3.1 Mediators of the inflammatory response

Induction of inflammation triggers the production of numerous inflammatory mediators which are chemical factors that are secreted by various cells that take part in the inflammatory process either directly and/or by responding to the inflammatory stimulus (Allam *et al*., 2008). Inflammatory mediators may act singly, in combination or in sequence to amplify the tissue's/organ's response to stimulus and influence the course of inflammation (Allam *et al*., 2008). Cellular mediators of inflammation can be produced either by specialized leukocytes (such as tissue-resident macrophages and mast cells) or by cells present in local tissues. Other mediators such as histamine and serotonin are preformed and stored in granules of mast cells, basophils and platelet (Medzhitov, 2008). Inflammatory mediators can be classified into (i) vasoactive amines including histamine and serotonin, which are produced in an all-or-none manner when mast cells and platelets degranulate causing increased vascular permeability and vasodilation, or vasoconstriction and (ii) vasoactive peptides including those that can be stored in active form in secretory vesicles such as substance P or those generated by proteolytic processing of inactive precursors in the extracellular fluid such as kinins, fibrinopeptide A, fibrinopeptide B and fibrin degradation products. Other mediators include i) those generated through proteolysis by Hageman factor, thrombin or plasmin which cause vasodilation and increased vascular permeability, (ii) compliment fragments produced by several pathways of compliment activation such as C3a, C4a and C5a which promote granulocyte and monocyte recruitment and induce mast-cell degranulation, thus affecting the vasculature, (iii) lipid mediators including eicosanoids and platelet-activating factors derived from phospholipids such as phosphatidylcholine that are present in the inner leaflet of cellular membranes, (iv) inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and many others produced by macrophages and mast cells (Medzhitov, 2008). Cytokines play several important roles in mediating the inflammatory response, including the activation of the endothelium and leukocytes and induction of acute phase response, (v) chemokines produced by many cell types and, which control leukocyte extravasation and chemotaxis towards affected tissues and (vi) proteolytic enzymes including elastin, cathepsins and matrix metalloproteinases which play important roles in host defence, tissue remodelling and leukocyte migration (Medzhitov, 2008). The tissues and cells whose functional states are specifically affected by the inflammatory mediators are the effectors.

3.2 Markers of inflammation

Since several circulating cells and chemical mediators participate in both acute and chronic inflammation, numerous markers that can be measured in clinical practice have been identified. These include acute phase proteins [C-reactive protein (CRP), fibrinogen and serum amyloid A (SAA)], haemostatic factors [plasminogen activator inhibitor-1 (PAI-1),

selectins, soluble inter-cell adhesion molecule-1 (sICAM-1), vascular cell adhesion molecule-1 (vCAM-1)] and pro- and anti-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-10 (Ridker *et al*., 2000; Danesh *et al*., 2004).

3.2.1 C-reactive protein

C-reactive protein (CRP) is an ideal and probably the most studied circulation marker of inflammation. It is a non-glycosylated 224-residue plasma protein produced by the liver, but also by smooth muscle cells and adipocytes (Sarwar *et al*., 2009). The synthesis of CRP is closely regulated by increased levels of circulating IL-6 (Casas *et al*., 2008; Sarwar *et al*., 2009). The mechanisms by which CRP mediates inflammation varies and includes increased expression of adhesion molecules, PAI-1 and monocyte attractant proteins, reduced nitric oxide availability and increased low density lipoprotein cholesterol (LDL-C) uptake by macrophages. Previously, available methods were less sensitive and were only able to identify acute phase responses of CRP, however recently, high sensitivity immunoassay methods that enable the measurement of circulating baseline levels of CRP and assessment of low-grade chronic inflammation are available (Sarwar *et al*., 2009). Several human studies indicated that increased concentrations of circulating CRP may serve as a direct biological marker or reveal an underlying association with risk of several disease conditions including active coronary disease, cancers, diabetes and chronic kidney disease (Berk *et al*., 1990; Goldberg, 2009; Ridker, 2009; Aleksandrova *et al*., 2010; Bertoni *et al*., 2010; Chaturvedi *et al.*, 2010; Fox *et al*., 2010).

3.2.2 Cytokines

Cytokines are a diverse group of soluble, short acting proteins, glycoproteins, and peptides produced by various immune- and vascular cell types such as activated lymphocytes and macrophages, endothelial cells, epithelial cells and connective tissue cells in response to inflammatory stimuli (Allam *et al*., 2008; Sprague and Khalil, 2009; Wu and Schauss, 2012). These proteins have a regulatory role in cellular immune responses and are also mediators of both acute and chronic inflammation. Cytokines may be classified into (i) pro-inflammatory cytokines, produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions, and (ii) anti-inflammatory cytokines. Tumour necrosis factor-alpha (TNF-α), IL-1 and IL-6 are the major pro-inflammatory cytokines that have been used *in vivo* as a marker of inflammation. First described in 1975, TNF-α is a member of the group of cytokines that stimulate the acute phase reaction (Anker and von Haehling, 2004; Wu and Schauss, 2012). Mainly produced by activated macrophages, mast cells, endothelial cells, adipose tissue, cardiac myocytes, neuronal cells, and fibroblasts, TNF-α exert its effects via TNF-α receptors (TNFR) which are expressed by all nucleated

cells (Anker and von Haehling, 2004). The TNFR are of two types, (i) TNFR-1 which are more abundantly expressed and appear to be the main signalling receptor and (ii) TNFR-2 which has a more protective role and is primarily expressed in haematopoietic cells with evidence suggesting it can modulate action of TNFR-1 on immune and endothelial cells (Bolger and Anker, 2000; Anker and von Haehling, 2004; von Haehling *et al*., 2004; Balkwill, 2009). Recent reports has indicated that TNF-*α* activates the transcription of NF-*κ*B, which regulates the expression of genes involved in inflammation, oxidative stress and endothelial dysfunction (Rimbach *et al*., 2000; Kumar *et al*., 2004; dela Paz *et al*., 2007; Zhang *et al*., 2009).

The IL-1 family lies at the centre of the innate immune and inflammatory response together with TNF-α and IL-6. These proteins exert control over inflammation at both the receptor and nuclear levels, with IL-1 family of receptors containing activators and suppressors of inflammation. The family is made up of 11 members with IL-1α and IL-1β as the most thoroughly studied *in vitro*, in animal models of disease and in humans (Dinarello, 2009). Interleukin-1α is a constitutively expressed protein cleaved by calpain while IL-1β is synthesized as pro-IL-1β before cleavage by caspase-1 to the active cytokine. Both IL-1α and IL-1β operate through the IL-1 type I receptor (IL-1RI) on a variety of cell types inducing expression of an array of pro-inflammatory genes, such as IL-6, TNF-α, IL-8, IL-17 and IL-1 itself (Mills and Dunne, 2009).

Interleukin-1β is a 17 kD protein which has been implicated as the key IL-1 member mediating acute and chronic inflammatory responses as well as autoimmune disorders (Kang and Weylandt, 2008; Ren and Torres, 2009). An important role of IL-1β is to mediate the inflammatory process by inducing the expression and synthesis of many inflammatory genes such as lipoprotein-associated phospholipase A_2 (PLA_2), cyclooxygenase-2 (COX-2) and iNOS, leading to increased production of pro-inflammatory eicosanoids (such as prostaglanding E2), platelet activating factor and nitric oxide (White *et al*., 2008, Kang and Weylandt, 2008; Dinarello, 2009). Also IL-1β increases the expression of adhesion molecules such as iCAM-1 on mesenchymal cells and vCAM-1 on endothelial cells and has also been implicated as an angiogenic factor (Voronov *et al*., 2003), playing a role in tumour metastasis and blood vessel formation (Dinarello, 2009). Interleukin-1β has important homeostatic functions in the normal organism, such as in the regulation of feeding, sleep, and temperature, however, its overproduction has been implicated in the pathophysiology of disease states such as rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, osteoarthritis, vascular disease, multiple sclerosis, and Alzheimer's disease (Dinarello, 1996; Braddock and Quinn, 2004, and Dinarello, 2004).

Interleukin-6, a pleiotropic 184 amino acid monomer, is one of the interleukins that can act both as a pro-inflammatory or anti-inflammatory cytokine and myokine (Sarwar *et al*., 2009; Wu and Schauss, 2012). It is usually produced by lymphocytes, macrophages, endothelial cells, fibroblasts, hepatocytes and neural tissues to stimulate immune response, especially during infection and after trauma, such as burns or other tissue damage leading to inflammation. The synthesis of IL-6 is tightly regulated and expressed at low levels in healthy individuals, however during infection, trauma or other stress, it is expressed at much higher concentrations and its level positively correlate with higher all cause mortality, unstable angina, left ventricular dysfunction, propensity to diabetes and its complications, hypertension, obesity, renal failure and several types of cancer (Abeywardena *et al*., 2009; Steiner *et al*., 2009; Fisman and Tenenbaum, 2010; Goicoechea *et al*., 2012). It is also the cytokine responsible for the up-regulation of synthesis and secretion of several downstream markers, including CRP and both are known to be correlated with a number of markers for insulin resistance syndrome and endothelial dysfunction (Abeywardena *et al*., 2009; Sarwar *et al*., 2009). Other cytokines involved in the mediation of inflammatory response includes the anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IFN-α and TGF-β which are all involved in the down-regulation of inflammatory reactions.

3.3 Role of oxidative stress in inflammatory response

Oxidative stress and tissue injury are major hallmarks in the pathophysiology of most chronic conditions, such as diabetes, cancers, neurodegenerative diseases and even aging. Several reports have indicated that the pathology of most of these conditions has inflammatory components and that inflammation and oxidative stress are interconnected (Khansari *et al*., 2009; Callewaert, 2012; Kundu and Surh, 2012). Reactive oxygen and nitrogen species generation have multiple effects on inflammation including direct cytotoxicity, a sensitising or preconditioning effect to a second insult, or involvement in the generation of pro-inflammatory mediators (Jaeschke, 2011). Production of reactive RONS is important to the regulation of innate immune responses. During acute inflammation, the interaction of the cellular immune system with endogenous or exogenous stimulus result in the activation of phagocytic cells such as neutrophils, leading to generation of a RONS-dependent respiratory burst that direct toxicity towards invading microbes (Martinon, 2010). Other sources of reactive oxygen species during acute or chronic inflammation include the mitochondrial respiratory chain, the metabolic cascade of arachidonic acid, protease-mediated enzyme xanthine oxidase, and other oxidases such as NADPH oxidase (Victor *et al*., 2004). The generation of low-level RONS during acute inflammation reactions, functions to destroy invading pathogen and foreign materials, and regulates a number of signalling pathways

including kinases, transcription factors, metabolic enzymes and protease. Evidence has suggested that when the production of RONS by resident macrophages and inflammatory leukocytes are uncontrolled and in excess, oxidative and nitrosative stress, as well as tissue injury occur (Kundu and Surh, 2008; 2012). Beyond its direct cytotoxicity to invading pathogens, the release of RONS (H_2O_2) by damaged tissues can form a decreasing concentration gradient that directs leukocytes recruitment at the site of tissue injury, demonstrating that RONS can orchestrate inflammatory responses in tissues (Niethammer *et al*., 2009; Martinon, 2010). Production of RONS by the pattern recognition receptors (PRRs) and toll-like receptors (TLRs) regulates activation of redox-regulated transcription factors such as NF-κB and AP-1 as well as modulate cytokines production (Iriti and Faoro, 2007; Ogier-Denis *et al*., 2008; Martinon, 2010). Cytokines such as TNF-α, IL-1β and IL-6, as well as chemokines such as CXCL8 (IL-8), CXCL2 (MIP-2) and CCL2 (MCP-1) can induce damage directly in target tissues and/or indirectly by recruiting and activating additional leukocytes in a process that amplified the inflammatory response (Laskin, 2009).

Tumour necrosis factor-α is the most notable of the pro-inflammatory cytokines and it has been implicated not only in inflammatory tissue injury but also in the regulation of apoptosis, acute-phase protein gene expression and cytochrome P450 activity (Laskin *et al*., 2011). Both TNF-α and IL-1 directly promote the accumulation of inflammatory cells in tissues by up-regulaing adhesion molecules and stimulating the endothelium to produce chemokines. Also TNF-α activates neutrophils and macrophages to produce RONS and in conjunction with IL-1 induced the release of pro-inflammatory mediators such as IL-6, platelet activating factors, prostaglandins, matrix metalloproteinase (MMP) and various chemokine from macrophages and other cell types (Mukhopadhyay *et al*., 2006; Bradley, 2008; Laskin *et al*., 2011). It has also been demonstrated that after an inflammatory stimulus, accumulation of damaged mitochondria precipitate an increase in RONS production, which in turn enhances inflammasome activation that is consistent with a secondary messenger model (Nakahira *et al*., 2011; Zhou *et al*., 2011).

4. Dietary modulation of oxidative stress and inflammation

Evidence has shown that oxidative stress and inflammation are closely linked via positive feedback mechanisms and are both associated with most chronic diseases, including CVD, obesity, diabetes, cancer and neurodegenerative diseases (Garcia-Bailo *et al*., 2011) (**discussed in previous sections**). Also, measurement of markers of oxidative stress, such as MDA, TBARS, 4-HNE and F2-isoprostanes, have been used to link oxidative stress and many chronic diseases (Dalle-Donne *et al*., 2006). Therefore, dietary factors that can positively modulate oxidative stress and inflammation may be an important public health tool

to reduce the burden of chronic diseases. Diet has been known as a risk factor for chronic diseases for many years, and the upsurge in cases of chronic diseases worldwide, in the past decades has been attributed in part, to the change from traditional, largely plant-based diet to the high-fat, energy dense diets with substantial content of animal fat (Roberts and Barnard, 2005). Also the Mediterranean diet characterized by frequent consumption of fruit, vegetables, legumes, cereals and fish; and low in meat and cheese is positively associated with a reduced risk of most chronic diseases including cardiovascular diseases, diabetes and cancer (Martinez-Gonzalez *et al*., 2009). Fruits, vegetables, spices and teas are excellent sources of fiber, vitamins and minerals, but they also contain components like polyphenols, terpenes, alkaloids, and phenolics which may provide substantial health benefits beyond basic nutritional functions (Aggarwal *et al*., 2006). Disease prevention using whole plant extracts, or isolated compounds from plant (phytochemicals), is gaining more scientific attention worldwide, because it is a cost-effective alternative to orthodox treatment, and the phytochemicals are presumed from anecdotal evidence to be safe, with low toxicity and general acceptance (Surh, 2003, Weng and Yen, 2012). Whole plant extracts as well as isolated molecules have been shown to possess antioxidant, anti-inflammatory, anticancer, and tumour inhibitory effects. The health promoting effects of these plants are proposed to be due to the polyphenolic antioxidant molecules which they contain. These molecules have shown antioxidant activity, free radical scavenging capacity, as well as ability to induce protective enzyme systems (Yao *et al*., 2004; De Kok *et al*., 2008; Mates *et al*., 2011). Plants often contain different polyphenolic phytochemicals, and it is the general assumption that the observed health effects of these plants may be due to the additive and/or synergistic effects of the complex mixture of these phytochemicals rather than the effect of a single molecule (Liu, 2003; 2004; de Kok *et al*, 2008).

4.1 Plant phenolics and polyphenols

Plant phenolics are one of the most widely dispersed groups of phytochemicals in the plant kingdom, with an excess of 8000 phenolic structures being reported (Crozier *et al*., 2009; Tsao, 2010). These phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups and can range from simple, low molecular weight, simple aromatic-ring compounds to large and complex tannins and derived polyphenols (Yao *et al*., 2004; Crozier *et al*., 2009). Phenolics are secondary metabolites produced during diverse physiological processes in the plants, such as growth, lignifications, pigmentation, pollination and defense against pathogens, predators and environmental stress (Balasundram *et al*., 2006; Crozier *et al*., 2009). All plant phenolics are derived from the pentose phosphate, shikimate and phenylpropanoid pathways, contributing to bitterness, astringency, colour, flavour, odour and oxidative stability in foods (Balasundram *et al*., 2006; Pandey and Rizvi,

2009). Phenolics are found primarily conjugated to one or more sugar moiety (which can be mono-, di-, or even oligosaccharides) and organic acids. The most common sugar residues found in phenolics is glucose, although others such as rhamnose, xylose, galactose, arabinose, as well as glucuronic and galacturonic acid may be found (Bravo, 1998). They can be classified into groups based on the number and arrangement of their carbon atoms. According to Crozier *et al*. (2009), plant phenolics occurring naturally in healthy plant tissue can be classified into (i) flavonoids and (ii) non-flavonoids. The major non-flavonoids of dietary significance include phenolic acids, phenolic alcohols, stilbenes and the lignans. Phenolic acids consist of two subgroups, i.e., the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic acid, *p*-hydroxybenzoic, and protocatechuic, vanillic and syringic acids, which have the C_6-C_1 structure in common. Gallic acid is the commonest phenolic acid and is found primarily as non-sugar galloyl esters in grapes, wine, mangoes, green tea and black tea (Bravo, 1998; Balasundram *et al*., 2006; Crozier *et al*., 2009). Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C_6–C_3), with caffeic, ferulic, *p*-coumaric and sinapic acids being the most common (Bravo, 1998). The stilbenes with a C_6-C_2-C_6 structure consist of two aromatic rings linked through a two carbon bridge with a double bond. The most popular of this group is resveratrol which is found mainly in red wine and pea nuts, and to a lesser extent in berries, red cabbage, spinach and certain herbs. The other group, flavonoids will be discussed in more detail below.

4.2 Flavonoids: types and structure

Flavonoids are widely distributed in food and beverages of plant origin, including teas, fruits, vegetables, spices, cocoa and wine that form part of human diet. Flavonoids in plants play a key role in growth and development (Taylor and Groteworld, 2005), plant-insect interactions (Simmonds, 2001), and protection against harmful radiation by absorbing UVB and scavenging ROS generated by UVB irradiation (Xu *et al*., 2008a, 2008b). Flavonoids are known to provide health promoting effects in humans that consume them. Reports have shown that flavonoids possess many useful health properties, including anti-inflammatory, oestrogenic, enzyme system modulation, antimicrobial, vascular and anti-tumour activity, however, the antioxidant activity is the most studied one that is attributed to flavonoids and has been found to be responsible for other biological activities in which the prevention of oxidative stress is beneficial (Yao *et al*., 2004; Lin and Weng, 2006; Pereira *et al*., 2009). Flavonoids also possess anti-cancer activity which has been shown to go beyond the antioxidant, free radical scavenging activity, but may involve mechanisms such as regulation of gene expression in cell proliferation, oncogenes, and tumour suppressor genes, induction

of cell cycle arrest and apoptosis, modulation of detoxification enzymes activity, stimulation of immune system and regulation of hormone metabolism (Lin and Weng, 2006).

Flavonoids are the largest class of polyphenols, with a diphenylpropane backbone, consisting of 15 carbon atoms with two aromatic rings linked by a three carbon bridge (C6-C3-C6). This structure, known as the flavan nucleus (**Figure 3**), has two benzene rings (A and B) joined by a three-carbon chain that form a pyran ring (C).

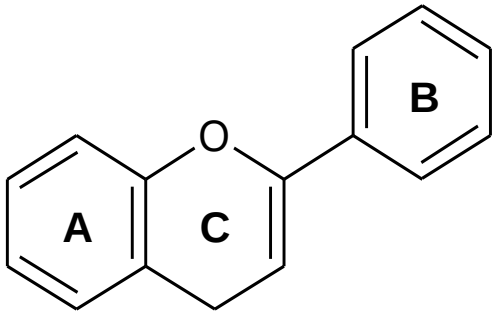

Figure 3: Flavan backbone of polyphenols (Source: Croizer *et al*., 2009)

They are ubiquitous in plants and are found especially in the epidermis of leaves and skin of fruits (Crozier *et al*., 2009) The flavan backbone is altered by various substitutions and functional groups in different positions to produce different subclasses of flavonoids. The main subclasses of flavonoids are flavones, flavonols, flavanols, anthocyanins, isoflavones, flavanones, chalcones and dihydrochalcones (Yao *et al*., 2004; Lin and Weng, 2006; Pereira *et al*., 2009; Croizer *et al*., 2009). Other classes include the coumarins, aurones, dihydroflavonols and flavan-3,4-diols. **Figure 4** depicts the structural skeletons of the different flavonoid groups while **Table 2** shows specific examples and food sources of the different flavonoids classes.

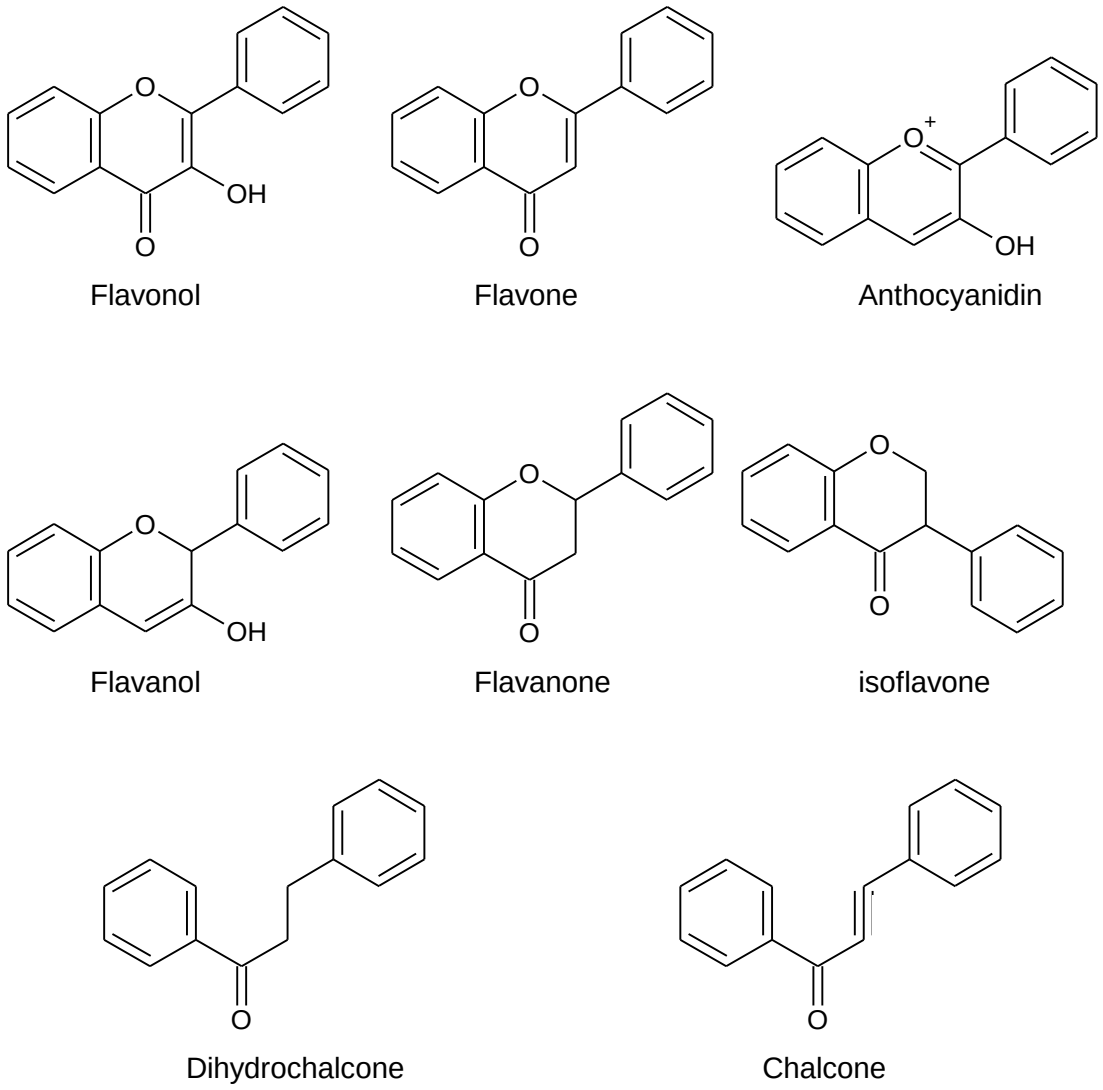

Figure 4: Structural skeleton of the different flavonoid groups (Source: Crozier *et al*., 2009).

Table 2: Different flavonoid classes, specific examples and food sources

Class	Compounds	Common dietary Sources
Flavonol	Kaempferol, Quercetin, Isorhamnetin, Myricetin	Apples, broccoli, onions, tea, berries, grapes, olives, chilli pepper, tomatoes, rooibos and beans
Flavone	Apigenin, Luteolin, Diosmetin, Tangeretin, Nobiletin	Olives, celery, parsely, chilli pepper, citrus, thyme, sage and lemon
Flavanol	Catechin, Epicatechin, Epigallocatechin	Tea, beans, apples, apricots, grapes, chocolate and berries
Flavanone	Hesperetin, naringenin	Bitter orange, grapefruit peel, citrus, lemon, and lime
Anthocyanidin	Cyanidin, delphinidin, malvidin, pelargonidin, petunidin	Apples, berries, cocoa, pomegranate, blueberry and red wine.
Isoflavone	Genistein, Daidzein, coumestan, coumestrol	Soybeans, lucerne, clovers
Chalcone	Naringenin chalcones	Tomato skin, juice and paste
Dihydrochalcone	Aspalathin, aspalalinin, phloretin	Rooibos, apples, and cider

4.3 Rooibos (Aspalathus linearis)

4.3.1 History, botany and morphology

Rooibos, *Aspalathus linearis* (Brum f) Dahlg. (Family Fabaceae; tribe Crotolarieae) is a hardy shrub that grows between 1.5 to 2 m high with bright green needle-shaped leaves with small, yellow flowers (Van Wyk and Gericke, 2000). After harvesting, the needle-like leaves and stem can be either bruised and fermented prior to drying or dried immediately (Mckay and Blumberg, 2007). The unfermented product remains green in colour and is referred to as green rooibos (**Figure 5B**). During fermentation the colour changes from green to red with oxidation of the constituent polyphenols with the final product often referred to as red tea or red bush tea (**Figure 5A**). The genus *Aspalathus* comprises about 278 species and is endemic to South Africa, showing high degree of polymorphism in terms of morphology, geographical distribution, ecology and phenolic constituents (Van Heerden *et al*., 2003). Rooibos has been consumed by locals for over 300 years, but it was unknown outside South Africa until 1904 when Benjamin Ginsberg, a Russian immigrant to South Africa recognised its potential and started trading with rooibos that he bought from the Khoi descendants and became the first exporter of rooibos (http://www.sarooibos.co.za/content/view/31/79). In the

1930's Dr P.F. Le Fras Nortier, a local doctor and amateur botanist realised the commercial potential of rooibos and initiated its cultivation in plantations by local farmers (http://www.sarooibos.co.za/content/view/31/79). By 1999/2000, rooibos production was between 4500 and 6000 metric tonnes with the domestic market absorbing 70-75% of the annual production (Wilson, 2005). Today, rooibos cultivation has reached a large scale of more than 12000 metric tonnes, serving both local and increasing international market demand (http://www.sarooibos.co.za/content/view/29/77).

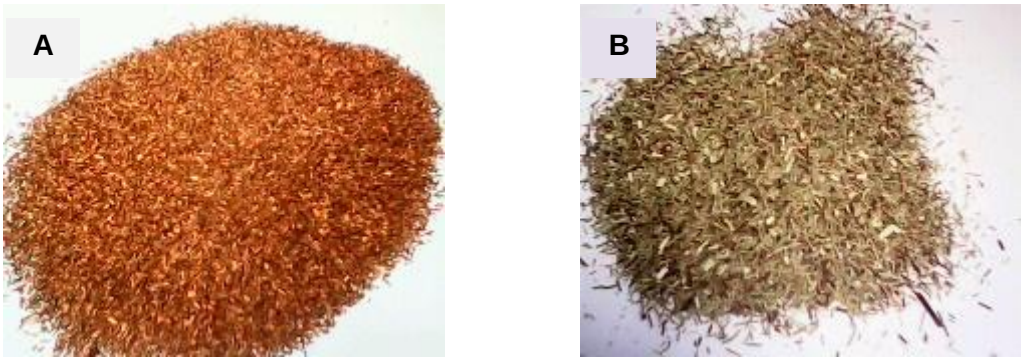

Figure 5: (A) Fermented rooibos and (B) Green (unfermented) rooibos

4.3.2 Phytochemical composition

The herbal beverage made from rooibos is naturally caffeine-free (Cheney and Scholtz, 1963; Morton, 1983) and low in tannin when compared to *Camellia sinensis* teas (Joubert *et al*., 2008a). The leaf tannin content of rooibos is reported to be about 3.2% to 4.4% (Reynecke *et al*., 1949; Blommaert and Steenkamp, 1978). Rooibos is unique in its monomeric flavonoid composition. It contains two unique compounds, namely aspalathin and aspalalinin. Aspalathin is a C-C linked dihydrochalcone glucoside (Koeppen and Roux, 1966; Rabe *et al*., 1994) and aspalalinin, a cyclic dihydrochalcone (Shinamura *et al.*, 2006) both of which are only isolated from rooibos. Rooibos is also one of the only three known sources of nothofagin, a 3-dehydroxydihydrochalcone glucoside (Joubert 1996; Joubert *et al*., 2009a). The other known sources of nothofagin are the heartwood of *Nothofagus fusca* (Hills and Inoue, 1967) and the bark of a Chinese medicinal plant, *Schoepfia chinensis* (Huang, C. *et al*., 2008).

Other flavonoids in rooibos include the C-C linked β-D-glucopyranosides such as flavones orientin and iso-orientin (Koeppen and Roux, 1965) as well as vitexin and isovitexin, both

flavone analogues of nothofagin (Rabe *et al*., 1994). The flavanones, dihydro-orientin and dihydroiso-orientin (Bramati et al., 2002), as well as hemiphlorin (Shinamura *et al*., 2006) have also been isolated from rooibos. Other flavones isolated from rooibos include chrysoeriol, luteolin and luteolin-7-o-glucoside while the flavonols quercetin, quercetin-3-o-robinoside, hyperoside, isoquercitrin and rutin are also present (Snyckers and Salemi, 1974; Rabe *et al*., 1994; Bramati *et al*., 2002; Kazuno *et al*., 2005; Shinamura *et al*., 2006; Krafczyk and Glomb, 2008). The presence of phenolic acids, lignans and the coumarin, esculentin (Rabe *et al*., 1994; Shinamura *et al*., 2006; Krafczyk and Glomb, 2008), as well as monomeric flavan-3-ol, (+)-catechin and oligomeric flavan-3-ol, procyanidin B3 and bis-fisetinidol-(4β,6:4β,8)-cathechin (Ferreira *et al*., 1995; Krafczyk and Glomb, 2008) have also been detected in rooibos. **Figure 6** shows the major flavonoids that have been identified from rooibos.

As a result of fermentation during processing of rooibos, the content of polyphenols in rooibos is reduced. Studies have found a higher percentage of total polyphenols, flavonoids and non-flavonoids in unfermented/green rooibos compared to fermented/red rooibos (Marnewick *et al*., 2000; Standley *et al*., 2001; Joubert *et al.,* 2005). These differences may be attributed to (i) the enzymatic and chemical modifications that occur during fermentation and (ii) to the processing methods used (Joubert, 1996; Standley *et al*., 2001). During fermentation, aspalathin present in unfermented rooibos is extensively oxidized to dihydroiso-orientin. Nothofagin, iso-orientin, isovitexin and vitexin are degraded as well, but to a lesser extent (Joubert, 1996; Joubert and Ferreira, 1996; Bramati *et al*., 2003; Krafczyk and Glomb, 2008). *In vitro* studies revealed that fermentation and processing also affect the antioxidant activities of rooibos, with results showing that antioxidant activity (measured by different free radical scavenging assays) decrease with fermentation (Von Gadow *et al*., 1997a; Standley *et al*., 2001; Joubert *et al*., 2004; Joubert *et al*., 2008b), an effect attributed to decreased total polyphenol content with fermentation.

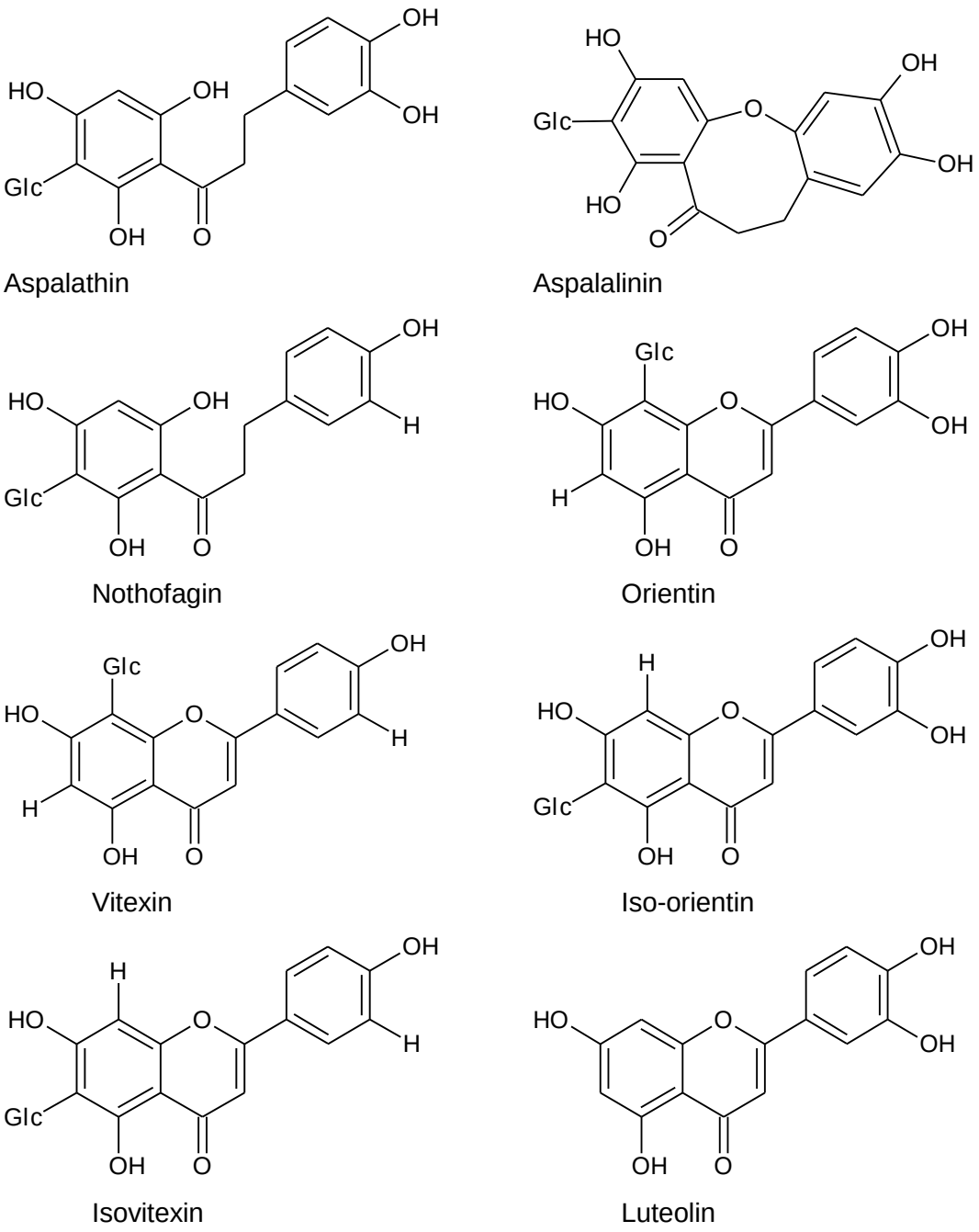

Figure 6: Some major flavonoids identified in rooibos (Glc = C-β-D-Glucosyl group) (Source: Joubert *et al*., 2008).

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4.3.3 Biological activities

The attractiveness and use of naturally occurring compounds including those derived from fruits, vegetables, teas, various herbs and spices as potential chemopreventive and chemotherapeutic agents is gaining world-wide appeal. Evidence abound that rooibos and/or its flavonoids hold great potential not only in the prevention but also in therapy of a wide variety of disease conditions. Since the first report on the biological activity of rooibos, an enormous body of work has revealed that rooibos extracts possess antioxidant, antimutagenic, anti-inflammatory, anti-diabetic, hepatoprotective, antimicrobial, and above all cancer preventive properties. The following sections will therefore, take a detailed look at the various biological and health promoting effects that has been shown for rooibos.

4.3.3.1 Antioxidant and lipid peroxidation inhibition activities

As a consequence of aerobic respiration or a result of exogenous factors such as smoking or exposure to pollutants, reactive oxygen species (ROS) and other free radicals including hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are constantly generated in living organisms (Droge, 2002). Although the controlled production of ROS has important physiological roles especially in energy production, phagocytosis, regulation of cell growth and cellular signalling, a high ROS production not counterbalanced by cellular antioxidant defence may result in oxidative stress. As previously mentioned, oxidative stress has been implicated in the pathogenesis of many disease conditions such as cancer, cardiovascular diseases, atherosclerosis, hypertension, ischeamia/reperfusion injury, diabetes mellitus, neurodegenerative disorders (Alzheimer's and Parkinson's disease), rheumatoid arthritis and aging (Finkel, 2005; Valko *et al*., 2007). Epidemiological and population-based evidence has led to the conclusion that diet is a key factor and a potential tool for the prevention and/control of chronic diseases (WHO, 2003; Darnton-Hill *et al*., 2004; Daar *et al*., 2007; Mirmiran *et al*., 2008). More specifically, epidemiological studies have revealed that diets rich in fruits, vegetables and teas lower the risk for certain chronic diseases because they are important sources of phenolic antioxidants (Hollman, 2001; Stoclet *et al*., 2004; Arts and Hollman, 2005; Fraser *et al*., 2007). Rooibos is a potent source of unique and beneficial antioxidants and is particularly rich in flavonoids, phenolic acids and other polyphenols, which are thought to contribute to its health benefits. Both *in vitro* and *in vivo* studies offer a unique opportunity to assess the contribution of the antioxidant properties of rooibos and rooibos polyphenols to the physiological effect of rooibos administration in different models of oxidative stress.

Several studies have confirmed that rooibos showed antioxidant activity both *in vitro* and *in vivo*. Extracts of fermented and unfermented rooibos, as well as rooibos flavonoids showed

in vitro antioxidant activity by scavenging free radicals (Von Gadow *et al*., 1997a, 1997b; Standley *et al*., 2001; Joubert *et al*., 2004; Joubert *et al*., 2008b; Snijman *et al*., 2009; Krafczyk *et al*., 2009). Aqueous extracts of fermented rooibos showed *in vitro* inhibition of lipid peroxidation in cell membranes using rabbit erythrocyte membrane, rat liver microsome and rat liver homogenates (Hitomi *et al*., 1999; Joubert *et al.,* 2008b), while methanolic extracts of fermented and unfermented rooibos inhibited microsomal lipid peroxidation (Marnewick *et al*., 2005). Rooibos was also shown to scavenge alkyl peroxyl radicals formed during lipid peroxidation (Akaike *et al*., 1995). Aqueous and methanolic extracts of rooibos inhibited peroxyl radical-induced DNA strand scission in a dose-dependent manner, with the aqueous extract being less effective compared with the methanolic extract (Lee and Jang, 2004). Treatment with fermented rooibos increased the viability of Chinese harmster fibroblast (V79-4) cells following H_2O_2-induced oxidative stress by increasing the activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) (Yoo *et al*., 2008).

The antioxidant effects shown by rooibos *in vitro* have been confirmed in many experimental animal model studies. Aqueous rooibos extract administration suppressed age-related accumulation of lipid peroxides in several regions of the rat brain (Inanami *et al*., 1995). Gastric intubation of luteolin, (a flavonoid found in rooibos, at 10 µmol/kg body weight) given 2 hr prior to γ-ray irradiation suppressed lipid peroxidation in mice bone marrow and spleen (Shimoi *et al*., 1996). In a study in vitamin E deficient rats, dietary supplementation of a freeze-dried hot water extract of rooibos, decreased the level of lipid peroxidation in the liver, lung, small intestine and stomach of the animals, although the changes were not significant (Hitomi *et al*., 2004). Rooibos extracts given for 10 weeks increased the antioxidant status of the CCl_4-treated rats by increasing α-tocopherol and reducing the liver concentrations of coenzyme Q while inhibiting the formation of malondialdehyde (Kucharskar *et al*., 2004). The ability of rooibos to improve the redox status has been shown by different studies in rats. Aqueous rooibos extracts (2%, w/v) increased the glutathione status in the liver (Marnewick *et al*., 2003), heart (Pantsi *et al*., 2011) and sperm (Awoniyi *et al*., 2012) of rats subjected to different models of chemical-induced oxidative stress.

4.3.3.2 Antimutagenic, antitumourigenic and anti-carcinogenic effects

The increasing mortality and morbidity arising from various cancers world-wide has made the search for an alternative strategy (such as the use of bioactive components from plants) in the prevention and management of cancers very imperative. Results from studies in recent decades have shown that bioactive compounds from plants have important roles in the prevention, and reducing the risks of chronic diseases, including cancer (Colic and Pavelic,

2002; Mehta *et al*., 2010). The beneficial effects shown by these compounds are attributed among others, to their antioxidant and free radical scavenging ability.

Rooibos is a rich and unique source of polyphenols, and the polyphenols present in rooibos being powerful antioxidants, may play important roles in the prevention of cancer by reducing damage to DNA in the cell, and modulating the promotion of cancer. Several studies have demonstrated the *in vitro* antimutagenic properties of both fermented and unfermented rooibos extracts using various test systems. Fermented aqueous rooibos extract (3.33%, w/v) significantly suppressed the number of chromosomal aberrations in Chinese hamster ovary cells induced by benzo[a]pyrene (BaP) and mitomycin C (MMC) in the presence or absence of rat liver microsomal enzymes (S9). The clastogen-suppressing effect was obtained when cells were exposed to rooibos before and/or after mutagen treatment (Sasaki *et al*., 1993). Aqueous extract of rooibos (2-10% of extract) suppressed oncogenic transformation of mouse fibroblast cells subjected to x-ray induced transformation in a dose-dependent manner (Komatsu *et al*., 1994). In another study, the effect of different concentrations of a fermented rooibos extract included in the culture medium, on growth and changes of growth parameters of cultured chick embryonic skeletal muscle cells, was investigated. It was discovered that the rooibos extract significantly inhibited cell proliferation as reflected by decreased DNA, RNA and protein contents in primary cell cultures of fibroblasts and myoblasts, in a dose dependent manner (Lamosova *et al*., 1997). The *in vitro* antimutagenic property of rooibos was further investigated by Marnewick *et al*. (2000). Both fermented and unfermented rooibos extracts (5, 10%, w/v) were significantly effective against 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1)-induced mutagenesis in the *Salmonella typhimurium* mutagenicity assay (tester strain TA98 and TA100) in the presence of metabolic activation with S9. However, poor inhibitory effects against the direct acting mutagens, methyl methanesulfonate, cumene hydroperoxide and H_2O_2 using tester strain TA102, were observed (Marnewick *et al*., 2000) using the same mutagenicity assay. Further studies, revealed that fermented rooibos exhibited a higher antimutagenic activity than unfermented rooibos against both AFB_1 and 2-AAF, although antimutagenic activity of both rooibos extracts were comparable to that of an extract prepared from *Camellia sinensis* (Van der Merwe *et al*., 2006). Using genetically engineered V79 Chinese hamster fibroblasts that expressed human CYP1A2, N(O)-acetyltransferase (hNAT2*4) and sulfotransferase (hSULT1A1*1), Platt *et al*. (2010) showed that rooibos moderately protects against the genotoxicity of 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) and the protection shown was comparable to that shown by green and black teas. In another study, aqueous extracts of unfermented rooibos inhibited cell proliferation of human oesophageal cancer cells (WHCO5) by affecting energy (ATP) production (Sissing, 2008). The antimutagenic properties of the

most prevalent flavonoids in rooibos (aspalathin and nothofagin) and their flavone derivatives were investigated by Snijman *et al*. (2007). The results showed that aspalathin, nothofagin and their structural flavonoid analogues displayed moderate antimutagenic properties while luteolin and to some extent, chrysoeriol, showed activities comparable to those of the green tea flavonoids, EGCG. In the same study, quercetin and isoquercitrin exhibited antimutagenic, pro-mutagenic and mutagenic effects in the presence or absence of metabolic activation.

The *in vitro* antimutagenic potential of rooibos extracts have also been substantiated by several animal model experiments. Intraperitoneal injection of fermented rooibos extract (1mL of 0.1%, w/v) 6 hr prior to MMC treatment reduced the induction of micronucleated reticulocytes (MNRETs) in peripheral blood of ICR male mice (Sasaki *et al*., 1993). The *ex vivo* antimutagenic potential of fermented and unfermented rooibos was established in a 10 week rooibos feeding study in male Fischer rats. It was discovered that liver cytosolic fractions from rats consuming fermented and unfermented rooibos, protected against AFB_1-induced mutagenesis in the *Salmonella typhimurium* assay with tester strain TA100. However, only liver cytosolic fraction from rats fed the unfermented rooibos showed protection against 2-AAF-induced mutagenesis in the same assay with tester strain TA98 (Marnewick *et al*., 2004). Also the activation potential of hepatic microsomal preparations from rats consuming rooibos were evaluated in the study, with rooibos reducing the activation of AFB_1, but not that of 2-AAF (Marnewick *et al*., 2004).

The antitumourigenic activity of rooibos was established in a two-stage mouse skin carcinogenesis assay. Topical application of ethanol/acetone soluble fractions of fermented and unfermented rooibos prior to tumour promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) on ICR mouse skin initiated with 7,12-dimethylbenz(a)anthracene (DMBA) significantly reduced the number and size of tumours per mouse, as well as delayed tumour development (Marnewick *et al*., 2005). More recently, a study in female SKH-1 hairless mice, showed that topical application of polyphenol-rich extracts of fermented and unfermented rooibos, prior to ultraviolet B-induced tumour promotion and after DMBA-initiated skin cancer, reduced the number of tumours per mouse by 91.39% and 75.37% respectively, and also the tumour volume by 97.28% and 90.74%, respectively (Petrova, 2010). Another study related to tumour promotion in mouse skin, showed that the expression of cyclooxygenase-2 (COX-2) in ICR mouse skin by TPA was significantly reduced by a methanolic extract of fermented rooibos (60, 300, 600 μg, topically applied prior to TPA exposure) (Na *et al*., 2004). A recent study which monitored the cancer modulating properties of hot water extracts of fermented and unfermented rooibos in a liver carcinogenesis model against fumonisin B_1 (FB_1)

promotion in male Fischer rat utilizing diethylnitrosamine as cancer initiator, showed that unfermented rooibos was more protective against FB_1-induced cancer promotion, by significantly reducing the total number and size of pre-neoplastic foci staining positively for the placental form of γ-glutamyl transferase in the liver, presumably by arresting their growth (Marnewick *et al*., 2009). A similar study investigated the protective effect of rooibos against methylbenzylnitrosamine-induced oesophageal cancer in male Fischer rats and reported that fermented and unfermented rooibos significantly reduced the number and size of papillomas, with rats drinking unfermented rooibos failing to develop larger papillomas (Sissing, 2008; Sissing *et al*., 2011). Results from these studies suggest that rooibos could be developed as a nutraceutical for the chemoprevention of liver and oesophageal cancer.

4.3.3.3 Hepatoprotective effects

Chronic hepatic disease represents one of the foremost health problems worldwide, with liver cirrhosis and drug-induced liver injury accounting for the ninth leading cause of death in the western and developing countries (Saleem *et al*., 2010). It is a well-known fact that the available synthetic drug to treat liver disorders might also cause further damage to the liver (Chen *et al*., 2011). Hence herbal drugs have become increasingly popular and their use is widespread. Hepatoprotective effects of rooibos have been reported in a number of studies. Consumption of rooibos protected against liver damage by suppressing the observed increase in plasma activities of AST, ALT, alkaline phosphatase, bilirubin, and resulted in a histological regression of steatosis and cirrhosis in the liver of rats challenged with carbon tetrachloride (CCl_4) (1 mL/kg, i.p.) twice a week for 10 weeks (Ulicna *et al*., 2003). Another study from the same laboratory also reported that rooibos supported the regeneration of rat liver after intoxication with carbon tetrachloride, and this protective effect was ascribed to the ability of rooibos to inhibit lipid peroxidation in the liver (Ulicna *et al*., 2008).

4.3.3.4 Immunomodulatory effects

The use of plant products as immune-stimulants is based on century-old tradition, and the current practice of ingesting phytochemicals to support the immune system and/or to fight infections is based on the observation that these compounds have a direct inhibitory effect on microbial organisms. The first report on the immunomodulatory activity of rooibos was by Kunishiro *et al*. (2001). The study examined the effects of a fermented rooibos extract on antigen-specific antibody production and cytokine generation *in vitro* and *in vivo*. The addition of rooibos extract at concentrations of 1-100 µg/mL stimulated the antibody response in murine splenocytes using anti-ovalbumin (anti-OVA) or sheep red blood cell (SRBC). However, the rooibos extract did not modify the non-specific antibody response elicited with a lipopolysaccharide in purified splenic β-cells. Also, the addition of a rooibos extract at

concentrations ranging from 10-1000 µg/mL, showed that rooibos increased antibody responses and improved cell survival through the stimulation of interleukin 2 (IL-2) in splenocytes primed with OVA and anti-CD3, while suppressing the generation of IL-4 in OVA-primed splenocytes (Kunishiro *et al*., 2001). A study reported that rooibos extracts showed remarkable immune modulating activity amongst 10 different kinds of tea extracts commonly consumed in Japan, when their specific antibody formation responses was investigated (Yamamoto, 2003). In the study, addition of a hot water extract of rooibos to mouse spleen cells cultured in OVA, enhanced OVA-specific IgM antibody production and induced NK cell cytocidal activity (Yamamoto, 2003). Another study from the same laboratory reported that an aqueous fraction obtained after column fractionation of a hot water extract of rooibos, showed augmenting effects on anti-OVA IgM production in OVA-stimulated murine splenocytes, which was associated with the production of IL-10. Furthermore, continuous ingestion of the rooibos fraction was found to increase the anti-OVA IgM level in the sera of OVA-immunized BALB/c mice (Ichiyama *et al*., 2007). A recent study investigated the *in vitro* effects of rooibos on biomarkers of specific immune pathways using whole blood culture assay. It was reported that rooibos extract addition to unstimulated whole blood cultures, induced higher IL-6, IL-10 and interferon gamma (IFN-γ) secretion, while its addition to stimulated whole blood culture induced higher IL-6, lower IL-10 and had no effect on IFN-γ secretion (Heindricks and Pool, 2010).

In vivo, the oral administration of a rooibos water extract to Wistar rats significantly restored the OVA-induced antibody production in the serum after cyclosporin A treatment and stimulated IL-2 generation in murine splenocytes (Kunishiro *et al*., 2001). Based on the results from this *in vivo* study, and those of the *in vitro* study previously described, the authors (Kunishiro *et al*., 2001) suggested that hot water extracts of rooibos may facilitate antigen-specific antibody production, through selective augmentation of IL-2 generation both *in vitro* and *in vivo*, proposing that rooibos consumption may be of value in the prophylaxis of diseases involving a severe defect in helper T cell (Th1) immune response such as cancer, allergies, AIDS and other infections.

4.3.3.5 Anti-inflammatory effects

Inflammation plays an important role in various diseases, such as rheumatoid arthritis, atherosclerosis and cardiovascular diseases, type 2 diabetes and cancers, which all shows a high prevalence globally. Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant-derived formulations, and the anti-inflammatory activity of several plant extracts and isolated compounds have been demonstrated scientifically (Mueller *et al*., 2010). A possible explanation for these anti-

inflammatory effects of plant extracts may be found in the interplay between oxidative stress and inflammation. ROS are not only involved in the occurrence of oxidative stress, but also in the promotion of inflammatory processes via activation of transcription factors such as NF-$_K$B and activator protein (AP)-1 which induce the production of cytokines like TNF-α (MacNee, 2001; Rahman, 2002).

Scientific studies reporting on the possible anti-inflammatory effects of rooibos and/or its flavonoids are sparse. A study in Japan reported that unfermented rooibos extract (1.6%, w/v) administration as the only source of drinking fluid for 8 weeks, reduced inflammation in dextran sodium sulphate (DSS)-induced colitis rats via an increased antioxidant activity (Baba *et al.,* 2009). There was a significant increase in serum SOD and urine 8-hydroxy-2´-deoxyguanosine levels in rooibos rats compared to the control and the DSS rats. Based on these findings, the authors concluded that rooibos may prevent DNA damage and inflammation by its antioxidative activity *in vivo* (Baba *et al*., 2009). The anti-inflammatory activity of a rooibos tea extract in DMSO (0.5 mg/mL) and two of its flavonoids (luteolin and quercetin) were investigated, together with other herbal extracts, in a study using a lipopolysaccharide-stimulated macrophage model. The study results revealed that incubation with a rooibos extract (0.5 mg/mL) significantly reduced the secretion of the pro-inflammatory cytokine, IL-6, by at least 25% and marginally reduced the expression of inducible nitric oxide synthase (iNOS). However, the secretion of the anti-inflammatory cytokine IL-10, was also reduced (Mueller *et al*., 2010). In the same study, rooibos flavonoids luteolin and quercetin at concentrations of 50 and 100 nM, reduced the secretion of IL-6 and TNF-α, while also inhibiting the expression of cyclooxygenase 2 (COX-2) and iNOS. The authors concluded that, diets rich in antioxidants such as rooibos may contribute to the reduction of inflammation and be preventive against related diseases.

4.3.3.6 Bronchodilatory, antispasmodic and blood pressure lowering effects

In an attempt to rationalize some of the medicinal uses of rooibos, aqueous extracts of this herbal tea were studied for possible bronchodilatory, anti-spasmodic and blood pressure lowering activities *in vitro* and *in vivo.* Rooibos extracts were shown to contain K_{ATP} channel-dependent bronchodilatory substances as it caused a dose-dependent relaxation of K^+-induced contraction in rabbit jujenum and aorta, as well as in guinea pig trachea, while a dose-dependent decrease in mean arterial blood pressure in rats, was also produced (Khan and Gilani, 2006). The selective bronchodilatory effect of rooibos was shared by one of its known flavonoids, chrysoeriol. A previous study, at the same laboratory in rabbit jujenum using rooibos flavonoids, chrysoeriol, orientin and vitexin showed that the compounds exhibited K_{ATP} channel opening properties (Gilani *et al*., 2006). In the same study, it was

reported that the rooibos extract showed a dose-dependent anti-diarrhoeal and anti-secretory effects against castor oil-induced diarrhoea and fluid accumulation in mice. The authors concluded that the rooibos extract exhibited a combination of dominant K_{ATP} channel activating and weak Ca^{2+} antagonist effects, owing to which it showed spasmolytic, antidiarrhoeal and antisecretory activities, thus providing a scientific basis for the medicinal use of rooibos in hyperactive gastrointestinal, respiratory and cardiovascular disease, with the potential for it to be developed as a remedy for the congestive airway disorders.

4.3.3.7 Anti-diabetic effects

Diabetes is one of the most common global diseases and affects approximately 200 million people worldwide (McCune and Johns, 2002; Buyukbalci and El, 2008), with the figure forecasted to rise to around 300 million people by 2025 (Pandey and Rizvi, 2009). Long term effects of diabetes include progressive development of specific complements such as retinopathy, nephropathy and neuropathy. People with diabetes are also at risk for cardiovascular, peripheral vascular and cerebrovascular diseases (Pandey and Rizvi, 2009). All these complications substantially increase the rates of morbidity and mortality associated with the disease and reduces the quality of life of the diabetic individuals. Several polyphenol-rich plants and/or plant extracts have been used for controlling diabetes (Erasto *et al*., 2005; Jung *et al*., 2006). Polyphenols may affect glycaemia through different mechanisms, including, inhibition of intestinal glucose absorption, increasing glucose transport and metabolism in muscle and/or stimulating insulin secretion (McCune and Johns, 2002; Gallagher *et al*., 2003; Kelble, 2005; Pandey and Rizvi, 2009). A report by Johnston *et al*. (2005) demonstrated that glucose uptake into cells under sodium-dependent conditions was inhibited by flavonoid glycosides and non-glycosylated polyphenols in polarised Caco-2 intestinal cells. Under sodium-free conditions, aglycones and non-glycosylated polyphenols inhibited glucose uptake, whereas glycosides inhibited the active transport of glucose (Han *et al*., 2007).

Investigation into the anti-diabetic properties of rooibos in streptozotocin-induced diabetic rats showed that administration of aqueous and alkaline extracts of rooibos to diabetic rats did not affect markers of diabetic status such as glucose, glycated haemoglobin and fructosamine, however biochemical markers characterizing hepatotoxic effects in plasma, advanced glycation end-products (AGEs) and malondialdehyde in plasma and in different tissues of the diabetic rats were reduced (Ulicna *et al*., 2006). Previously, an *in vitro* study by Kinae *et al*. (1994) reported that a freeze-dried extract of fermented rooibos suppressed the formation of glycated albumin, including AGEs in a mixture of D-glucose and human serum albumin. Quercetin, a flavonol found in rooibos in small concentrations, has been shown to

mediate the inhibition of the facilitated diffusion of glucose transporter 2 (GLUT2) in Chinese hamster ovary cells (Song *et al*., 2002). A recent study demonstrated, for the first time, the hypoglycaemic and anti-diabetic activity of aspalathin in type 2 diabetic model db/db mice. Purified aspalathin from a fermented rooibos extract, increased dose-dependently and significantly the glucose uptake by L6 myotubes at concentrations of 1-100 µM, irrespective of insulin absence and also increased insulin secretion from cultured RIN-5F cells at 100 µM. Also in the study, dietary aspalathin (0.1-0.2%, g/kg diet) suppressed the increase in fasting blood glucose levels for 5 weeks and improved impaired glucose tolerance at 30, 60, 90 and 120 minutes in db/db mice (Kawano *et al*., 2009). The results from this study presented the first evidence of the beneficial effects of aspalathin on glucose homeostasis in type 2 diabetes, through the stimulation of glucose uptake in muscle tissues, and insulin secretion from pancreatic β-cells. In another study, a fermented aqueous extract, and an aspalathin-enriched unfermented extract of rooibos, were reported to lower the elevation in blood glucose observed, in an STZ-induced hyperglycaemic Wistar rat's model (Joubert *et al*., 2010). The same study also reported that, the elevated blood glucose observed in a diet-induced model of type 2 diabetes in vervet monkeys was lowered by a fermented aqueous extract of rooibos. Some authors, have suggested that, since rooibos and/or aspalathin are capable of scavenging intracellular reactive oxygen species (ROS), then the anti-diabetic potential of rooibos may be due to its antioxidative function, which may be involved in the activation of insulin-stimulated glucose uptake and hence, modulation of glucose homeostasis observed in the studies (Hanhineva *et al*., 2010).

4.3.3.8 Antimicrobial effects

The antimicrobial properties of rooibos are widely acknowledged. Nakano *et al*. (1997a; b) reported that alkaline extracts of fermented rooibos suppressed the cytopathic effects of HIV infected MT-4 cells *in vitro*, however, the hot water extract was not effective. Hot water extracts of fermented and unfermented rooibos showed growth inhibition against a host of bacteria including *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Streptococcus mutans, Escherichia coli*, and the yeast *Saccharomyces cerevisae*, with the fermented extract showing more potency (Scheepers, 2001). Growth inhibition in the case of *Escherichia coli,* was dose dependent and bacteriostatic when liquid cultures were used. When a solid medium zone inhibition method was used, the antimicrobial property of an aqueous rooibos extract (1.5%, w/v) was demonstrated against the bacteria *Bacillus cereus* and *Micrococcus luteus* and the yeast *Candida albicans*, however, no inhibitory activity could be demonstrated for *Escherichia coli*, *Pseudomonas aeruginosa* and *Lactobacillus acidophilus* (Almajano *et al*., 2008). In another study, Coetzee *et al*. (2008) demonstrated that aqueous extracts of unfermented rooibos used at 10 mg/mL, inhibit *Escherichia coli*

growth in liquid cultures by as much as 60-85% after 6 h, but the inhibition was reduced by about 25-50% after 24 h with further reduction thereafter. Result from the same study also demonstrated the antifungal activity of rooibos against the plant pathogen *Botrytis cinerea* by reducing spore germination of the fungus by 33%. A recent study investigated the antibiofilm activity of a rooibos extract, together with 14 other selected herbs, spices, beverages and commercially important medicinal plants on *Listeria monocytogenes*. Results obtained showed that the rooibos extract successfully inhibited cell attachment of both clinical and ATCC isolates by about 70%. The rooibos extract also exhibited good antibiofilm activity by inhibiting biofilm growth and development (>70%) of preformed *Listeria monocytogenes* isolates and also reducing metabolic activity and biomass of the bacteria isolates (Sandasi *et al*., 2010).

4.3.4 Human studies

Reports examining the health benefits of rooibos in humans have been scarce and limited. However, various health promoting effects, including, antioxidant and oxidative stress modulation, inhibition of post-prandial oxidative stress, inhibition of angiotensin converting enzymes (ACE's), lipid profile modulation, promotion of hair growth, and anti-wrinkle activity, among others, have been reported for rooibos in humans.

The first human study with rooibos was conducted by Hesseling *et al*. (1979), when they studied the effects of rooibos compared with black tea and water on iron absorption in 30 healthy young men. The study revealed that rooibos consumption (200 mL for 14 days) did not have a deleterious effect on the iron status parameters, such as haemoglobin, ferritin, transferrin, serum iron and iron binding capacity, compared with the control group taking water. A more recent parallel intervention study, involving 175 primary school children in South Africa, revealed that consumption of 200 mL servings of rooibos twice daily for 16 weeks did not have any adverse effects on the iron status parameters including serum ferritin, transferrin, the total iron binding capacity and the transferrin saturation (Breet *et al*., 2005).

A study to test the potential anti-histaminic effects of rooibos was conducted in seven patients diagnosed with either asthma or hay fever. Ingestion of fermented rooibos or topical application of a rooibos poultice did not produce any anti-allergenic activities (Hesseling and Joubert, 1982). The dermatological potential of rooibos was demonstrated in a study that revealed the consumption of a diluted infusion of rooibos at least once a week is beneficial to patients with dermatological diseases. In this study, rooibos consumption decreased the incidence of recurrent *Herpes simplex* and human papilloma virus infection, while patients

with atopic dermatitis were successfully treated, resulting in a decrease in itching sensation. Also, rooibos consumption inhibited the infiltration of neutrophils associated with Behcet's disease, *Psoriasis vulgaris* and *Acne pustulosa* (Shindo and Kato, 1991). The anti-wrinkle activity of rooibos was demonstrated in a recent study, when a commercial extract of a rooibos blend with *Camelia sinensis* extract, was topically applied to female skin. Results obtained from the study, indicated that the extract reduced skin wrinkles by 10%, however, no effect was shown on skin smoothness, roughness and scaliness (Chuarienthong *et al*., 2010). Also, Glynn (2010) demonstrated that a blend of botanical extracts containing unfermented rooibos, when topically applied to male, promoted hair growth by increasing hair density, number of anagen follicles and hair growth rate.

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Sauter (2005) investigated the effect of an aspalathin-enriched extract of unfermented rooibos (15% aspalathin) on the plasma antioxidant status of 20 subjects who were given an oral dose (250 mg/tablet) twice daily for 14 days with diet restrictions to ensure low flavonoid intake during the study period. Rooibos consumption did not show any effect on the antioxidant status biomarkers monitored, except a slight decrease in the antioxidant status when the xanthine/xanthine oxidase assay was used. In another study, the effect of unfermented rooibos consumption on the antioxidant status of workers who were occupationally exposed to lead was investigated in a randomized placebo-controlled 8 week intervention trial. The study monitored the indices of lead exposure [blood lead (PbB), erythrocyte porphyrins (EP), and delta-aminolevunilic acid dehydratase (ALAD)] and antioxidant status markers (SOD, GSH and MDA) in the erythrocytes and plasma. Rooibos consumption did not have any effect on the mean blood lead and erythrocyte porphyrin levels (lead exposure status) but there was a modulation of antioxidant status of the lead-exposed workers as shown by the decreased MDA levels and increased SOD activity and GSH levels in the plasma and erythrocytes of these factory workers (Nikolova *et al*., 2007). More recently, a study by Francisco (2010) showed that fermented rooibos consumption modulated postprandial glycemia, lipemia and oxidative stress in healthy subjects after an intake of a standardised fat meal. Consumption of rooibos by the subjects improved their redox status by increasing the total GSH and significantly lowering the level of conjugated dienes and TBARS after 6 hours. Also the levels of total cholesterol, LDL-cholesterol and triacylglycerol as well as high sensitive C-reactive proteins (hs-CRP) were all decreased in the same study. Guerreiro *et al.* (2010), revealed that consumption of a rooibos infusion with added glucose (25g) did not change the glycemic response when compared to the reference solution (water with 25g of glucose) after 2 hours in a Caucasian population of young male and female adults aged 17 to 24 years, showing that rooibos is a healthy beverage for obese and diabetic patients. An acute, cross-over design intervention study involving 15 healthy

volunteers consuming 500 mL of either water, fermented or unfermented rooibos, showed that the plasma antioxidant capacity using the TRAP assay, increased significantly with both rooibos extracts, reaching a peak 1 hour post consumption. No change in triacylglycerol, cholesterol or uric acid levels were observed with any of the treatments, while a transitory increase in glycaemia observed at 30 minutes was linked to a glucose upload (Villano *et al*., 2010). Marnewick *et al*. (2011) showed that consumption of 6 cups of fermented rooibos herbal tea for 6 weeks improved the blood lipid profile and reduced oxidative stress by decreasing lipid peroxidation and improving the redox status (GSH:GSSG) of adults at risk of CVDs. This study provided the first clinical evidence in humans of the ability of rooibos to modulate oxidative stress in adults at risk for developing heart disease.

A recent randomized, three phase cross-over study investigated the effect of green tea, black tea and rooibos on the activity of the angiotensin-converting enzyme (ACE) and nitric oxide (NO) level in healthy volunteers (Persson *et al*., 2010). Study subjects received a single oral dose of 400 mL green tea, black tea or rooibos, with the activity of ACE and NO concentration measured at 0, 30, 60, and 180 minutes. The oral intake of a single dose (400 mL) of rooibos significantly inhibited ACE activity after 30 and 60 minutes, while no significant modulation was seen for green and black teas. When subjects were divided into subgroups according to ACE genotype, those with genotype II and ID showed significant inhibition of ACE activity after intake of rooibos. Rooibos consumption did not have any effect on NO concentration, blood pressure and heart rate (Persson *et al*., 2010). The results of this study is highly significant, since ACE inhibitors are the first-line treatment of hypertension and thus common drugs used for cardiovascular diseases, suggesting that rooibos may have anti-hypertensive and cardiovascular effect through inhibition of ACE activity.

Another randomized cross over design study evaluated the effects of rooibos, bottled water and a carbohydrate beverage on blood and urinary markers of hydration after acute dehydration in collegiate wrestlers, by measuring urine specific gravity (Usg), urine osmolality (Uosm), plasma osmolality (Posm) and plasma volume (Pvol), pre and post dehydration and at 1-h after rehydration. Results from the study revealed that after consuming a volume of fluid equal to 100% of the body fluid loss during exercise, Posm and Uosm in the rooibos and water group were significantly lower at the 1-h time point when compared with the baseline. The same trend was found for Usg and plasma volume shift, however, the interaction was not statistically significant. The finding of this study therefore demonstrated that rooibos was no more effective than plain water in promoting rehydration in collegiate wrestlers after acute dehydration (Utter *et al*., 2010).

4.3.5 Absorption, metabolism and bioavailability of rooibos and its flavonoids

The ability of bioactive molecules, as well as drugs to produce a biological effect depends on their ability to enter the target cell. Even though a compound has strong antioxidative or other biological activities *in vitro*, it would have little biological effect *in vivo* if little or none of the compounds reached the target tissues. Evidence have shown that flavonoids and other polyphenols easily penetrate all cultured cells, including those from peripheral organs, such as the heart, lung, breast, prostate, liver, kidney and brain. Reason for this is that with the exception of hepatic and intestinal cells, cultured cells usually have low expression of enzymes [Cytochrome P450 system, UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs)] capable of metabolizing dietary compounds and drugs. Therefore, for most cell culture studies, there is no limitation in cellular uptake due to poor membrane penetration or possible efflux transporters and/or metabolic instability (Walle, 2007). However, when polyphenols are administered orally to animals or humans, very little or none of these compounds appear in the systemic circulation. The reason is the very high expression of in particular the UGTs and SULTs in the small intestine and liver, through which all the oral dose will pass, resulting in low bioavailability (Walle, 2007). **Table 3** shows the main factors affecting the bioavailability of dietary polyphenols in humans.

Table 3: **Main factors affecting the bioavailability of dietary polyphenols in humans (source: D'Archivio *et al*., 2010).**

External factors	Environmental factors (*i.e.*, sun exposure, degree of ripeness); food availability
Food processing related factors	Thermal treatments; homogenization; lyophylization; cooking and methods of culinary preparation; storage
Food related factors	Food matrix; presence of positive or negative effectors of absorption (*i.e.*, fat, fibre)
Interaction with other compounds	Bonds with proteins (*i.e.*, albumin) or with polyphenols with similar mechanism of absorption
Polyphenols related factors	Chemical structure; concentration in food; amount introduced
Host related factors	Intestinal factors (*i.e.*, enzyme activity; intestinal transit time; colonic microflora) Systemic factors (*i.e.*, gender and age; disorders and/or pathologies; genetics; physiological condition)

Most polyphenols exist in plants in glycosidic forms. The major naturally occurring glycoside conjugates of polyphenols occurring in plants, are the *O*-glycosides with sugars *O*-linked at phenolic hydroxyl groups, forming a carbon-oxygen-carbon bond which are acid labile (Prasian and Barnes, 2007). Most polyphenolic *O*-glycosides undergo intestinal hydrolysis by intestinal glucosidases/hydrolases, to release the respective aglycones, both from the host (Day *et al*., 2000) and intestinal bacteria (Hur *et al*., 2000). Because the aglycones and their metabolites are more hydrophobic, they are more efficiently transported across the wall of the gastrointestinal tract than their respective glucosides and are thus converted both in the gut wall and the liver, as well as at peripheral tissue sites into phase I and phase II metabolites (Prasian and Barnes, 2007). Aspalathin, the major flavonoid of rooibos is characterized as a C-glycosyldihydrochalcone, with the glucose directly linked to the flavonoid nucleus via an acid-resistant and largely enzyme-resistant, C-C bond (Koeppen and Roux, 1966). Since a great number of rooibos flavonoids are C-glycosides, transformation into their corresponding aglycones with more favourable absorption molecular characteristics, by human intestinal bacteria will be an important factor in their bioavailability. The bioavailability of aspalathin as a major flavonoid of rooibos and one of its most potent antioxidants *in vitro* is of the utmost importance for its physiological relevance (Joubert *et al*., 2009).

At present, literature on the absorption, bioavailability and metabolism of rooibos and its major flavonoids, aspalathin, nothofagin, orientin, and isoorientin are sparse and limited,

although numerous studies have been conducted on the flavonols, quercetin and luteolin, both of which occur at very low levels in rooibos. A study investigated the *in vitro* transport of pure aspalathin solutions and extracts from unfermented rooibos across intestinal epithelial cells using the Caco-2 cell line and human skin (Huang, M. *et al*., 2008). Results revealed a concentration dependent transport of aspalathin across the intestinal epithelium, while the percutaneous penetration of both, pure compound and extract, across human skin membrane was low. Also the transport of aspalathin was lower for the pure compound when compared with the extract from unfermented rooibos (Huang, M. *et al*., 2008). This according to the authors, may be attributed to, other phytoconstituents present in the extract. Kreuz *et al*. (2008) investigated the metabolism of aspalathin to identify the parent compound and related metabolites in urine and plasma after daily oral administration of rooibos (157-167 mg aspalathin/kg body weight) to pigs over a period of 11 days. Results from the study showed that no aspalathin or its metabolites were found in the plasma and this was attributed to their extensive binding to plasma proteins. In the urine, six substances including aspalathin and its metabolites, methylated aspalathin, glucuronidated aspalathin, glucuronidated and methylated aspalathin, a glucuronidated aglycone of aspalathin, as well as a metabolite of eriodictyol glucoside (dihydro-isoorientin) were identified. These results confirmed the absorption and conjugation of aspalathin in the small intestine as a C-glycoside, as well as being cleaved in an aglycone and sugar moiety. However, the liberation of the aglycone and biotransformation of aspalathin to a flavanone glycoside are minor metabolic pathways (Kreuz *et al*., 2008). Since the aglycone of aspalathin and its flavanone glycoside, dihydro-isoorientin, were shown to be present as conjugates in this study, Joubert *et al*. (2009) suggested a metabolic pathway for aspalathin (**Figure 7**) based on that of phloretin (Blaut *et al*., 2003), neohesperidin chalcone (Braune *et al*., 2005) and eriodictyol (Zhang *et al*., 2007).

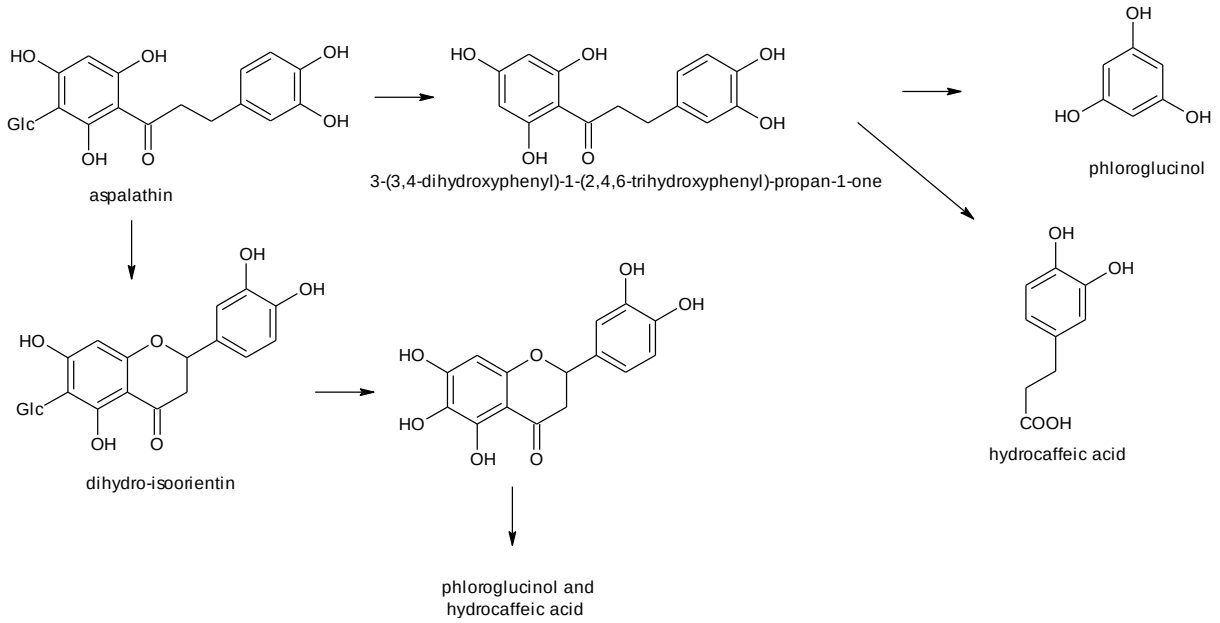

Figure 7: Proposed metabolic pathway of aspalathin degradation (source: Joubert *et al*., 2009)

Recent data from a study involving 10 volunteers ingesting 500 mL of either fermented or unfermented rooibos revealed that, no rooibos flavonoid metabolite could be detected in the plasma. However, in a 24 hr urine sample of these volunteers, eight metabolites were identified, including O-linked methyl, sulphate and glucuronide metabolites of aspalathin and an eriodictyol-O-sulphate (Stalmach *et al*., 2009). The main compound excreted was an O-methyl-aspalathin-O-glucuronide following ingestion of the unfermented drink and eriodictyol-O-sulphate after ingestion of fermented rooibos drink, with overall metabolite level excreted being 82 and 352 nmol, accounting for 0.09% and 0.22% of the flavonoids in the fermented and unfermented rooibos drink, respectively (Stalmach *et al*., 2009). Based on the data obtained, the authors concluded that the dihydrochalcones and flavanone C-glucosides in unfermented and fermented rooibos are poorly bioavailable with metabolites being absent in the plasma and only trace amounts being excreted in urine 24 h after consumption. Another study to characterise the absorption and metabolism of aspalathin in humans, identified and quantified aspalathin metabolites in urine after oral administration of an aspalathin-rich unfermented rooibos extract. Methylated metabolites of aspalathin with and without glucuronidation were detected in the urine of subjects, demonstrating that deglycosylation is not a prerequisite for aspalathin absorption (Courts and Williamson, 2009).

Van der Merwe *et al*. (2010) investigated the *in vitro* metabolism of aspalathin and nothofagin by monitoring the formation of glucuronyl and sulphate conjugates by subcellular rat liver fractions obtained from induced and un-induced male Fischer rats. Results showed that glucuronidation of both aspalathin and nothofagin lead to the formation of one major and one minor glucuronidated product, of both flavonoids after incubation with Aroclor 1254-induced microsomes, while only one minor product was identified for aspalathin and nothofagin with un-induced microsomes. Sulphation yielded one minor conjugated product for aspalathin with both induced and uninduced rat liver cytosolic fractions but nothofagin was not sulphated indicating that the cathecol is required for sulphation or that the sulphated product is unstable under *in vitro* and *in vivo* conditions (Van der Merwe *et al*., 2010). Further results from the same study identified the 4-OH or 3-OH position on the A-ring of aspalathin as the likely sites of conjugation, while for nothofagin, the 4-OH position on the A-ring and the 6´-OH position of the B-ring seem to be involved. Also online HPLC antioxidant assay utilizing $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, showed that glucuronidation of aspalathin eradicated its antioxidant properties under the assay conditions (Van der Merwe *et al*., 2010). Despite the loss of radical scavenging properties by aspalathin metabolites, the conjugated products are likely, as in the case of quercetin conjugates (Day *et al*., 2000; Zhang *et al*., 2007) to exhibit some other biological properties.

Despite the poor bioavailability of rooibos flavonoids, a recent study confirmed the presence of unmetabolised aspalathin and other minor flavonoids of rooibos in human plasma (Breiter *et al*., 2011). In the study, male healthy volunteers consumed either 500mL of unfermented rooibos (2%, w/v) or 500 mL of an isolated active fraction (0.1%, w/v) dissolved in water. In addition to unmetabolised aspalathin and nothofagin, seven metabolites of both aspalathin and nothofagin were identified in the urine of these volunteers. They included four conjugated metabolites of aspalathin (sulphated, glucuronidated, methylated-glucuronidated and methylated forms), two glucuronidated aglycone metabolites of aspalathin and one glucuronidated nothofagin metabolite. In the plasma aspalathin, orientin, isoorientin, (S)-eriodictyol-8-C-glucoside,vitexin and an isomer of rutin were all detected unchanged (Breiter *et al*., 2011). Though rooibos flavonoids are poorly bioavailable, aspalathin and nothofagin are both likely to accumulate in tissues and body fluids and still exert biological activities in vivo due to being available in both conjugated and unconjugated forms.

4.4 Oil palm (Elaeis guineensis): Origin, distribution and other uses

Oil palm (*Elaeis guineensis*) originated from the tropical rainforest region of West and Central Africa, between latitude 12^{o}N and 10^{o}S, on a coastal belt of about 200-300 km stretching from Senegal to North Angola (Lim, 2012). The oil palm, is a plant of the Palmae

(Arecaceae) family, cultivated throughout the world, from mild temperate to warm temperate, and sub-tropical to tropical climates and it's regarded as one of the most rapidly expanding plants in the world, especially, with the phenomenon growth in Southeast Asia, particularly in Indonesia and Malaysia (Oguntibeju *et al*., 2012). Malaysia has led the world palm oil production and export until 2006, when it was surpassed by Indonesia. Presently, Indonesia is the world's largest producer and exporter of palm oil. Data from the Food and Agricultural Organisation (FAO) has shown that palm oil production in Indonesia increased by over 400% between 1994-2004 and the Indonesian Palm Oil Association has predicted that production could rise to 25 million metric tonnes by the end of 2012 (GAPKI, 2012). Both Indonesia and Malaysia jointly account for 87% of the world palm oil production, while Thailand, Nigeria and Colombia account for 2% each, with the remaining 7% shared by other countries (**Table 4**).

Table 4: World palm oil production as of 2006 (Source: Crutchfield, 2007)

Countries	'000 Metric Tonnes	%
Indonesia	15900	44
Malaysia	15881	43
Thailand	820	2
Nigeria	815	2
Colombia	711	2
Others	2718	7

Apart from oil production, the oil palm tree and some of its products are finding other industrial uses. Sreekala *et al*. (2002) reported that hybridizing palm oil empty fruit bunch fibres with phenol-formaldehyde-based composite reinforced with glass, strengthen the mechanical performance of the composite, making it a useful material in aerospace, automotive and office materials industries. Oil palm shell charcoal treated with chitosan, can be used for bioremediation of heavy metal from industrial waste water (Normanbhay and Palanisamy, 2005), while the oil palm shell can also be used for the adsorption of hydrogen sulphide emitted from sewage treatment facility (Guo *et al*., 2007). Other uses of oil palm and its products, include the use of (i) ash derived from oil palm waste incineration as a cement replacement material (Tay and Show, 1995), (ii) palm kernel meal in animal livestock feeds (Onwudike, 1986; Sundu *et al*., 2006) and (iii) palm oil mill effluent as organic fertilizer (Nwoko and Ogunyemi, 2010). Palm wine, obtained by tapping the sap from the stem just

below the apex of a standing or felled palm is a popular beverage that is consumed in Africa, it is reported to contain about 4.3 and 3.4% of sucrose and glucose respectively, and it's an important source of vitamin B complex among its other health benefits (Lim, 2012). The potential of palm oil as an alternative biofuel has also been reported, since it has been demonstrated that between 40-70% of palm oil can be converted to a range of hydrocarbons such as gasoline, diesel and light gases (Leng *et al*., 1999).

4.4.1 Palm oil

Two different types of oil are obtained from the oil palm plant. Crude palm oil (CPO) is the principal oil component (accounting for about 73%) and it's extracted from the fleshy mesocarp of the oil palm fruit (**Figure 8)**, and palm kernel oil (PKO), extracted from the oil-rich endosperm (**Figure 8)** of the fruit which accounts for about 32% of the oil (Oguntibeju *et al*., 2012). Palm kernel oil is a low melting point oil, made up of about 92% saturated fatty acids which are mainly lauristic and myristic acid (Edem, 2002). The palm kernel oil is a non-drying oil and its products find uses either alone or in blends with other oils in making confectionary fats, biscuit dough, and filling creams, cake icings, ice cream, mayonnaise, coffee creamers, margarine and many other food products (Lim, 2012). Also PKO can be used to manufacture soaps, soap powders and detergents, while the pressed cake obtained from the kernel after oil extraction, is an important ingredient in livestock feeds (Edem, 2002).

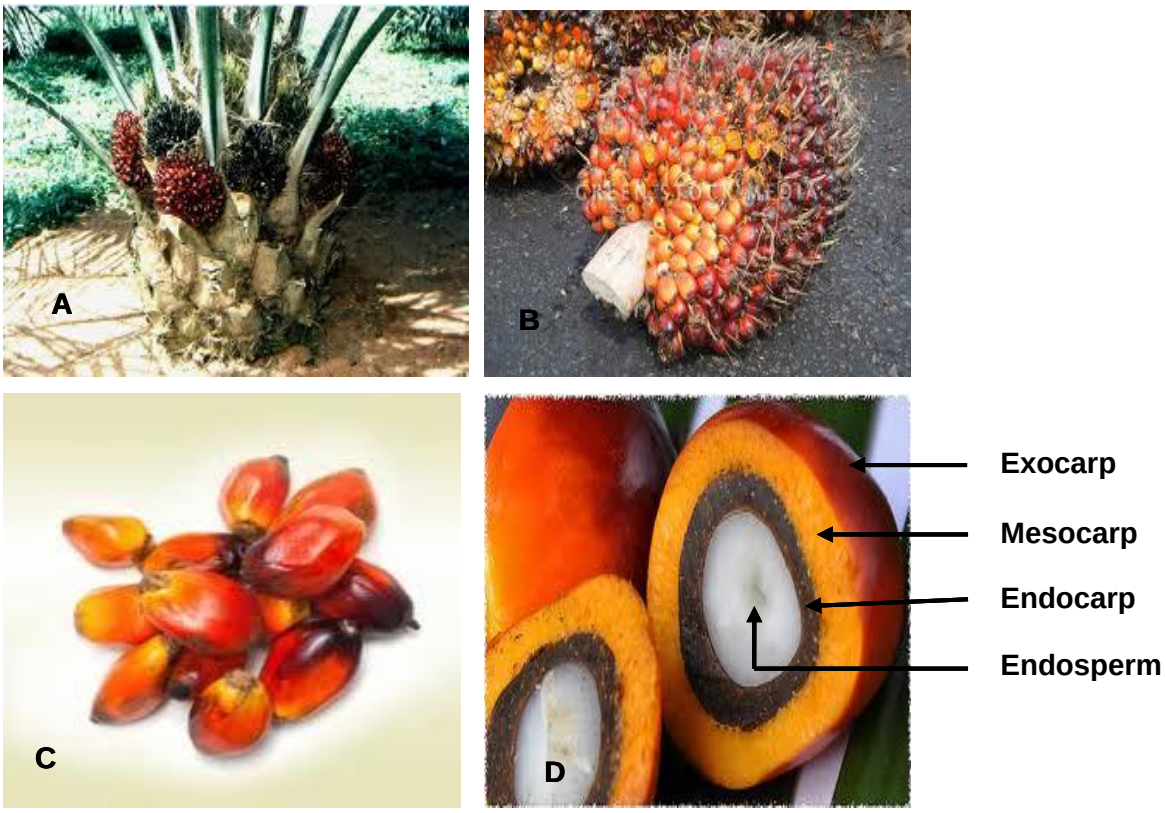

Figure 8: (A) an oil palm tree with matured fruit (B) an oil palm fruit bunch (C) oil palm fruits (D) a cut section of the oil palm fruit showing different part from where CPO (mesocarp) and PKO (endocarp) are produced.

Palm oil is a lipid extract from the fleshy orange-red mesocarp of the fruits of the oil palm tree which contain 45-55% oil. It is the second largest vegetable oil in terms of world production and consumption, and many economist has predicted that palm oil will be the leading international traded oil by the end of 2012 (Crutchfield, 2007). The characteristic red colour of palm oil is due to the abundance of carotenoids (700-1000 ppm) in the crude oil. In Africa, the palm oil is unrefined and it is used in the crude and unprocessed form. The average Malaysian crude palm oil, contains some undesirable non-glyceride components such as free fatty acids (about 3.5%), moisture, impurities and trace metals which are all detrimental to the stability of the palm oil (Nagendran *et al*., 2000). The crude palm oil is subjected to a refining process to remove the undesirable components rendering the oil more stable. Refining can either be physical or chemical, and both processes involve high-temperature deodorization and de-acidification [physical (250° to 300°C), chemical (220° to 240°C)] under vacuum of 3 to 5 torr (Nagendran *et al*., 2000). The product obtained after either physical or

chemical refining, is referred to as refined, bleached and deodorized palm oil (RBDPO). Although the high temperature and vacuum used during deodorization and de-acidification are necessary for the removal of as much of the undesirable components in the crude palm oil as possible, the conditions also result in the removal of some of the tocopherols and tocotrienols and the destruction of all the carotenoids present in crude palm oil. To retain the vitamin E and carotenoids, crude palm oil is subjected to a modified physical refining process (**Figure 9**) which involves pretreatment of the oil by degumming and bleaching, followed by deodorization and deacidification by molecular distillation (Ooi *et al*., 1991). The product from the modified refining process is comparable to RBDPO and meets all international quality specification for refined oils, while still retaining more than 80% of the vitamin E and carotenoids that are destroyed by the conventional physical and chemical refining process (Nagredan *et al*., 2000). This product still retains its red colour and it is known as red palm oil (RPO) and research has shown that its carotenoid content and quality parameters are stable for nine months when stored at 30^{o}C, and for more than one year at less than 10^{o}C (Choo et *al*., 1993). As a result of dry fractionation, an olein (liquid) and stearin (solid) fraction can be obtained from RBDPO and RPO. Palm olein is the most popular frying oil in Southeast Asia and it is considered as the gold standard in frying (Lim, 2012).

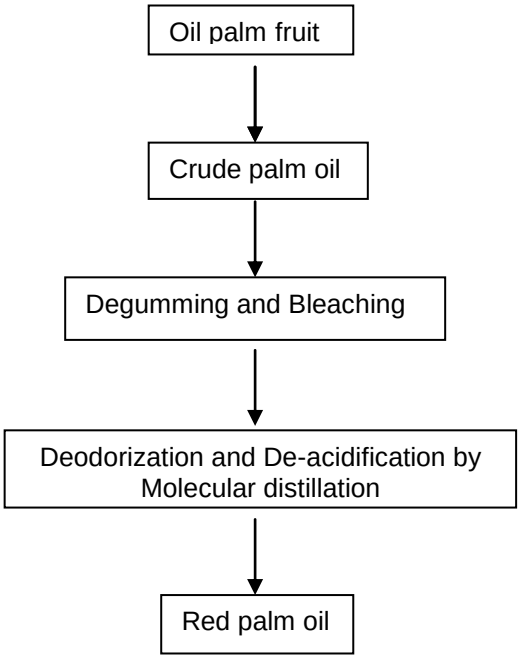

Figure 9: Schematic diagram showing the processing of crude palm oil to red palm oil

4.4.2 Composition of red palm oil

Red palm oil is made up of about 90% triglycerides, with a minor proportion of di- and monoglycerides as well as phytonutrients. Red palm oil is unique in that it contains an equal amount of saturated and unsaturated fatty acids, with about 44% palmitic acid, 5% stearic acid (both saturated), 40% oleic acid (monounsaturated), 10% linoleic acid and 0.4% α-linoleic acid (both polyunsaturated), with natural fat soluble tocopherol, tocotrienol and carotenoids, which may act as antioxidants (Edem, 2002, Engelbrecht *et al.,* 2009). With concentrations up to 30 times that of carrots and 300 times that of tomatoes, red palm oil is the richest natural food source of carotenoids. The characteristic orange-red colour of RPO is due to the abundance of carotenoids (700-1000 ppm) in the crude oil. In the refined red palm oil, as much as 80% of the carotene and vitamin E originally present in crude palm oil, is retained through a modified refining process of degumming and bleaching, de-acidification and de-odorization using molecular distillation as mentioned previously. The refined oil contains 500 ppm carotenes of which 90% is present as α-carotene (37%) and β-carotene (47%). Lycopene represent 1.5% of the carotenoids and cis-α-carotene 6.9% with all other minor carotenes making up the difference (Van Rooyen *et al.,* 2008). The carotenoids together with vitamin E, function as biological antioxidants which neutralise highly reactive

radicals (·OH) and free fatty peroxyl radicals to less active species, thus protecting cells against oxidative damage (Krinsky, 1992, Packer, 1992, Edem, 2002). Red palm oil is also a rich source of vitamin E (about 560-1000 ppm) (Sundram and Gapor, 1992, Edem, 2002), with 70% of the vitamin E content in the form of tocotrienols (mainly as α-, β- and γ-tocotrienols), while tocopherols account for the remaining 30% (Al Saqer *et al.,* 2004). Red palm oil is the only vegetable oil available in the world market in appreciable quantities that is rich in tocotrienols (Cottrell, 1991, Gu *et al.,* 1997). It was reported by Serbinova *et al* (1991), that tocotrienols in RPO can be 40-60 times more potent as antioxidants than tocopherols. Other studies have revealed that the most abundant tocotrienol in RPO is γ-tocotrienol, and that it is a potent antioxidant that is hypocholesterolemic, and also prevent platelet aggregation (Helub *et al*., 1989; Qureshi *et al*., 1991).

Other minor components found in RPO are phytosterols [such as sitosterol (60%), stigmasterol (24%), campsterol (13%), and cholesterol (3%)], phospholipids, glycolipids, coenzyme Q (Q10), and squalene (Loganathan *et al*., 2010). Phytosterols are lipophilic and are converted *in vivo* via a series of enzymatic, side-chain cleavage reactions into cholesterol, which is a major precursor of steroid hormones (Ebong *et al*., 1999). Squalene is a naturally occurring, fat soluble triterpene with antioxidant activity. Evidence has shown that it possess anti-cancer as well as cardio-protective properties (Loganathan *et al*., 2010), and that when present at high concentrations *in vivo*, it can bring about a negative feedback inhibition of HMG-COA reductase, the rate limiting enzyme in the biosynthesis of cholesterol (Hassan, 1988). Apart from the fat soluble antioxidants found in palm oil, studies have shown that the palm fruit also contains several phenolic compounds including gallic, chlorogenic, gentisic, coumaric and caffeic acids, as well as catechins, hesperidin, narirutin, and 4-hydroxyl benzoate, all of which have appreciable radical scavenging and antioxidant ability (Tan *et al.,* 2001; Loganathan *et al*., 2010; Atawodi *et al*., 2011). **Table 5** shows the different phytonutrients in red palm oil and their health benefits.

Table 5: Phytonutrient in red palm oil and reported health benefits (Loganathan *et al*., 2010)

Palm phytonutrient	Health benefits
Vitamin E (600-1000 ppm)	• Anti-cancer effects • Anti-angiogenesis • Antioxidant • Anti-atherosclerosis • Anti-ageing • Inhibition of cholesterol biosynthesis • Cardio-protection • Anti-diabetic effects
Carotenoids (500-700 ppm)	• Pro-vitamin A activity • Cardio-protection • Anti-cancer effects
Phytosterols (300-620 ppm)	• Cholesterol lowering effects
Squalene (250-540 ppm)	• Cardio-perotection • Inhibition of cholesterol biosynthesis • Anti-cancer effects
Phospholipids (20-100 ppm)	• Brain development • Energy endurance • Eases digestion, nutrition and absorption
Co-enzyme Q10 (10-80 ppm)	• Enhance cellular energy production • Antioxidative defense mechanism • Cardio-protection • Anti-cancer
Polyphenolics (40-70 ppm)	• Inhibition of cholesterol biosynthesis • Anti-cancer •Aids circulation problems

4.4.3 Health benefits

4.4.3.1 Hypocholesterolemic effects

The outcry against the consumption of palm oil was due to the fear that the high concentration of saturated fatty acid (SFA) in palm oil will have a negative effect on lipid profiles, especially total cholesterol and LDL-cholesterol levels. However, numerous studies have shown that this fear is misplaced and that the SFA in palm oil are mostly neutral and may even have a positive effect on lipid profiles. This hypocholesterolemic effect is due to a number of reasons. Whether or not a dietary fat will influence the blood cholesterol level depends on the intramolecular fatty acid distribution in the triacylglycerol (TAG) backbone. The preferential absorption of fatty acids and subsequent elevation or reduction effects on blood cholesterol will depend on the position of the SFA and unsaturated fatty acid (UFA) side chains on the TAG backbone. The conformation of the TAG in palm oil is such that UFAs are usually located on position 2 in about 87% of the molecules, while SFA occupy position 1 and 3, leading to absorption of more UFA than SFA (Bester *et al*., 2010a; Lim, 2012). Also, the most abundant SFA in palm oil is palmitic acid and research has shown that compared to lauric and myristic acid, it does not have a negative impact on serum lipid profiles, especially in normocholesterolemic young men (Hayes and Khosla, 1992, Khosla and Hayes, 1994; Sundram *et al*., 1994; Choudhury *et al*., 1995). Tocotrienols found in abundance in palm oil are not only potent antioxidants, but also inhibit the enzyme HMG-COA reductase, which is the rate limiting enzyme in cholesterol biosynthesis in the liver, thus exerting hypocholesterolemic effect (Zhang *et al*., 2003; Bester *et al*., 2010a; Lim, 2012). Red palm oil has SFA and UFA in nearly equal concentrations, so any deleterious effects of the SFA are countered by the UFA present in the palm oil (Khosla, 2006). Since the fatty acid composition, tocotrienol and carotenoid content of RPO is similar to that of crude palm oil, it is therefore pertinent to note that RPO should have similar effects as crude palm oil, on the serum lipid profile. Several rodent and human studies have shown that consumption of a RPO diet is hypocholestrolemic, and even exerts positive effects on blood lipid profile. Very recent studies in rodents have shown that red palm oil supplementation exhibit either positive effects (decrease total cholesterol, triglyceride, LDL-Cholesterol, and increase HDL-cholesterol) on the serum lipid profile (Wilson *et al*., 2005; Oluba *et al*., 2008; Salinas *et al*., 2008; Budin *et al*., 2009; Ajuwon *et al*., 2011) or remains neutral (Ayeleso *et al*., 2012). Various studies in humans using mildly hypercholesterolemic to normocholesterolemic younger subjects, also indicated that the cholesterol raising ability of palm oil was either muted or disappeared (Ng *et al*., 1991; Qureshi *et al*., 1995; Sundram *et al*., 1997; Zhang *et al*., 2003). Results from all these studies, therefore show that RPO consumption is not detrimental to health, but may have potential health benefits.

4.4.3.2 Benefits in cardiovascular diseases

Cardiovascular diseases are among the leading cause of mortality worldwide, accounting for approximately 17 million deaths annually (Halpin *et al*., 2010). Healthy diets, especially consumption of diets rich in natural antioxidants have been suggested as one of the modifiable risk factors to reduce the impact of CVD. Palm oil is a vegetable oil with a cocktail of antioxidants with proven health benefits. Scientific evidence has shown that palm oil and its phytonutrient components have cardiac protective effects (Esterhuyse *et al*., 2005a; 2005b; Bester *et al*., 2006; 2010a; 2010b; Engelbrecht *et al*., 2009). Increased serum levels of lipoproteins are strong risk factors for cardiovascular conditions such as hypertension, coronary heart disease and stroke, and several studies have shown that palm oil consumption does not have a negative effect on blood lipid profiles, but it's rather hypocholesterolemic or may even be neutral.

Various studies have shown that palm oil and its antioxidant phytonutrients are anti-atherogenic. Hornstra *et al*. (1988) showed that feeding PO to rabbits for 18 months cause the lowest extent of atherosclerosis compared with fish oil, linseed oil and olive oil. In another feeding study that compared the effect of PO and sunflower seed oil, it was discovered that PO, despite containing 50% SFA, did not increase arterial thrombosis tendency, but reduced platelet aggregation when compared with highly polyunsaturated sunflower oil (Rand *et al*., 1988). Another study in which patients consumed γ-tocotrienol and α-tocopherol-enriched fraction of PO for 2 years, showed that there is a reduction in carotid artery restenosis in patients with carotid atherosclerosis (Kooyenga *et al*., 1997; Lim, 2012). Comparing atherogenic potential of RPO and RBDPO, Kritchevsky (2000) showed that RPO is less atherogenic than RBDPO, and this effect was ascribed to the presence of vitamins and carotenes that are present in RPO. In a study using postmenopausal rats, it was discovered that feeding fresh RPO protects the aorta and that such protection is lost with repeated heating of the RPO (Adam *et al*., 2009). Peroxisome proliferators-activated receptors (PPARs) are ligand regulated transcription factors, which play important role in the prevention and development of atherosclerosis via regulation of energy metabolism and inflammation. Evidence from the work of Li *et al*. (2010) showed that tocotrienol-enriched RPO prevents atherosclerosis by modulating the activity of PPARα, PPARγ and PPARδ.

The effects of PO and its antioxidant components on endothelial dysfunction and blood pressure have also been reported. According to Oguntibeju *et al*. (2012), RPO supplementation can be used as a tool in preventing vascular resistance and improve endothelium-dependent relaxation, which have been implicated in the development and pathogenesis of hypertension. Osim *et al*. (1996) demonstrated a beneficial effect of PO on

blood pressure. In the study, rats fed a fresh PO diet for 14 weeks have mean arterial pressure (MAP) that is comparable with those of the control rats, although feeding rats with an oxidized PO diet for the same period showed an elevated level of MAP. Red palm oil supplementation decreased oxidative stress and reduced the elevation in mean arterial blood pressure induced by buthionine sulfoximine (BSO) in rats by mechanisms involving changes in endothelium-derived factors (Ganafa *et al*., 2002). In a follow-up study involving high salt fed rats, it was established that RPO supplementation for four weeks attenuated the progression of salt-induced hypertension and mortality, by mechanisms involving reduction in oxidative stress and/or improvement in endothelium dependent relaxation and a reduction in vascular resistance and remodelling induced by the high salt (Bayorh *et al*., 2005). Two recent studies that investigated the effect of fresh RPO and repeatedly heated RPO on blood pressure, discovered that feeding fresh RPO for six months, either lowered blood pressure (Ng *et al*., 2012) or had no detrimental effects on blood pressure, but rather resulted in the elevation of NO content and reduced the contractile response to phenylephrine (Jaarin *et al*., 2011). Feeding repeatedly heated RPO for six months, elevated blood pressure in both studies, and this according to the authors, could be associated with an increased vascular reactivity and reduction in NO levels (Jaarin *et al*., 2011), as well as an adverse vascular remodelling and induction of VCAM-1 expression on endothelial cells (Ng *et al*., 2012).

A number of studies have also shown RPO and/or its minor constituents offer protection against ischaemia/reperfusion injury. Palm oil vitamin E supplementation was shown to increase post-ischaemic functional recovery, prevent LDH leakage and formation of lipid peroxidation products as well as suppress the decrease in ATP and creatinine phosphate levels in rats subjected to 40 minutes of global ischaemia (Serbinova *et al*., 1992). The positive effect shown by palm oil vitamin E was adduced to the synergistic interactions between the tocotrienol and tocopherol components of palm oil, since α-tocopherol was found to be less effective when supplemented alone. Das *et al*. (2008) compared the cardioprotective ability of the different isomers of tocotrienol (α, δ, and γ) against that of a tocotrienol-rich fraction (TRF) of palm oil. Data from the study revealed that supplementation of all the isomers provided cardioprotection, evidenced by a reduced infarct size and improvement of post-ischaemic ventricular function. Results also showed that the γ-isoform resulted in the best cardioprotection, followed by the α- and δ-isoform. The observed beneficial effects shown by the isomers on post-ischaemic ventricular function were inversely related to the degree of protection of the proteosome (Das *et al*., 2005; 2008). Dietary palm olein oil supplementation for 30 days attenuated oxidative stress associated with ischaemic-reperfusion injury in isolated rat hearts by increasing the myocardial activities of catalase,

SOD and glutathione peroxidase, with a concomitant decrease in TBARS level (Narang *et al*., 2004).

More recent studies at the Experimental Antioxidant Research Laboratory (Cape Peninsula University of Technology, South Africa), investigated the effects of dietary RPO supplementation on ischaemic-reperfusion injury and proposed some mechanisms for the positive effects observed. In one study, long-Evans rats were fed an RPO diet (7 g/kg diet) for six weeks. It was observed that RPO protected the heart against the consequences of global ischaemia via the activation of the NO-cGMP pathway and/or changes in PUFA composition during ishaemia-reperfusion (Esterhuyse *et al*., 2005a). In a follow-up study (Esterhuyse *et al*., 2005b), the activation of the NO-cGMP and inhibition of the cAMP pathway were proposed as the mechanisms responsible for the protection and improved reperfusion aortic output observed when RPO was supplemented in the diet of cholesterol fed rats. Using an isoenergetic diet, Bester *et al*. (2006) showed that RPO could offer protection against ischaemia-reperfusion injury, irrespective of the fat content of the diet, as indicated by improved aortic output recovery accompanied by an increase in cGMP. Still investigating the mechanism of functional recovery, Engelbrecht *et al* (2006) studied the regulation of mitogen-activated protein kinases (MAPKs) and PKB/Akt in rats fed a diet containing RPO for 6 weeks. The results showed that the protection and improved aortic output recovery observed was associated with an increased p38 and PKB/Akt phosphorylation, decreased c-Jun NH_2-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) phosphorylation and attenuation of the poly(ADB-ribose) polymerase (PARP) cleavage. Similar studies by the same group in rats fed a hypercholesterolemic diet showed that RPO supplementation improved functional recovery via a down-regulation of p38 and JNK while ERK phosphorylation was increased (Engelbrecht *et al*., 2009). Also a reduction in caspase-3 and PARP cleavage was observed, showing that RPO supplementation protected cholesterol-fed rat hearts against ischaemic-reperfusion injury by activating small kinases pathways and inhibiting apoptosis pathways. More evidence has shown that the beneficial effect of RPO during ischaemia-reperfusion injury may be mediated by the phosphatidylinositol-3-kinase (PI3-K) signaling pathway (Engelbrecht *et al*., 2009). Two recent reports from the group also showed that RPO supplementation attenuated ishaemic/reperfusion–mediated injury by reducing infarct size in normally fed rats (Bester *et al*., 2010b) and in cholesterol-fed rats (Szucs *et al*., 2011).

4.4.3.3 Anticancer effects

Accumulating evidence in the recent past has demonstrated that RPO and/or its phytoconstituents, especially carotenoids and tocotrienols, have potent anticancer effects *in vitro* and in experimental and several human cancers. The first evidence that palm oil may have anticancer ability came from the work of Sylvester *et al*. (1986), when they fed diets high in animal fats or vegetable fats (containing palm oil) to rats before and during carcinogen (7.5 mg; 7, 12-dimethylbenz (a) anthracene, DMBA) administration and observed that the vegetable oil diet did not enhance tumourigenesis in the rats. In a separate study few years later, Sundram *et al*. (1989) comparing the effect of different vegetable oils [corn oil (CO), soya bean oil (SBO), crude palm oil (CPO), refined, bleached, deodorized palm oil (RBDPO) and metabisulfite-treated palm oil (MCPO)] on DMBA-induced mammary tumourigenesis, observed that high palm oil diets did not promote DMBA-induced mammary tumourigenesis as rats fed CO and SBO had a higher tumour incidence and significantly more tumours at autopsy, when compared to rats fed the three palm oil diets. The protective effect shown by the palm oil diets was thought then to be due to the fact that linoleic acid, which was known to promote mammary carcinogenesis, was found in only trace amounts in palm oil (Nesaretnam *et al*., 2012). However, the first evidence to show that the tocopherols and tocotrienols present in palm oil may be responsible for the antitumourigenic ability of palm oil was provided by Nesaretnam *et al*. (1992) when he showed that palm oil stripped of vitamin E, promoted tumourigenesis in rats treated with DMBA. Subsequent studies have shown that the tocotrienols are the active form of vitamin E against cancer. According to a study by Goh *et al*. (1994), γ- and δ-tocotrienol-derived from palm oil, exhibited a strong activity against tumour promotion *in vitro* by inhibiting EBVEA (Epstein Barr Virus early antigen) expression in Raji cells induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), an activity that is absent in α- and γ-tocopherols, dimers of γ-tocotrienols and γ-tocopherols.

Several other in vitro studies have demonstrated the antiproliferative and apoptotic effects of the tocotrienol-rich fraction (TRF) of palm oil using different cell lines including, normal, preneoplastic, neoplastic and highly malignant mouse mammary epithelial cells (Mcintyre *et al*., 2000a, 2000b; Sylvester and Shah, 2002), human breast cancer (oestrogen receptor negative MDA-MB-435) cell line (Nesaretnam *et al*., 1995), estrogen-responsive (ER+)-MCF7 human breast cancer cells (Nesaretnam *et al*., 1998) and human colon RKO cells (Agarwal *et al*., 2004). More recent studies indicated that a TRF of palm oil is capable of inhibiting cellular proliferation by inducing cell cycle arrest and apoptosis selectively in human prostate cancer cells (Srivastava and Gupta, 2006). Feeding RPO diets at 7% and 14% in an AIN-93G based diet were effective in inhibiting the formation of azoxymethane (AOM)-induced aberrant foci in male Fisher 344 rats, showing that RPO may have a beneficial effect

in reducing the incidence of colon cancer (Boateng *et al*., 2006). Evidence has also shown that palm oil possesses anti-skin tumour activity. In the study, topical application of palm oil an hour before application of TPA, resulted in a reduction in both tumour incidence and tumour yield, compared to TPA-treated animals. Also a reduction in malignant tumour incidence was observed in palm oil treated animals, and the authors proposed that the mechanism of the observed effect may involve the inhibition of tumour promoter-induced epidermal ornithine decarboxylase (ODC) activity, [(3)H]thymidine incorporation and cutaneous oxidative stress (Kausar *et al*., 2003).

Evidence also shows that palm oil carotenoids may have *in vivo* and *in vitro* anticarcinogenic ability. Tan and Chu (1991) used benzo(a)pyrene (BaP) metabolism as a probe for chemical carcinogenesis and studied the *in vitro* and *in vivo* effects of palm oil carotenoids (β-carotene, α-carotene and canthaxanthin) on BaP metabolism in the rat hepatic cytochrome P450 monooxygenase system. Their results showed that the anticarcinogenic activity is in the order β-carotene > α-carotene > canthaxanthin. In a previous study, Murakoshi *et al*. (1989) demonstrated that α-carotene caused a dose- and time- dependent inhibition of the proliferation of human neuroblastoma GOTO cells, with the inhibitory ability of α-carotene found to be about ten times that of β-carotene. In a follow-up study, the same authors showed that the inhibitory effects of palm oil α-carotene on spontaneous liver carcinogenesis in C3H/Hc male mice, was greater than that of β-carotene (Murakoshi *et al*., 1992). Also *in vivo*, Manorama *et al*. (1993) demonstrated that RPO effectively inhibited chemical carcinogenesis in rats more than RBDPO, and the effectiveness of RPO was attributed to its carotenoid content. Recent *in vivo* studies showed that TRF caused a delayed onset in tumour formation and a reduction in tumour size in the treatment group compared with control when supplemented to athymic mice that were inoculated with human breast cancer cells (Nesaretnam *et al*., 2012). According to Healy *et al*. (2009), γ-tocotrienols from palm oil suppressed tumour growth in mice implanted with human breast cancer cells, by activating the ER-stress apoptotic pathway leading to growth arrest and apoptosis in the cancer cells. A very recent novel finding demonstrated the ability of palm oil tocotrienols to upregulate the expression of IL-24 mRNA gene that inhibits tumour growth and induces apoptosis in cancer cells without any adverse effect on normal cells (Selvaduray *et al*., 2010).

The anticancer effects of palm oil tocotrienols have been demonstrated in some clinical studies involving breast cancer patients. Researchers from the Malaysian Palm Oil Board (MPOB) demonstrated a 65% higher concentration of tocotrienols in the adipose tissues of patients with benign breast lumps, compared with those with malignant tumours and opine that the higher concentrations of tocotrienols in patients with benign tumours may be

associated with protective effects of tocotrienols (Nesaretnam *et al*., 2007). A clinical trial in Malaysia in which tocotrienol was combined with tamoxifen in women with primary oestrogen receptor-positive breast cancer for 5 years, revealed that the risk of death due to breast cancer was decreased by 70%, while the risk of recurrence was 20% lower in patients receiving combine tocotrienol and tamoxifen treatment compared with those receiving tamoxifen alone, suggesting that tocotrienol and tamoxifen work synergistically to reduce the risk of dying and recurrence of breast cancer (Nesaretnam *et al*., 2010).

4.4.3.4 Antioxidant and oxidative stress modulation

Since oxidative stress has been implicated in the pathogenesis of many disease states, the use of natural and synthetic molecules with antioxidant property has become a novel chemoprevention approach to tackle some of these conditions. RPO consists of a cocktail of many antioxidant compounds and the antioxidant effects of RPO has been ascribed mainly to the synergistic action of the carotenoids and vitamin E, as well as other minor constituents such as lycopene, coenzyme Q10, and squalene (Van Rooyen *et al*., 2008). The tocopherols, tocotrienols and carotenoids found in RPO are lipophilic chain breaking antioxidants and singlet oxygen quenchers, and their antioxidant effects may be rooted in their ability to donate phenolic hydrogens (electrons) to lipid peroxyl radicals.

Many studies have shown that RPO and its extracts have the ability to inhibit lipid peroxidation *in vitro* and *in vivo*. Farombi and Britton (1999) compared the antioxidant effectiveness of α- and β-carotene from palm oil in an organic solution of egg yolk phosphatidylcholine (EYPC) using lipid soluble 2,2′-azobis-(2,4-dimethyl valeronitrile) (AMVN) as peroxyl radical generator. Data from the study showed that both α- and β-carotene inhibited the formation of phosphatidyl choline hydroperoxide and TBARS, however, α-carotene better attenuated the peroxyl radical-dependent lipid peroxidation than β-carotene. Dietary palm olein oil supplementation for 30 days attenuated oxidative stress associated with ischaemic-reperfusion injury (Narang *et al*., 2004) and oxidative stress induced by isoproterenol (Narang *et al*., 2005) by augmenting cardiac antioxidant enzymes (CAT, SOD and GPx), inhibiting TBARS elevation and protecting against isoproterenol-induced myocardial necrosis in rats. Wu and Ng (2007) reported that red palm oil extract was able to prevent $FeCl_2$-ascorbic acid-induced lipid peroxidation in rat liver and brain homogenates. Cadmium-induced ocular tissue lipid peroxidation was also inhibited by RPO in rabbits (Eriyamremu *et al*., 2008). Tocotrienol-rich fraction of RPO was reported to inhibit the level of MDA and protein carbonyl production in the pancreas (Budin *et al*., 2011) and the level of MDA and 4-hydroxynonenal in the plasma and aorta (Budin *et al*., 2009) of streptozotocin-induced diabetic rats. In a previous study, we demonstrated that red palm oil

supplementation was able to protect against lead-induced oxidative stress in male Wistar rats by augmenting tissue antioxidant enzymes and total GSH level as well as inhibiting the formation of TBARS (Ajuwon *et al*., 2010). Lee *et al*. (2009) showed that supplementation of TRF of palm oil improved performance and reduced exercise-induced oxidative stress in forced swimming rats. Another study from the same laboratory examined the protective effect of TRF from palm oil on CCl_4-induced oxidative hepatotoxicity in rats. Data from the study showed that daily oral administration of TRF for eight weeks prevented elevation of ALT, AST, TBARS, triacylglycerol and total cholesterol levels, as well as augmented CAT, SOD and GPx activities in CCl_4-treated rats. The results suggested that TRF exert effective protection against CCl4-induced hepatotoxicity and its mechanism of action could be ascribed to the modulation of oxidative status in the liver of the rats (Lee *et al*., 2010). All these antioxidant effects can be ascribed to the synergistic interaction occurring *in vivo* with the various antioxidant constituents present in RPO.

4.4.3.5 Other health benefits

Some studies have also highlighted other health benefits of palm oil. Palm oil vitamin E was found to have gastroprotective effects by causing healing of ethanol-induced gastric lesions in Sprague Dawley rats (Jaarin *et al*., 2000), while Nafeeza *et al*. (2002) showed that TRF from palm oil and tocopherol were effective equally in preventing aspirin-induced gastric lesions. Another study demonstrated that TRF of palm oil and all tocotrienol isoforms (α, γ, and δ) possessed anti-inflammatory effects by inhibiting the production of IL-6 and NO in lipopolysaccharide-induced RAW264.7 macrophages (Yam *et al*., 2009). Other health benefits shown by palm oil and/or its minor constituents include antidiabetic (Budin *et al*., 2009; 2011) and neuroprotective (Sen *et al*., 2010) effects.

4.5 Synergistic effects of dietary antioxidants

Most of the proposed health benefits and chemopreventive effects shown by many medicinal plants have been attributed to the additive and synergistic interactions of the various phytochemicals they contain, rather than the effect of a single substance. In cancer chemoprevention, recent evidence is pointing to the fact that, specific combinations of phytochemicals, may actually be more effective in protecting against cancer, than isolated compounds, with results from studies showing combinatorial effects, where a single agent is inactive (Blot *et al*., 1993; Van Breda *et al*., 2008; De Kok *et al*., 2008). A number of studies have also reported enhanced chemoprevention due to synergistic effects of combinations of various polyphenols. Chemopreventive synergism has been observed between epigallocatechin gallate (EGCG) isolated from green teas, and cucurmin, in the inhibition of malignant and pre-malignant human oral epithelial cells (Khafif *et al*., 1998). Zhou *et al*.

(2003; 2004) demonstrated a synergistic inhibition of tumourigenicity, final tumour weight and metastasis to lymph nodes by green tea and a soy phytochemical concentrate (SPC) combination in human prostate tumour in mice. Evidence also exists that polyphenols and antioxidant vitamins may result in the synergistic modulation of lipid peroxidation, oxidative stress, and co-oxidation of dietary antioxidants (Gorelik *et al*., 2005).

However, to the best of our knowledge, no scientific study has reported on the possible synergistic effects of rooibos and red plam oil and the current study addresses this specific void.

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CHAPTER 3

The role of chronic rooibos and red palm oil feeding on the endogenous antioxidant system in male Wistar rats

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The role of chronic rooibos and red palm oil feeding on the endogenous antioxidant system in male Wistar rats

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Abstract

The present study was aimed at investigating the role of chronic feeding of an aqueous fermented rooibos extract, red palm oil (RPO) or their combination on biochemical parameters and the endogenous antioxidant system in male Wistar rats. Forty rats were randomized into four groups (n=10) and fed daily either standard rat chow (SRC) and water, SRC and the aqueous rooibos extract (2% w/v), SRC and RPO (200 µL/day) with water, or SRC and RPO (200 µL/day) with rooibos (2% w/v). The experiment lasted for 22 weeks. Chronic feeding of these plant extracts or their combination for 22 weeks did not induce any adverse hepatic or renal effects, as shown by serum levels of liver and kidney function markers [alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin (ALB), creatinine (CREA), blood urea nitrogen (BUN) and uric acid). This was corroborated by histopathology analyses which showed that liver tissue from the three supplementation groups displayed normal hepatic histoarchitecture similar to that of the control animals. Chronic feeding of RPO alone for 22 weeks did not influence the antioxidant/oxidant balance in the blood and liver significantly. Feeding rooibos alone for 22 weeks significantly ($P<0.05$) reduced malondialdehyde (MDA) levels in the plasma, while significantly increasing catalase (CAT), glutathione reductase (GR) and reduced glutathione (GSH) levels in the liver. Combined feeding of rooibos extract and RPO modulated the endogenous antioxidant system by significantly ($P<0.05$) increasing the plasma total polyphenol, plasma total antioxidant capacity, whole blood GSH and GSH/GSSG ratio, while also significantly ($P<0.05$) reducing liver MDA and whole blood GSSG levels. In conclusion, data from this study suggest that chronic feeding of an aqueous fermented rooibos extract alone, or in combination with RPO modulates the endogenous antioxidant system in Wistar rats and this modulation may

involve an additive or synergistic interaction between the phytochemicals in the aqueous rooibos extract and RPO.

Keywords: Endogenous antioxidant, rooibos, red palm oil, glutathione redox status

1. Introduction

Convincing experimental evidence has established oxidative stress as either a cause or consequence of several disease states, including cardiovascular diseases (CVD), obesity, diabetes, cancer, neurodegenerative diseases such as Parkinson's and Alzheimer's disease, as well as aging (Khensari *et al*., 2009; Chen *et al*., 2011; Lappas *et al*., 2011; Otani, 2011; Malireddy *et al*., 2012). Oxidative stress refers to the cytopathological consequences of a disturbance in regular cellular and molecular function caused by an imbalance between the production of free radicals (reactive species) and the natural antioxidant ability of cells resulting in oxidative damage to macromolecules (Small *et al*., 2012). These free radicals, including reactive oxygen species (ROS), reactive nitrogen species (RNS) and other oxidants, are the mediators of oxidative stress. Though, reactive oxygen and nitrogen species (RONS) are generally considered as highly reactive and cytotoxic molecules, recent evidence is suggesting that besides their noxious effects, they participate in important physiological processes such as defense against pathogens, signal transduction and apoptosis (Droge, 2002; Valko *et al*., 2007). Reactive oxygen and nitrogen species, including superoxide radical anion ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals produced during oxidative stress, are highly reactive and they can attack important macromolecules within their vicinity, resulting in damage to important cell structures, such as lipids and membranes, proteins and nucleic acids (Valko *et al*., 2007; Franco and Panayiotidis, 2009). Lipid peroxidation as a major consequence of oxidative stress-induced damage may disrupt cellular functions and membrane integrity, leading to pathophysiological alterations and cell death (Cheeseman, 1993; Shieh *et al*., 2010). Also, impairment of the antioxidant defense system of the cell, including inactivation/inhibition of antioxidant enzymes, as well as an alteration of the glutathione (GSH) redox status, that is crucial for cellular thiol-antioxidant defense system and cellular metabolic machinery, is another consequence of oxidative stress (Malireddy *et al*., 2012).

To prevent redox imbalance and oxidative damage that may arise as a result of oxidative stress, living cells have developed a biological defence system, consisting of an array of enzymatic and non-enzymatic antioxidants, to prevent damage due to oxidative stress.

However, overproduction of RONS, with subsequent oxidative stress may overwhelm the endogenous antioxidant defence system, as is the case during the onset or progression of a disease state (Dalle-Donne *et al*., 2006; Fisher-Wellman and Bloomer, 2009). Therefore dietary antioxidant therapy are seriously sought after and regarded as a promising strategy to strengthen the cellular antioxidant defence system and prevent oxidative stress-mediated cellular injury. Chemoprevention using whole plant extracts is gaining more scientific attention worldwide, partly because the plants are sources of bioactive phytochemicals, with antioxidant, disease prevention and therapeutic properties (Malireddy *et al*., 2012), and also because experimental and epidemiological studies have provided convincing evidence that diets rich in fruits, vegetables, teas and spices are associated with lower risk of chronic diseases (Aggarwal *et al*., 2006; Asif *et al*., 2011).

Rooibos is a unique South African herbal tea produced from the leaves and stems of *Aspalathus linearis* Dahlg. (Leguminosae). It is naturally caffeine-free and has a low tannin content when compared to *Camellia sinensis* teas, but high in unique antioxidant polyphenols (Joubert *et al*., 2008; Marnewick, 2009). Rooibos has a flavonoid profile that is distinctly different from those found in *Camellia sinensis*, including the β-dihydroxy-dihydrochalcone glucoside aspalathin, and its cyclic counterpart aspalalinin (**Figure 1**), both of which are unique to rooibos (Rabe *et al*., 1994; Shimamura *et al*., 2006), the dihydrochalcone nothofagin, as well as flavonols including orietin, iso-orientin, vitexin, isovitexin, luteolin, quercetin and chrysoeriol among others (Rabe *et al*., 1994; Bramati *et al*., 2002; Joubert *et al*., 2008).

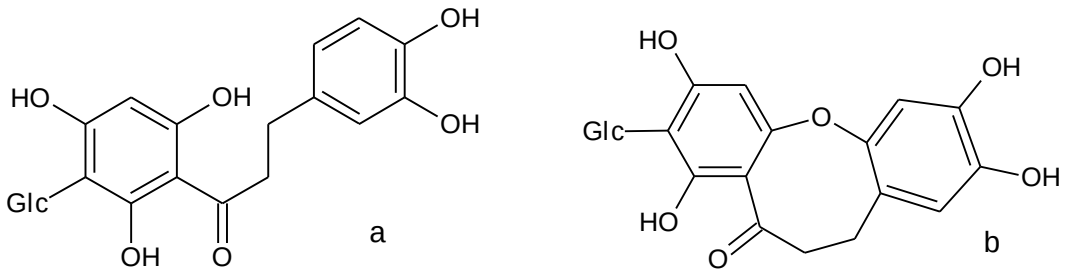

Figure 1: (a) Aspalathin, (b) aspalalinin, two unique flavonoids found only in *Aspalathus linearis*

Recent studies have shown rooibos to modulate oxidative stress by preventing lipid peroxidation and increasing blood glutathione levels in rat tissues and in humans (Marnewick *et al*., 2003; Nikolova *et al*., 2007; Marnewick *et al*., 2011; Awoniyi *et al*., 2012), and exert

hepatoprotective effects in acute and chronic liver damage in rats (Ulicna *et al*., 2003; 2008). Furthermore, rooibos has been documented to improve the glutathione redox status *in vivo* (Marnewick *et al*., 2003, 2011; Pantsi *et al*., 2011, Awoniyi *et al*., 2012).

Red palm oil (RPO) is the edible oil obtained from crude palm oil extracted from the oil palm plant, (*Elaeis guineesis*), after a modified refining process, involving degumming and bleaching, followed by deacidification and deodorisation by molecular distillation (Nagendran *et al*., 2000). It is an antioxidant-rich oil, with almost equal proportions of saturated (mostly palmitic acid) and unsaturated (mostly oleic and linoleic acid) fatty acids (Sambanthamurthi *et al*., 2000). Red palm oil is made up of a cocktail of fat soluble antioxidants, including vitamin E (70% as tocotrienols and 30% as tocopherols), carotenoids (mainly α- and β-carotene), as well as lycopene, squalene and coenzyme Q10 (Sundram *et al*., 2003; Al Saqer *et al*., 2004). The beneficial health effects of RPO have been demonstrated in various studies. Red palm oil has been shown to be hypocholesterolemic, or have a neutral effect on serum total cholesterol (Wlison *et al*., 2005; Budin *et al*., 2009), protect hearts subjected to ischaemia/reperfusion injury (Esterhuyse *et al*., 2006, Engelbrecht *et al*., 2009; Bester *et al*., 2010) and inhibit lipid peroxidation and modulate oxidative stress in different models of oxidant-mediated injury (Narang *et al*., 2005; Eriyamremu *et al*., 2008; Budin *et al*., 2009; 2011).

Most of the reports on the beneficial effects of both rooibos and RPO are based on experimental studies in rodent model of diseases or in animals with extensive pathology. Very limited, if not non-existent reports are available on the effect of rooibos and/or RPO supplementation in healthy animals. Furthermore, most experimental and intervention studies on rooibos and RPO involved either acute or sub-chronic feeding. Therefore, this study investigated the response of the endogenous antioxidant system in apparently healthy Wistar rats, to long-term/chronic feeding of rooibos and RPO. Also, since it has been reported that whole plant extracts have far more profound effects than isolated phytochemicals, because of the synergistic interactions of the cocktail of phytochemicals in whole extracts, we hypothesized that chronic feeding of rooibos and RPO together, may result in the synergy of their observed effects.

2. Materials and Methods

2.1 Chemicals

The chemicals L-ascorbic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulfonate (ABTS), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), fluorescein sodium salt, formaldehyde, Folin Ciocalteu's phenol reagent, gallic

acid, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), hesperidin, histological grade formaldehyde, 6-hydroxydopamine, metaphosphoric acid, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-thiobarbituric acid (TBA) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), iron chloride hexahydrate ($FeCl_3.6H_2O$), and potassium persulfate were obtained from Sigma–Aldrich (Johannesburg, South Africa). Diethylenetriaminepentaacetic acid (DETAPAC), 4-(dimethylamino)-cinnamaldehyde (DMACA) and malondialdehyde bis (diethyl acetal) (MDA), glacial acetic acid, trifluoroacetic acid, sulfuric acid (H_2SO_4), hexane, methanol (MeOH), ethanol (EtOH), dichloromethane, tetrahydrofuran, acetone, and hydrochloric acid (HCl) were purchased from Merck (Johannesburg, South Africa). All other reagents used were of analytical grade.

2.2 Red palm oil and rooibos herbal tea preparations

Fermented rooibos (superior grade) plant material was a generous gift from Rooibos Limited (Mr Arend Redelinghuys, Clanwilliam, South Africa). An aqueous extract (2% w/v) of rooibos (RTE) was prepared by the addition of freshly boiled tap water to leaves and stems at a concentration of 2g/100 mL (Marnewick *et al*., 2003). The mixture was allowed to stand at room temperature for 30 minutes with constant stirring, filtered and dispensed into water bottles. The aqueous rooibos extract was fed to rats *ad libitum* and fresh rooibos was prepared every second day. The RPO used in this study (Carotino^{TM} baking fat) was supplied by Carotino SDN BHD (company number: 69046-T), Johar-Bahru, Malaysia, and was fed to the rats orally (200 µL, equivalent to 7g/kg diet) on a daily basis in the morning, before the animals has access to the standard rat chow.

2.3 Animal treatment and experimental design

Forty pathogen-free, male Wistar rats weighing 220 ± 24 g were used in this study. The animals were obtained and housed at the Primate Unit of Stellenbosch University (Tygerberg Campus, South Africa). The rats were housed individually in stainless steel wired-bottom cages fitted with polypropylene houses under controlled environment, maintained at a temperature of between 22-24^{o}C, with a 12 h light dark cycle and 50-54% humidity. The rats were fed standard rat chow (SRC) *ad libitum* and had free access to tap water or the aqueous rooibos extract. The animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health Publication no. 80-23, revised 1978). The study protocol was approved by CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee (Ethics Certificate no:

CPUT/HAS-REC 2010/A003). After acclimatization in the experimental animal holding facility for 1 week, the rats were randomized into four groups of 10 animals each, and treated for 22 weeks as follows:

Group I (control group): Fed SRC with access to water as the sole source of drinking fluid for the duration of the study.
Group II (rooibos group): Fed SRC with access to rooibos (2% w/v) as the sole source of drinking fluid for the duration of the study.
Group III (red palm oil group): Fed SRC and red palm oil [200 µL (equivalent to 7g/kg diet) per day], with access to water as only source of drinking fluid for the duration of the study.
Group IV (rooibos + red palm oil group): Fed SRC and red palm oil (200 µL per day), with access to rooibos (2% w/v) as the only source of drinking fluid for the duration of the study.

The general conditions of the rats were monitored daily throughout the study and body weights recorded weekly and at sacrifice (end of 22 weeks). Fluid intake was monitored at intervals of 2 days throughout the study. At the end of the experimental period, after an overnight fast, animals in all the groups were sacrificed under sodium pentobarbital anaesthesia (0.15 mL/100g body weight, i.p.). Approximately 8 ml of blood was collected via the abdominal aorta and this was aliquoted into tubes with (EDTA) and without anticoagulant to obtain plasma and serum, respectively. Plasma/serum was separated immediately by centrifugation at 5 000 g for 5 min at 4^{o}C. The liver was removed, washed twice with ice-cold PBS (10 mM phosphate buffered saline pH 7.2) to remove residual blood, blotted to dry, and weighed. A slice of the liver sample was taken and fixed in 10% buffered formaldehyde solution for histological examination. The remaining liver tissue was immediately snap frozen in liquid nitrogen and stored at -80^{o}C for biochemical analysis.

2.4 Histopathological analysis

Histopathologic analyses of the liver samples were carried out at the Department of Anatomy and Histology, Stellenbosch University (Tygerberg Campus, South Africa). Formalin-fixed liver tissues were washed in tap water, dehydrated in serial ethanol, cleared in xylene and embedded in paraffin. A 3-5 µm thick section was made from the paraffin-embedded block and stained with haematoxylin and eosin for microscopic assessment. The slides were examined under light microscopy by a pathologist who was blind to the protocol of the study.

2.5 Soluble solids, total polyphenols, flavonol and flavanol content of the aqueous rooibos extract

The soluble solids content of the rooibos extract was determined gravimetrically (twelve repetitions) after drying 1 mL aliquots of the extract at 70^{o}C for 24 hours. The total polyphenol content of the aqueous rooibos extract was determined using the Folin Ciocalteu's phenol reagent according to the method described by Singleton *et al*. (1999) and results expressed as mg gallic acid equivalents/mg soluble solids. The flavanol content of the aqueous rooibos extract was determined colorimetrically at 640 nm using p-dimethylaminocinnamaldehyde (DMACA) according to the method of Treutter (1989). Results were expressed as mg catechin standard equivalents/mg soluble solids. The flavonol/flavones content was determined spectrophotometrically at 360 nm and results were expressed as mg quercetin standard equivalents/mg soluble solids (Mazza *et al*., 1999).

2.6 In vitro total antioxidant capacity of rooibos extract and red palm oil

2.6.1 Oxygen radical absorbance capacity assay

This assay measures the antioxidant capacity of plant and biological samples as a rate of the peroxyl radical-generated decline in the fluorescence of fluorescein. The oxygen radical absorbance capacity (ORAC) of rooibos extract and RPO was determined according to a method described by Ou *et al*. (2001) with some modifications. Briefly, 12 µL of diluted sample or trolox standard was mixed with 138 µL of fluorescein (14 µM) and 50 µL of AAPH (4.8 mM) added, to initiate the free radical attack. Fluorescence (excitation 485, emission 538) was recorded, every 1 min for 2 hr in a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µM Trolox equivalents (TE)/L or µM Trolox equivalents (TE)/g.

2.6.2 Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) of the aqueous rooibos extract and RPO was determined according to the method described by Re *et al*. (1999). This method measures the radical scavenging ability of antioxidants against ABTS$^{\bullet+}$. The ABTS$^{\bullet+}$ solution was prepared 24 h before use by mixing ABTS salt (8 mM) with potassium peroxodisulfate (140 mM) and then storing the solution in the dark until the assay could be performed. The ABTS$^{\bullet+}$ solution was diluted with distilled water (1:20) to give an absorbance of 1.50 at 734 nm. Briefly, 25 µL of sample or trolox standard was mixed with 275 µL ABTS$^{\bullet+}$ solution in a 96-well clear plate. The plate was incubated for 30 min at room temperature and the absorbance read at 734 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µM TE/L or µM TE/g.

2.6.3 Ferric ion reducing antioxidant power assay

The ferric reducing ability (FRAP) of the rooibos extract and RPO was determined using the method described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 100 mM HCl) and $FeCl_3.6H_2O$ (20 mM) in a ratio of 10:1:1, v/v/v). Briefly, 10 µL of sample or ascorbic acid (AA) standard was added to 300 µL FRAP reagent in a 96-well clear plate. The plate was incubated at room temperature for 30 min, and absorbance read at 593 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µmol AAE/L or µmol AAE/ g.

2.7 High performance liquid chromatography analysis of the aqueous rooibos extract

The aqueous rooibos extract was filtered (Whatman no 4) and chromatographically separated on an Agilent Technologies 1200 series HPLC system according to an adapted method described by Bramati *et al*. (2002). The HPLC system consisted of a G1315C diode array and multiple wavelengths detector, a G1311A quaternary pump, a G1329A autosampler, and a G1322A degasser. A 5 µm YMC-Pack Pro C18 (150 mm x 4.6 mm i.d.) column was used for separation and acquisition was set at 287 nm for aspalathin and 360 nm for other components. The mobile phases consisted of water (A) containing 300 µL/L trifluoroacetic acid and methanol (B) containing 300 µL/L trifluoroacetic acid. The gradient elution started at 95% A, changing to 75% A after 5 min and to 20% A after 25 min and back to 95% A after 28 min. The flow rate was set at 0.8 mL/min, the injection volume was 20 µL and the column temperature was set at 23^{o}C. Peaks were identified based on the retention time of the standards and confirmed by comparison of the wavelength scan spectra (set between 210 nm and 400 nm).

2.8 High performance liquid chromatography analysis of RPO

2.8.1 Vitamin E content of RPO

Vitamin E in RPO was determined as isoforms of tocopherol and tocotrienol. Extraction was done by shaking 1 g of RPO in 5 ml of absolute ethanol for 30 min, followed by centrifugation at 5000 g for 10 min. An aliquot of the top vitamin E layer (20 µL) was injected into a chromatographic system (Agilent Technology 1200 series), using an analytical column YMC-Pack Pro C18 (150 x 4.6 mm, i.d.) with the UV-visible wavelength detector set at 296 nm. The mobile phase consisted of A (acetonitrile:methanol:isopropanol:water; 45:45:5:5, v/v) and B (acetonitrile:methanol:isopropanol; 50:45:5, v/v) and elution was carried out at a flow rate of 1mL/min. Mobile phase A was programmed to B within 10 min and this condition maintained for another 15 min before returning to the original condition. The content of

tocopherols and tocotrienols were quantified by comparing the retention time and/or peak area with standards (Iqbal *et al*., 2007).

2.8.2 Carotenoids content of RPO

Carotenoids from RPO were extracted with tetrahydrofuran:dichloromethane (1:1, v/v) and analysed on an Agilent Technology 1200 series HPLC with the visible detector set at 450 nm according to a modified method of Rautenbach *et al*. (2010). Twenty microlitre of extracted samples were injected automatically into the column (YMC-Pack Pro C30, 250 x 4.6 mm i.d., room temperature) and isocratic elution performed on a mobile phase consisting of methanol:acetone (9:1, v/v) with flow rate set at 1 mL/min. Peaks were identified based on the retention time of the α- and β-carotene standards.

2.9 Preparation of liver homogenates

The liver tissue was homogenized on ice in 10 volumes of 50 mM phosphate buffer containing 1 mM EDTA and 0.5% Triton X-100 (pH 7.5). The homogenate was transferred into tubes and centrifuged at 10 000 g for 10 min at 4^{o}C. The supernatant was collected, divided into aliquots, and stored at -80^{o}C until used for analyses of antioxidant capacities, lipid peroxidation, activity of antioxidant enzymes and glutathione status. Protein content of biological samples (erythrocytes and liver homogenate) was determined using the BCA protein assay kit supplied by Pierce (Illinois, USA).

2.10 Antioxidant capacity of plasma and liver samples

To avoid protein interference in antioxidant capacity assays, sub-samples of plasma and liver homogenates were precipitated with 0.5 M perchloric acid (1:1, v/v) and centrifuged at 10 000 g for 10 min at 4^{o}C. Supernatants were collected as protein free fractions (Roble-Sanchez *et al*., 2011). Plasma total polypnenol, as well as ORAC, TEAC and FRAP assays (plasma and liver) were performed as previously described for the rooibos extract and RPO in section 2.6 (Benzie and Strain, 1996; Re *et al*., 1999; Singleton *et al*., 1999; Ou *et al*., 2001).

2.11 Liver and kidney function markers

Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, lactate dehydrogenase (LDH), albumin, total protein, creatinine (CREA), blood urea nitrogen (BUN) and uric acid were measured on a Medica EasyRA automated clinical chemistry analyser (Medica Corporation Bedford, Mass., USA), using standard commercial kits (Medica Corporation Bedford, Mass., USA).

2.12 Oxidative stress and antioxidant status biomarkers

2.12.1 Plasma and hepatic lipid peroxidation

Lipid peroxidation was assessed by measurement of conjugated dienes (CDs) and malondialdehyde (MDA). Plasma and liver MDA were assayed as MDA-TBA adducts using HPLC with a UV-visible detector according to a method of Khoschsorur *et al*. (2000). Conjugated dienes were estimated according to the method of Recknagel and Glende (1984). After the initial extraction of the lipid content from the plasma and liver homogenates, the lipid residues were dissolved in cyclohexane and CDs were measured spectrophotometrically at 234 nm and results expressed as nmol/L or nmol/g tissue in plasma and liver, respectively.

2.12.2 Hepatic and erythrocyte antioxidant enzymes activity

Catalase (CAT) activity in the erythrocytes and liver homogenates were determined according to the method described by Aebi (1984), in which the rate of decomposition of hydrogen peroxide was measured at 240 nm. The activity of catalase was calculated using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ and results expressed as µmole H_2O_2 consumed/min/mg protein. The activity of superoxide dismutase (SOD) was determined according to the method of Crosti *et al*. (1987), and SOD activity expressed as U/mg protein. Glutathione peroxidase (GPx) activity was determined according to the method of Ellerby and Bredesen (2000). The activity of GPx was calculated using the mmolar extinction coefficient of 6.22 and results expressed as nmol NADPH oxidized per min per mg protein. *G*lutathione reductase (GR) was assayed by a method of Staal *et al*. (1969) and result expressed as µmol NADPH oxidized per min per mg protein using the mmolar extinction coefficient of 6.22.

2.12.3 Hepatic and whole blood glutathione redox status

The total glutathione (GSH and GSSG) was measured according to the method described by Asensi *et al*. (1999). Aliquots of whole blood without (for GSH determination) or with 3 mM freshly prepared M2VP (for GSSG determination) were precipitated with 5% (w/v) metaphosphoric acid (MPA), while liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 10 000g for 10 min, 50 µL of supernatant (from whole blood or liver homogenate) was added to 50 µL of glutathione reductase (1U) and 50µL of 0.3mM DTNB. The reaction was initiated by addition of 1 mM NADPH to a final volume of 200 µL. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as

standards. GSH concentration was calculated as the difference between total glutathione and 2GSSG.

2.13 Statistical analysis

Values were expressed as mean ± SD. Data were tested for normality using the Kolmogorov–Smirnof test and Levene's Test for Equality of variances. Differences between group means were estimated using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for all pairwise comparisons. The Kruskal-Wallis test, a non-parametric analogue to the one-way ANOVA was used to test for group differences when data was not normally distributed. Results were considered statistically significant at $P<0.05$, or marginally significant at $P<0.1$. All the statistics were carried out using MedCalc v 12.2.1 software (MedCalc software bvba, Mariakerke, Belgium).

3. Results

3.1 Phytochemical content and antioxidant capacity of plant materials

The phytochemical content and *in vitro* antioxidant capacity of the aqueous rooibos extract and RPO are shown in **Table 1** and **2,** respectively. The total polyphenolic content of the rooibos extract is 1.06 ± 0.00 mg GAE/mL, 63% of which are made up of flavonols and flavanols. HPLC analysis of the rooibos extract revealed the presence of peaks consistent with patterns showed by standards including aspalathin, iso-orientin, orientin, rutin/hyperoside and others (**Figures 2 and 3**). Quantitatively, the HPLC analysis showed aspalathin (29.98 ± 0.08 µg/mL), iso-orientin (25.98 ± 0.52 µg/mL), orientin (18.61 ± 0.13 µg/mL) and hyperoside/rutin (14.55 ± 2.26 µg/mL) were the main flavonoids in the aqueous rooibos extract (Table 1). The total polyphenolic content of RPO used is 0.17 ± 0.00 mg GAE/100g (**Table 2**). The tocopherol content of the oil is 98 µg/g, while the tocotrienol content is 386 µg/g. The total carotene content (α and β) is 53 µg/g. the in vitro antioxidant capacity of the RPO, measured as ORAC, FRAP and TEAC values are 175 ± 15.19 µmol TE/100g, 12.30 ± 0.28 µmol AAE/100g and 0.16 ± 0.00 µmol TE/100g, respectively (**Table 2**).

Table 1: Phenolic content and *in vitro* antioxidant capacity of the aqueous rooibos extract

Constituents	Concentration
Soluble solids (mg/mL)	2.85 ± 0.39
Total polyphenol (mg GAE/mL)	1.06 ± 0.00
Flavonol (mg QE/ mL)	0.48 ± 0.01
Flavanol (mg CE/mL)	0.19 ± 0.01
Aspalathin (µg/mL)	29.98 ± 0.08
Orientin (µg/mL)	18.61 ± 0.13
Iso-orientin (µg/mL)	25.98 ± 0.52
Vitexin (µg/mL)	6.07 ± 1.05
Iso-vitexin (µg/mL)	7.18 ± 0.20
Hyperoside/rutin (µg/mL)	14.55 ± 2.26
Quercetin (µg/mL)	0.89 ± 0.18
Luteolin (µg/mL)	0.22 ± 0.06
Chrysoeriol (µg/mL)	0.25 ± 0.00
ORAC (µmol AAE/mL)	1.69 ± 0.03
FRAP (µmol TE/mL)	5.20 ± 0.04
TEAC (µmol TE/mL)	4.79 ± 0.33

Values are mean ± SD. Soluble solid is a mean of 12 determinations while other parameters are mean of 5 determinations. AAE (ascorbic acid equivalent), CE (catechin equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent), TE (trolox equivalent).

Table 2: Phytochemical constituents, fatty acid content and *in vitro* antioxidant capacity of the red palm oil used in the study

Constituents	Concentration
α-tocopherol (µg/g)	71.28 ± 2.30
β/γ-tocopherol(µg/g)	6.20 ± 0.94
δ-tocopherol (µg/g)	20.70 ± 1.66
α-tocotrienol (µg/g)	102.36 ± 1.52
β/γ-tocotrienol (µg/g)	227.48 ± 2.74
δ-tocotrienol (µg/g)	56.46 ± 1.55
α-carotene (µg/g)	23.74 ± 1.16
β-carotene (µg/g)	29.34 ± 2.92
Total polyphenol (mg GAE/100g)	0.17 ± 0.00
ORAC (µmol TE/100g)	175.00 ± 15.19
FRAP (µmol AAE/100g)	12.30 ± 0.28
TEAC (µmol TE/100g)	0.16 ± 0.00
SFA (%)	51
MUFA (%)	38
PUFA (%)	11

Values are mean ± SD for (n=5). AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent), SFA (saturated fatty acid), MUFA (mono-unsaturated fatty acid), PUFA (poly-unsaturated fatty acid).

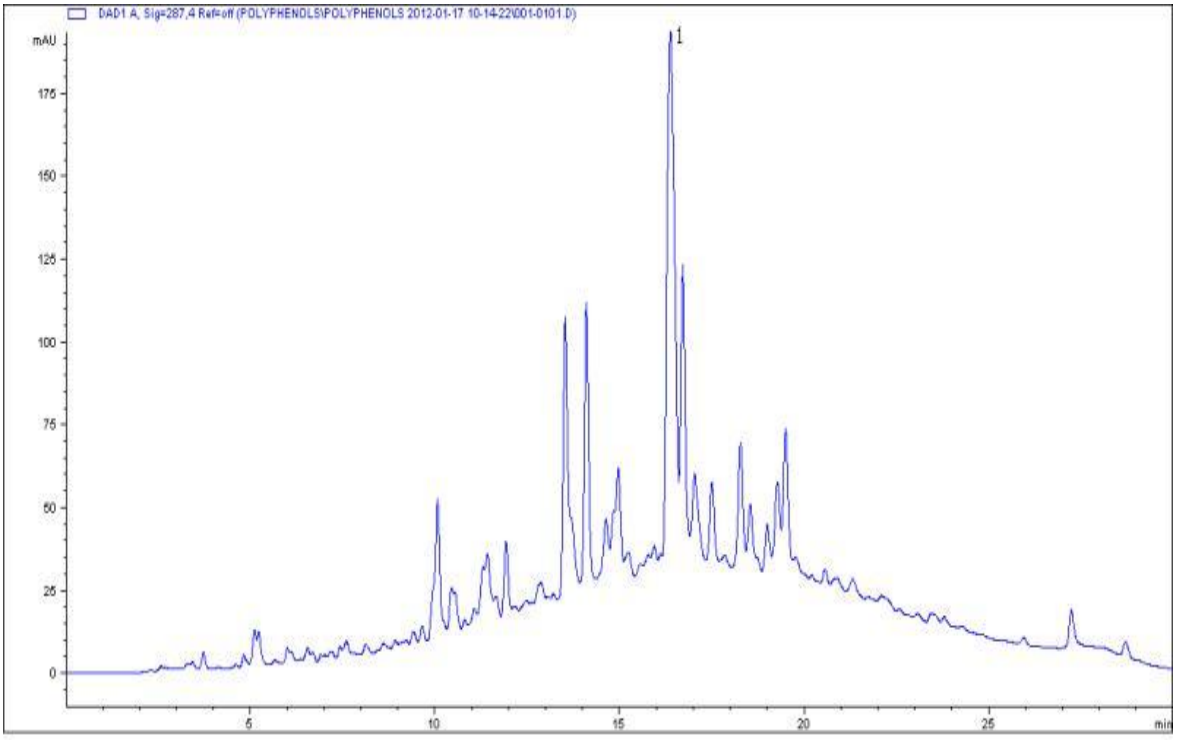

Figure 2: HPLC chromatogram (287 nm) of aqueous rooibos extract used showing peak 1 for aspalathin.

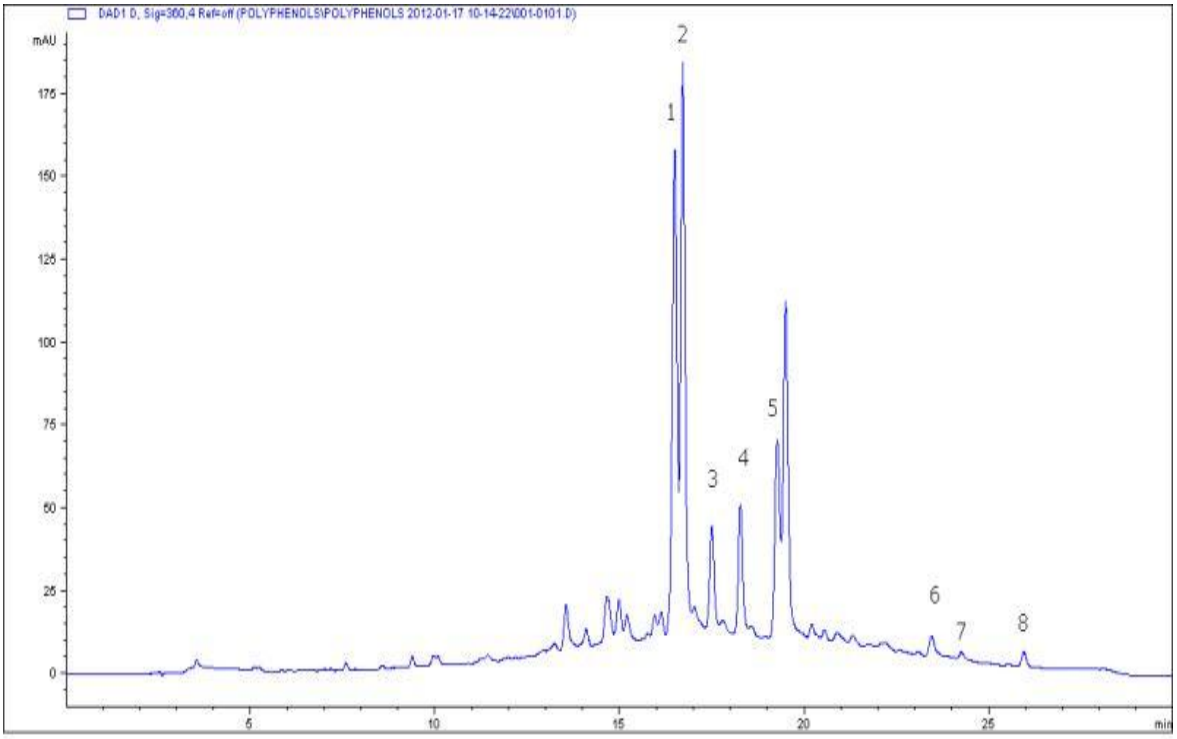

Figure 3: HPLC chromatogram (360 nm) of aqueous rooibos extract showing peaks for other flavonoids. 1, orientin; 2, iso-orientin; 3, vitexin; 4, isovitexin; 5, hyperoside/rutin; 6, quercetin; 7, luteolin; 8, chrysoeriol.

3.2 Daily intake profile of rooibos and red palm oil

The daily phenolic, vitamin E and carotene intakes of the various rat groups consuming the aqueous rooibos and RPO are shown in **Table 3**. Daily water intake between the control group and the group fed RPO alone was similar (P>0.05). Rooibos intake, total phenolic, flavonol and flavanol intakes per day between the two groups that were fed rooibos were not significantly different (P>0.05). The daily vitamin E and carotene intakes between the group fed RPO alone and the group fed combination of rooibos and RPO were also similar (P>0.05).

Table 3: Daily phenolic, vitamin E and carotenoid intakes of the various rat groups consuming the aqueous rooibos extract and RPO

Treatment	Water/rooibos intake/day/ 100 g BW (mL)	Total phenolic intake (mg GAE/ day/100 g BW)	Flavonol intake (mg QE/ day/ 100 g BW)	Flavanol intake (mg CE/day/ 100 g BW)	Vitamin E intake/day (µg/100g BW)	α-Carotene intake/day (µg/100g BW)	β-Carotene intake/day (µg/100g BW)
Control	6.63 ± 0.67	ND	ND	ND	ND	ND	ND
RTE	6.83 ± 0.31	7.22 ± 0.33	2.91 ± 0.13	1.10 ± 0.05	ND	ND	ND
RPO	6.89 ± 0.82	ND	ND	ND	17.50 ± 1.66	0.86 ± 0.08	1.06 ± 0.10
RTE + RPO	6.70 ± 0.32	7.09 ± 0.34	2.85 ± 0.14	1.08 ± 0.05	16.52 ± 0.75	0.81 ± 0.04	1.00 ± 0.05

Calculations of the total phenolic, flavonol and flavanol intakes were calculated based on the soluble solid intake obtained from the average rooibos consumption per day. Values are mean ± SD (n=10). ND (not determined), BW (body weight), CE (catechin equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent), RTE (aqueous rooibos extract), RPO (red palm oil).

3.3 Body and liver weight change

The effect of chronic feeding of rooibos, RPO or their combination on body weight gain, liver weight and relative liver weight is shown in **Table 4**. Chronic feeding of rooibos, RPO or their combination for 22 weeks, did not have any deleterious effect on the body weight gains across all the groups, however, consuming these plant products alone or in combination did significantly ($P<0.05$) decrease the absolute liver weight and the relative liver weight of the rats when compared to the control animals.

Table 4: Effect of chronic feeding of aqueous rooibos extract, RPO or their combination on body weight gain, liver weight, and relative liver weight of experimental rats

Treatment	Body weight gain (g)	Liver weight (g)	Relative liver weight (%)
Control	294.00 ± 39.61	13.62 ± 1.32	2.81 ± 0.15
RTE	285.80 ± 22.06	11.77 ± 1.87*	2.46 ± 0.32*
RPO	281.40 ± 32.40	11.30 ± 1.37*	2.39 ± 0.16*
RTE + RPO	281.90 ± 21.40	11.75 ± 1.48*	2.42 ± 0.17*

Values are mean ± SD (n=10). *Significantly different from control at $P<0.05$. RTE (aqueous rooibos extract), RPO (red palm oil).

3.4 Liver and kidney function markers and histopathology

Table 5 shows the effect of chronic feeding of rooibos and RPO on markers of liver and kidney functions. The serum levels of hepato-specific enzymes (ALP, ALT, AST and LDH) remained similar to that of the control, after feeding with the rooibos extract, RPO or their combination for 22 weeks. Data from the histopathological examination of liver tissues showed that none of the feeding regimens had a deleterious effect on the liver, with the liver tissues showing normal histoarchitecture (**Figure 4**). Also the levels of ALB, BUN, CREA and uric acid which can be used to assess damage to the kidney, was unaffected by chronic feeding of the rooibos extract, RPO or their combination for 22 weeks.

Table 5: Effect of chronic feeding of aqueous rooibos extract, RPO or their combination on markers of liver and kidney functions.

Treatment	Control	Rooibos	RPO	Rooibos + RPO
ALB (g/L)	33.14 ± 0.88	33.74 ± 0.60	34.72 ± 1.05	33.78 ± 1.10
ALP (U/L)	74.11 ± 11.16	68.05 ± 11.55	67.28 ± 11.73	66.25 ± 10.76
ALT (U/L)	91.07 ± 21.24	82.46 ± 21.73	95.11 ± 27.60	82.64 ± 13.29
AST (U/L)	145.37 ± 19.53	126.83 ± 45.46	143.01 ± 42.89	122.99 ± 21.37
LDH (U/L)	262.84 ± 73.20	192.86 ± 86.00	216.00 ± 71.05	200.68 ± 35.52
CREA (µmol/L)	30.50 ± 2.63	31.06 ± 2.87	30.50 ± 2.89	32.78 ± 1.47
BUN (mmol/L)	5.94 ± 0.50	6.21 ± 0.47	6.26 ± 0.52	6.04 ± 0.53
Uric Acid (µmol/L)	121.00 ± 21.31	94.89 ± 28.38	100.11 ± 34.79	95.11 ± 24.94

Values are mean ± SD (n=7-10). ALB (albumin), ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), BUN (blood urea nitrogen), CREA (creatinine), LDH (lactate dehydrogenase).

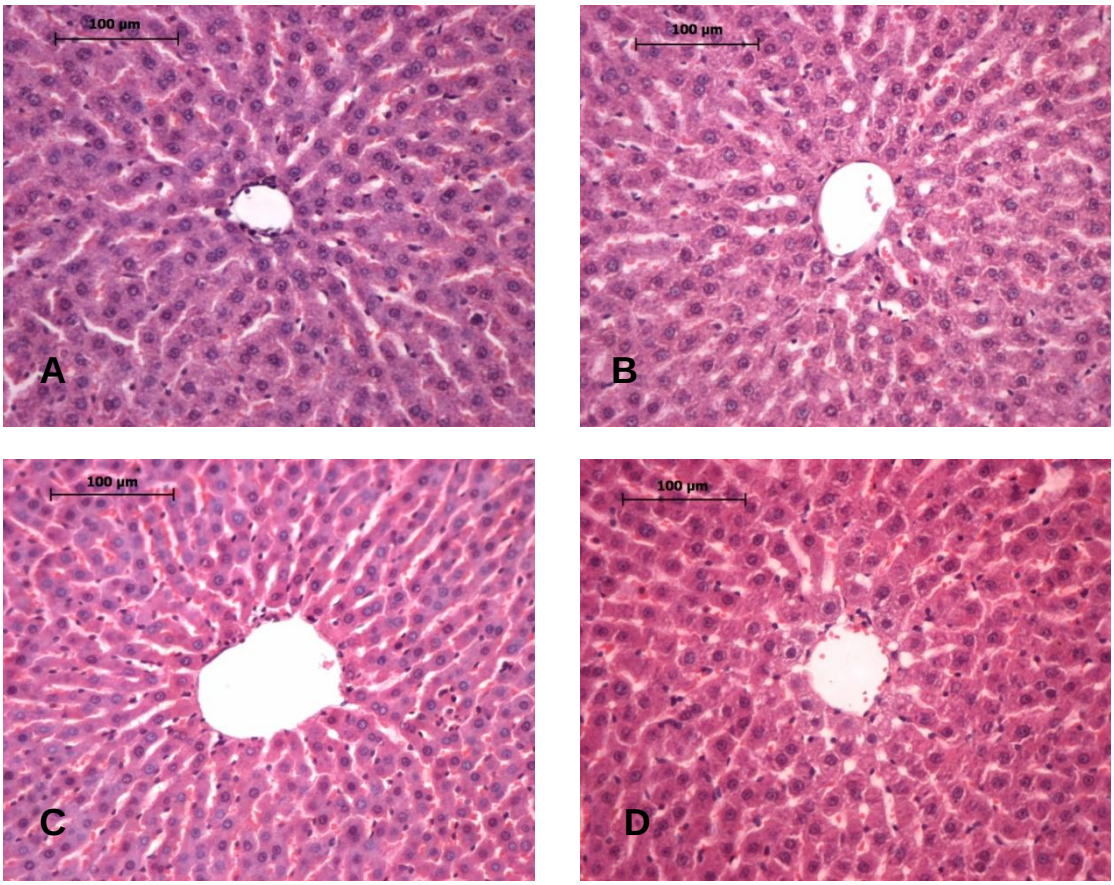

Figure 4: Hematoxylin and eosin stained liver sections showing the effect of chronic feeding of aqueous rooibos, red palm oil or their combination on liver histoarchitecture. (A) control group, (B, C and D) rooibos, red palm oil and rooibos + red palm oil group respectively, showing normal histoarchitecture of the liver.

3.5 Plasma and hepatic antioxidant capacity

Effect of chronic feeding of aqueous rooibos extract, RPO or their combination on the antioxidant capacity in the plasma and liver is presented in **Table 6**. When considering the plasma, total polyphenol and TEAC status were unaffected by chronic feeding of the aqueous rooibos extract or RPO when compared with control. However, combined feeding of the rooibos extract and RPO significantly ($P<0.05$) increased the total polyphenol content and TEAC status. The ORAC and FRAP status of the rats remained similar ($P>0.05$) to those of the control group after being fed the aqueous rooibos extract, RPO or their combination for 22 weeks.

In the liver, the ORAC, TEAC and FRAP status in the groups chronically fed the aqueous rooibos extract, RPO or their combination was not statistically ($P>0.05$) different when compared to the control group.

Table 6: Effects of chronic feeding of aqueous rooibos extract, RPO or their combination on total polyphenol and antioxidant capacity in the plasma and liver of male Wistar rats

	Plasma				Liver		
Treatment	**Total polyphenol (mg GAE/L)**	**TEAC (µmol TE/L)**	**ORAC (µmol TE/L)**	**FRAP (µmol AAE/L)**	**TEAC (µmol TE/g tissue**	**ORAC (µmol TE/g tissue)**	**FRAP (µmol AAE/g tissue)**
Control	64.44 ± 3.75	6130 ± 360	1412 ± 265	268.67 ± 30.65	65.38 ± 6.72	17.23 ± 1.69	2.85 ± 0.30
RTE	67.28 ± 5.32	6200 ± 268	1614 ± 321	266.37 ± 15.63	66.30 ± 3.12	16.14 ± 1.85	2.87 ± 0.35
RPO	63.92 ± 3.40	6252 ± 244	1463 ± 395	260.35 ± 18.49	63.03 ± 2.11	16.37 ± 1.89	3.03 ± 0.35
RTE + RPO	71.77 ± 4.39*	6586 ± 344*	1626 ± 405	271.74 ± 17.36	65.08 ± 4.46	16.91 ± 1.43	3.03 ± 0.39

Values are mean ± SD (n=10). *Significantly different from control at $p<0.05$. FRAP (ferric reducing ability of plasma), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity), RTE (aqueous rooibos extract), RPO (red palm oil), AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent).

3.6 Erythrocyte and hepatic antioxidant enzymes

Figures 5-8 show the results of the effect of chronic feeding of rooibos and RPO on erythrocyte and hepatic antioxidant enzymes. In the plasma, the activities of SOD, GPx and GR remained unchanged (P>0.05) as a result of chronic feeding of the aqueous rooibos extract, RPO or their combination, when compared to the control group (**Figures 5a, 6a & 5b, 6b**). The activity of CAT in rats chronically fed the rooibos extract or RPO alone was similar (P>0.05) to that of the control. However, combined feeding of the rooibos extract and RPO significantly (P<0.05) increased the erythrocyte CAT activity when compared to the control group (**Figure 5b**).

Hepatic SOD and GPx remain unchanged across all treatment groups when compared to control group (**Figures 7a & 8a**). Feeding the aqueous rooibos extract alone or combined with RPO significantly (P<0.05) increased the CAT and GR activity, when compared with the control group (**Figures 7b & 8b**).

3.7 Plasma and hepatic lipid peroxidation

Lipid peroxidation was assessed as conjugated dienes (CDs) and malondialdehyde (MDA) levels (**Figures 9 and 10**). Plasma CDs remained unchanged (P>0.05) compared to control rats when aqueous rooibos extract alone was consumed. Feeding RPO alone, or combined with rooibos extract to the rats, significantly (P<0.05) reduced the level of CDs when compared to the control (**Figure 9a**). The level of MDA was marginally (P<0.1) lowered as a result of chronic feeding of aqueous rooibos extract alone, when compared to the control group consuming water. Rats consuming RPO alone or combined with the rooibos extract for 22 weeks had MDA values that were similar (P>0.05) to those of the control animals (**Figure 9b**).

In the liver, the level of CD was not affected by chronic feeding of the aqueous rooibos extract or RPO. However, combined supplementation of the aqueous rooibos extract and RPO significantly (P<0.05) elevated hepatic CD levels when compared to control rats (**Figure 10a**). Hepatic MDA was also not affected by chronic feeding of rooibos extract or RPO alone, however, when both extract were fed together, hepatic MDA was significantly (P<0.05) lowered when compared to the control group (**Figure 10b**).

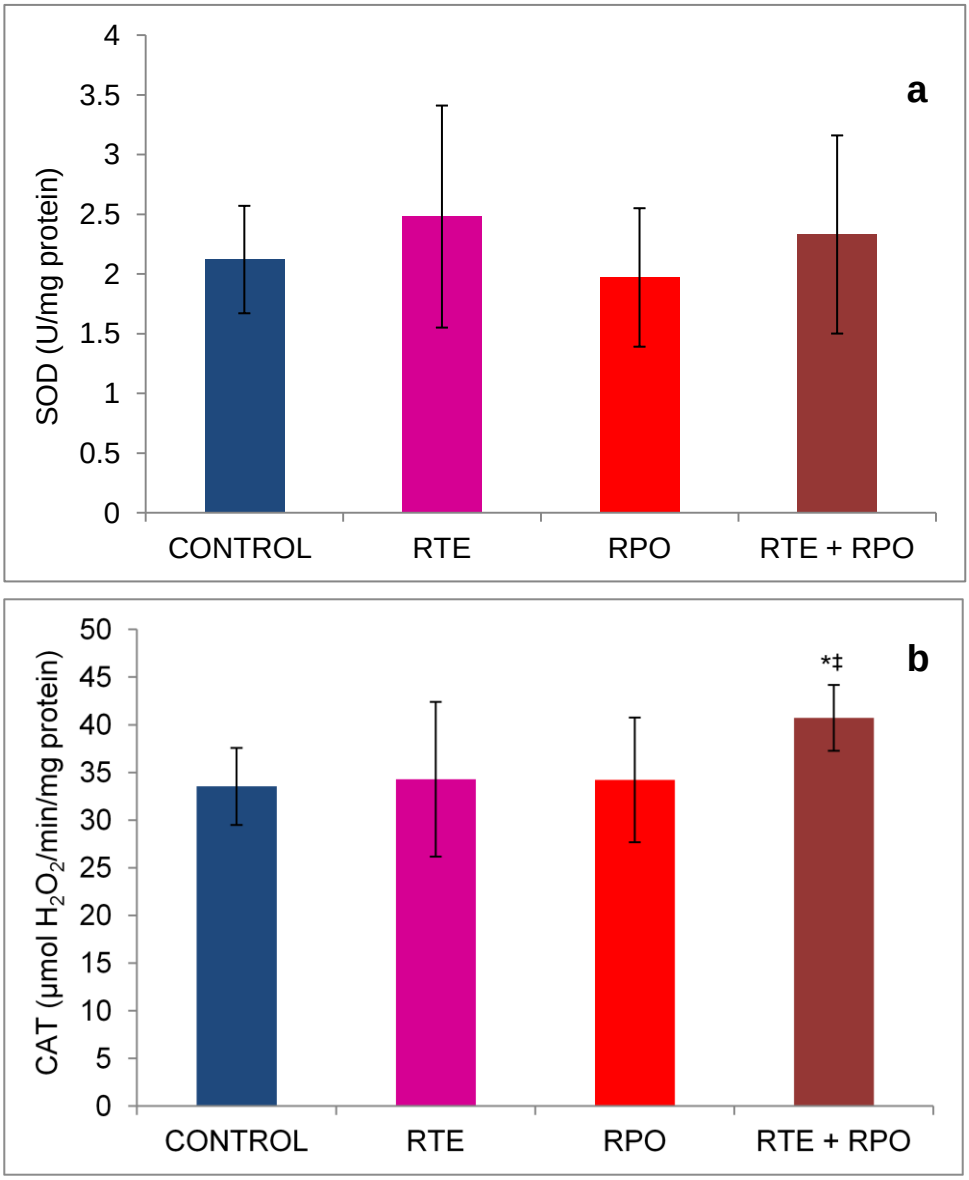

Figure 5: Effect of chronic consumption of rooibos, RPO or their combination on (a) SOD and (b) CAT activity in the plasma. Bars represent mean ± SD (n=7-10). *Significantly different from control group at P<0.05. ‡Significantly different from RTE and RPO group at P<0.05. RTE (aqueous rooibos extract), RPO (red palm oil). CAT (catalase), SOD (superoxide dismutase).

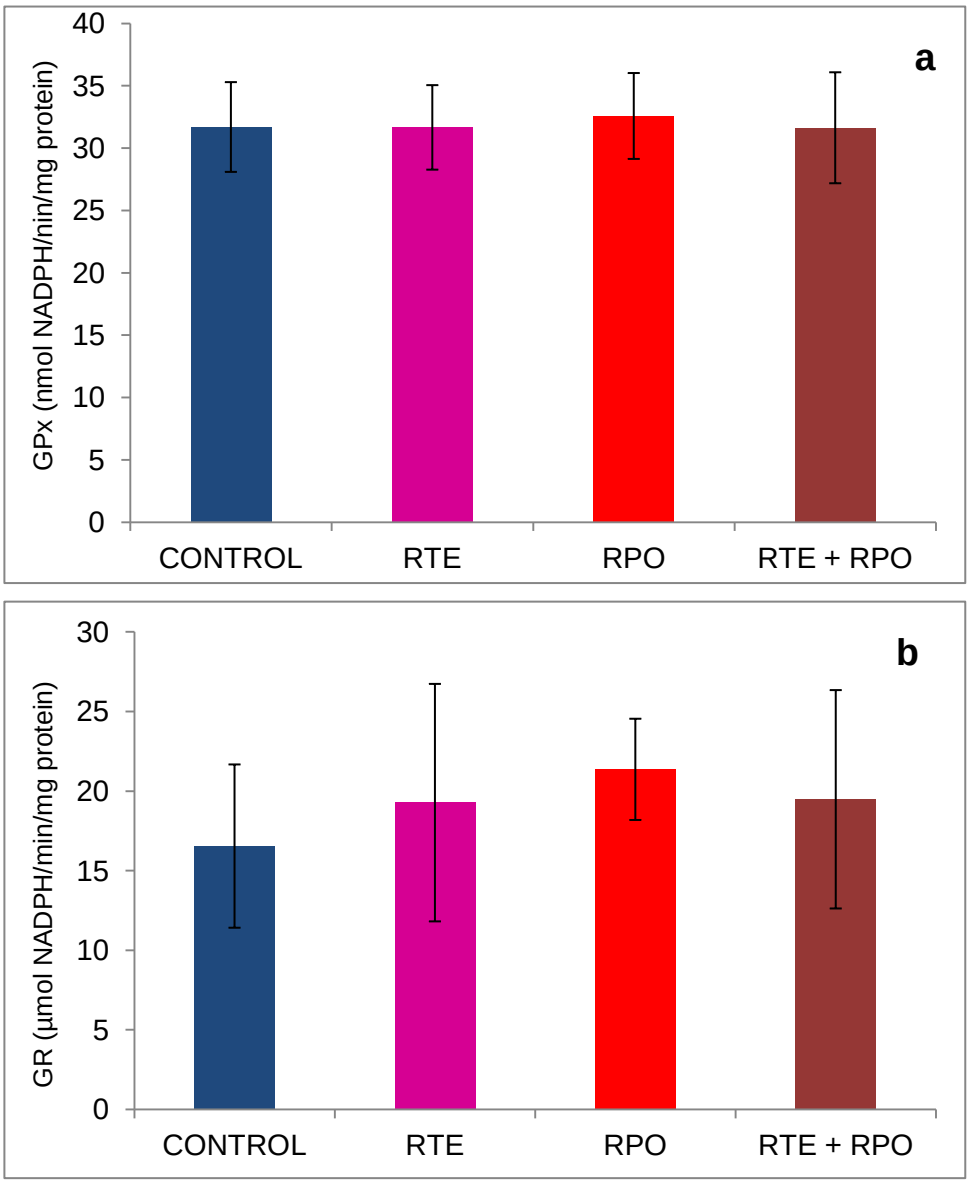

Figure 6: Effect of chronic consumption of rooibos, RPO or their combination on (a) GPx and (b) GR activity in the plasma. Bars represent mean ± SD (n=7-10). RTE (aqueous rooibos extract), RPO (red palm oil). GPx (glutathione peroxidase), GR (glutathione reductase).

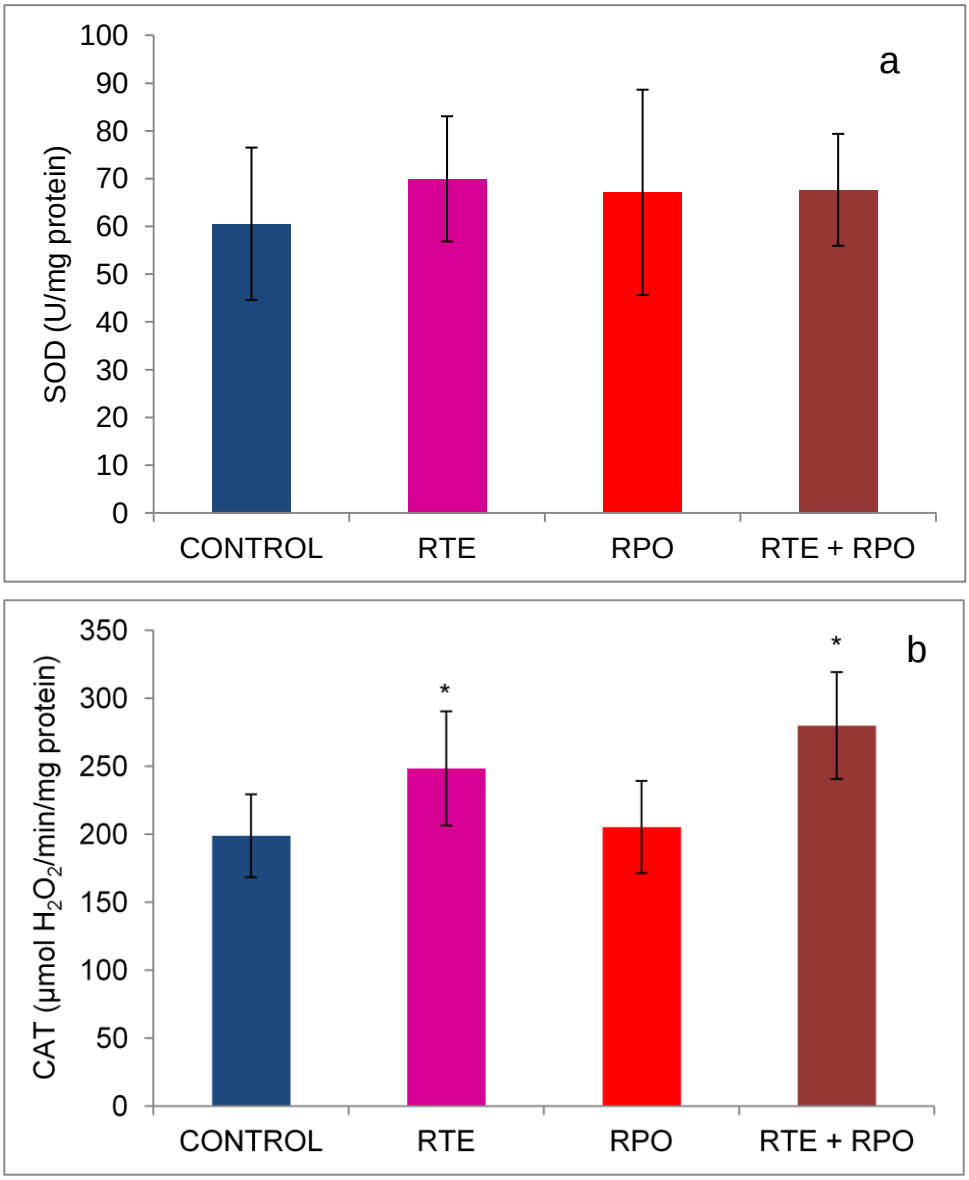

Figure 7: Effect of chronic consumption of rooibos, RPO or their combination on (a) SOD and (b) CAT activity in the liver. Bars represent mean ± SD (n=7-10). *Significantly different from control at P<0.05. RTE (aqueous rooibos extract), RPO (red palm oil). CAT (catalase), SOD (superoxide dismutase).

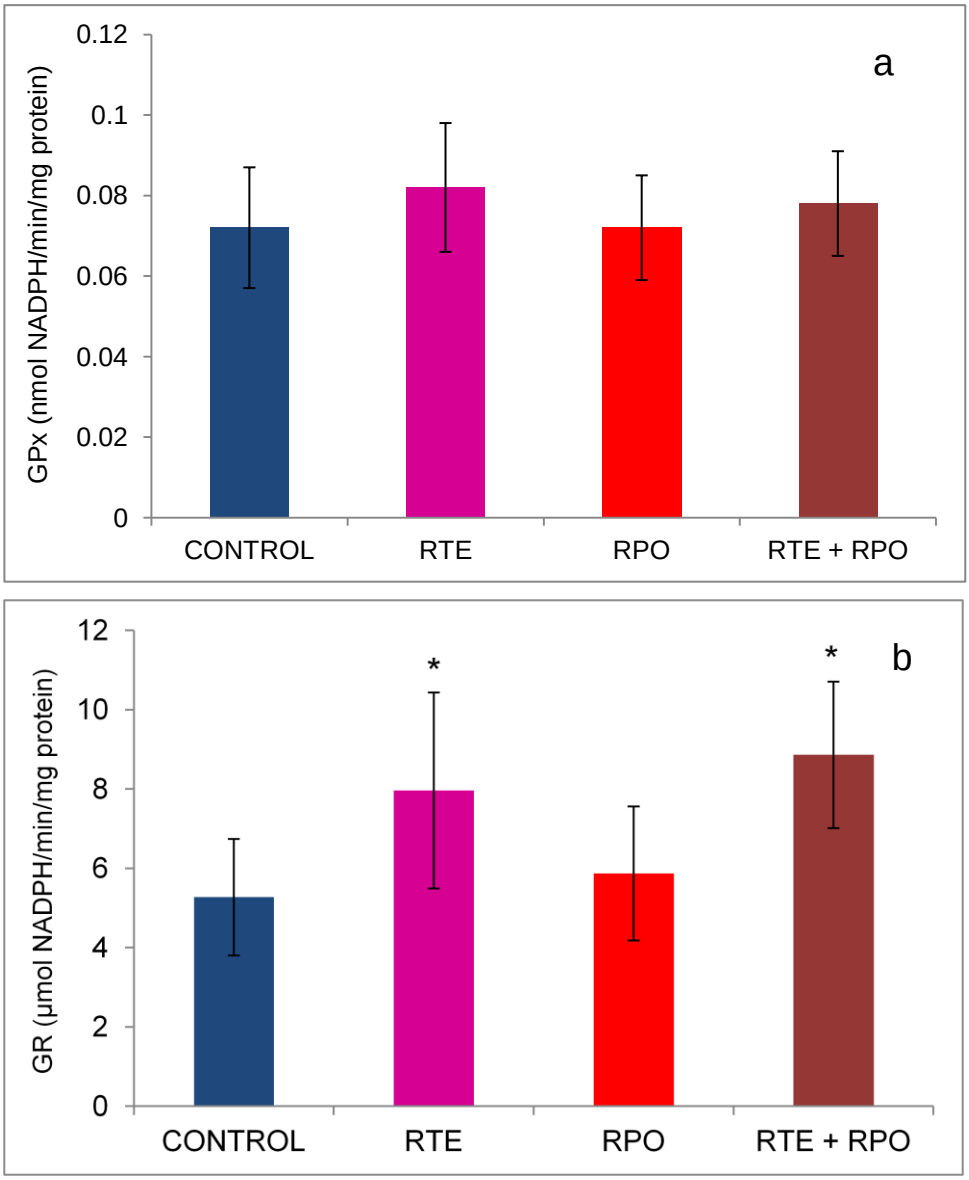

Figure 8: Effect of chronic consumption of rooibos, RPO or their combination on (a) GPx and (b) GR activity in the liver. Bars represent mean ± SD (n=7-10). *Significantly different from control at p<0.05. RTE (aqueous rooibos extract), RPO (red palm oil). GPx (glutathione peroxidase), GR (glutathione reductase).

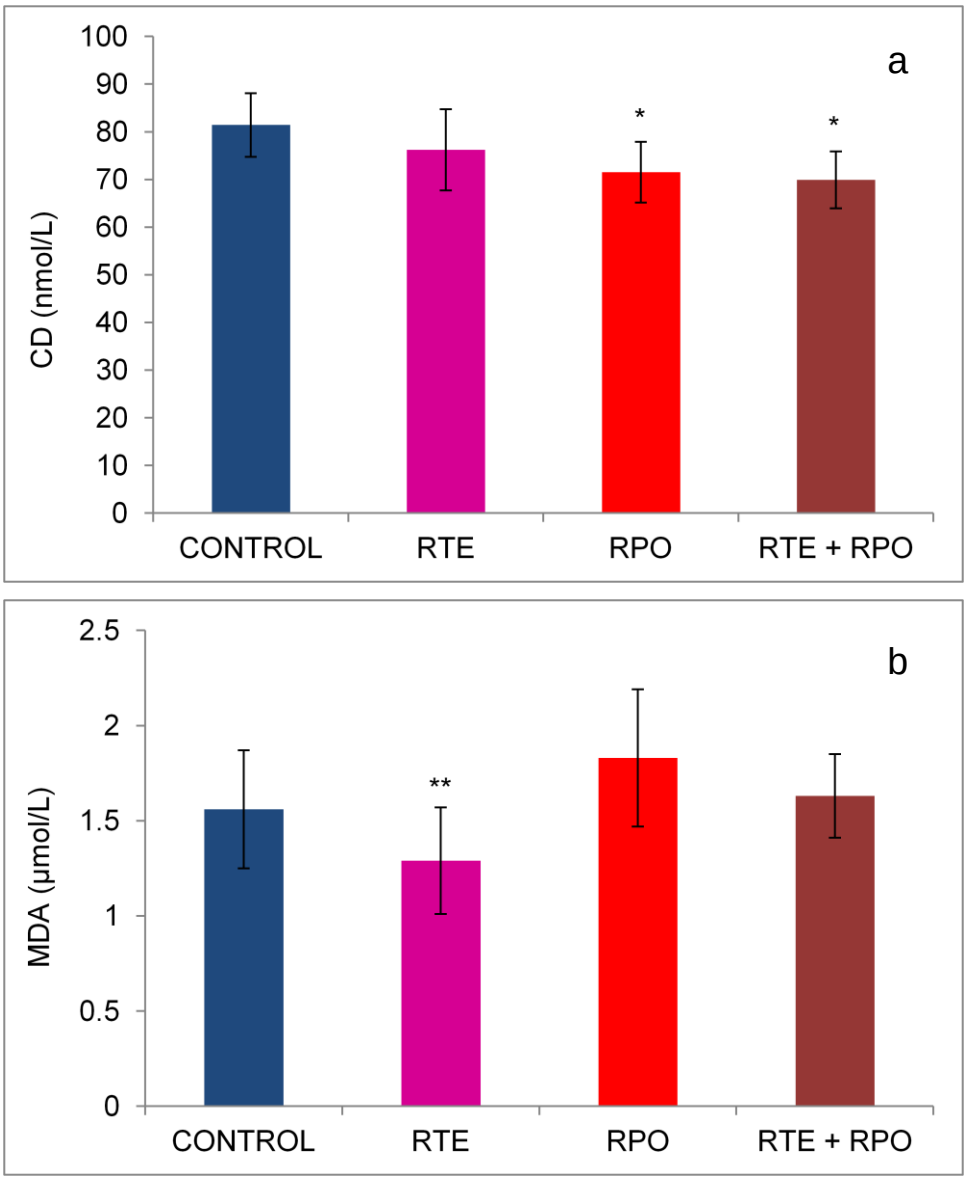

Figure 9: Effect of chronic consumption of rooibos, RPO or their combination on plasma (a) CD and (b) MDA in Wistar rats. Bars represent mean ± SD (n=7-10). *Significantly different from control group at P<0.05. **Marginally different from control group at P<0.1. CD (conjugated dienes), MDA (malondialdehyde), RTE (aqueous rooibos extract), RPO (red palm oil).

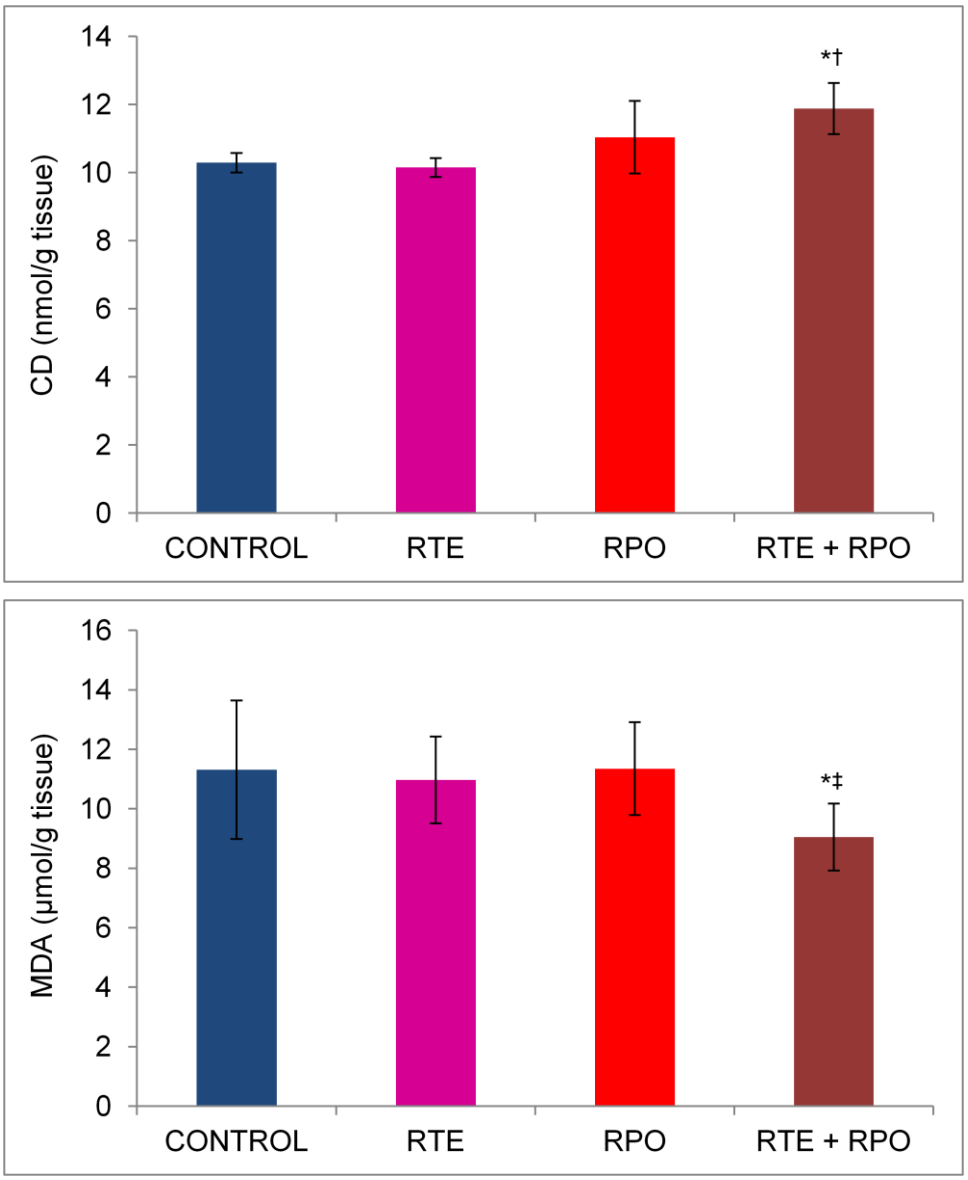

Figure 10: Effect of chronic consumption of rooibos, RPO or their combination on hepatic (a) CD and (b) MDA in Wistar rats. Bars represent mean ± SD (n=7-10). *Significantly different from control group at P<0.05). †Significantly different from RTE group at P<0.05. ‡Significantly different from RTE and RPO group at P<0.05. CD (conjugated dienes), MDA (malondialdehyde), RTE (aqueous rooibos extract), RPO (red palm oil).

3.8 Whole blood and hepatic glutathione status

Table 7 shows the glutathione status across all experimental groups. In the blood, consumption of the aqueous rooibos extract alone, or together with RPO for 22 weeks, significantly ($P<0.05$) increased the level of reduced glutathione (GSH) when compared to the control. Rats that were fed RPO alone for 22 weeks had GSH values that were comparable to those of the control. Oxidized glutathione (GSSG) levels were elevated significantly ($P<0.05$) as a result of chronic feeding of rooibos extract or RPO. When the rooibos extract and RPO were consumed together, the observed increase in GSSG was reduced to a level that was comparable to that of the control rats. The GSH/GSSG ratio of rats consuming the rooibos extract or RPO was similar to those of the control rats. However, when the rooibos extract and RPO were supplemented together for 22 weeks, there was a significant ($P<0.05$) increase in the GSH/GSSG ratio when compared to control.

Hepatic GSH was significantly ($P<0.05$) decreased by chronic feeding of RPO compared to control. The levels of GSH remain unchanged when aqueous rooibos extract was consumed either alone, or in combination with RPO. Chronic feeding of aqueous rooibos extract alone or together with RPO significantly ($P<0.05$) decreased the liver GSSG levels when compared to the control levels. There was a trend towards an increase in the GSH/GSSG ratio in rats consuming rooibos alone, or in combination with RPO, although not significant ($P>0.05$).

Table 7: Effects of chronic feeding of aqueous rooibos extract, RPO or their combination on glutathione status in whole blood and liver of all experimental male Wistar rats

	Whole blood			Liver		
Treatment	**GSH (µmol/L)**	**GSSG (µmol/L)**	**GSH:GSSG ratio**	**GSH (µmol/g wet liver)**	**GSSG (µmol/g wet liver)**	**GSH:GSSG ratio**
Control	728.76 ± 85.47	269.59 ± 17.49	2.71 ± 0.29	9.19 ± 0.76	0.35 ± 0.08	27.79 ± 6.30
RTE	893.67 ± 86.33*	288.30 ± 12.97*	3.11 ± 0.37	8.42 ± 1.12	0.25 ± 0.09*	32.39 ± 13.84
RPO	770.13 ± 95.06	294.33 ± 13.03*	2.70 ± 0.38	7.23 ± 1.11*	0.30 ± 0.05	24.75 ± 6.53
RTE + RPO	915.28 ± 90.98*	283.20 ± 15.85	3.75 ± 0.71*	8.53 ± 1.23	0.24 ± 0.06*	34.12 ± 5.68§

Values are mean ± SD of 8-10 rats per group. GSH (reduced glutathione), GSSG (oxidized glutathione). *Significantly different from control group ($P<0.05$). §Significantly different from RPO group ($P<0.05$). GSH (reduced glutathione), GSSG (oxidized glutathione). RTE (aqueous rooibos extract), RPO (red palm oil).

4. Discussion

The sensitive balance between the pro-oxidant and antioxidant systems in the body is of utmost importance when determining the state of health, well-being and survival of organisms. A loss in the functional activity of the antioxidant systems, as a result of overproduction of reactive free radicals or depletion of the antioxidant molecules, might result in the disruption of the pro-oxidant to antioxidant ratio, creating a state of oxidative stress, which has been implicated as either a cause or consequence of many pathologies (Dalle-Donne *et al*., 2006; Fisher-Wellman and Bloomer, 2009). Reactive oxygen species (ROS) are generated as by-products of several intracellular pathways during normal metabolism, however their generation may be increased during infection, inflammation and exposure to environmental pollutants (Oto *et al*., 2011). In healthy organisms, a wide range of endogenous antioxidants (including cellular antioxidant enzymes, such as CAT, SOD, GPx and GR, as well as reduced glutathione) exist to counterbalance the generation of ROS and peroxides generated intracellularly, as well as in detoxification of xenobiotics of exogenous origin (Halliwell and Gutteridge, 2007; Valko *et al*., 2007). The fact that oxidative stress has been implicated in the pathophysiology of most disease states suggest that augmenting the cellular antioxidant defense system may be a promising and pragmatic approach to prevent or slow down the progression of disease states in which oxidative stress have been implicated.

Epidemiological findings and experimental data from animal and human studies have shown that diets rich in plant-derived foods, containing high levels of natural antioxidants, may contribute to reduced mortality from diseases (Alia *et al*., 2003; Lopez-Lazaro, 2009), thus attention is now centred on dietary phytochemicals not only as an effective intervention in disease onset and progression, but also as an intervention for sustaining and promoting overall health. Fruits, vegetables, herbal teas and spices are rich sources of dietary phytochemicals, which individually or in combination, may benefit health (Yahia, 2010). The health benefits shown by fruits, vegetables, herbal teas and spices have been attributed to the presence of essential dietary micronutrients, fibres, antioxidant vitamins, trace elements, and polyphenolic compounds, mostly flavonoids (Yahia, 2010).

In this study, the effects of a sustained feeding of two antioxidant-rich natural plant products on the endogenous antioxidant system in male Wistar rats were investigated. Specifically, the activities of antioxidant enzymes and the glutathione redox status were measured in the liver and blood of these rats. Prior to the feeding experiment, the phytochemical composition as well as the *in vitro* antioxidant capacity of the aqueous rooibos extract and red palm oil

were quantified in order to determine whether the phenolic and antioxidant content of the plant extracts played an important role in their observed health effects. In accordance with previously published studies, HPLC quantification of the rooibos extract showed that aspalathin is the major flavonoids present in rooibos, along with others such as iso-orientin, orientin, vitexin, iso-vitexin and hyperoside/rutin (Rabe *et al*., 1994; Bramati *et al*., 2002; Shimamura *et al*., 2006). Different isoforms of vitamin E, α- and β-carotene were also quantified in the RPO used in this study.

Literature on whether consumption of a polyphenol-rich diet will lead to an increase in plasma total polyphenol levels and plasma total antioxidant capacity (TAC) has been inconclusive, with some studies reporting an increase (Leontowicz *et al*., 2003; Gorinstein *et al*., 2006), while others have reported no increase (Alia *et al*., 2003; Kim *et al*., 2008; Garcia-Solis *et al*., 2008). The assays for antioxidant activity have been suggested to lack specificity (Halliwell, 2009), and that no single measurement of TAC is sufficient, but rather the use of a battery of assay methods. Thus, for the current study, it was decided to measure the plasma and hepatic antioxidant capacity using three well known TAC (ORAC, FRAP and TEAC) assays. Our results showed that chronic consumption of aqueous rooibos extract or RPO for 22 weeks did not increase the plasma total polyphenol or plasma and liver TAC measured as ORAC, FRAP or TEAC. However, when the rooibos extract and RPO were fed together, the combination resulted in a significant increase in plasma total polyphenol and plasma TAC measured only as TEAC. Some studies in humans have shown that the plasma total polyphenol content parallels the plasma TAC after the intake of polyphenol-rich diets (Cao *et al*., 1998; Serafini *et al*., 2003; Henning *et al*., 2004; Torabian *et al*., 2009; Wang *et al*., 2012), suggesting that phenolic compounds are the major contributors to the plasma TAC, a fact that we also suspected may be true in animals. The TEAC assay is based on the inhibition of the absorbance of the ABTS radical cation ($ABTS^{\bullet+}$) by antioxidants. The $ABTS^{\bullet+}$ radical can be solubilised in both aqueous and organic media, allowing the assay to measure lipophilic antioxidants like carotenes and tocopherols (Karadag *et al*., 2009) that are found in RPO. Based on this, we can assume that the TEAC assay is more sensitive and more applicable to our study, since we supplemented with plant extracts that are rich in both hydrophilic and lipophilic antioxidants. The fact that the combined supplementation resulted in an increase in plasma total polyphenol levels and plasma TAC measured as TEAC, showed that there is a positive interaction in the ability of both extracts to increase the plasma polyphenol and TAC. This interaction we propose to be additive rather than synergistic based on the magnitude of the increase.

Results from this study showed that chronic supplementation of the aqueous rooibos extract, RPO or their combination for 22 weeks did not have any deleterious effects on the growth of the rats, as body weight gains remained similar across all treatment groups. However, all the rats in the treatment groups maintained lower absolute- and relative liver weights compared to the control rats, and no clear reason could be offered to explain this. Furthermore, no mortality was recorded throughout the 22 weeks of supplementation and fluid intakes in all the supplementation groups were similar to those of the control group. A widely used measure of safety of medicinal and plant extracts in literature is to determine their effect on serum levels of liver and kidney function markers. Increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) are used as surrogate markers for hepatic injury. In this study, we observed that chronic feeding of aqueous rooibos extract, RPO or their combination for 22 weeks resulted in no significant changes in the serum levels of ALP, ALT, AST and LDH and that it was similar to those of the control animals. The same trend was also observed in the serum levels of kidney function markers (albumin, creatinine, blood urea nitrogen, and uric acid), that were also similar to those of the control group consuming water. Therefore our result showed that chronic feeding of rooibos, RPO or their combination for 22 weeks did not exhibit adverse hepatic or renal effects. The result of the effect of rooibos, RPO or their combination on liver functions was further corroborated by the results of the histopathological examination of the liver tissues, which showed that liver tissues from the supplementation groups exhibited normal histoarchitecture similar to those of the control. This observation from our study becomes all the more important, because a recent case study of a possible adverse hepatic effect of rooibos was reported (Sinisalo *et al*., 2010), although this was in a patient diagnosed with a low grade B-cell malignancy, Waldenstrom's macroglobulinemia, six years earlier. Previous studies have reported on the safety of rooibos (Ulicna *et al*., 2003; 2006; 2008; Marnewick *et al*., 2011), a fact that was confirmed by the current study.

Increased ROS generation during oxidative stress may result in lipid peroxidation, which causes considerable alteration in the functions and structural organization of the cell membrane. Lipid peroxidation is a well-known mechanism of toxicity exhibited by many xenobiotics (Jaeschke *et al*., 2002; Akhgari *et al*., 2003; Awodele and Alade, 2012). It is expected that antioxidant supplementation will augment the cell's endogenous antioxidant defence system, and therefore inhibit lipid peroxidation. Results from this study showed that in the plasma, chronic feeding of RPO alone or together with the aqueous rooibos extract, significantly lowered the plasma level of CDs. When considering the plasma malondialdehyde levels, chronic rooibos extract supplementation alone significantly lowered the MDA levels, while other treatment regimens were ineffective. In the liver, CD levels were

unaffected by either chronic supplementation of rooibos extract or RPO. However, chronic feeding of rooibos extract and RPO combined actually increased the hepatic CD levels. It has been reported that dietary antioxidants such as carotenoids and flavonoids (found in RPO and rooibos) may act as pro-oxidants (at certain levels) and induce an oxidative stress microenvironment under certain conditions (Halliwell, 2008; Pamplona and Costantini, 2010). The pro-oxidant effect is said to be beneficial, since imposing a mild oxidative stress, might induced an upregulation of the antioxidant defence system and xenobiotic metabolising enzymes, resulting in overall cytoprotection (Fahey and Kensler, 2007; Halliwell, 2009; Tang and Halliwell, 2010). Hepatic MDA did not respond to chronic feeding of either aqueous rooibos extract or RPO, but the combined feeding actually lowered the MDA levels, pointing to an additive or synergistic effect of the two extracts.

Determination of the activity of antioxidant enzymes as a marker of protective role of chronic feeding of the rooibos extract, RPO or their combination, did not show any major effect in our study. In the erythrocytes, we observed a significant increase only in the activity of CAT as a result of combined feeding of the rooibos extract and RPO, while the other three enzymes (SOD, GPx and GR) remained unchanged by any of the feeding protocols. Hepatic CAT and GR activities were increased by chronic feeding of the rooibos extract alone or in combination with RPO. Other feeding regimens did not elicit any effect on any of the antioxidant enzymes. Evidence from literature has shown that the effect of the consumption of polyphenol- and other antioxidant-rich diets on the activity of antioxidant enzymes remain contradictory. Some authors have reported no increase after consumption of a polyphenol-rich diet containing red wine, green tea, as well as fruits and vegetables in humans (Van der Gaag *et al*., 2000; Van der Berg *et al*., 2001; Young *et al*., 2002), while others have reported an increase in erythrocyte GPx and GR after consumption of a grape skin extract diet for one week (Young *et al*., 2000). Rat feeding experiments revealed that green tea leaves caused an increase in SOD and CAT activity (Lin *et al*., 1998), thyme oil and thymol prevented age-induced decline in GPx and SOD in ageing rat brain (Youdim and Deans, 2000), lycopene fed to young female rats increased erythrocyte activity of SOD, GPx and GR (Breinholt *et al*., 2000), while gavage of natural flavonoids to female rats decreased the activity of CAT, GPx and GR (Breinholt *et al*., 1999). These studies show that the responses of the antioxidant enzymes do not follow set patterns, but could be diet-, tissue- and species-specific (Crawford *et al*., 2000).

Reduced glutathione (GSH) is the major non-protein thiol in living organisms and plays an important role in coordinating cellular antioxidant defence (Ilaiyaraja and Khanum, 2011). Results from the current study showed that chronic feeding of rooibos alone or in

combination with RPO for 22 weeks actually increased the GSH level in the blood, while feeding RPO alone did not show any significant effect. The increased GSH level may be ascribed to the ability of flavonoids in rooibos to increase GSH synthesis by up-regulating the mRNA expression of γ-glutamylcysteine synthetase (γ-GCS), the rate limiting enzyme in the GSH biosynthetic pathway, since previous studies have shown that polyphenolic compounds from plants increased the γ-GCS activity and GSH contents (Jeon *et al*., 2003; Chen *et al*., 2004; Moskaug *et al*., 2005) and the rooibos extract has a significantly higher total polyphenol content when compared to the RPO in the current study. The oxidised glutathione levels were increased in the blood by the chronic feeding of the rooibos extract and RPO, while combined feeding of both extracts, did not cause any increase and levels were similar to that of the control animals. Consequently, the combined feeding of the extracts also significantly increased the GSH/GSSG ratio, pointing to a positive interaction between the rooibos extract and RPO that might be additive or synergistic. Hepatic GSH levels were significantly reduced by chronic feeding of RPO alone for 22 weeks, while the rooibos extract, when fed alone or together with RPO, maintained a similar GSH and GSSG level as the control. Consequently, the GSH/GSSH ratio in the liver remained unchanged across all treatment groups compared with control.

In conclusion, the present results suggest that chronic feeding of RPO alone to rats for 22 weeks, while not inducing any hepatic or renal toxic effect does not influence the antioxidant/oxidant balance (redox status) in the blood and liver of the rats significantly, although several studies have reported on the antioxidant and oxidative stress modulating effect of RPO (Narang *et al*., 2005; Eriyamremu *et al*., 2008; Budin *et al*., 2009; 2011). However it should be noted that all these studies were conducted in animals that were either pre- or post-exposed to an oxidative stress inducer. Our results also show that chronic feeding of rooibos extract alone for 22 weeks did not induce any adverse hepatic or renal effect, while consumption of the fermented rooibos extract alone was able to modulate the antioxidant/oxidant balance by reducing MDA formation in the plasma and increasing CAT, GR activity and GSH levels in the liver. The observed health effect shown by many medicinal plants have been attributed to the additive or synergistic interaction of the various phytochemicals they contained, rather than to one single phytochemical (De Kok *et al*., 2008). Evidence also exists that polyphenols and antioxidant vitamins may result in the synergistic modulation of lipid peroxidation, oxidative stress, and co-oxidation of dietary antioxidants (Gorelik *et al*., 2005). The results from our study show that apart from modulating the antioxidant/oxidant balance, chronic feeding of aqueous rooibos extract and RPO together for 22 weeks exhibited a tendency towards a positive interaction that might be additive or synergistic, when considering the increased plasma total polyphenol content and

TAC, inhibition of hepatic MDA and improvement of the GSH redox status. It is pertinent to say that since this study was conducted in apparently healthy rats, future studies should explore supplementation of these extracts in rats under extensive pathology or those that are pre- or post exposed to an oxidative stress inducer to fully understand the mechanisms behind this observed protective effects.

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CHAPTER 4

Protective effects of rooibos (*Aspalathus linearis)* and/or red palm oil (*Elaeis guineensis*) supplementation on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats

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Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats

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Abstract

The possible protective effects of an aqueous rooibos extract (*Aspalathus linearis*), red palm oil (RPO) (*Elaeis guineensis*) and/or their combination on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatotoxicity in Wistar rats was investigated. Male Wistar rats, (n=10/group) were randomly divided into eight groups receiving either standard rat pellets (SRP) or SRP and RPO (7 g/kg diet) and having access to either water or an aqueous rooibos extract (2%, w/v) as the sole source of drinking fluid for 8 weeks. During the last two weeks, oxidative stress was induced by daily *t*-BHP (30 µmol/100 g body weight, i.p.) or vehicle control injections. *Tert*-butyl hydroperoxide caused a significant ($P<0.05$) elevation in conjugated dienes (CD) and malondialdehyde (MDA) levels (markers of lipid peroxidation) paralleled with significant ($P<0.05$) decreases in glutathione (GSH) and GSH:GSSG redox status, and inducing varying changes in activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) in the blood and liver. This apparent oxidative injury was associated with evident gross histopathological changes in liver architecture and elevated levels of serum marker enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Supplementation with rooibos, RPO and/or their combination, significantly ($P<0.05$) decreased CD and MDA levels in the liver and were effective in reducing the serum level of ALT, AST and LDH. Likewise, the changes observed in the activities of CAT, SOD, GPx and GR as well as impairment in redox status in the erythrocytes and liver were all reversed by supplementation with rooibos, RPO and/or their combination. Though we observed protective effects when rooibos and RPO were supplemented concomitantly, the observed effects were neither additive nor synergistic. Data from this study suggested that rooibos and RPO either

supplemented alone or combined, are capable of alleviating *t*-BHP-induced oxidative hepatotoxicity and that the mechanism of this protection may involve inhibition of lipid peroxidation and modulation of antioxidants and the redox status.

Key words: Rooibos, red palm oil, tert-butyl hydroperoxide, oxidative hepatotoxicity

1. Introduction

The liver is a target organ for toxic substances because the hepatocytes that make up the majority of the liver structure are very active in the metabolism of xenobiotics. During detoxification of xenobiotics, reactive oxygen and nitrogen species (RONS) are generated which can result in oxidative or nitrosative stress. Both ROS and RNS are products of normal cellular metabolism and they may be deleterious or beneficial species. At low/moderate concentrations, ROS/RNS are involved in physiological roles including cell signalling, defence against infectious agents and induction of mitogenic responses (Droge, 2002; Valko *et al*., 2007). However, overproduction of ROS arising from mitochondrial electron transport chain or excessive stimulation of NADPH result in oxidative stress, a deleterious process that can lead to damage to important cell structures, including lipids and membranes, proteins and DNA (Valko *et al*., 2007; Franco and Panayiotidis, 2009). *Tert*-butyl hydroperoxide (*t*-BHP) is a well known oxidant that has been used as a model to investigate mechanisms of cellular damage caused by oxidative stress (Rush *et al*., 1985; Kim *et al*., 2007; Hwang *et al*., 2011; Yang *et al*., 2012). It can be metabolized to peroxyl and alkoxyl radicals by cytochrome P-450 in the hepatocytes, which in turn can initiate lipid peroxidation, producing loss of membrane fluidity and mediating DNA damage (Martin *et al*., 2001; Hwang *et al*., 2011), which are known phenomena of oxidative stress in cells and/or tissues. Oxidative stress has been associated with cellular injury seen in many pathological conditions. In humans, oxidative stress is involved in many disease conditions, such as neurodegenerative disorders including Parkinson's and Alzheimer's disease, cardiovascular diseases, diabetes and cancers (Valko *et al*., 2007; Franco and Panayiotidis, 2009; Ziech *et al*., 2010). Also evidence suggests that RONS are involved in the normal aging process as well as in age-related diseases (Finkel, 2003).

Under normal circumstances several endogenous protective mechanisms have evolved in mammalian cells to limit free radicals and the damage caused by them. There are several antioxidant defense systems, including the action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as well as non-enzymatic molecules, including reduced glutathione (GSH), ceruloplasmin and transferin

(Fridovich, 1998; Halliwell and Gutteridge, 2007). However, since this endogenous protection may not be sufficient when the formation of free radicals is excessive, especially during chronic disease conditions, additional protective mechanisms via dietary antioxidants, are of great importance. The role of natural antioxidants, especially those derived from plants in modifying various health challenges is gaining a lot of attention with scientific evidence showing that vegetables, fruits and teas have protective effects on and promote health (Joshipura *et al.,* 1999, Cox *et al.,* 2000; Asif, 2011; Das *et al*., 2012; Habauzit and Morand, 2012). The main factor that is probably responsible for these protective effects by fruits, vegetables and teas, is the high content of polyphenolic antioxidants which they contain (Cao *et al.,* 1996, Dreosti, 1996).

Rooibos (*Aspalathus linearis*) (Brum f) Dahlg. (Family Fabaceae; Tribe Crotolarieae) and red palm oil (RPO), from the fruit of the oil palm tree (*Elaeis guineensis*) Jacq. (Family Arecaceae), are two plant extracts exhibiting high antioxidant capacities. Rooibos herbal tea is made from the leaves and stems of the rooibos (*Aspalathus linearis*) plant, a shrubby legume that is indigenous to the Cedarberg Mountains around Clamwilliam and its surrounding area, north of Cape Town in the Western Cape Province of South Africa. Its popularity as a health/functional beverage is increasing worldwide, partly because it is caffeine free (Cheney and Scholtz, 1963), low in tannin content when compared to *Camellia sinensis* teas (Joubert *et al*., 2008) and also because it is high in antioxidant and bioactive phytochemicals (Marnewick, 2009). Polyphenolic constituents identified in rooibos include aspalathin (major polyphenol and unique only to rooibos), nothofagin, quercetin, rutin, isoquercitrin, orientin, luteolin, vitexin and crysoeriol (Rabe *et al.,* 1994; Bramati *et al.,* 2002). The antioxidant properties of rooibos have been confirmed both *in vitro* and *in vivo* (Marnewick *et al*., 2003; Joubert *et al*., 2004, Nikolova *et al*., 2007; Marnewick *et al*., 2011). Rooibos has been shown to be antimutagenic (Marnewick *et al*., 2000; 2004), cancer modulating (Marnewick *et al*., 2005; 2009; Sissing *et al*., 2011), anti-inflammatory (Baba *et al*., 2009), antidiabetic (Kawano *et al*., 2009), cardio-protective (Pantsi *et al*., 2011) as well as modulating oxidative stress (Nikolova *et al*., 2007; Marnewick *et al*., 2011; Awoniyi *et al*., 2012).

Red palm oil is a lipid extract from the fleshy orange-red mesocarp of the fruits of the oil palm tree. It is unique in that it contain an equal amount of saturated and unsaturated fatty acids, with about 44% palmitic acid, 5% stearic acid (both saturated), 40% oleic acid (monounsaturated), 10% linoleic acid and 0.4% α-linoleic acid (both polyunsaturated), with natural fat soluble tocopherol, tocotrienol and carotenoids which may act as antioxidants (Sambanthamurthi *et al.,* 2000, Edem, 2002). Apart from the fat soluble antioxidants found in

palm oil, studies have shown that RPO also contains several phenolic compounds, including gallic, chlorogenic, gentisic, coumaric and caffeic acids, as well as catechins, hesperidin, narirutin, and 4- hydroxyl benzoate, all of which have appreciable radical scavenging and antioxidant ability (Tan *et al.,* 2001; Loganathan *et al*., 2010; Atawodi *et al*., 2011). The health benefits of RPO have been highlighted in feeding experiments using different animal models. Red palm oil positively modulates the serum lipid profile when fed to experimental rats (Oluba *et al*., 2008; Budin *et al*., 2009). Researchers have also reported on the protective effect of RPO in reducing oxidative stress (Oguntibeju *et al*., 2010) and being associated with better recovery and protection of hearts subjected to ischaemia/reperfusion injury (Esterhuyse *et al.,* 2006; Van Rooyen *et al.,* 2008; Engelbrecht *et al.,* 2009).

Though, several studies have investigated the health potential of rooibos and red palm oil individually, to the best of our knowledge, there has been no report of a comparative study on these two herbal extracts. It is against this background that we tested the hypothesis that rooibos in a commonly used concentration as consumed by humans and red palm oil would have a synergistically positive effect on biomarkers of oxidative stress and ameliorate hepatotoxicity induced by *t*-BHP in male Wistar rats.

2. Materials and Methods

2.1 Chemicals

The chemicals L-ascorbic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulfonate (ABTS), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), fluorescein sodium salt, formaldehyde, Folin Ciocalteu's phenol reagent, gallic acid, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), hesperidin, histological grade formaldehyde, 6-hydroxydopamine, mangiferin, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-thiobarbituric acid (TBA) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), iron chloride hexahydrate ($FeCl_3$.6H2O), potassium persulfate and tert-butyl hydroperoxide were obtained from Sigma–Aldrich (Johannesburg, South Africa). Diethylenetriaminepentaacetic acid (DETAPAC), 4-(dimethylamino)-cinnamaldehyde (DMACA) and malondialdehyde bis (diethyl acetal) (MDA), glacial acetic acid, trifluoroacetic acid, sulfuric acid (H_2SO_4), hexane, methanol (MeOH), ethanol (EtOH), dichloromethane, tetrahydrofuran, acetone, and hydrochloric acid (HCl) were purchased from Merck (Johannesburg, South Africa). All other reagents used were of analytical grade.

2.2 Plant materials and rooibos herbal tea preparations

Fermented rooibos (superior grade) herbal tea was a generous gift from Rooibos Limited (Mr Arend Redelinghuys, Clanwilliam, South Africa). Rooibos herbal tea was prepared at a concentration customarily used for tea making purposes (Marnewick *et al*., 2003). An aqueous extract of rooibos (RTE) was prepared by the addition of freshly boiled tap water to tea leaves at a concentration of 2g/100 mL. The mixture was allowed to stand at room temperature for 30 minutes with constant stirring, filtered and dispensed into water bottles. The aqueous rooibos extract was fed to rats *ad libitum* and fresh tea was prepared every second day. The RPO used in this study (Carotino^{TM} baking fat) was supplied by Carotino SDN BHD (company number: 69046-T), Johar-Bahru, Malaysia. The rats were fed 200 µL (equivalent to 7g/kg diet) of the Carotino baking fat orally every day.

2.3 Animal treatment and experimental design

Eighty, pathogen-free, male Wistar rats (240 ± 23 g) and standard rat pellets were obtained from the Primate Unit of Stellenbosch University (Tygerberg Campus, South Africa). The animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Medical Research Council and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health Publication no. 80-23, revised 1978). The protocol for the study was approved by CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee (Ethics Certificate no: CPUT/HAS-REC 2010/A003). The rats were housed individually in stainless steel wired-bottom cages fitted with polypropylene houses in an experimental animal holding facility kept at a temperature of between 22-24^{o}C, with a 12 h light dark cycle and 50% humidity. The rats were fed standard rat pellets (SRP) *ad libitum* and had free access to tap water or the rooibos herbal tea extract. After acclimatization in the experimental animal holding facility for 1 week, the 80 animals were randomized into eight groups (n=10/group) as shown in **Table 1.** Oxidative stress was induced by intraperitoneal (i.p.) injection of *t*-BHP (30 µmol/100 g body weight) daily for the last two weeks of the 8 week study (Kumar and Muralidhara, 2007). Fluid intake was monitored at an interval of 2 days for the duration of the study period. The general conditions of the rats were monitored daily throughout the study and body weights recorded weekly and at sacrifice. At the end of the experimental period, fasted (16 h) animals in all the groups were euthanized by i.p. injection of sodium pentobarbital (0.15 ml/100 g bw). About 8 ml of blood was collected via the abdominal aorta and this was aliquoted into collection tubes with (EDTA) and without anticoagulant to obtain plasma and serum, respectively. Plasma/serum was separated immediately by centrifugation at 5 000 g for 5 min at 4^{o}C. The liver was excised, washed twice with ice-cold PBS (10 mM phosphate buffered saline pH 7.2) to remove residual blood, blotted to dry and weighed. A slice of the liver sample was taken

and fixed in 10% buffered formaldehyde solution for histological examination. The remaining liver tissue was immediately frozen in liquid nitrogen and stored at -80^{o}C for biochemical analyses.

Table 1: Animal treatment and experimental design

Groups	Treatments		
	RTE (2% w/v)	**RPO (7 g/kg diet)**	***t*-BHP (30 µmol/100 g body weight)**
Negative control (water)	-	-	-
Positive control (*t*-BHP)	-	-	+
RTE	+	-	-
RPO	-	+	-
RTE + RPO	+	+	-
RTE + *t*-BHP	+	-	+
RPO + *t*-BHP	-	+	+
RTE + RPO + *t*-BHP	+	+	+

t-BHP (*tert*-butyl hydroperoxide), RTE (aqueous rooibos extract), RPO (red palm oil),

2.4 Histopathological examinations

Histopathological examinations were performed at the Department of Anatomy and Histology, Stellenbosch University (South Africa). Formalin-fixed liver tissues were embedded in paraffin and cut into sections (3-5 µm thickness) and stained with haematoxylin and eosin (H & E). Examination of the stained tissue sections was done by a pathologist, who was blinded to the protocol of the study.

2.5 Preparation of soluble liver fraction

A 10% (w/v) homogenate of liver tissue was prepared in 50 mM NaH_2PO_4 containing 1 mM EDTA and 0.5% Triton-X (pH 7.5) and centrifuged at 10000 g for 10 min at 4^{o}C. The supernatant was collected and stored at -80^{o}C until used for analyses of antioxidant capacities, lipid peroxidation, activity of antioxidant enzymes and glutathione redox status. Protein content of samples (erythrocyte and liver homogenate) was determined using the BCA protein assay kit supplied by Pierce (Illinois, USA).

2.6 Soluble solids, total polyphenols, flavonol and flavanol content determination

The soluble solids content of the rooibos extract was determined gravimetrically (fifteen repetitions) after drying a 1 mL aliquot of the extract at 70^{o}C for 24 hours. The total polyphenol content of the aqueous rooibos extracts was determined using the Folin Ciocalteu's phenol reagent according to the method described by Singleton *et al*. (1999). Briefly, 125 µL of 0.2N Folin reagent and 100 µL of 7.5% Na_2CO_3 were added to 25 µL of aqueous rooibos extract in a clear 96-well plate. The mixture was allowed to stand at room temperature for 2 hr and absorbance read at 765 nm in a Multiskan Spectrum plate reader (Thermo Fisher scientific, Waltham, Mass., USA). Results were expressed as mg gallic acid equivalents/mg soluble solids. The flavanol content of the aqueous rooibos extract was determined colorimetrically at 640 nm using *p*-dimethylaminocinnamaldehyde (DMACA) according to the method of Treutter (1989) and results were expressed as mg catechin equivalents/mg soluble solids. The flavonol/flavones content was determined spectrophotometrically at 360 nm and results were expressed as mg quercetin equivalents/mg soluble solids (Mazza *et al*., 1999).

2.7 Determination of antioxidant capacity

2.7.1 Oxygen radical absorbance capacity (ORAC) assay

Sub samples of plasma and liver homogenates were first deproteinised using 0.5M perchloric acid (1:1,v/v), centrifuged at 10 000 g for 10 min and the resultant supernatant stored at -80^{o}C prior to analysis (Robles-Sanchez *et al*., 2011). The ORAC of the rooibos extract and protein free samples of plasma and liver was determined according to a fluorometric method described by Ou *et al*. (2001). The reaction mixture consisted of 12 µL of diluted protein-free sample (1:10 with 75 mM phosphate buffer, pH 7.4) and 138 µL of fluorescein (14 µM) which was used as a target for free radical attack. The reaction was initiated by the addition of 50 µL AAPH (4.8 mM) and the fluorescence (excitation 485, emission 538) recorded every 5 min for 2 hr in a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). The ORAC values were calculated using regression equation $y = ax^2 + bx + c$ between Trolox concentration (µM) and the area under the curve. Results were expressed as µM Trolox equivalents (TE)/L or µM Trolox equivalents (TE)/g tissue.

2.7.2 Trolox equivalent antioxidant capacity (TEAC) assay

The trolox equivalent antioxidant capacity of the aqueous rooibos extract was determined according to the method described by Re *et al*. (1999). The $ABTS^{\bullet+}$ solution was prepared 24 h before use by mixing ABTS salt (8 mM) with potassium peroxodisulfate (140 mM) and the solution stored in the dark until the assay could be performed. The $ABTS^{\bullet+}$ solution was diluted 1:20 with distilled water to give an absorbance of 1.50 at 734 nm. Each sample

(25 µL) was mixed with 275 µL $ABTS^+$ solution in a 96-well clear plate. The plate was read after 30 min incubation at room temperature in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Trolox was used as the standard and results were expressed as µM TE/L or µM TE/g tissue.

2.7.3 Ferric ion reducing antioxidant power (FRAP) assay

The ferric reducing ability of the rooibos extract, plasma and liver samples was determined using the method described by Benzie and Strain (1996). Briefly, 10 µL of sample was mixed with 300 µL FRAP reagent in a 96-well clear plate. The FRAP reagent was a mixture (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 100 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 min, the plate was read at a wavelength of 593 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Ascorbic acid (AA) was used as the standard and the results were expressed as µmol AAE/L or µmol AAE/g tissue.

2.8 High performance liquid chromatography analysis of aqueous rooibos extract

The rooibos tea extract was filtered (Whatman no 4) and chromatographically separated on an Agilent Technologies 1200 series HPLC system according to an adapted method described by Bramati *et al*. (2002). The HPLC system consisted of a G1315C diode array and multiple wavelengths detector, a G1311A quaternary pump, a G1329A autosampler, and a G1322A degasser. A 5 µm YMC-Pack Pro C18 (150 mm x 4.6 mm i.d.) column was used for separation and acquisition was set at 287 nm for aspalathin and 360 nm for other components. The mobile phases consisted of water (A) containing 300 µL/L trifluoroacetic acid and methanol (B) containing 300 µL/L trifluoroacetic acid. The gradient elution started at 95% A changing to 75% A after 5 min and to 20% A after 25 min and back to 95% A after 28 min. The flow rate was set at 0.8 mL/min, the injection volume was 20 µL and the column temperature was set at 23^{o}C. Peaks were identified based on the retention time of the standards and confirmed by comparison of the wavelength scan spectra (set between 210 nm and 400 nm).

2.9 High performance liquid chromatography analysis of RPO

2.9.1 Vitamin E content of RPO

Vitamin E in RPO was extracted by shaking 1 g of RPO in 5 mL of absolute ethanol for 30 min, followed by centrifugation at 3500 g for 10 min. The top vitamin E layer was analyzed on an Agilent Technology 1200 series HPLC system with the visible wavelength detector set at 296 nm. Twenty microlitre of sample was injected into the column (YMC-Pack Pro C18, 150

x 4.6 mm i.d., room temperature) and elution performed with a mobile phase consisting of A (acetonitrile:methanol:isopropanol:water; 45:45:5:5, v/v) and B (acetonitrile:methanol: isopropanol; 50:45:5, v/v) at a flow rate of 1mL/min. Mobile phase A was programmed to B within 10 min and this condition was maintained for another 15 min before returning to the original conditions. The contents of tocopherols and tocotrienols were quantified by comparing the retention time and/or peak area with standards (Iqbal *et al*., 2007).

2.9.2 Carotenoid content of RPO

Carotenoids from RPO were extracted with tetrahydrofuran:dichloromethane (1:1, v/v) and analysed on an Agilent Technology 1200 series HPLC with the visible detector set at 450 nm according to a modified method of Rautenbach *et al*. (2010). Twenty microlitre of extracted samples were injected automatically into the column (YMC-Pack Pro C30, 250 x 4.6 mm i.d., room temperature) and isocratic elution performed on a mobile phase consisting of methanol:acetone (9:1, v/v) with flow rate set at 1 mL/min. Peaks were identified based on the retention time of the α- and β-carotene standards.

2.10 Liver function tests

Serum alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) were analysed using a Medica EasyRA automated clinical chemistry analyser (Medica Corporation Bedford, Mass., USA) and standard diagnostic kits (Medica Corporation Bedford, Mass., USA).

2.11 Oxidative status biomarkers

2.11.1 Lipid peroxidation

Lipid peroxidation was assessed by measurement of conjugated dienes (CDs) and malondialdehyde (MDA). Plasma and liver MDA were determined by HPLC using a method adapted from Khoschsorur *et al*. (2000). Briefly, plasma or liver homogenates (100 µL) were mixed with ortho-phosphoric acid (0.44 M, 0.75 mL), aqueous TBA (42 mM, 0.25 mL) and water (twice distilled, 0.45 mL). The mixture was heated in a boiling water bath for 60 min. After cooling on ice, alkaline methanol (50 ml methanol + 4.5 ml 1 M NaOH) was added (1:1). The samples were centrifuged at 3 500 g for 3 min at 4^{o}C. To 1 mL supernatant, 500 µL of n-hexane was added and centrifuged at 15000 g for 40 sec and the supernatant collected. The neutralized reaction mixture (50 µL) was then chromatographed on an Agilent 1200 series HPLC. A 5 µm YMC-PackPro C18 (150 mm x 4.6 mm i.d.) column was used for separation with 60:40 (v/v) 50 mM phosphate buffer, pH 6.8-methanol as mobile phase. The flow rate was 1 mL min^{-1}. Fluorometric detection was performed with excitation at 532 nm and emission at 552 nm. The peak of the MDA-TBA adduct was calibrated with an MDA standard

processed in exactly the same way as the samples. Conjugated dienes were estimated according to the method of Recknagel and Glende (1984). Briefly, 405 µL of chloroform-methanol mixture (2:1 v/v) was added to 100 µL of plasma or liver homogenates. The mixture was vortexed for 60 s and centrifuged at 10 000 g for 10 min at 4^{o}C. The top aqueous layer was discarded and 200 µL of the bottom chloroform layer was taken in a clean eppendorf tube and dried under nitrogen gas for 10 min. Cyclohexane (1 mL) was added to the tube and vortexed for 60 s. Two hundred microlitre of the mixture was taken into a clear 96-well plate and the absorbance was read at 234 nm against a cyclohexane blank in a Multiskan Spectrum plate reader (Thermo Fisher scientific, USA). The concentration of CD was calculated using the equation 1.

$$\text{CD} = \frac{\left(A_{234s} - A_{234b}\right)}{\varepsilon} \times 10 \text{ (nmol CD/ml)}$$

Where:

A_{234s} = absorbance of sample at 234 nm

A_{234b} = absorbance of blank at 234 nm

ε = extinction coefficient = 2.95 x 10^4

Quoted ε is always based on a 1cm cuvette; since 300 µL in a plate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

Equation 1: Calculation of conjugated dienes in various samples

2.11.2 Antioxidant enzyme activity assays

2.11.2.1 Catalase

Catalase (CAT) activity in the erythrocytes and liver homogenates were determined using the method described by Aebi (1984). In a clear 96-well plate, 5 µL of sample and 170 µL of 50 mM potassium phosphate, pH 7.0 was added followed by 50 µL of 0.1% hydrogen peroxide in 50 mM potassium phosphate (pH 7.0) to initiate the reaction. The rate of decomposition of hydrogen peroxide was measured at 240 nm for 2 min in 15 s intervals in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Catalase activity (µmole H_2O_2 consumed/min/µg protein) was determined using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.

2.11.2.2 Superoxide dismutase

The activity of superoxide dismutase (SOD) was determined according to the method of Crosti *et al*. (1987). The reaction mixture in a 96 well plate consisted of 15 µL of sample, 170 µL of 0.1 mM DETAPAC in 50 mM sodium phosphate buffer (pH 7.4) and 20 µL of 1.6

mM 6- hydroxydopamine which initiated the reaction. The reaction was measured at 490 nm for 4 min at 30 s intervals and SOD activity expressed as U/mg of protein.

2.11.2.3 Glutathione peroxidase

The activity of glutathione peroxidase (GPx) was determined according to the method of Ellerby and Bredesden (2000) modified for a microplate reader. To the assay mixture containing 210 µL of assay buffer (50 mM potassium phosphate, 1 mM EDTA pH 7.0), 2.5 µL of GR (0.1 U/mL), 2.5 µL of GSH (0.1 M), 5 µL of NADPH (7.5 Mm), 2.5 µL of sodium azide (100 mM) and 5 µL of erythrocyte or liver homogenate, 25 µL of H_2O_2 (15 mM) was added. The rate of H_2O_2-dependent oxidation of NADPH was immediately monitored at 340 nm for 2 min at 30 s intervals. The activity of GPx was calculated using the extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ and results were expressed as nmol NADPH oxidized/min/µg protein.

2.11.2.4 Glutathione reductase

The activity of glutathione reductase (GR) was determined by a method of Staal *et al*. (1969) modified for a microplate reader. Briefly, to the assay mixture containing 20 µL of sample and 200 µL of assay buffer (50 mM sodium phosphate and 25 mM of EDTA, pH 8.0) were added 20 µL of 12.5 mM GSSG and 10 µL of 3 mM NADPH. The rate of oxidation of NADPH was immediately monitored at 340 nm for 3 min at 30 s intervals. The activity of GR was calculated using the extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ and results were expressed as µmol NADPH oxidized/min/µg protein.

2.11.3 Glutathione status analysis

The total glutathione (GSH and GSSG) was measured according to the method described by Asensi *et al*. (1999). Aliquot of erythrocyte without (GSH) or with 3 mM freshly prepared M2VP (GSSG) were first deproteinized by 5% (w/v) metaphosphoric acid (MPA), while liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 10 000g for 10 min, 50 µL of supernatant (from whole blood or liver homogenate) was added to 50 µL of glutathione reductase (1U) and 50 µL of 0.3mM DTNB. The reaction was initiated by addition of 1 mM NADPH to a final volume of 200 µL. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards.

2.12 Statistical analysis

Values were expressed as mean ± SEM. Data were tested for normality using the Kolmogorov–Smirnof Test and Levene's Test for Equality of variances. Differences between

group means were estimated using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for all pairwise comparisons. The Kruskal-Wallis Test, a non-parametric analogue to the one-way ANOVA was used to test for group differences when data was not normally distributed. Results were considered statistically significant at $P<0.05$ and marginally significant at $P<0.1$. All the statistics were carried out using MedCalc v 12.2.1 software (MedCalc software bvba, Mariakerke, Belgium).

3. Results

3.1 Phenolic content and antioxidant capacity of aqueous rooibos tea extract

Before the commencement of the study, the total phenolic content and *in vitro* antioxidant capacity of the rooibos extract were determined and the results are shown in **Table 2**. The total phenolic content of the aqueous rooibos extract is 0.30 ± 0.01 mg GAE/mg soluble solid of which the flavonoids accounts for 68%. **Table 3** and **Figure 1** show the HPLC quantification and daily intake of flavonoids in the rooibos extract. Aspalathin, iso-orientin and orientin were the major flavonoids consumed by the rats, with other notable flavonoids including vitexin, iso-vitexin, hyperoside/rutin and trace amount of quercetin, luteolin and chrysoeriol.

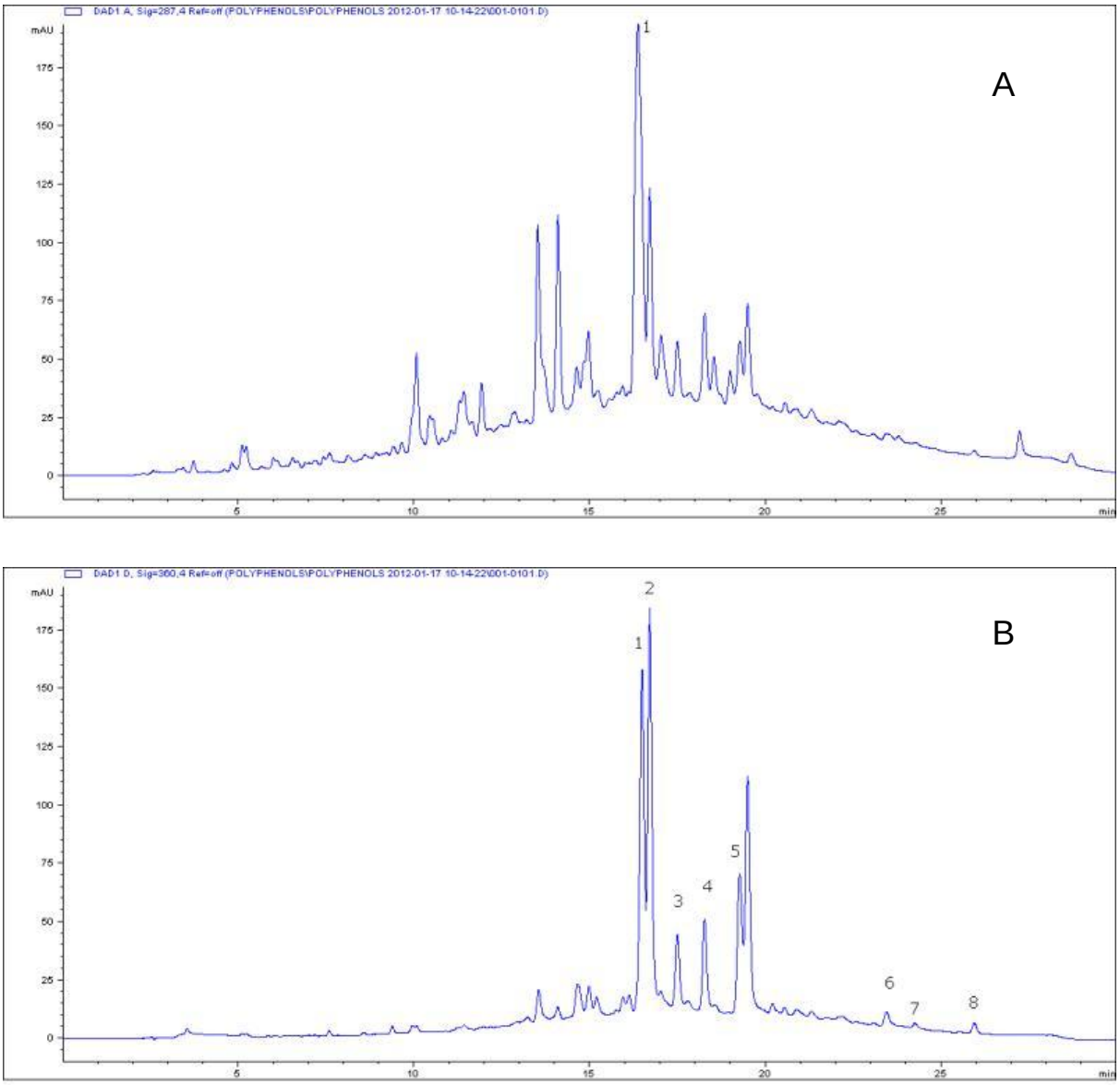

Figure 1: HPLC chromatogram of flavonoids in aqueous rooibos extract used in the study. A (287nm), 1, aspalathin; B (360 nm), 1, orientin; 2, iso-orientin; 3, vitexin; 4, isovitexin; 5, hyperoside/rutin; 6, quercetin; 7, luteolin; 8, chrysoeriol.

Table 2: Phenolic content and antioxidant capacity of aqueous rooibos (2%, w/v) extract

Soluble solids (mg/mL)	Total phenolic content (mg gallic acid equivs/mg soluble solids)	Flavonol content (mg quercetin equivs/mg soluble solids)	Flavanol content (mg cathechin equivs/mg soluble solids)	FRAP (µmol AAE/mL)	TEAC (µmol TE/mL)	ORAC (µmol TE/mL)
2.743 ± 0.26	0.303 ± 0.006	0.159 ± 0.004	0.058 ± 0.002	4.90 ± 0.35	5.22 ± 0.22	14.72 ± 1.57

Values are mean ± SEM. Soluble solids content is a mean of 15 determinations while other parameters are mean of 6 determinations. AAE (ascorbic acid equivalent), TE (trolox equivalent), FRAP (ferric reducing ability of the plasma), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity).

Table 3: HPLC quantification of flavonoids in aqueous rooibos tea extract consumed by rats

Soluble solids (mg/mL)		2.74 ± 0.26	
Phenolic compound	**Concentration (µg/mL)**	**(%) of Soluble solids**	**Daily intake (mg/100 g BW)**
Aspalathin	28.32 ± 1.65	1.03 ± 0.06	0.24 ± 0.01
Orientin	16.94 ± 1.67	0.62 ± 0.06	0.15 ± 0.01
Isoorientin	23.64 ± 2.34	0.86 ± 0.09	0.20 ± 0.02
Vitexin	6.06 ± 0.61	0.22 ± 0.02	0.05 ± 0.01
Isovitexin	6.50 ± 0.68	0.24 ± 0.02	0.06 ± 0.01
Hyperoside/Rutin	14.55 ± 1.30	0.53 ± 0.05	0.13 ± 0.01
Quercetin	0.89 ± 0.11	0.03 ± 0.003	0.01 ± 0.001
Luteolin	0.22 ± 0.03	0.01 ± 0.001	Trace amount
Chrysoeriol	0.23 ± 0.02	0.01 ± 0.001	Trace amount

Values are mean ± SEM (n=4). BW (body weight)

3.2 Fluid and phenolic intake of rats consuming the aqueous rooibos extract

The fluid and phenolic intakes per day of the experimental rats consuming the rooibos extract are presented in **Table 4**. Water intake per day was similar between the negative and positive control rats. Rooibos intake across all groups consuming rooibos was also not different ($P>0.05$), except for rats subjected to *t*-BHP injection and consuming a combination of rooibos and RPO that had a significantly lower ($P<0.05$) daily fluid intake. As a result, the total phenolic and flavonoids intakes in this group of rats, were also significantly lower ($P<0.05$) when compared to the other groups consuming rooibos.

Table 4: Fluid and Phenolic intake of rats fed aqueous rooibos tea extract for a period of 8 weeks.

Treatment	Water/rooibos intake/day/100 g BW (ml)	Total phenolic intake (mg gallic acid equivs/day/100 g BW)	Flavonol intake (mg quercetin equivs/day/100 g BW)	Flavanol intake (mg catechin equivs/day/100 g BW)
Negative control (water)	9.29 ± 0.25[a]	ND	ND	ND
Positive control (*t*-BHP)	8.53 ± 0.20[a]	ND	ND	ND
RTE	8.90 ± 0.26[a]	7.38 ± 0.21[a]	3.87 ± 0.11[a]	1.41 ± 0.04[a]
RPO	8.83 ± 0.21[a]	ND	ND	ND
RTE + RPO	8.81 ± 0.20[a]	7.31 ± 0.17[a]	3.83 ± 0.09[a]	1.39 ± 0.03[a]
RTE + *t*-BHP	8.99 ±0.32[a]	7.45 ± 0.26[a]	3.91 ± 0.1[a]	1.42 ± 0.05[a]
RPO + *t*-BHP	8.63 ± 0.33[a]	ND	ND	ND
RTE + RPO + *t*-BHP	7.72 ± 0.15[b]	6.40 ± 0.13[b]	3.36 ± 0.07[b]	1.22 ± 0.02[b]

ND not determined. Calculations of the total phenolic, flavonol and flavanol intakes were calculated based on the soluble solid intake obtained from the average rooibos consumption per day. Values are mean ± SEM (n=10). Mean followed by different superscript are significantly different at P<0.05. RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (tert-butyl hydroperoxide).

3.3 HPLC quantification of antioxidants in red palm oil and their daily intake

The different isomers of vitamin E and carotene quantified in the RPO used, as well as their average daily intakes are shown in **Table 5**. Tocotrienols accounted for 80% of the vitamin E present in the RPO used in this study. β-carotene accounted for 55% of the carotene, while α-carotene accounted for the remaining 45%.

Table 5: Daily intakes, vitamin E and carotene content of RPO

Constituent	Concentration (µg/g RPO)	Daily intake (µg)
α-Tocotrienol	102.36 ± 0.68	17.91 ± 0.12
β/γ-Tocotrienol	227.48 ± 1.22	39.81 ± 0.21
δ-Tocotrienol	56.46 ± 0.69	9.88 ± 0.12
α-Tocopherol	71.28 ± 1.03	12.47 ± 0.18
β/γ-Tocopherol	6.20 ± 0.42	1.09 ± 0.07
δ-Tocopherol	20.70 ± 0.74	3.62 ± 0.13
α-Carotene	23.74 ± 0.52	4.15 ± 0.09
β-Carotene	29.34 ± 1.30	5.14 ± 0.23

Values are mean ± SEM (n= 5).

3.4 Body and liver weight changes

During the study, rats in all the experimental groups did not show any deleterious effects and no mortality was recorded. Estimated food intakes in all the treatment groups remained unchanged. The total body weight gain, absolute liver weight and relative liver weight are shown in **Table 6**. The total body weight gain was lower in the positive control group compared to the negative control group, but the decrease was not significant ($P>0.05$). Liver weights and relative liver weights were also similar in the positive and negative control groups. Rats consuming rooibos, RPO or their combination without *t*-BHP injection, maintained their body weights, liver weights and relative liver weights comparable ($P>0.05$) to that of the negative control group, suggesting that rooibos and RPO had no adverse effects on the rats growth responses. The total body weight gain, liver weight and relative liver weight of all *t*-BHP-treated rats consuming rooibos either alone or in combination with RPO were similar ($P>0.05$) to those of the positive control. However, rats injected with *t*-BHP and consuming RPO alone had a significantly lower ($P<0.05$) relative liver weight compared with the positive control rats.

Table 6: Effects of rooibos and RPO consumption on body weight gain, liver weight, and relative liver weight in all experimental rats.

Treatment	Body weight gain (g)	Liver weight (g)	Relative liver weight (%)
Negative control (water)	150.64 ± 3.56	10.78 ± 0.30	2.96 ± 0.10
Positive control (*t*-BHP)	127.90 ± 6.48	10.90 ± 0.47	3.12 ± 0.09
RTE	150.15 ± 8.67	11.93 ± 0.47	3.14 ± 0.10
RPO	133.54 ± 5.26	10.45 ± 0.37	2.82 ± 0.07
RTE + RPO	138.25 ± 7.15	11.27 ± 0.40	2.83 ± 0.07
RTE + *t*-BHP	152.33 ± 5.54	12.47 ± 0.51	3.23 ± 0.11
RPO + *t*-BHP	134.80 ± 7.37	10.19 ± 0.32	2.65 ± 0.05[#]
RTE + RPO + *t*-BHP	138.88 ± 3.08	11.51 ± 0.34	2.81 ± 0.07

Values are mean ± SEM (n=10). [#]Significantly different vs positive control (P<0.05). RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (tert-butyl hydroperoxide).

3.5 Biochemical markers of liver function

The plasma hepatic marker enzyme levels of all treatment groups are presented in **Figures 2, 3 and 4**. Intraperitoneal injection of *t*-BHP for 2 weeks caused abnormal liver functions in treated rats. The level of plasma hepato-specific enzymes such as alanine amino transferase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH), were significantly increased (P<0.05). Tert-butyl hydroperoxide exposure brought about a 2.79, 2.70 and 2.11 fold increase in the level of ALT, AST and LDH respectively, when compared to the negative control rats. Rooibos and RPO, when supplemented individually to rats without *t*-BHP treatment, did not have any significant effect (P>0.05) on the level of ALT, AST and LDH when compared with the negative control group. Upon oxidative stress-induction with *t*-BHP, supplementation with rooibos extract significantly (P<0.05) lowered the observed increases in ALT, AST and LDH by 39, 33 and 32%, respectively, while the reduction brought about by RPO constituted 40, 50 and 47%, respectively. Rats consuming a diet supplemented with RPO and rooibos as drinking fluid (RTE + RPO group), without t-BHP treatment, showed a significant (p<0.05) increase in ALT and AST levels when compared with negative control rats drinking water. However, when rooibos and RPO were supplemented simultaneously in *t*-BHP exposed animals (RTE + RPO + *t*-BHP group), a significant decrease (p<0.05) was observed for these liver marker enzymes (ALT, AST and LDH) when compared to positive control rats.

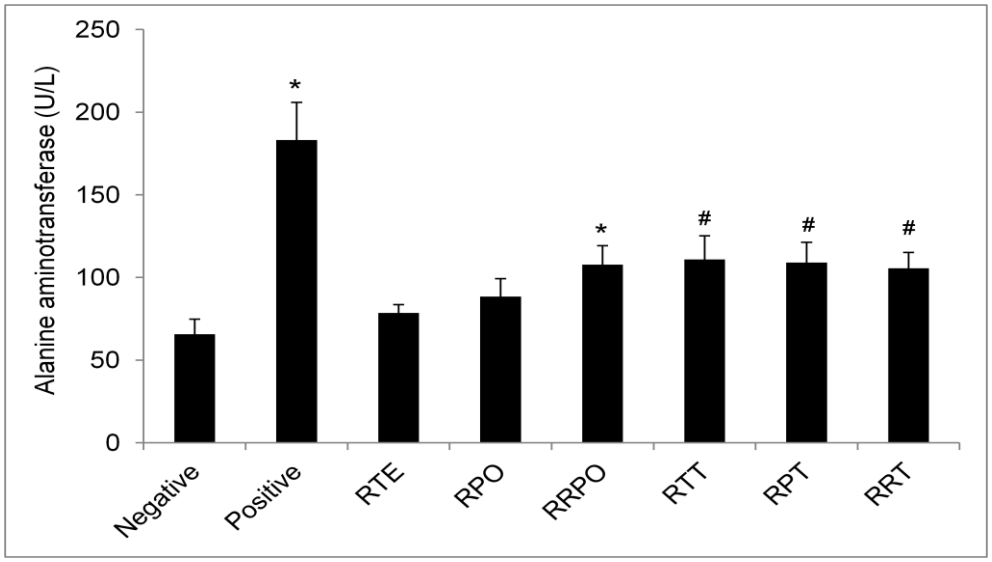

Figure 2: Effects of rooibos and RPO consumption on serum alanine aminotransferase (ALT) level in all experimental rats. Bars represent mean ± SEM of 7-10 rats. *Significantly different from negative control group (P<0.05). #Significantly different from positive control (*t*-BHP) group (P<0.05). RTE (rooibos), RPO (red palm oil), RRPO (rooibos + RPO), RTT (rooibos + *t*-BHP), RPT (red palm oil + *t*-BHP), RRT (rooibos + red palm oil + *t*-BHP).

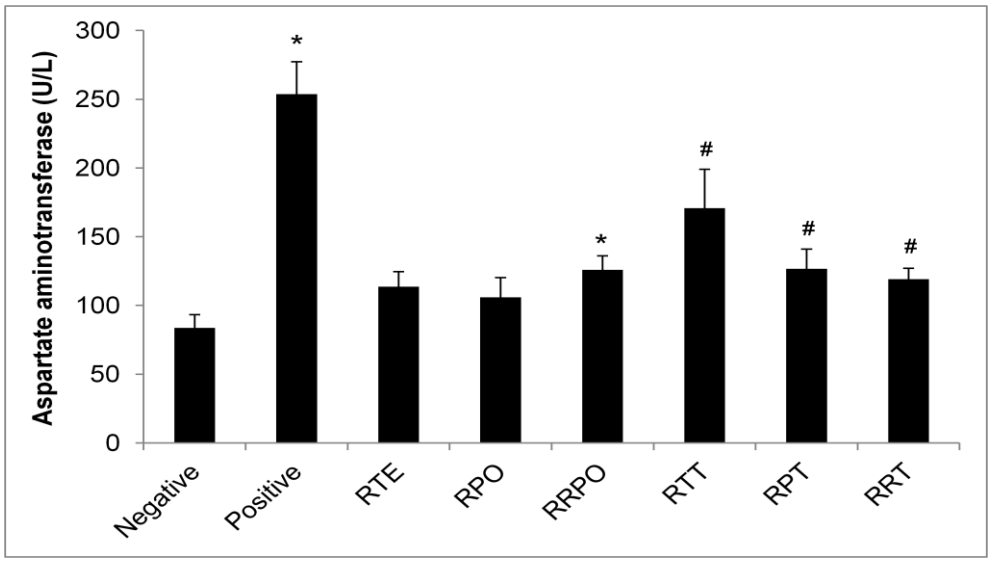

Figure 3: Effects of rooibos and RPO consumption on serum aspartate aminotransferase (AST) level in all experimental rats. Bars represent mean ± SEM of 7-10 rats. *Significantly different from negative control group (P<0.05). #Significantly different from positive control (*t*-BHP) group (P<0.05). RTE (rooibos), RPO (red palm oil), RRPO (rooibos + RPO), RTT (rooibos + *t*-BHP), RPT (red palm oil + *t*-BHP), RRT (rooibos + red palm oil + *t*-BHP).

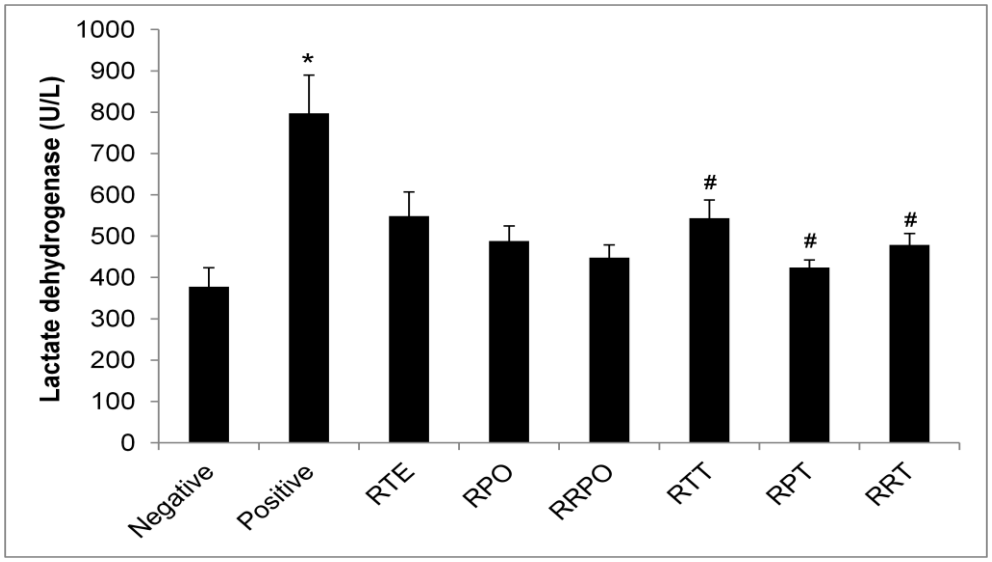

Figure 4: Effects of rooibos and RPO consumption on serum lactate dehydrogenase (LDH) level in all experimental rats. Bars represent mean ± SEM of 7-10 rats. *Significantly different from negative control group (P<0.05). #Significantly different from positive control (*t*-BHP) group (P<0.05). RTE (rooibos), RPO (red palm oil), RRPO (rooibos + RPO), RTT (rooibos + *t*-BHP), RPT (red palm oil + *t*-BHP), RRT (rooibos + red palm oil + *t*-BHP).

3.6 Histopathological observations

Figure 5 shows the liver histoarchitecture of the different experimental groups, examined by conventional light microscopy in H & E stained sections. **Figure 5A** shows the liver section of a negative control rat revealing normal architecture of hepatic cells with granulated cytoplasm and uniform nuclei. Rats consuming rooibos, RPO or their combination without *t*-BHP treatment also exhibited normal histological architecture as shown in **Figures 5 (B-D)** respectively. Treatment with *t*-BHP resulted in alteration of liver histoarchitecture, evidenced by hepatocyte degeneration, with massive lymphocyte infiltration and mononuclear cell aggregation **(Figure 5E)**. Rats consuming rooibos, RPO or their combination with *t*-BHP treatment exhibited almost normal hepatocellular architecture, with slight lymphocyte infiltration **(Figures 5E-5H).**

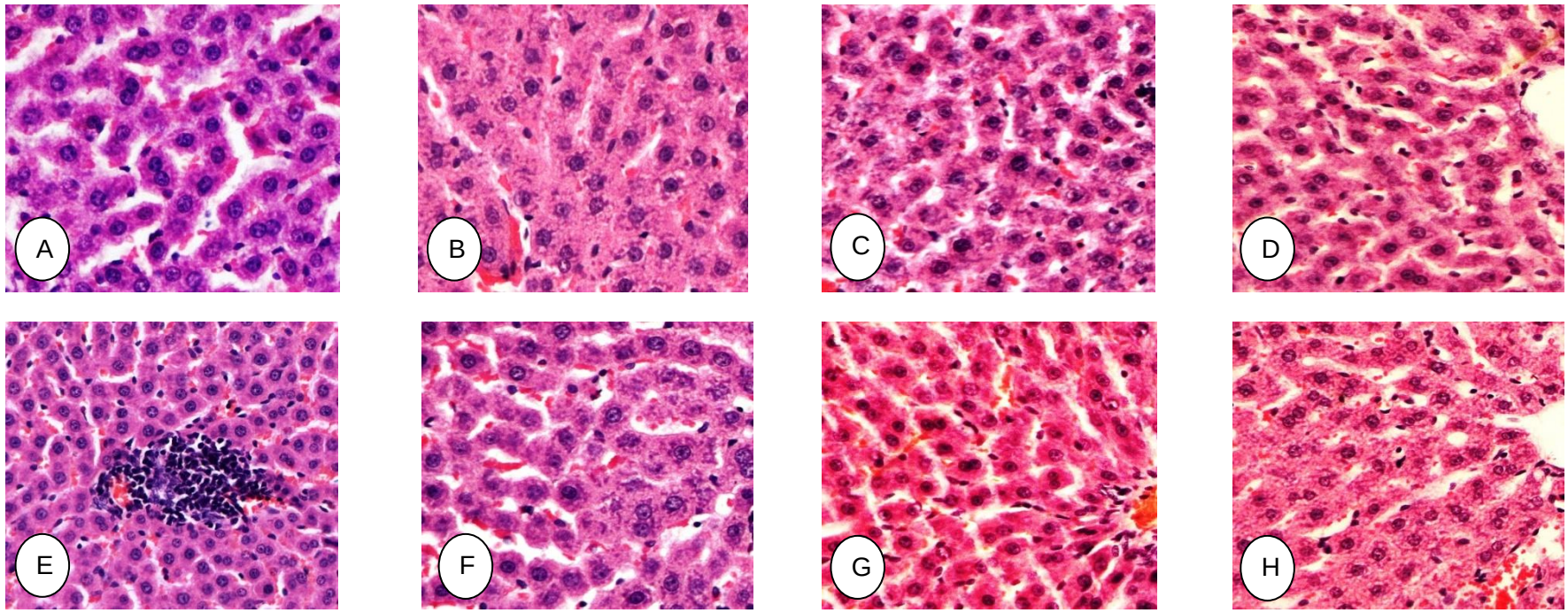

Figure 5: Histopathology of the liver showing (A-D) normal architecture with granulated cytoplasm and uniform nuclei of negative control, rooibos, RPO or their combination respectively (H&E, X20). (E) positive control (*t*-BHP treated rats) showing hepatocyte degeneration with massive lymphocyte and mononuclear cellular aggregation (H&E, X20). (F-H) rats pre-treated with rooibos, RPO or their combination before *t*-BHP treatment, showing almost normal hepatocellular architecture with slight lymphocyte infiltration (H&E, X 20).

3.7 Antioxidant capacity of plasma and liver

The antioxidant capacity of plasma and liver samples were assessed as total polyphenol content, FRAP and ORAC activities (**Table 7**). Treatment with *t*-BHP resulted in a significant ($P<0.05$) decrease in the level of plasma total polyphenols when compared to the rats consuming water (negative control). The consumption of the rooibos extract and RPO either alone or in combination did not restore these induced levels. Rats consuming the rooibos extract and RPO alone or in combination, without *t*-BHP treatment also showed a significant ($P<0.05$) decrease in their plasma levels of total polyphenols when compared to negative control rats. When considering the antioxidant capacity of the plasma, treatment with *t*-BHP caused a significant ($P<0.05$) decrease in the ORAC values, but not the FRAP values while co-treatment with rooibos alleviated this decrease and caused a significant ($P<0.05$) enhancement of the plasma ORAC, with no such effect for RPO either alone or when combined with the rooibos extract. No significant differences were shown in the FRAP activity of plasma of rats consuming the rooibos, RPO or combination (with/without *t*-BHP treatment). In the liver, *t*-BHP treatment resulted in a significant ($P<0.05$) decrease in ORAC values but not in FRAP values. Co-treatment with rooibos, RPO or their combination did not reverse the decrease. No significant differences were shown in the hepatic FRAP levels of rats consuming, rooibos, RPO or their combination, with or without *t*-BHP treatment.

Table 7: Effects of aqueous rooibos, RPO or their combination on total polyphenol content and antioxidant capacity of plasma and liver of all experimental rats.

	Plasma			Liver	
Treatment	**Total polyphenol content (mg GAE/L)**	**ORAC (µmol TE/L)**	**FRAP (µmol AAE/L)**	**ORAC (µmol TE/g tissue)**	**FRAP (µmol AAE/g tissue)**
Negative control (water)	65.27 ± 2.71	1934.32 ± 101.82	204.85 ± 31.02	15.20 ± 0.39	2.01 ± 0.06
Positive control (*t*-BHP)	51.11 ± 1.48*	1535.97 ± 50.60*	185.81 ± 11.15	11.51 ± 0.59*	1.99 ± 0.05
RTE	55.38 ± 2.05*	2082.34 ± 88.11	291.75 ± 52.82	14.29 ± 1.23	2.08 ± 0.07
RPO	45.97 ± 1.33*	1284.86 ± 42.14*	210.96 ± 21.18	13.18 ± 0.39*	2.16 ± 0.04
RTE + RPO	56.43 ± 2.60*	1437.41 ± 90.66*	207.84 ± 14.48	14.50 ± 0.75	2.04 ± 0.05
RTE + *t*-BHP	55.66 ± 3.92*	1721.08 ± 153.85#	243.04 ± 28.98	9.99 ± 1.04*	2.21 ± 0.05
RPO + *t*-BHP	54.53 ± 2.98*	1296.03 ± 74.88*	207.08 ± 19.47	10.00 ± 0.68*	2.11 ± 0.04
RTE + RPO + *t*-BHP	54.37 ± 2.43*	1505.31 ± 95.46*	203.91 ± 18.92	10.65 ± 0.85*	2.07 ± 0.04

Values are mean ± SEM of 7-10 rats per group. *Significantly different from negative control group ($P<0.05$). #Significantly different from positive control group ($P<0.05$). ORAC (oxygen radical absorbance capacity), FRAP (ferric ion reducing antioxidant power), RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (*tert*-butyl hydroperoxide). AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent).

3.8 Antioxidant enzymes activity

The effects of aqueous rooibos extract, RPO and/or their combination on antioxidant enzymes activities in erythrocytes and the liver of all experimental rats is presented in **Table 8.** In the erythrocyte, *t*-BHP treatment marginally ($P<0.1$) increased the activity of CAT by about 36%, while the activities of GR, SOD and GPx were significantly ($P<0.05$) reduced by 63, 68 and 52% respectively, when compared with the negative control rats. Rats consuming the rooibos extract, RPO or their combination without *t*-BHP treatment, had significantly ($P<0.05$) decreased CAT activity, but showed no significant ($P>0.05$) differences in the activities of GR, SOD and GPx when compared with negative control rats. Consumption of rooibos, alone or in combination with RPO, reversed the changes induced by *t*-BHP by significantly ($P<0.05$) lowering the CAT, and increasing the GR, SOD and GPx activities compared with the positive control rats. Red palm oil consumption alone, by *t*-BHP treated rats, significantly decreased CAT, and increased GR and GPx activities, but showed no significant difference ($P>0.05$) in the SOD activity when compared with the positive control rats.

Hepatic CAT and GPx were reduced marginally ($P<0.1$) and significantly ($P<0.05$) respectively, while GR activity was increased ($P<0.05$) significantly, in the positive control group when compared with the negative control group. Rats consuming the rooibos extract or RPO without *t*-BHP treatment, showed activities of hepatic CAT, GR, SOD and GPx comparable to those of the negative control rats, while the consumption of rooibos extract and RPO together in these rats, significantly ($P<0.05$) increased CAT and GPx activities when compared with the negative control rats. Consumption of the rooibos extract and RPO, either alone or in combination with *t*-BHP treatment, significantly ($P<0.05$) increased GPx and decreased GR activity in the liver when compared with positive control rats. Only the combined supplementation of the rooibos extract and RPO was able to significantly ($P<0.05$) increase CAT activity in these rats when compared with those of the positive control. The activity of SOD remained unchanged in the liver when *t*-BHP treated rats (positive control) were compared with negative control rats, and also when *t*-BHP treated rats consuming rooibos extract, RPO or their combination, were compared with the positive control rats.

Table 8: Effects of aqueous rooibos extract, RPO and/or their combination on antioxidant enzymes activities in erythrocyte and liver of all experimental rats.

	Erythrocytes				Liver			
Treatment	**CAT**	**GR**	**SOD**	**GPx**	**CAT**	**GR**	**SOD**	**GPx**
Negative (water)	0.64 ± 0.05	0.56 ± 0.06	5.45 ± 1.71	1.76 ± 0.17	198.90 ± 9.17	17.03 ± 0.50	37.46 ± 4.05	28.41 ± 1.84
Positive (*t*-BHP)	0.87 ± 0.11**	0.21 ± 0.05*	1.72 ± 0.59*	0.85 ± 0.23*	178.31 ± 5.63**	20.14 ± 1.14*	46.98 ± 3.18	22.10 ± 1.04*
RTE	0.22 ± 0.03*	0.85 ± 0.14	5.96 ± 0.76	1.74 ± 0.14	202.88 ± 5.08	16.16 ± 0.50	38.66 ± 3.04	35.01 ± 1.44*
RPO	0.41 ± 0.02*	0.44 ± 0.05	2.54 ± 0.34	1.62 ± 0.37	197.79 ± 6.23	16.47 ± 0.40	31.14 ± 1.77	29.91 ± 1.19
RTE + RPO	0.20 ± 0.02*	0.42 ± 0.09	7.42 ± 1.56	2.02 ± 0.22	233.26 ± 4.34*	16.43 ± 0.70	34.35 ± 3.49	42.40 ± 4.02*
RTE + *t*-BHP	0.30 ± 0.02*#	0.44 ± 0.05#	4.12 ± 0.89#	1.67 ± 0.14#	195.51 ± 6.39	16.25 ± 0.30#	52.10 ± 3.34	31.45 ± 1.93#
RPO + *t*-BHP	0.43 ± 0.17*#	0.43 ± 0.05#	1.13 ± 0.25	1.55 ± 0.09#	186.11 ± 6.01	16.38 ± 0.40#	38.14 ± 1.86	32.75 ± 2.28#
RTE + RPO + *t*-BHP	0.45 ± 0.05*#	0.40 ± 0.07#	7.21 ± 1.20#	2.10 ± 0.41#	199.37 ± 8.20#	16.37 ± 0.40#	36.19 ± 2.39	37.64 ± 3.10#

Values in columns are mean ± SEM for 7-10 rats per group. *Significantly different from negative control ($P<0.05$). **Marginally different from negative control ($P<0.1$). #Significantly different from positive control ($P<0.05$). CAT (catalase, µmol H_2O_2 consumed/min/ µg protein in the erythrocyte or µmol H_2O_2 consumed/min/ mg protein in the liver), GR (glutathione reductase, µmol NADPH oxidized/min/µg protein in the erythrocyte or µmol NADPH oxidized/min/mg protein in the liver), SOD (superoxide dismutase, U/µg protein in erythrocyte or U/mg protein in the liver), GPx (glutathione peroxidase, nmol NADPH oxidized/min/µg protein in the erythrocyte or nmol NADPH oxidized/min/mg protein in the liver). RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (tert-butyl hydroperoxide).

3.9 Lipid peroxidation

The effects of the aqueous rooibos extract, RPO or their combination on markers of lipid peroxidation in the plasma and liver of all experimental rats are presented in **Table 9**. In the plasma, the CD levels of *t*-BHP treated rats (positive control) were significantly ($P<0.05$) higher than those of the negative control rats, however, the MDA levels remained unchanged among these two groups. Rats consuming the rooibos extract without *t*-BHP treatment, exhibited similar levels of CD and MDA when compared to rats consuming water (negative control). However, RPO alone or combined with rooibos without *t*-BHP treatment, caused a significant ($P<0.05$) increase in the level of conjugated dienes, but not MDA when compared to the negative control. Consuming the rooibos extract, RPO or their combination with *t*-BHP treatment, significantly ($P<0.05$) increased plasma CD levels, but MDA remain unchanged in these rats when compared with positive control rats.

In the liver, treatment with *t*-BHP resulted in a significant ($P<0.05$) and marginal ($P<0.1$) increase in CD and MDA respectively when compared to the rats consuming water (negative control). Rats consuming the rooibos extract, RPO or their combination without *t*-BHP treatment exhibited similar levels of CD but a significantly lowered level of MDA when compared to the negative control rats. The increase in CD and MDA levels observed in the liver of *t*-BHP treated rats were significantly ($P<0.05$) reduced as a result of supplementation with rooibos extract, RPO or their combination.

Table 9: Effects of aqueous rooibos extract, RPO and/or their combination on markers of lipid peroxidation in the plasma and liver of all experimental rats.

	Plasma		Liver	
Treatment	**CD (nmol/L)**	**MDA (µmol MDA/L)**	**CD (nmol/g tissue)**	**MDA (µmol MDA/g tissue)**
Negative control (water)	71.67 ± 2.43	2.44 ± 0.09	7.29 ± 0.15	0.37 ± 0.09
Positive control (*t*-BHP)	89.75 ± 1.30*	2.72 ± 0.16	8.98 ± 0.12*	0.62 ± 0.04**
RTE	72.60 ± 1.24	2.55 ± 0.07	7.54 ± 0.17	0.10 ± 0.01*
RPO	108.89 ± 13.4*	2.52 ± 0.11	7.50 ± 0.12	0.10 ± 0.004*
RTE + RPO	102.11 ± 4.91*	2.49 ± 0.07	7.44 ± 0.17	0.11± 0.003*
RTE + *t*-BHP	101.69 ± 5.18$^{\#}$	2.61 ± 0.11	7.56 ± 0.21$^{\#}$	0.10 ± 0.01*$^{\#}$
RPO + *t*-BHP	103.67 ± 4.67$^{\#}$	2.45 ± 0.13	7.98 ± 0.22$^{\#}$	0.16 ± 0.06*$^{\#}$
RTE + RPO + *t*-BHP	106.83 ± 2.15$^{\#}$	2.42 ± 0.09	7.79 ± 0.11$^{\#}$	0.11 ± 0.01*$^{\#}$

Values in columns are mean ± SEM of 8-10 rats per group. *Significantly different from negative control (P<0.05). **Marginally different from negative control (P<0.1). $^{\#}$Significantly different from positive control (P<0.05). CD (conjugated diene), MDA (malondialdehyde). RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (tert-butyl hydroperoxide).

3.10 Glutathione redox status

The glutathione redox status of the different treatment groups is presented in **Table 10**. In the erythrocytes, the GSSG levels remained similar across all treatment groups. Treatment with *t*-BHP significantly (P<0.05) depleted the GSH and resultant GSH/GSSG ratio by 70 and 75% respectively when compared to the negative control group. Rats consuming the rooibos extract or RPO alone without *t*-BHP treatment exhibited similar level of GSH and GSH/GSSG ratio when compared to rats consuming water (negative control). Co-supplementation of the rooibos extract and RPO in rats without *t*-BHP treatment, significantly (P<0.05) increased the GSH levels and GSH/GSSG ratio when compared to the negative control rats. Supplementation of rooibos extract, RPO or their combination to *t*-BHP-treated rats, was able to reverse the observed impairment in GSH redox status by significantly (P<0.05) increasing the GSH levels and GSH/GSSG ratio to that comparable to levels found in rats drinking water (negative control).

Hepatic GSH level and GSH/GSSG ratio were significantly (P<0.05) reduced, while GSSG remained unchanged in rats treated with *t*-BHP compared to negative control rats.

Consumption of the rooibos extract alone, or combined with RPO, without *t*-BHP treatment significantly ($P<0.05$) increased GSH level and GSH/GSSG ratio, but significantly ($P<0.05$) decreased GSSG when compared to negative control rats. Rats consuming RPO alone, without *t*-BHP treatment, exhibited similar GSH levels, but a significantly ($P<0.05$) decreased GSSG level and increased GSH/GSSG ratio when compared to the negative control animals consuming water. Supplementation of rooibos extract, RPO or their combination to *t*-BHP treated rats, resulted in a significantly ($P<0.05$) increased GSH level and GSH/GSSG ratio, parallel with a decreased GSSG level, when compared to positive control rats. The level of improvement observed in the redox status of this group of rats is comparable to what was obtained in negative control rats consuming water.

Table 10: Effects of aqueous rooibos extract, RPO and/or their combination on glutathione status in the erythrocyte and liver of all experimental rats

	Erythrocyte			Liver		
Treatment	**GSH (µmol/µg protein)**	**GSSG (µmol/µg protein)**	**GSH:GSSG**	**GSH (µmol/g wet liver)**	**GSSG (µmol/g wet liver)**	**GSH:GSSG**
Negative control (water)	0.210 ± 0.037	0.121 ± 0.012	1.70 ± 0.20	6.13 ± 0.09	0.46 ± 0.08	18.52 ± 1.57
Positive control (*t*-BHP)	0.064 ± 0.012*	0.150 ± 0.014	0.41 ± 0.06*	3.84 ± 0.39*	0.56 ± 0.09	8.54 ± 1.63*
RTE	0.190 ± 0.025	0.126 ± 0.011	1.59 ± 0.25	7.82 ± 0.47*	0.23 ± 0.03*	32.54 ± 4.35*
RPO	0.195 ± 0.024	0.109 ± 0.005	1.75 ± 0.16	5.89 ± 0.39	0.23 ± 0.04*	33.05 ± 6.48*
RTE + RPO	0.321 ± 0.027*	0.117 ± 0.003	2.72 ± 0.18*	8.61 ± 0.52*	0.22 ± 0.04*	54.27 ± 10.35*
RTE + *t*-BHP	0.159 ± 0.026$^{\#}$	0.112 ± 0.005	1.42 ± 0.20$^{\#}$	7.28 ± 0.52$^{*\#}$	0.32 ± 0.04$^{\#}$	24.76 ± 2.96$^{\#}$
RPO + *t*-BHP	0.182 ± 0.023$^{\#}$	0.109 ± 0.004	1.65 ± 0.19$^{\#}$	5.84 ± 0.29$^{\#}$	0.14 ± 0.01$^{\#}$	41.47 ± 2.58$^{\#}$
RTE + RPO + *t*-BHP	0.240 ± 0.022$^{\#}$	0.126 ± 0.008	1.89 ± 0.13$^{\#}$	7.72 ± 0.27$^{\#}$	0.37 ± 0.03$^{\#}$	22.92 ± 3.24$^{\#}$

Values are mean ± SEM of 8-10 rats per group. *Significantly different from negative control (P<0.05). $^{\#}$Significantly different from positive control (P<0.05). GSH (reduced glutathione), GSSG (oxidised glutathione). RTE (aqueous rooibos extract), RPO (red palm oil), *t*-BHP (tert-butyl hydroperoxide).

4. Discussion

Tert-butyl hydroperoxide is a membrane permanent pro-oxidant that has been extensively employed as a model for investigating the mechanism of cell injury initiated by oxidative stress in a variety of systems (Rush *et al*., 1985; Yen *et al*., 2004; Hwang *et al*., 2011; Yang *et al.*, 2012). Metabolism of *t*-BHP either by cytochrome P450 or haemoglobin triggers the generation of harmful free radicals such as alkoxyl and peroxyl radicals in the hepatocytes and erythrocytes. The free radicals readily cross cellular membranes and lead to formation of highly reactive hydroxyl radicals which can initiate lipid peroxidation, affect cell membrane integrity, damage protein, DNA and result in cell injury in hepatocytes and rat liver (Guidarelli *et al*., 1997; Kim *et al*., 2007; Hwang *et al*., 2009). An alternative metabolic pathway for *t*-BHP is its rapid conversion by GSH catalyzed by GPx to produce t-butanol and GSSG. The GSSG is then recycled back to GSH by the enzyme GR, resulting in NADPH oxidation. The depletion of GSH and the oxidation of NADPH are associated with Ca^{2+} homeostasis, a critical event in *t*-BHP-induced toxicity (Lee *et al*., 2008; Yang *et al*., 2012).

A way of preventing free radical-mediated cellular injuries is to augment the oxidative defense capacity of the cell through intake of antioxidants. Recently, much attention has focused on the health beneficial role of naturally occurring antioxidants in biological systems. Phenolic phytochemicals derived from plants are being considered to play an important role as physiologically functional foods and are being utilized for treatment and prevention of clinical diseases related to oxidative stress, even though their modes of action may still not be fully understood (Lee *et al*., 2004). The beneficial effects of these compounds are attributed to the antioxidant and free radical scavenging properties of their various components such as polyphenols and flavonoids (Cao *et al*., 1996; Dreosti *et al*., 1996). Rooibos (*Aspalathus linearis*) and red palm oil (RPO), from the fruit of the oil palm tree (*Elaeis guineensis*) are two such plant extracts exhibiting high antioxidant capacity.

Rooibos is an important source of antioxidants due to its rich flavonoid content with numerous studies reporting on its health benefits. Its antioxidant (Marnewick *et al*., 2003; Kucharska *et al*., 2004), anti-inflammatory (Baba *et al*., 2009), anti-diabetic (Kawano *et al*., 2009) as well as ability to modulate oxidative stress (Ulicna *et al*., 2006; Nikolova *et al*., 2007; Marnewick *et al*., 2011; Awoniyi *et al*., 2012) have been demonstrated in animal models and human studies. Red palm oil is rich in cocktail of lipid soluble antioxidants such as α- and β-carotene, lycopene, tocopherols (α, β, γ and δ isoform), tocotrienols (α, β, γ and δ isoform) and coenzyme Q_{10} (Van Rooyen *et al*., 2008; Oguntibeju *et al*., 2012). *In vivo*

experiments using various animal models have revealed that RPO has many health benefits including protection against oxidative stress (Ebong *et al.*, 1999; Budin *et al*., 2009), modulation of serum lipid profile (Oluba *et al*., 2008; Budin *et al*., 2009) and protection of the heart against ischaemia/reperfusion injury (Esterhuyse *et al*., 2006; Engelbrecht *et al*., 2009; Van Rooyen *et al*., 2008).

In the current study, an aqueous rooibos extract and RPO were investigated to determine a possible protective effect either individually or combined against *t*-BHP-induced oxidative hepatotoxicity in Wistar rats. HPLC quantification of the aqueous rooibos extract used in this study yielded aspalathin as the major flavonoid present in rooibos (Table 2) which is in accordance with previously published studies (Rabe *et al*., 1994; Bramati *et al*., 2002; Joubert *et al*., 2008; Marnewick, 2009). Other constituents quantified include orientin, iso-orientin, vitexin, isovitexin, rutin, and trace quantities of quercetin, luteolin and chrysoeriol. HPLC quantification of the RPO used in this study, also yielded isoforms of tocopherols and tocotrienols, as well as α- and β-carotene in fractions that is in accordance to previously published works (Sambanthamurthi *et al*., 2000; Edem, 2002).

Evaluation of the total antioxidant capacity (TAC) of food has become a standard and this is due largely to the renewed interest in health benefits of foods, supplements and plants with high antioxidant potentials (Stevenson and Lowe, 2009). While there is no universally accepted measure, the oxygen radical absorbance capacity (ORAC) (Ou *et al*., 2001) and the ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996) are two of the most popular TAC assays. In the current study, it was observed that the plasma total polyphenol content was significantly reduced in all treatment groups compared to the negative control group. Treatment with *t*-BHP lowered the TAC measured as ORAC in the plasma and the liver. Supplementation with rooibos alone restored the ORAC depletion caused by *t*-BHP treatment in the plasma, with no effect exhibited in the liver. Feeding RPO alone or in combination with rooibos resulted in no net increase in TAC assessed either as ORAC or FRAP in the plasma or liver. In fact, RPO supplementation either alone or in combination tends to lower the TAC of both plasma and liver. Reports on whether supplementation of polyphenol-enriched diets will increase plasma total polyphenols and TAC in rats has been controversial. Apple and pear peels (Leontowicz *et al*., 2003), as well as raw and boiled garlic (Gorinstein *et al*., 2006) were reported to enhance plasma total polyphenol and TAC while intake of cranberry powder and mango did not produce any such effect (Kim *et al*., 2008; Garcia-Solis *et al*., 2008). Previous studies, using different rodent models, have reported that rooibos supplementation, did not increase the TAC measured as ORAC

(Marnewick *et al*., 2003; Sauter, 2004). Also, Marnewick *et al*. (2011) reported that consumption of six cups of rooibos daily for six weeks did not enhance the plasma antioxidant capacity in adult humans who are at the risk of developing cardiovascular diseases. The assays for antioxidant capacity have been suggested to lack specificity and their estimates are not likely to indicate any resultant changes in plasma antioxidant capacity (Lotito and Frei, 2006; Halliwell, 2009). Therefore, this may account for the reason why in the current study, there is no change in plasma antioxidant capacity even in the group consuming rooibos. Furthermore, the plasma antioxidant capacity is a fasting measurement; therefore it may not represent the active antioxidant pool since the half-lives of all the individual compounds, including polyphenols and non-polyphenols, may fluctuate (Marnewick *et al*., 2011). In addition the 12 h fasting period may have a more pronounced effect on the non-phenolic antioxidants making the antioxidant capacity to remain unchanged or diminished regardless of increased polyphenol consumption. Previous reports have indicated that the antioxidant capacity of a compound is dependent upon reaction media (Foti and Ruberto, 2001; Huang *et al*., 2005). Therefore, an organic solvent based ORAC assay would be ideal for RPO which is rich in lipophilic antioxidants. However, fluorescein, used as the fluorescent probe in the ORAC assay is not sufficiently lipid soluble and its fluorescence intensity in a non-polar organic solvent is low. Therefore, this may account for the very low ORAC values observed in the RPO supplemented groups.

In recent years, medicinal plants and herbs are getting great attention as important sources of bioactive substances, with health beneficial effects. However, a great limitation to the use of medicinal plants and herbs is the issue of safety and toxicity. Damage to the liver is a widely used indicator of toxicity of medicinal plants and herbs *in vivo* (Willet *et al*., 2004; Saad *et al*., 2006). The aminotransferases (ALT and AST) and LDH are among serum marker enzymes of hepatic function, with their increase in the serum indicating hepatic damage. The supplementation of rooibos, RPO and/or their combination to normal rats did not result in any toxicity or adverse effects as indicated by the levels of the serum aminotransferases and LDH. Results from this study further confirmed that *t*-BHP-induced hepatotoxic effects manifested by a significant increase in activity of liver function marker enzymes ALT, AST and LDH in the serum of *t*-BHP treated rats. These observations are in accordance with those obtained by previous studies (Valentao *et al*., 2004; Yen *et al*., 2004; Hwang *et al*., 2008; Oh *et al*., 2012). Alanine amino transferase, AST and LDH are cytoplasmic and the rise in their serum levels are attributed to damaged structural integrity of the liver and as a result these enzymes are released into the blood circulation after the rupture of the plasma membranes (Yen *et al*., 2004; Shaarawy *et al*., 2009). The

t-BHP-induced hepatic damage observed was confirmed by histopathology examination of the *t*-BHP-treated rats which revealed severe hepatic degeneration and hepatocyte vacuolation, as well as massive lymphocyte and mononuclear cellular aggregation. Rooibos and RPO supplementation either individually or combined in *t*-BHP treated rats, significantly reduced the elevated levels of ALT, AST and LDH. The diminished levels of these serum enzymes can be ascribed to a stabilizing effect of the rooibos and RPO phyto-constituents on the plasma membrane of the hepatocytes, as well as repair of the damaged hepatic tissues, probably brought about by the stimulation of hepatocellular protein synthesis and accelerated regeneration of the hepatocytes (Das and Sarma, 2009). Histopathological examination of livers from *t*-BHP-treated rats whose diet were supplemented with rooibos and RPO, revealed enhanced hepatocellular architecture with slight lymphocyte infiltration, which is a clear manifestation of the hepatoprotective effects of rooibos and RPO. This result is consistent with previous findings that have been reported in different experimental models of rats exposed to other toxicants where rooibos or RPO have been supplemented (Ulicna *et al*., 2003; 2006; 2008; Ebong *et al*., 1999; Adeneye and Benebo, 2007; Jaffri *et al*., 2011).

Oxidative stress, manifested as lipid peroxidation has been implicated in the mechanism of various types of cell injury. It has been hypothesized that one of the principal causes of *t*-BHP-induced liver injury is the formation of lipid peroxides by free radical derivatives (alkoxyl and peroxyl radicals) (Hwang *et al*., 2009). In the current study, the *t*-BHP-induced lipid peroxidation was assessed by determining the levels of conjugated dienes (CD) and malondialdehyde (MDA). Plasma, as well as hepatic CD levels were significantly increased, while hepatic MDA levels were marginally increased by the *t*-BHP treatment. Supplementation with rooibos, RPO or their combination effectively inhibited this observed increase in the liver. The elevation in CD and MDA levels in the *t*-BHP-treated group in this study may be due to either overproduction of alkoxyl and peroxyl radicals or their accumulation resulting from dysfunction of antioxidant systems during the repeated exposure to *t*-BHP. Previous reports have indicated that rooibos reduced age-related lipid peroxide accumulation (measured as TBARS) in the brain of over-age rats consuming the tea for 21 months and inhibited MDA formation in rat tissues and liver microsomal preparations (Inanami *et al*., 1995; Marnewick *et al*., 2005; Ulicna *et al*., 2003, 2006). Recent reports in humans also revealed that rooibos consumption significantly decreased plasma MDA levels in lead factory workers (Nikolova *et al*., 2007) and also significantly lowered plasma CD and MDA levels in adults at the risk for cardiovascular diseases taking 6 cups of rooibos per day for 6 weeks (Marnewick *et al*., 2011). The ability of rooibos to protect against lipid peroxidation may involve one or more of several different antioxidant properties

exhibited by rooibos or synergistic interactions of its different phenolic constituents. The protective effect may be due to the ability of rooibos phenolic constituents not only to bind lipid peroxides, but also their ability to inhibit the lipid peroxidation cascade, either by acting as a sacrificial antioxidant or as a chelator of transition metals that promote lipid peroxidation (Gutteridge and HalliwelL 1999; Nijveldt *et al*., 2001, Awoniyi *et al*., 2012). Also the protective effect may be associated with the inhibition of cytochrome P450-mediated metabolism of *t*-BHP to active toxic radicals that initiate lipid peroxidation. RPO is a rich source of lipid soluble antioxidants including tocopherols, tocotrienols and carotenes. Previous reports have highlighted the ability of RPO and its extracts to inhibit lipid peroxidation both *in vitro* and *in vivo*. Wu and Ng (2007) reported that a red palm oil extract is able to prevent $FeCl_2$-ascorbic acid-induced lipid peroxidation in rat liver and brain homogenates. Cadmium-induced ocular tissue lipid peroxidation was also inhibited by RPO in rabbits (Eriyamremu *et al*., 2008) while a tocotrienol-rich fraction of RPO was reported to inhibit the level of MDA and protein carbonyl production in the pancreas (Budin *et al*., 2011) and the level of MDA + 4-hydroxynonenal in the plasma and aorta (Budin *et al*., 2009) of streptozotocin-induced diabetic rats. The potential of RPO to prevent lipid peroxidation induced by *t*-BHP in this study can be attributed to contributions of its lipid antioxidants (tocopherols, tocotrienols and carotenoids), and this may be rooted in their ability to donate phenolic hydrogen (electrons) to lipid peroxyl radicals (Budin *et al*., 2009). Tocopherols, tocotrienols and carotenes found in RPO are lipophilic, chain breaking antioxidants which can exert their actions in the hydrophobic lipid core of membranes, thereby protecting the cell membranes from lipid peroxidation induced by *t*-BHP. Supplementation of the combination of rooibos and RPO also reduced *t*-BHP-induced production of CD and MDA in this study, however the level of reduction was similar to that observed for treated rats consuming either rooibos or RPO alone with no additional protection.

Closely related to lipid peroxidation are the antioxidant enzymes including SOD, CAT and GPx which are produced by mammalian cells as a defence against ROS generation (Halliwell and Gutteridge, 2007). Scientific evidence has revealed that oxidative stress mediated by toxic injuries, is associated with change in antioxidant enzyme levels and that the specific responses of antioxidant enzymes do not follow set patterns but are stress-, tissue- and species-specific (Crawford *et al*., 2000). In the current study, a marginal increase in the activity of CAT and a significant decrease in the activities of SOD, GR and GPx in the erythrocyte of *t*-BHP treated rats were observed. Consumption of rooibos alone or combined with RPO reversed the changes in activities of the antioxidant enzymes induced by *t*-BHP in the erythrocytes. Red palm oil alone, in the diet of the *t*-BHP treated rats, restored the

changes in the activity of CAT, GR and GPx. In the liver, the activities of CAT and GPx were marginally and significantly decreased respectively, while GR activity was increased, with SOD unaffected by the *t*-BHP treatment. Rooibos, RPO or their combination reversed the changes induced in the activities of GR and GPx, but only the combination was effective in augmenting CAT activity. Superoxide dismutase is the first enzyme in the ROS detoxification process and it converts superoxide radicals to H_2O_2. The decrease in SOD activity observed in the erythrocytes of *t*-BHP treated rats in this study, can be adduced to the depletion or inactivation of the enzyme as a result of ROS generation (Kono and Fridovich, 1982), which in turn resulted in the initiation and propagation of lipid peroxidation, which would have contributed to the observed increase in CD and MDA levels discussed earlier. Scavenging of H_2O_2 produced by SOD, is the primary role of CAT and the increased CAT activity observed in the erythrocytes of *t*-BHP treated rats could be a compensatory mechanism attributed to the resultant increased formation of H_2O_2 by SOD and/or up-regulation of expression of gene encoding for CAT. The fact that both GPx and GR activities were decreased in the erythrocytes lead us to speculate that the metabolism of *t*-BHP in the erythrocytes is via both the cytochrome P450 generation of alkoxyl and peroxyl radicals and the direct detoxification by GSH. The reduction in activity of GPx can be ascribed to its use in catalysing the oxidation of GSH, resulting in the formation of GSSG, which is then reduced to GSH by GR, resulting in NADPH oxidation (Kim *et al*., 2007). In the liver, our observation of a decrease in GPx and an increase in GR activity, while SOD activity was not affected, suggest that organic alkoxyl and peroxyl radicals may not be involved in *t*-BHP-induced oxidative stress in the liver. The activities of these enzymes were modulated to a varying degree in *t*-BHP-treated rats consuming either, rooibos, RPO or their combination. Previously, Marnewick *et al*. (2009) reported that an aqueous rooibos extract modulated the changes observed in the activities of antioxidant enzymes in rats subjected to diethyl nitrosamine (DEN)-initiated and fumonisin B_1 (FB_1)-promoted hepatocarcinogenesis. Another study reported that changes in the activity of SOD and CAT observed in the epididymal sperm of rats subjected to *t*-BHP treatment, was reversed by rooibos supplementation (Awoniyi *et al*., 2012). Another report has also shown that RPO supplementation increased the observed decrease in CAT and SOD activity induced by cadmium in the ocular tissue of rabbit (Eriyamremu *et al*., 2008). In the current study, the observed modulation by rooibos, RPO and/or their combination could be attributed to the natural antioxidants present in them. The flavonoids present in rooibos as well as the tocopherol, tocotrienol and carotenoids present in RPO, may quench free radicals generated by *t*-BHP and/or up- or down-regulate the transcription of antioxidant enzyme genes, all which may result in the increase or decrease in their synthesis.

The fact that glutathione (GSH) is involved in defense reactions against oxidative stress as an antioxidant, is widely acknowledged (Sies, 1997; Ajuwon *et al*., 2011; Lushchak, 2012). Glutathione is the predominant non enzymatic intracellular antioxidant (Morimoto *et al*., 2008), and participates in the removal of free radicals (including H_2O_2, superoxide anion, alkoxyl and peroxyl radicals), maintenance of membrane protein thiols and it is also a substrate for GPx and GR (Naik *et al*., 2011). Present in the cells in both the reduced (GSH) and oxidized (GSSG) forms, but because of the action of the NADPH-dependent enzyme GR, the cellular content of glutathione is predominantly in favour of GSH under normal physiologic conditions (Shaik and Mehvar, 2006). In agreement with previous reports (Kim *et al*., 2007; Hwang *et al*., 2008; Oh *et al*., 2012; Yang *et al*., 2012), the current study revealed that *t*-BHP treatment resulted in a reduction in GSH levels both in the erythrocytes and liver, while GSSG level was only increased in the liver. The oxidation of GSH to GSSG is a sensitive marker of oxidative stress and under condition of increased stress, the GSH:GSSG ratio decreases either due to increased GSSG or decreased GSH levels (Dickinson and Forman, 2002). In this study, *t*-BHP treatment also resulted in a reduction in GSH:GSSG ratio in the liver and erythrocytes. Supplementation with rooibos, RPO and/or their combination reversed the reduction in GSH levels and GSH:GSSG ratio, observed in *t*-BHP treated rats both in the erythrocytes and liver. Recently, Pantsi *et al*. (2011) reported that a fermented rooibos supplementation restored the decrease in GSH levels and GSH:GSSG ratio in the hearts of rats subjected to ischaemia/reperfusion injury. Similarly, Marnewick *et al*. (2011) also showed that drinking six cups of rooibos per day for six weeks increased the GSH levels and GSH:GSSG ratio in adults at risk for developing cardiovascular disease, while Awoniyi *et al*. (2012) reported that rooibos supplementation in *t*-BHP treated rats enhance the epididymal sperm GSH levels. The significant increase in GSH level due to rooibos consumption may be attributed to the phenolic antioxidants in rooibos ability to improve the redox/antioxidant status of the cell resulting in an enhanced endogenous detoxification capacity. The polyphenols in rooibos may quench free radicals produced by *t*-BHP, sparring GSH and hence lowering the vulnerability of the cells to further oxidative stress. Another intriguing possibility for the observed GSH increase is that rooibos polyphenols may up-regulate the expression of γ-glutamylcysteine synthetase (γ-GCS), which is the rate limiting enzyme in the synthesis of GSH. Previous studies have shown that polyphenolic compounds from plants increased the γ-GCS activity and GSH contents (Jeon *et al*., 2003; Chen *et al*., 2004; Moskaug *et al*., 2005), although no study has yet been conducted to determine if rooibos or its flavonoids can increase γ-GCS mRNA expression. That RPO supplementation restored the observed impairment in the redox status observed

in *t*-BHP challenged rat could be ascribed to its vitamin E and carotene constituents. Tocopherols, tocotrienols and carotenes in RPO are able to quench peroxyl radicals generated by *t*-BHP biotransformation by donating hydrogen from their phenolic hydroxyl group to the peroxyl radical thereby forming a stable radical species (Rimbach *et al*., 2010) and thus spare GSH and protect the cells from oxidative stress. RPO could also increase the biosynthesis of GSH because previous *in vitro* and *in vivo* studies have indicated that α-tocopherol (Masaki *et al*., 2002; Masaki and Sakurai, 2007) and β-carotene (Takeda *et al*., 2008; Katsuura *et al.,* 2009) increased intracellular GSH levels by up-regulating the mRNA expression of γ-GCS.

One of the hypotheses we set out to investigate in this study is whether co-supplementation of rooibos and RPO to *t*-BHP-challenged rats will result in a synergy of their protective effects. Our results indicated that co-supplementation of rooibos and RPO actually protect against t-BHP-induced hepatotoxicity as demonstrated by reduction in level of liver function marker enzymes, inhibition of CD and MDA formation, reversal of changes in antioxidant enzymes and increase in intracellular GSH level and GSH:GSSG ratio in t-BHP treated rats. However, the level of protection shown is only equal to that of either rooibos or RPO, thus any synergy in their combine protective effects could not be shown.

5. Conclusion

The result of the present investigation suggest that antioxidant-rich rooibos, RPO and/or their combination, showed efficient protective action against *t*-BHP-induced oxidative hepatotoxicity in rats. This is demonstrated by their ability to (i) reverse the increase in liver function marker enzymes (ALT, AST and LDH), (ii) prevent lipid peroxidation by reducing the levels of CD and MDA, (iii) modulate changes in activity of antioxidant enzymes and (iv) restore the redox status by increasing the GSH levels and GSH:GSSG ratio in *t*-BHP treated rats. The effect observed when rooibos and RPO were supplemented in combination, although protective, were not synergistic. Both rooibos and RPO are rich in antioxidant compounds, therefore, the effects observed for each extract are proposed to be as a result of synergistic interaction of all the compounds in each extract. The protective biochemical function of naturally occurring antioxidants in biological system and their mechanisms of action are gaining more attention. This study therefore provides biological evidence supporting the use of rooibos and RPO as an adjuvant therapy for the prevention and treatment of liver disorders, however, a series of well controlled clinical intervention studies are needed to explore this possibility further.

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CHAPTER 5

Rooibos and/or red palm oil supplementation attenuates oxidative stress and inflammatory responses in lipopolysaccharide-induced endotoxemic rats

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Rooibos and/or red palm oil supplementation attenuates oxidative stress and inflammatory responses in lipopolysaccharide-induced endotoxemic rats

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Abstract

The protective effects of an aqueous rooibos extract, red palm oil and/or their combination on lipopolysaccharide-induced hepatic endotoxemia in male Wistar rats were investigated. Rats were injected with lipopolysaccharide (LPS) (0.5 mg/kg body weight) after oral feeding of an aqueous rooibos extract, red palm oil and/or their combination for four weeks. Liver function marker enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)], cytokine levels (TNF-α, IL-1β, IL-6 and IL-10), oxidative stress indices [conjugated dienes (CD), malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR)] and redox status markers (GSH, GSSG and GSH/GSSG ratio) were monitored in the rats. LPS injection induced a marked hepatic damage evident by significantly ($P<0.05$) elevated levels of ALT, AST and LDH in the serum. Hepatic CD, MDA and GSSG were significantly ($P<0.05$) increased, with a resultant significant ($P<0.05$) decrease in GSH/GSSG ratio in the LPS challenged rats. Furthermore, varying changes were induced in the activities of antioxidant enzymes both in the blood and liver, while the hepatic levels of TNF-α, IL-1β and IL-6 were all increased significantly ($P<0.05$), as a result of the LPS challenge. Supplementation with rooibos and red palm oil in combination caused a decrease in the LPS-induced elevated levels of ALT, AST and LDH, comparable to levels found in the negative control rats. The observed increases in hepatic CD, MDA, GSSG as well as the decrease in GSH/GSSG ratio, were all reversed ($P<0.05$) by rooibos, red palm oil and/or their combination. Also the elevation in TNF-α and IL-6 and the changes observed in the activities of antioxidant enzymes were all restored after consumption of these plant extracts. In conclusion, our results suggest that rooibos, red palm oil and/or their combination

attenuates LPS-induced hepatic endotoxemia possibly by preventing oxidative stress, improving the GSH redox status and inhibiting the formation of pro-inflammatory cytokines.

Keywords: cytokines, oxidative stress, lipopolysaccharide, rooibos, red palm oil.

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1. Introduction

Lipopolysaccharide (LPS), an endotoxin, is a major glycolipid component of the outer cell wall of gram-negative bacteria, and it is made up of a polysaccharide O-chain and a lipid-A moiety, which is the biologically active region of the endotoxin embedded within the bacterial membrane (Larrosa *et al*., 2011). Endotoxemia-induced toxicity is characterised by injury to various organs, including liver, kidney and the brain, and it has been implicated as a contributing factor to bacterial infection resulting in sepsis, which is one of the major causes of morbidity and mortality in intensive care units (Sebai *et al*., 2009). As a result of exposure, LPS elicits a systemic inflammatory process, which triggers an acute phase response in the host that is characterised by fever, leucocytosis, thrombocytopenia, changed metabolic responses and redox status impairment (Sebai *et al*., 2009; Abdel-Salam *et al*., 2012).

The consequences of the disturbed intracellular redox balance includes the generation of excessive reactive oxygen and nitrogen species (RONS), induction of lipid peroxidation, DNA and protein damage, depletion of intracellular stores of endogenous antioxidants and inhibition of antioxidant enzymes (Sebai *et al*., 2008). RONS are also suggested to mediate the systemic inflammatory response during sepsis and other bacterial infections via activated macrophages, which are among the prime mediators and are mostly responsible for cellular and molecular pathogenesis of sepsis (Kaur *et al*., 2006b). Activated macrophages produce cytokines, such as tumour necrosis factor-α (TNF-α), interleukins (IL)-1, IL-6, IL-8, and IL-12 and other pro-inflammatory molecules, including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide (NO) (Noguchi *et al*., 2003; Victor *et al*., 2003; Sun *et al*., 2006). The production of these toxic mediators has been related to most of the toxicities in LPS-induced injury in the liver and systemic circulation (Hartung and Wendel, 1991).

Evidence has shown that LPS causes oxidative stress, by inducing an increase in lipid peroxidation and depletion in hepatic endogenous antioxidants such as reduced glutathione as well as inhibits antioxidant enzyme activities (Kaur *et al*., 2006a, 2006b; Kao *et al*., 2009; Bharrhan *et al*., 2010). The involvement of oxidative stress in the injury associated with LPS

suggests that dietary antioxidants may enhance the efficacy of treatment protocols designed to mitigate LPS-induced endotoxemia. Medicinal plants, fruits, vegetables, spices and teas are drawing a lot of attention because of their demonstrated health benefits. Several studies have reported that phytochemicals in fruits, vegetables, spices and teas possess a high number of protective biological properties, including antioxidant, anti-inflammatory and other beneficial effects (Navindra, 2008; Darvesh *et al*., 2010; Krzyzanowski *et al*., 2010). Rooibos herbal tea and red palm oil (RPO) are two such antioxidant-rich plant extracts.

Rooibos (*Aspalathus linearis*) (Brum f) Dahlg. (Family Fabaceae; Tribe Crotolarieae) is a shrubby legume that is indigenous to the mountainous area of Clanwilliam in the Western Cape Province of South Africa, and is traditionally used to make an herbal beverage that is naturally caffeine-free, low in tannin and rich in unique polyphenolic antioxidants (Cheney and Scholtz, 1963; Joubert *et al*., 2008; Marnewick, 2009). Several polyphenolic constituents have been identified in rooibos, with the β-hydroxy-dihydrochalcone glucosides, aspalathin and nothofagin, the most abundant (Rabe *et al*., 1994; Bramati *et al*., 2002; Joubert *et al*., 2008; Marnewick, 2009). Others includes the cyclic dihydrochalcone, aspalalinin (Shimamura *et al*., 2006) and flavonoids such as orientin, iso-orientin, vitexin, isovitexin, rutin, qurcetin, isoquercitrin and luteolin (Rabe *et al*., 1994; Bramati *et al*., 2002; Kazuno *et al*., 2005; Joubert *et al*., 2008). Due to its rich content of different compounds with antioxidant and other health properties, rooibos is gaining more attention worldwide because of its potential for clinical uses. Aqueous extracts of rooibos have been shown to possess antioxidant activities *in vitro* (Joubert *et al*., 2004; Yoo *et al*., 2008). *In vivo* evidence has shown that rooibos extracts possess anti-carcinogenic and anti-tumorigenic effects (Marnewick *et al*., 2005; Marnewick *et al*., 2009; Petrova, 2009; Sissing *et al*., 2011), as well as being able to modulate oxidative stress by inhibiting lipid peroxidation and augmenting the glutathione status in rat sperm (Awoniyi *et al*., 2012) and humans with an occupational risk and at the risk of developing cardiovascular diseases (Nikolova *et al*., 2007; Marnewick *et al*., 2011). The immunomodulatory effects of rooibos have been previously reported both *in vitro* and *in vivo* (Kunishiro *et al*., 2001; Hendricks and Pool, 2010). Baba *et al*. (2009) reported that rooibos may prevent inflammation by reducing oxidative DNA damage in a dextran sodium sulphate (DSS)-induced colitis model in rats. Another recent study also showed that rooibos and two of its flavonoids (luteolin and quercetin) were able to reduce the secretion of pro-inflammatory cytokine IL-6 and TNF-α using a lipopolysaccharide-stimulated macrophage model (Mueller *et al*., 2010).

Red palm oil is a lipid extract obtained from the fleshy orange-red mesocarp of the fruits of the oil palm tree (*Elaeis guineensis*, Jacq. Family Arecaceae). It is a unique oil, with equal amounts of saturated and unsaturated fatty acids, an excellent source of fat soluble antioxidants such as the tocopherols, tocotrienols and carotenoids (Sambanthamurthi *et al.,* 2000; Edem, 2002; Engelbrecht *et al.,* 2009) and is the richest natural food source of carotenoids containing about 500 ppm of which 90% are made up of α-carotene (37%) and β-carotene (47%), with other minor carotenoids such as lycopene and cis-α-carotene also present (Van Rooyen *et al*., 2008). About 70% of the vitamin E content of red palm oil is in the form of tocotrienols (mainly as α-, β- and γ-tocotrienols), while tocopherols account for the remaining 30% (Al-Saqer *et al.,* 2004). Recent evidence has also shown the oil palm fruit to contain several phenolic compounds including gallic-, chlorogenic-, gentisic-, coumaric- and caffeic acids, as well as catechins, hesperidin, narirutin, and 4- hydroxyl benzoate, all of which have appreciable radical scavenging and antioxidant ability (Tan *et al.,* 2001; Loganathan *et al*., 2010; Atawodi *et al*., 2011). The beneficial role of red palm oil has been demonstrated in various animal experimental models. Studies in rodents have shown that red palm oil supplementation exhibited either positive effects (decreased total cholesterol, triglyceride, LDL-cholesterol, and increase HDL-cholesterol) on the serum lipid profile (Wilson *et al*., 2005; Oluba *et al*., 2008; Salinas *et al*., 2008; Budin *et al*., 2009; Ajuwon *et al*., 2011) or remained neutral (Ayeleso *et al*., 2012). The ability of red palm oil to protect the heart that has been subjected to ischaemia/reperfusion injury has been demonstrated *ex vivo* (Esterhuyse *et al*., 2005, 2006; Engelbrecht *et al*., 2009; Bester *et al*., 2010). Demonstrated antioxidant and oxidative stress modulating effects in different rodent models of oxidative injury (Narang *et al*., 2005; Eriyamremu *et al*., 2008; Lee *et al*., 2009; Ajuwon *et al*., 2010; Budin *et al*., 2011) are known for RPO, while the anti-inflammatory effect of inhibiting the production of IL-6 and NO in lipopolysaccharide-induced RAW264.7 macrophages (Yam *et al*., 2009) by a tocotrienol-rich fraction of palm oil, has also been reported.

Literature on the *in vivo* anti-inflammatory effects of rooibos, are limited, and to the best of our knowledge, there are no reports available on the anti-inflammatory effect of RPO in experimental animals. Prompted by this paucity of data, we investigated the protective effects of rooibos, RPO or their combination, on LPS-induced endotoxemia in Wistar rats. We also tested the hypothesis that the protective effect of rooibos and RPO observed may be due to the synergistic interaction between these antioxidant-rich extracts.

2. Materials and Methods

2.1 Chemicals

The chemicals L-ascorbic acid, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulfonate (ABTS), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), fluorescein sodium salt, formaldehyde, Folin Ciocalteu's phenol reagent, gallic acid, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), hesperidin, histological grade formaldehyde, 6-hydroxydopamine, lipopolysaccharide (*Escherichia coli*, serotype 0111:B4), metaphosphoric acid, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-thiobarbituric acid (TBA) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), iron chloride hexahydrate ($FeCl_3$.6H2O), and potassium persulfate were obtained from Sigma–Aldrich (Johannesburg, South Africa). Diethylenetriaminepentaacetic acid (DETAPAC), 4-(dimethylamino)-cinnamaldehyde (DMACA) and malondialdehyde bis (diethyl acetal) (MDA), glacial acetic acid, trifluoroacetic acid, sulfuric acid (H_2SO_4), hexane, methanol (MeOH), ethanol (EtOH), dichloromethane, tetrahydrofuran, acetone, and hydrochloric acid (HCl) were purchased from Merck (Johannesburg, South Africa). All other reagents used were of analytical grade.

2.2 Plant materials and rooibos herbal tea preparations

Fermented rooibos (superior grade) plant material was a generous gift from Rooibos Limited (Mr Arend Redelinghuys, Clanwilliam, South Africa). An aqueous extract (2%, w/v) of rooibos (RTE) was prepared by the addition of freshly boiled tap water to tea leaves at a concentration of 2g/100 mL (Marnewick *et al*., 2003). The mixture was allowed to stand at room temperature for 30 minutes with constant stirring, filtered and dispensed into water bottles. The aqueous rooibos extract was fed to rats *ad libitum* and fresh tea prepared every second day. The RPO used in this study (Carotino^{TM} baking fat) was supplied by Carotino SDN BHD (company number: 69046-T), Johar-Bahru, Malaysia, and was fed to the rat orally (200 μL, equivalent to 7g/kg diet) daily in the morning, before the animals were fed the standard rat chow.

2.3 Animal treatment and experimental design

Fifty pathogen-free, male Wistar rats weighing 290 ± 14 g were obtained from the Primate Unit of Stellenbosch University (Tygerberg Campus, South Africa). The rats were housed individually in stainless steel wired-bottom cages fitted with polypropylene houses in an experimental animal holding facility maintained at a temperature of between 21-24^{o}C, with a

12 h light dark cycle and 50% humidity. The rats were fed standard rat chow (SRC) *ad libitum* and had free access to tap water or the aqueous rooibos extract. The animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health Publication no. 80-23, revised 1978). The study protocol was approved by CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee (Ethics Certificate no: CPUT/HAS-REC 2010/A003). After acclimatization in the experimental animal holding facility for 1 week, the rats were randomized into five groups of 10 animals each, and treated for 28 days as follows: **Group I** (negative control group) and **II** (positive control group), fed SRC and received water as the sole source of drinking fluid. **Group III** (RTE + LPS group), fed SRC and received aqueous rooibos extract (2%, w/v) as the sole source of drinking fluid. **Group IV** (RPO + LPS group) fed SRC and RPO (200 µL) daily and received water as the sole source of drinking fluid. **Group V** (RTE + RPO + LPS group), fed SRC and RPO (200 µL) daily and received aqueous rooibos extract as the sole source of drinking fluid. On the 27th day of the experiment, the rats were injected with either 0.1 mL of PBS vehicle (**Group I**) or 0.1 mL of LPS (0.5 mg/kg bw, i.p., Ohsaki *et al*., 2006), (**Groups II-V**) to induce endotoxemia. **Figure 1** shows the experimental design followed in the study. The general conditions of the rats were monitored daily throughout the study and body weights recorded weekly and at sacrifice. Fluid intake was monitored at intervals of 2 days for the duration of the study period. At the end of the experimental period, fasted animals in all the groups were sacrificed 16 hours after the last LPS injection under sodium pentobarbital anaesthesia (0.15 ml/100g body weight, i.p.). Approximately 8 ml of blood was collected via the abdominal aorta and this was aliquoted into tubes with (EDTA) and without anticoagulant to obtain plasma and serum, respectively. Plasma/serum was separated immediately by centrifugation at 5 000 x g for 5 min at 4^{o}C. The liver was excised, washed twice with ice-cold PBS (10 mM phosphate buffered saline pH 7.2) to remove residual blood, blotted to dry, weighed and immediately snap frozen in liquid nitrogen and stored at -80^{o}C for biochemical analysis.

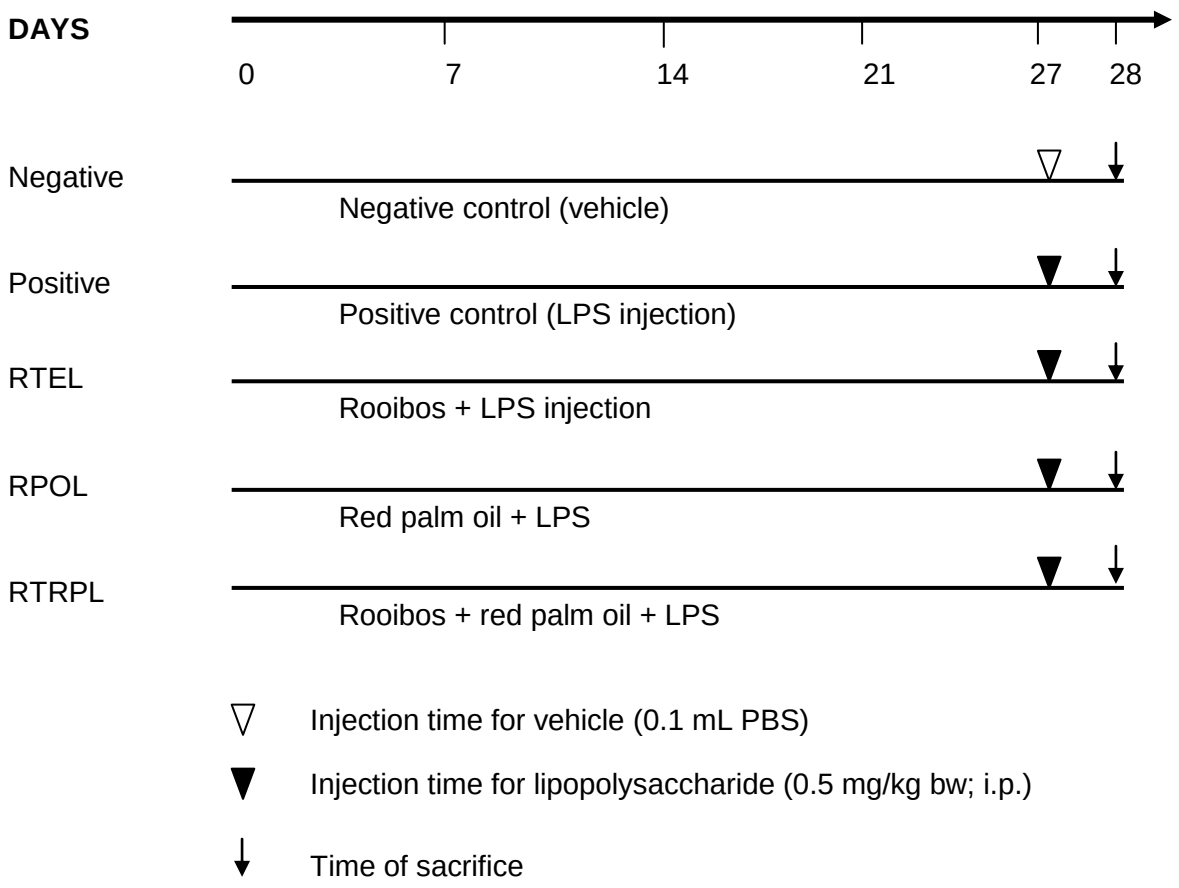

Figure 1: Schematic diagram illustrating the experimental design utilized to investigate the protective effects of rooibos and RPO on LPS-induced hepatic endotoxemia in Wistar rats. LPS (lipopolysaccharide, E coli serotype), RTEL (RTE + LPS), RPOL (RPO + LPS), RTRPL (RTE + RPO + LPS).

2.4 Soluble solids, total polyphenols, flavonol and flavanol content of the aqueous rooibos extract

The soluble solids content of the rooibos extract was determined gravimetrically (twelve repetitions) after drying 1 mL aliquots of the extract at 70^{o}C for 24 hours. The total polyphenol content of the aqueous rooibos extract was determined using the Folin Ciocalteu's phenol reagent according to the method described by Singleton *et al*. (1999) and results expressed as mg gallic acid equivalents/mg soluble solids. The flavanol content of the aqueous rooibos extract was determined colorimetrically at 640 nm using p-dimethylaminocinnamaldehyde (DMACA) according to the method of Treutter (1989). Results were expressed as mg catechin standard equivalents/mg soluble solids. The flavonol/flavones content was determined spectrophotometrically at 360 nm and results were expressed as mg quercetin standard equivalents/mg soluble solids (Mazza *et al*., 1999).

2.5 Total antioxidant capacity of rooibos extract and red palm oil

2.5.1 Oxygen radical absorbance capacity assay

This assay measures the antioxidant capacity of plant and biological samples as a rate of the peroxyl radical-generated decline in the fluorescence of fluorescein. The oxygen radical absorbance capacity (ORAC) of rooibos extract and RPO was determined according to a method described by Ou *et al*. (2001) with some modifications. Briefly, 12 µL of diluted sample or trolox standard was mixed with 138 µL of fluorescein (14 µM) and 50 µL of AAPH (4.8 mM) added, to initiate the free radical attack. Fluorescence (excitation 485, emission 538) was recorded, every 1 min for 2 hr in a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µM Trolox equivalents (TE)/L or µM Trolox equivalents (TE)/g.

2.5.2 Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) of the aqueous rooibos extract and RPO was determined according to the method described by Re *et al*. (1999). This method measures the radical scavenging ability of antioxidants against $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ solution was prepared 24 h before use by mixing ABTS salt (8 mM) with potassium peroxodisulfate (140 mM) and then storing the solution in the dark until the assay could be performed. The $ABTS^{\bullet+}$ solution was diluted with distilled water (1:20) to give an absorbance of 1.50 at 734 nm. Briefly, 25 µL of sample or trolox standard was mixed with 275 µL $ABTS^{\bullet+}$ solution in a 96-well clear plate. The plate was incubated for 30 min at room temperature and the absorbance read at 734 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µM TE/L or µM TE/g.

2.5.3 Ferric ion reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of the rooibos extract and RPO was determined using the method described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 100 mM HCl) and $FeCl_3.6H_2O$ (20 mM) in a ratio of 10:1:1, v/v/v). Briefly, 10 µL of sample or ascorbic acid (AA) standard was added to 300 µL FRAP reagent in a 96-well clear plate. The plate was incubated at room temperature for 30 min, and absorbance read at 593 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µmol AAE/L or µmol AAE/ g.

2.6 High performance liquid chromatography analysis of aqueous rooibos extract

The aqueous rooibos extract was filtered (Whatman no 4) and chromatographically separated on an Agilent Technologies 1200 series HPLC system according to an adapted method described by Bramati *et al*. (2002). The HPLC system consisted of a G1315C diode array and multiple wavelengths detector, a G1311A quaternary pump, a G1329A autosampler, and a G1322A degasser. A 5 µm YMC-Pack Pro C18 (150 mm x 4.6 mm i.d.) column was used for separation and acquisition was set at 287 nm for aspalathin and 360 nm for other components. The mobile phases consisted of water (A) containing 300 µL/L trifluoroacetic acid and methanol (B) containing 300 µL/L trifluoroacetic acid. The gradient elution started at 95% A, changing to 75% A after 5 min and to 20% A after 25 min and back to 95% A after 28 min. The flow rate was set at 0.8 mL/min, the injection volume was 20 µL and the column temperature was set at 23^{o}C. Peaks were identified based on the retention time of the standards and confirmed by comparison of the wavelength scan spectra (set between 210 nm and 400 nm).

2.7 High performance liquid chromatography analysis of RPO

2.7.1 Vitamin E content of RPO

Vitamin E in RPO was determined as isoforms of tocopherol and tocotrienol. Extraction was done by shaking 1 g of RPO in 5 ml of absolute ethanol for 30 min, followed by centrifugation at 5000 g for 10 min. An aliquot of the top vitamin E layer (20 µL) was injected into a chromatographic system (Agilent Technology 1200 series), using an analytical column YMC-Pack Pro C18 (150 x 4.6 mm, i.d.) with the UV-visible wavelength detector set at 296 nm. The mobile phase consist of A (acetonitrile:methanol:isopropanol:water; 45:45:5:5, v/v) and B (acetonitrile:methanol:isopropanol; 50:45:5, v/v) and elution was carried out at a flow rate of 1mL/min. Mobile phase A was programmed to B within 10 min and this condition maintained for another 15 min before returning to the original condition. The content of tocopherols and tocotrienols were quantified by comparing the retention time and/or peak area with standards (Iqbal *et al*., 2007).

2.7.2 Carotenoids content of RPO

Carotenoids from RPO were extracted with tetrahydrofuran:dichloromethane (1:1, v/v) and analysed on an Agilent Technology 1200 series HPLC with the visible detector set at 450 nm according to a modified method of Rautenbach *et al*. (2010). Twenty microlitre of extracted samples were injected automatically into the column (YMC-Pack Pro C30, 250 x 4.6 mm i.d., room temperature) and isocratic elution performed on a mobile phase consisting of

methanol:acetone (9:1, v/v) with flow rate set at 1 mL/min. Peaks were identified based on the retention time of the α- and β-carotene standards.

2.8 Preparation of liver homogenates

The liver tissue was homogenized on ice in 10 volumes of 50 mM phosphate buffer containing 1 mM EDTA and 0.5% Triton-X (pH 7.5). The homogenate was transferred into tubes and centrifuged at 10 000 g for 10 min at 4^{o}C. The supernatant was collected, divided into aliquots, and stored at -80^{o}C until used for analyses of antioxidant capacities, lipid peroxidation, activity of antioxidant enzymes and glutathione status. Protein content of samples (erythrocyte and liver homogenate) was determined using the BCA protein assay kit supplied by Pierce (Illinois, USA).

2.9 Antioxidant capacity of plasma and liver samples

To avoid protein interference in antioxidant capacity assays, sub-samples of plasma and liver homogenates were precipitated with 0.5 M perchloric acid (1:1, v/v) and centrifuged at 10000 g for 10 min at 4^{o}C. Supernatants were collected as protein free fractions (Robles-Sanchez *et al*., 2011). Plasma total polypnenol, as well as ORAC, TEAC and FRAP assays (plasma and liver) were performed as previously described for the rooibos extract and RPO in section 2.5 (Benzie and Strain, 1996; Re *et al*., 1999; Singleton *et al*., 1999; Ou *et al*., 2001).

2.10 Liver function tests

Serum alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) were analysed using a Medica EasyRA automated clinical chemistry analyser (Medica Corporation Bedford, Mass., USA) and standard diagnostic kits (Medica Corporation Bedford, Mass., USA).

2.11 Oxidative stress and antioxidant status biomarkers

2.11.1 Plasma and hepatic lipid peroxidation

Lipid peroxidation was assessed by measurement of conjugated dienes (CDs) and malondialdehyde (MDA). Plasma and liver MDA were assayed as MDA-TBA adducts using HPLC with a UV-visible detector according to a method of Khoschsorur *et al*. (2000). Conjugated dienes were estimated according to the method of Recknagel and Glende (1984). After the initial extraction of the lipid content from the plasma and liver homogenates, the lipid residues were dissolved in cyclohexane and CDs were measured

spectrophotometrically at 234 nm and results expressed as nmol/L or nmol/g tissue in plasma and liver respectively.

2.11.2 Hepatic and erythrocyte antioxidant enzymes activity

Catalase (CAT) activity in the erythrocyte and liver homogenates were determined according to the method described by Aebi (1984), in which the rate of decomposition of hydrogen peroxide was measured at 240 nm. The activity of catalase was calculated using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ and results expressed as µmole H_2O_2 consumed/min/µg protein. The activity of superoxide dismutase (SOD) was determined according to the method of Crosti *et al*. (1987), and SOD activity expressed as U/mg protein. Glutathione peroxidase (GPx) activity was determined according to the method of Ellerby and Bredesen (2000). The activity of GPx was calculated using the mmolar extinction coefficient of 6.22 and results expressed as nmol NADPH oxidized per min per µg protein. *G*lutathione reductase (GR) was assayed by a method of Staal *et al*. (1969) and result expressed as µmol NADPH oxidized per min per µg protein using the mmolar extinction coefficient of 6.22.

2.11.3 Hepatic and whole blood glutathione redox status

The total glutathione (GSH and GSSG) was measured according to the method described by Asensi *et al*. (1999). Aliquot of whole blood without (GSH) or with 3 mM freshly prepared M2VP (GSSG) was precipitated with 5% (w/v) metaphosphoric acid (MPA), while liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 10,000g for 10 min, 50 µl of supernatant (from whole blood or liver homogenate) was added to 50 µL of glutathione reductase (1U) and 50µL of 0.3mM DTNB. The reaction was initiated by addition of 1 mM NADPH to a final volume of 200 µL. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards. GSH concentration was calculated as the difference between total glutathione and 2GSSG.

2.12 Multiplex cytokine Analysis

Sub samples of livers were homogenized in 10 volumes of phosphate buffered saline (10 mM PBS, pH 7.2) and centrifuged twice at 15000 x g for 15 min at 4^{o}C. The level of 4 inflammatory markers TNF-α, IL-1β, IL-6 and IL-10 were determined in these homogenates using customized Milliplex kits (Merck Millipore, St Charles, Missouri, USA), on the Bio Plex platform (Bio Plex^{TM}, Bio Rad, Laboratories, Hercules, USA) following the manufacturer's instructions. Following previous optimizations, homogenates were assayed undiluted, in a

blinded manner. All analyte levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all the analytes ranged from 3.2-10000 pg/ml. The analyses of the bead median fluorescence intensities were done using the Bio-Plex Manager software (version 4.1.1).

2.13 Statistical analysis

Values were expressed as mean ± SD. Data were tested for normality using the Kolmogorov–Smirnof test and Levene's Test for Equality of variances. Differences between group means were estimated using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for all pairwise comparisons. The Kruskal-Wallis test, a non-parametric analogue to the one-way ANOVA was used to test for group differences when data was not normally distributed. Results were considered statistically significant at $P<0.05$, or marginally significant at $P<0.1$. All the statistics were carried out using MedCalc v 12.2.1 software (MedCalc software bvba, Mariakerke, Belgium).

3. Results

3.1 Phenolic and carotenoid content and antioxidant capacity of the plant materials

Table 1 shows the results for the phenolic content and *in vitro* antioxidant capacity of the rooibos extract. Total phenolic content is 0.372 mg GAE/mg SS of which the flavonols and flavanols account for 45 and 17%, respectively. The antioxidant capacity of the rooibos extract determined as ORAC, FRAP and TEAC values are also shown in Table 1. **Figure 2** shows the HPLC profile of the aqueous rooibos extract, with aspalathin (29.98 ± 0.08 µg/mL) (287 nm) as the most abundant flavonoid. Other major flavonoids include iso-orientin (25.98 ± 0.52 µg/mL), orientin (18.61 ± 18.61 µg/mL), hyperoside/rutin (14.55 ± 2.26 µg/mL), vitexin (6.07 ± 1.05 µg/mL), and iso-vitexin (7.18 ± 0.20 µg/mL). Minor peaks corresponding to chrysoeriol, luteolin and quercetin are also shown on the chromatogram **(Figure 3)**. The vitamin E, carotenoid content and *in vitro* antioxidant capacity of the RPO used in this study are presented in **Table 2**. The total vitamin E content of the RPO is 484.48 µg/g of which the tocotrienols accounts for 80%. The total carotene content is 53.08 µg/g, and β-carotene accounts for 55%.

Table 1: Phenolic contents and antioxidant capacity of the aqueous rooibos extract

Constituents	Concentration
Soluble solids (mg/mL)	2.85 ± 0.39
Total polyphenol (mg GAE/mL)	1.06 ± 0.00
Flavonol (mg QE/ mL)	0.48 ± 0.01
Flavanol (mg CE/mL)	0.19 ± 0.01
Aspalathin (µg/mL)	29.98 ± 0.08
Orientin (µg/mL)	18.61 ± 0.13
Iso-orientin (µg/mL)	25.98 ± 0.52
Vitexin (µg/mL)	6.07 ± 1.05
Iso-vitexin (µg/mL)	7.18 ± 0.20
Hyperoside/rutin (µg/mL)	14.55 ± 2.26
Quercetin (µg/mL)	0.89 ± 0.18
Luteolin (µg/mL)	0.22 ± 0.06
Chrysoeriol (µg/mL)	0.25 ± 0.00
ORAC (µmol AAE/mL)	1.69 ± 0.03
FRAP (µmol TE/mL)	5.20 ± 0.04
TEAC (µmol TE/mL)	4.79 ± 0.33

Values are mean ± SD. Soluble solid is a mean of 12 determinations while other parameters are mean of 5 determinations. AAE (ascorbic acid equivalent), CE (catechin equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent), TE (trolox equivalent).

Table 2: Phytochemical constituents and antioxidant capacity of the red palm oil used in the study

Constituents	Concentration
α-tocopherol (µg/g)	71.28 ± 2.30
β/γ-tocopherol(µg/g)	6.20 ± 0.94
δ-tocopherol (µg/g)	20.70 ± 1.66
α-tocotrienol (µg/g)	102.36 ± 1.52
β/γ-tocotrienol (µg/g)	227.48 ± 2.74
δ-tocotrienol (µg/g)	56.46 ± 1.55
α-carotene (µg/g)	23.74 ± 1.16
β-carotene (µg/g)	29.34 ± 2.92
Total polyphenol (mg GAE/100g)	0.17 ± 0.00
ORAC (µmol TE/100g)	175.00 ± 15.19
FRAP (µmol AAE/100g)	12.30 ± 0.28
TEAC (µmol TE/100g)	0.16 ± 0.00
SFA (%)	51
MUFA (%)	38
PUFA (%)	11

Values are mean ± SD for (n=5). AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent) SFA (saturated fatty acid), MUFA (mono-unsaturated fatty acid) PUFA (poly-unsaturated fatty acid).

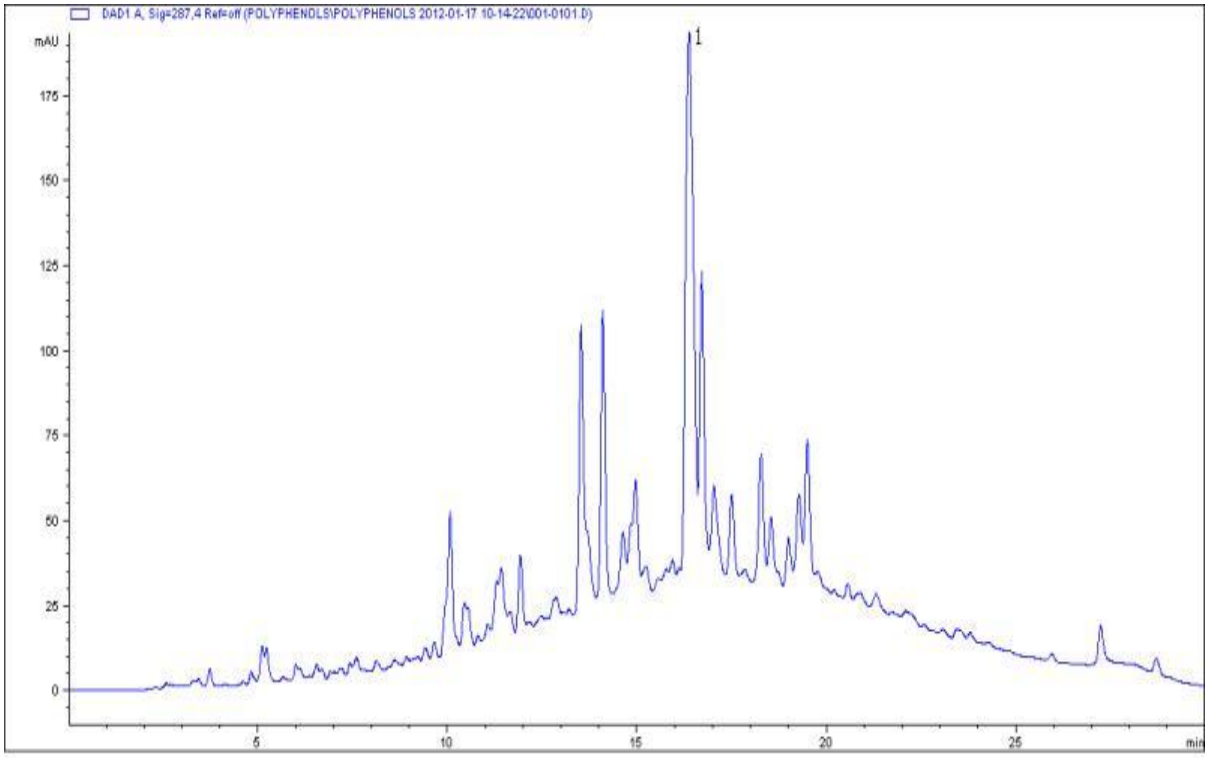

Figure 2: HPLC chromatogram (287 nm) of aqueous rooibos extract used showing peak 1 for aspalathin.

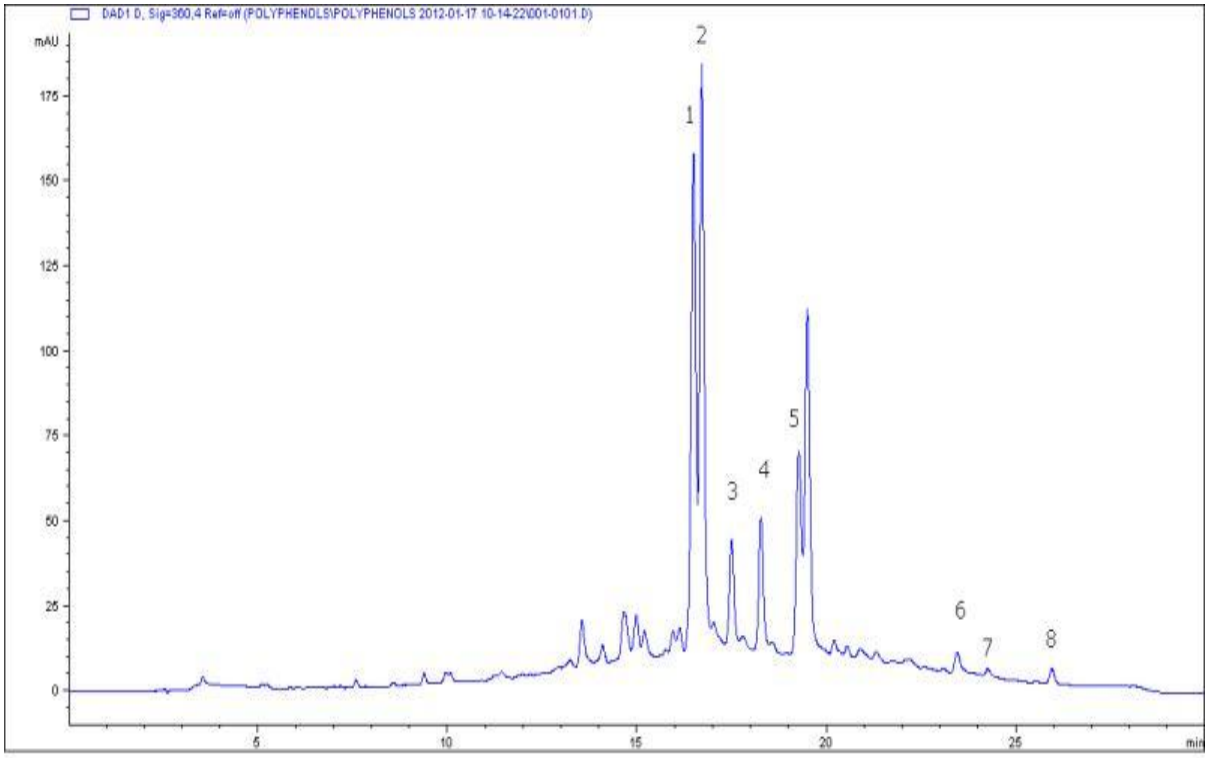

Figure 3: HPLC chromatogram (360 nm) of aqueous rooibos extract showing peaks for other flavonoids. 1, orientin; 2, iso-orientin; 3, vitexin; 4, isovitexin; 5, hyperoside/rutin; 6, quercetin; 7, luteolin; 8, chrysoeriol.

3.2 Daily fluid, phenolic, carotenoid and vitamin E intakes

The daily fluid, phenolic, carotenoid and vitamin E intakes of rats consuming the aqueous rooibos extract and/or RPO are presented in **Table 3**. There are no significant ($P>0.05$) differences in the daily water consumption between the positive control and negative control rats, and also between the LPS-treated rats consuming RPO and the positive control rats. Daily rooibos, total phenolic, flavonol and flavanol intakes did not differ between the two groups (RTE + LPS vs RTE + RPO + LPS) consuming rooibos. Tocopherol, tocotrienol, as well as α- and β-carotene contents, were significantly ($P<0.05$) higher in LPS-treated rats consuming RPO alone, compared to LPS-treated rats consuming the combination.

3.3 Body and liver weight change

Consuming rooibos, RPO or their combination, without LPS treatment did not have any adverse effect on the body weight gains, liver weights and relative liver weights of the rats when compared with the negative control rats (data not shown). The effects of consumption of rooibos, RPO or their combination, on the body weight gain, liver weight and relative liver weight in all experimental rats is shown in **Table 4**. No mortality was recorded in the LPS-challenged groups within the 16 hours before sacrifice. Intraperitoneal injection of LPS with or without rooibos, RPO or their combination, did not have any adverse effects on the body weight gain, liver weight or relative liver weight of the rats when compared to the negative control rats.

Table 3: Daily phenolic, vitamin E and carotenoid intakes of the various rat groups consuming aqueous rooibos extract and RPO

Treatment	Water/Rooibos intake/day/ 100 g BW (mL)	Total phenolic intake (mg GAE/ day/100 g BW)	Flavonol intake (mg QE/ day/ 100 g BW)	Flavanol intake (mg CE/day/ 100 g BW)	Tocopherol intake/day (µg/100g BW)	Tocotrienol intake/day (µg/100g BW)	α-Carotene intake/day (µg/100g BW)	β-Carotene intake/day (µg/100g BW)
Negative control	10.44 ± 1.06	ND	ND	ND	ND	ND	ND	ND
Positive control (LPS)	10.68 ± 0.76	ND	ND	ND	ND	ND	ND	ND
RTE + LPS	9.92 ± 0.49	10.51 ± 0.52	4.23 ± 0.21	1.60 ± 0.08	ND	ND	ND	ND
RPO + LPS	10.75 ± 0.82	ND	ND	ND	4.63 ± 0.24	18.20 ± 0.94	1.11 ± 0.06	1.38 ± 0.07
RTE + RPO + LPS	9.62 ± 0.63	10.18 ± 0.67	4.10 ± 0.27	1.55 ± 0.10	4.37 ± 0.20†	17.21 ± 0.78†	1.06 ± 0.05†	1.30 ± 0.06†

Calculations of the total phenolic, flavonol and flavanol intakes were calculated based on the soluble solid intake obtained from the average rooibos consumption per day. Values are mean ± SD (n=10). †Significantly different from RPO + LPS group at $P<0.05$. ND (not determined), BW (body weight), CE (catechin equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent), LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

Table 4: Effects of rooibos and RPO consumption on body weight gain, liver weight, and relative liver weight in all experimental rats

Treatment	Body weight gain (g)	Liver weight (g)	Relative liver weight (%)
Negative control	89.21 ± 17.31	11.83 ± 0.82	3.15 ± 0.22
Positive control (LPS)	91.19 ± 11.57	12.36 ± 1.25	3.36 ± 0.43
RTE + LPS	91.61 ± 6.20	12.64 ± 0.65	3.30 ± 0.14
RPO + LPS	89.94 ± 7.93	12.06 ± 1.44	3.23 ± 0.27
RTE + RPO + LPS	91.22 ± 7.26	12.46 ± 1.36	3.17 ± 0.30

Values are mean ± SD (n=10). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

3.4 Serum levels of liver function enzymes

Table 5 shows the effect of consumption of rooibos, RPO or their combination on serum levels of liver function marker enzymes in all experimental rats. There was a significant elevation ($P<0.05$) in the serum levels of ALT, AST and LDH with the LPS challenge when compared to the negative control, resulting in a 56, 82 and 50% increase, respectively. Consumption of the rooibos extract (without LPS challenge) did not show any negative effect on the liver function marker enzymes, while RPO consumption (without LPS challenge) significantly ($P<0.05$) increased the AST level (refer to addendum A). Consumption of the rooibos extract alone with the LPS challenge, marginally ($P<0.1$) reduced the levels of ALT when compared to the positive control, while consumption of RPO either alone, or combined with rooibos in LPS-challenged rats, significantly ($P<0.05$) reduced the ALT levels, also when compared to the positive control. Consumption of either the rooibos extract or RPO, (with LPS challenge) marginally reduced the levels of serum AST when compared to the positive control. However, when both extracts were consumed together in LPS-challenged rats, a significant ($P<0.05$) reduction in the serum levels of AST was noted. Serum LDH levels were significantly ($P<0.05$) lowered in LPS-challenged rats when rooibos and RPO were given either alone or combined.

Table 5: Effects of rooibos and/or red palm oil supplementation on liver function marker enzymes in all experimental rats

Treatments	ALT (U/L)	AST (U/L)	LDH (U/L)
Negative control	105.69 ± 28.93	88.98 ± 12.53	234.92 ± 74.55
Positive control (LPS)	165.29 ± 39.45*	162.15 ± 38.18*	351.36 ± 81.64*
RTE + LPS	133.95 ± 39.60$^{\#\#}$	132.09 ± 39.14$^{\#\#}$	252.67 ± 76.62$^{\#}$
RPO + LPS	123.09 ± 53.26$^{\#}$	132.01 ± 25.47$^{\#\#}$	250.06 ± 89.34$^{\#}$
RTE + RPO + LPS	90.27 ± 19.71$^{\#}$	115.14 ± 32.89$^{\#}$	246.88 ± 29.31$^{\#}$

Values are mean ± SD (n=7-10) *Significantly different from negative control (P<0.05). **Marginally different from negative control (P<0.1). $^{\#}$Significantly different from positive control (P<0.05). $^{\#\#}$Marginally different from positive control (P<0.1). ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

3.5 Plasma and liver antioxidant capacity

The antioxidant capacity of plasma and liver homogenates were assessed as total polyphenol content, FRAP, TEAC and ORAC values (**Table 6**). LPS injection significantly (P<0.05) reduced the plasma total polyphenol content, with rooibos supplementation able to reverse this reduction to a level comparable to that of the negative control. Consumption of RPO either alone or in combination with rooibos, did not show any improved effect on the plasma total polyphenol content in LPS-challenged rats. The plasma ORAC of rats challenged with LPS was not significantly different (P>0.05) from that of the negative control rats, regardless of whether the animals were taking rooibos, RPO and/or their combination or not. Also none of the treatments showed any significant effect on the plasma FRAP and TEAC when compared with the negative control. When considering the liver, the antioxidant capacity (FRAP, TEAC and ORAC values) was not significantly (P>0.05) altered with the LPS challenge while supplementation with rooibos, RPO and/or their combination showed a similar trend.

Table 6: Effects of aqueous rooibos, RPO and/or their combination on total polyphenol and antioxidant capacity in plasma and liver of all experimental rats

	Plasma				Liver		
Treatment	**Total polyphenol (mg GAE/L)**	**ORAC (µmol TE/L)**	**TEAC (µmol TE/L)**	**FRAP (µmol AAE/L)**	**ORAC (µmol TE/g tissue)**	**TEAC (µmol TE/g Tissue)**	**FRAP (µmol AAE/g tissue)**
Negative control	96.7 ± 9.5	1792 ± 167	7505 ± 362	125.8 ± 14.5	19.1 ± 4.3	54.7 ± 3.7	2.9 ± 0.3
Positive control (LPS)	73.4 ± 6.8*	1499 ± 375	7294 ± 780	124.4 ± 27.1	17.4 ± 4.1	55.9 ± 3.2	2.6 ± 0.1
RTE + LPS	85.7 ± 12.2$^{\#}$	1708 ± 529	7359 ± 484	129.6 ± 16.7	18.5 ± 4.5	56.5 ± 6.2	2.7 ± 0.2
RPO + LPS	74.7 ± 4.2	1567 ± 265	7279 ± 424	148.9 ± 33.7	17.7 ± 4.5	57.2 ± 3.3	2.9 ± 0.2
RTE + RPO + LPS	79.8 ± 6.8	1739 ± 326	7499 ± 185	127.7 ± 15.4	17.5 ± 3.0	57.1 ± 5.7	2.8 ± 0.2

Values are mean ± SD (n=7-10) *Significantly different from negative control (P<0.05). $^{\#}$Significantly different from positive control (P<0.05). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil). AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent). FRAP (ferric reducing ability of plasma), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity).

Table 7: Effects of aqueous rooibos extract, RPO and/or their combination on antioxidant enzymes activities in erythrocyte and liver of all experimental rats

	Erythrocytes				Liver			
Treatment	**CAT**	**GR**	**SOD**	**GPx**	**CAT**	**GR**	**SOD**	**GPx**
Negative control	0.21 ± 0.05	0.15 ± 0.01	22.35 ± 3.82	0.18 ± 0.03	0.11 ± 0.01	3.99 ± 1.12	55.01 ± 5.27	0.16 ± 0.02
Positive control (LPS)	0.22 ± 0.02	0.09 ± 0.04*	22.25 ± 3.25	0.14 ± 0.02*	0.23 ± 0.03*	2.69 ± 0.62**	42.06 ± 6.26*	0.12 ± 0.02*
RTE + LPS	0.20 ± 0.04	0.12 ± 0.05*	19.24 ± 1.72	0.16 ± 0.03$^{\#\#}$	0.16 ± 0.03$^{\#}$	3.37 ± 0.79	57.62 ± 6.92$^{\#}$	0.15 ± 0.01$^{\#}$
RPO + LPS	0.21 ± 0.03	0.11 ± 0.03*	18.42 ± 1.81	0.13 ± 0.02*	0.18 ± 0.02$^{\#}$	3.31 ± 1.56	46.62 ± 7.09*	0.15 ± 0.03$^{\#}$
RTE + RPO + LPS	0.23 ± 0.03	0.11 ± 0.03*	23.16 ± 2.71	0.17 ± 0.02$^{\#\#}$	0.20 ± 0.02$^{\#}$	3.66 ± 1.49$^{\#\#}$	52.60 ± 7.22$^{\#}$	0.17 ± 0.03$^{\#}$

Values in columns are mean ± SD (n=7-10). *Significantly different from negative control (P<0.05). **Marginally different from negative control (P<0.1). $^{\#}$Significantly different from positive control (P<0.05). $^{\#\#}$Marginally different from positive control (P<0.1). CAT (catalase, µmol H_2O_2 consumed/min/µg protein), GR (glutathione reductase, µmol NADPH oxidized/min/µg protein), SOD (superoxide dismutase, U/mg protein), GPx (glutathione peroxidase, nmol NADPH oxidized/min/µg protein). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

3.6 Erythrocyte and hepatic antioxidant enzymes

The results of consuming the rooibos extract, RPO and/or their combination on the antioxidant enzyme activities in the erythrocytes and liver are shown in **Table 7**. In the erythrocytes, the LPS challenge resulted in a significant ($P<0.05$) decrease in GR and GPx enzyme activities when compared to the negative control, with no effect on CAT and SOD activities, while none of the plant extracts could alleviate this induced-decrease.

In the liver, a significant ($P<0.05$) increase in CAT activity was observed in the LPS-treated (positive control) rats, when compared to negative control rats. Consuming rooibos, RPO or their combination, significantly ($P<0.05$) decreased the observed LPS-induced increase in CAT activity. The activities of SOD and GPx were significantly ($P<0.05$) reduced in LPS-treated rats when comparing to negative control rats, while that of GR was marginally ($P<0.1$) reduced. LPS-treated rats consuming a combination of the rooibos extract and RPO, marginally ($P<0.01$) restored the decrease in GR when compared with positive control rats. Consuming rooibos either alone or together with RPO significantly ($P<0.05$) improved the reduction in SOD activity induced by LPS. Also consuming rooibos, RPO or their combination, with the LPS challenge, significantly increased the GPx activity when compared to the positive control group.

3.7 Plasma and hepatic lipid peroxidation

Table 8 shows the effect of supplementation of the rooibos extract, RPO and/or their combination on plasma and hepatic lipid peroxidation in all experimental rats. Plasma CDs remained unchanged in LPS-challenged rats when compared to negative control rats. The combined consumption of rooibos and RPO with LPS challenge, significantly ($P<0.05$) increased the CDs levels when compared with positive control rats. When considering the plasma MDA levels, LPS challenge, significantly ($P<0.05$) elevated the levels by 22% when compared to negative control rats, while treatment with rooibos or RPO significantly ($P<0.05$) decreased the MDA levels to that of the negative control rats. The combined consumption of rooibos and RPO by the LPS-challenged rats, caused a further decrease in the MDA levels to values that were significantly lower ($P<0.05$) than those of the negative control rats.

When considering the liver, hepatic CD and MDA levels were both significantly ($P<0.05$) elevated (23 and 43%, respectively) in LPS-challenged rats compared with negative control rats. Feeding rooibos, RPO and/or their combination to LPS-challenged rats, significantly ($P<0.05$) lowered the increase in CDs by 7, 13 and 12%, respectively and for MDA by 22, 14 and 28%, respectively (Table 8).

Table 8: Effects of aqueous rooibos extract, RPO and/or their combination on markers of lipid peroxidation in the plasma and liver of all experimental rats.

	Plasma		Liver	
Treatment	**CD (nmol/L)**	**MDA (µmol/L)**	**CD nmol/g tissue)**	**MDA (µmol/g tissue)**
Negative control	85.79 ± 5.81	1.96 ± 0.21	10.38 ± 1.12	64.83 ± 5.46
Positive control (LPS)	88.40 ± 8.56	2.40 ± 0.20*	12.78 ± 0.79*	92.65 ± 7.57*
RTE + LPS	85.11 ± 8.17	1.76 ± 0.32$^{\#}$	11.84 ± 0.47$^{\#}$	72.27 ± 7.79$^{\#}$
RPO + LPS	92.18 ± 6.34	1.61 ± 0.38$^{\#}$	11.12 ± 0.34$^{\#}$	79.03 ± 13.91$^{\#}$
RTE + RPO + LPS	117.60 ± 7.04$^{\#}$	1.27 ± 0.22$^{\#}$	11.20 ± 0.42$^{\#}$	66.56 ± 5.79$^{\#}$

Values in columns are mean ± SD (n=8-10). *Significantly different from negative control (P<0.05). $^{\#}$Significantly different from positive control (P<0.05). CD (conjugated dienes), MDA (malondialdehyde). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

3.8 Whole blood and hepatic glutathione status

The glutathione status across all treatment groups is presented in **Table 9**. In the blood, the LPS challenge resulted in a significantly (P<0.05) increased oxidized glutathione (GSSG) level by 18%, while depleting the reduced glutathione (GSH) level as well as the GSH/GSSG ratio by 34 and 39%, respectively, when compared with the negative control rats. Supplementation with rooibos or RPO to LPS-challenged rats restored the GSH levels comparable to those of the negative control rats, while not showing any effect on GSSG levels. Consuming rooibos and RPO together in LPS-challenged rats, significantly increased (P<0.05) GSSG levels when compared to that of the positive control rats. Feeding rooibos alone or in combination with RPO augmented the GSH/GSSG ratio to values statistically (P>0.05) similar to those of the negative control rats.

Although the hepatic GSH levels were not affected by the LPS challenge or any of the other supplementation regimens when compared with the negative control, hepatic GSSG levels were significantly (P<0.05) elevated. Rooibos, RPO and/or their combination when consumed by the LPS-challenged rats, significantly (P<0.05) lowered the observed increase, to values comparable to that of negative control rats. AS a result, a significant (P<0.05) decrease in hepatic GSH/GSSG ratios were shown when compared to the negative control rats. Feeding rooibos alone, or together with RPO resulted in an improvement of the GSH/GSSG ratio to values that are similar to those found in the negative control.

Table 9: Effects of aqueous rooibos extract, RPO and/or their combination on glutathione status in whole blood and liver of all experimental rats.

	Whole blood			Liver		
Treatment	GSH (µmol/L)	GSSG (µmol/L)	GSH:GSSG ratio	GSH (µmol/g wet liver	GSSG (µmol/g wet liver)	GSH:GSSG ratio
Negative control	906.11 ± 173.54	173.47 ± 34.25	4.76 ± 1.22	6.38 ± 0.58	0.32 ± 0.10	20.16 ± 6.77
Positive control (LPS)	596.04 ± 135.59*	205.29 ± 8.69*	2.90 ± 0.65*	6.37 ± 0.83	0.44 ± 0.09*	14.02 ± 2.95*
RTE + LPS	862.60 ± 112.25#	202.92 ± 17.52	4.27 ± 0.59#	7.41 ± 0.75	0.35 ± 0.08#	21.98 ± 5.34#
RPO + LPS	766.47 ± 159.70#	211.84 ± 10.52	3.57 ± 0.79	6.20 ± 0.97	0.37 ± 0.09#	17.27 ± 3.92
RTE + RPO + LPS	829.58 ± 113.47#	232.69 ± 30.82#	4.02 ± 0.85#	6.72 ± 0.81	0.30 ± 0.04#	22.83 ± 3.50#

Values are mean ± SD of 8-10 rats per group. GSH (reduced glutathione), GSSG (oxidized glutathione). *Significantly different from negative control ($P<0.05$). #Significantly different from positive control ($P<0.05$). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

3.9 Hepatic cytokine levels

Figures 4-7 show the effect of rooibos, RPO and/or their combination on hepatic cytokine levels in experimental rats. A marginally significant (P<0.1) increase in TNF-α levels was observed in the livers of LPS-treated rats compared with negative control rats **(Fig. 4)**. Consuming rooibos, RPO and/or their combination, significantly (P<0.05) reduced the LPS-induced elevation in TNF-α to levels comparable to those observed in the negative control rats. LPS challenge also significantly (P<0.05) increased the levels of other pro-inflammatory cytokines, IL-1β **(Figure 5)** and IL-6 **(Figure 6)** when compared to the negative controls. Supplementation with rooibos, RPO and/or their combination was able to lower the LPS-induced increase in IL-6 levels to that of the negative control rats, while no effect was seen in the IL-1β levels of these rats that consumed the plant extracts. The hepatic levels of the anti-inflammatory cytokine IL-10 **(Figure 7)** was similar across all treatment groups, including the positive and negative control groups as well.

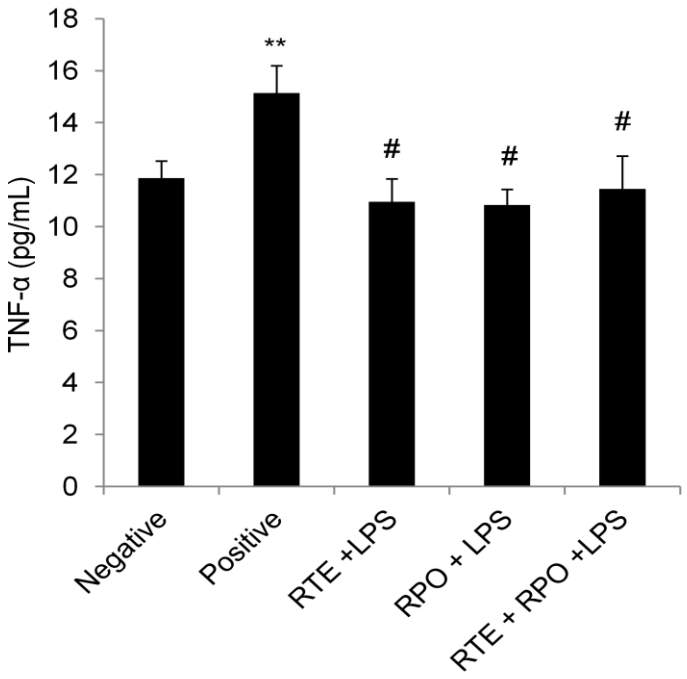

Figure 4: Effect of rooibos, RPO or their combination on LPS-induced TNF-α secretion in the liver. Bars represent mean ± SD (n=7-10). [**]Marginally different from negative control at P<0.1. [#]Significantly different from positive control at P<0.05. LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

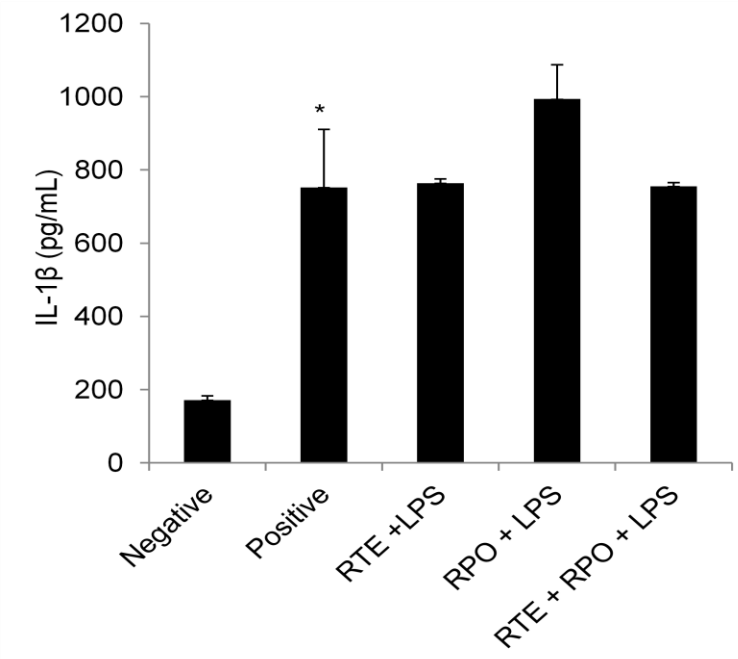

Figure 5: Effect of rooibos, RPO or their combination on LPS-induced IL-1β secretion in the liver. Bars represent mean ± SD (n=7-10). *Significantly different from negative control at P<0.05. LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

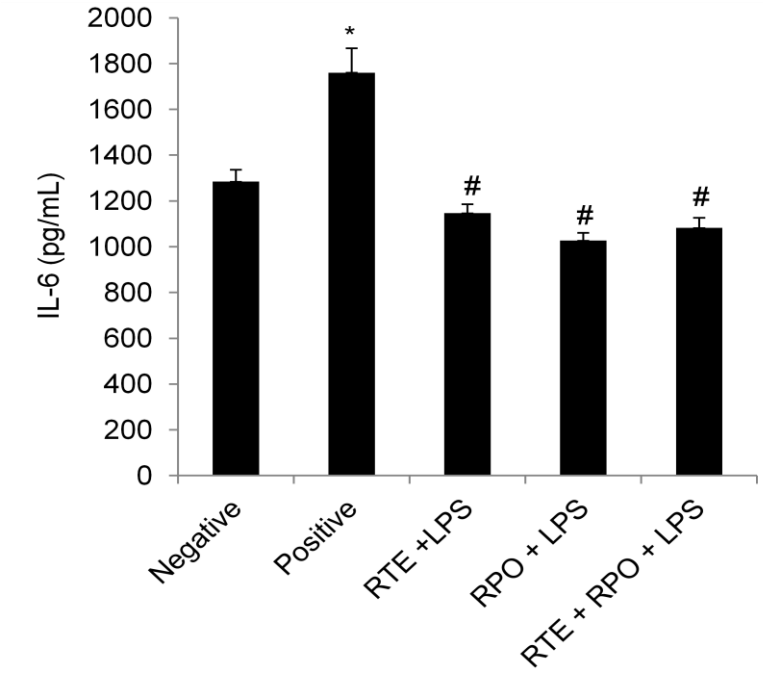

Figure 6: Effect of rooibos, RPO and/or their combination on LPS-induced IL-6 secretion in the liver. Bars represent mean ± SD (n=7-10). *Significantly different from negative control at P<0.05. #Significantly different from positive control at P<0.05. LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

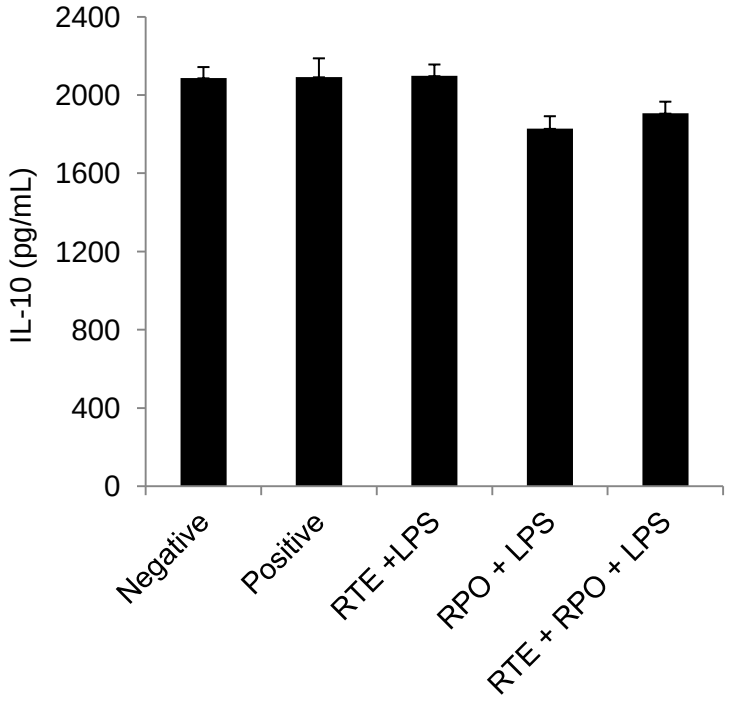

Figure 7: Effect of rooibos, RPO or their combination on LPS-induced IL-10 secretion in the liver. Bars represent mean ± SD (n=7-10). LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).

4. Discussion

The focus of this study was to investigate the acute effects of LPS-induced hepatic oxidative stress and inflammatory responses, and the possible protection offered by oral administration of an aqueous rooibos extract, RPO, or a combination of the two plant extracts. The liver is a target organ for most xenobiotics, because of its role in metabolism and detoxification. It plays an important and central role in the regulation of entry and metabolism of LPS upon exposure. The binding of LPS to CD14/LPS-binding protein and Toll-like receptor-4 (TLR4) on the surface of Kupffer cells, results in the release of chemical mediators such as superoxide, nitric oxide and the pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Lu *et al*., 2005; Cheng *et al*., 2006). Mostly, the hepatic and systemic toxicities of LPS have been attributed to the formation of these pro-inflammatory cytokines and reactive oxygen species (ROS) such as superoxide and NO. Apart from the excessive production of ROS as a result of the activation of Kupffer cells, migration of polymorphonuclear leukocytes (PMNs) into the liver also constitutes another source of ROS (Kaur *et al*., 2006a). Oxidative stress is a well-known mechanism of LPS-induced hepatic injury, and the redox imbalance produced may result in depletion of endogenous antioxidants such as the antioxidant enzymes and alteration of GSH redox status. Thus, augmenting the antioxidant defense system becomes imperative, especially during infections or periods of chronic oxidative insult. Whole extracts

or isolated compounds from plants are popular applications to reverse and/or prevent hepatotoxicity and oxidative stress produced by noxious agents, such as LPS and these beneficial effects have been attributed to their antioxidant and anti-inflammatory properties (Pilkhwal *et al*., 2010; Sharifudin *et al*., 2012).

In this study, it was observed that a single injection of LPS did not have a negative effect on the body weight gain, absolute liver weight and relative liver weight of the rats. However, LPS injection resulted in hepatic injury as indicated by an elevation in the levels of serum aminotransferases (ALT and AST) and lactate dehydrogenase (LDH), which are all circulating markers of hepatocyte injury. The hepatic function marker enzymes are cytoplasmic in nature but are usually leaked into circulation when liver damage occurs due to an alteration in membrane permeability (Hassan *et al*., 2012; Pari and Shagirtha, 2012). This observation is in accordance with several earlier reports which have shown that LPS induces hepatic damage, and as a consequence, increases the level of serum aminotransferases (Kaur *et al*., 2006a; 2006b; Kao *et al*., 2009; Sebai *et al*., 2010; Abdel-Salam *et al*., 2012). Results from the current study showed, that supplementation of rooibos and RPO in combination for 4 weeks prior to the LPS challenge, reversed the damage to the liver. Previous reports have demonstrated the hepatoprotective ability of some polyphenol-rich plant extracts such as *Artemisia absinthium* (Amat *et al*., 2010), *Salvia plebeia* (Qu *et al*., 2009), *Eucalyptus globulus* (Sugimoto *et al*., 2011) and *Hibiscus sabdariffa* (Kao *et al*., 2009), as well as isolated compounds such as curcumin (Kaur et al., 2006a; 2006b), resveratrol (Sebai *et al*., 2010), vitamin E (Bharrhan *et al*., 2010) and α- and γ-tocopherol (Chung *et al*., 2010) in ameliorating LPS-induced hepatic injury. In our study, prior supplementation of rooibos or RPO could not effectively attenuate liver dysfunction induced by LPS, which may be due to the relative short duration of supplementation. However, the protection observed when both were fed together may indicate an additive interaction between the phytoconstituents of each extract, leading to the maintenance of the integrity of the hepatocyte membrane.

Results from a previous study have shown activation of Kupffer cells during endotoxemic episodes to result in the secretion of a wide variety of cytokines, including TNF-α, IL-1β and IL-6 (Bharrhan *et al*., 2010). Up-regulation of cytokine production during LPS-induced endotoxemia is a well-known phenomenon, and evidence has shown that increased levels of pro-inflammatory cytokines from neutrophils in the liver were associated with liver cell damage (Hsieh *et al*., 2008). Tumour necrosis factor-α and IL-6 are two key cytokines involved in tissue damage during sepsis, although some reports have suggested that TNF-α is the central proximal mediator which regulates other subsequent events (Jaeschke, 2000;

Enomoto *et al*., 2003; Hagar, 2009). TNF-α is directly cytotoxic to the hepatocytes, activating NFκB of hepatocytes, Kupffer cells and endotheliocyte, to up-regulate the expression of chemokines such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and further worsening inflammatory injury to hepatocytes (Yao and Yue, 2005; Qu *et al*., 2009). In the present study, LPS induced a marginal increase in hepatic TNF-α, while significantly increasing the level of IL-1β and IL-6. Feeding rooibos, RPO and/or their combination for 4 weeks prior to LPS challenge, was able to reverse the increase in TNF-α and IL-6 observed in the liver to a level that is comparable to what was seen in untreated control animals. However no reversal was seen in the level of IL-1β with any of the plant extract supplementations. Although Hendricks and Pool (2010), in an *in vitro* study reported that rooibos tea induced a higher IL-6 and a lower IL-10 when added to endotoxin-stimulated white blood cells, literature supporting the inhibition of cytokines by rooibos, RPO and/or their combination does not exist. Thus, we are tempted to speculate that the protective effect shown by rooibos, RPO and/or their combination on cytokine formation in this study, may be due to the plethora of antioxidant compounds they contain, since previous reports have shown that flavonoids found in rooibos such as luteolin, rutin and quercetin (Takahashi *et al*., 2001; Kotanidou *et al*., 2002) and vitamin E, α- and γ-tocopherol (Bharrhan *et al*., 2010, Chung *et al*., 2010), all important antioxidants of RPO, reduced LPS-induced expression of cytokines. We also propose that the mechanism for the observed reduction in hepatic TNF-α and IL-6 may involve the ability of antioxidant phytochemicals present in rooibos and RPO, to inhibit the activation of Kupffer cells by LPS, leading to inhibition of TNF-α synthesis and subsequently synthesis of IL-6. Though the level of NFκB was not measured in this study, given its central role in signaling and the inflammatory cascade, it is proposed that inhibiting the activation of NFκB by rooibos, RPO and/or their constituents, may be an additional mechanism for the observed decrease in hepatic TNF-α and IL-6 level. IL-10 is an anti-inflammatory cytokine produced by monocytes and lymphocytes, while previous reports have indicated that it is antagonistic to TNF-α in its modulation of the inflammatory response (Jaffer *et al*., 2010). IL-10 has been reported to down-regulate TNF-α, as well as other cytokines production, by suppressing their gene expression in an autocrine-like feedback loop (Oberholzer *et al*., 2002). However in this study, we observed that the level of IL-10 was similar across all LPS treatment groups, indicating that the inhibition of TNF-α formation observed with rooibos and RPO feeding is independent of the activation of feedback loop of IL-10.

Nitric oxide (NO) and superoxide anion ($O_2^{\bullet -}$) are two free radicals secreted during activation of Kupffer cells under LPS insult. NO reacts with $O_2^{\bullet -}$ to form peroxynitrite which is a potent cytotoxic, oxidative agent that can elicit lipid peroxidation (Halliwell and Gutteridge, 2007;

Pilkhwal *et al*., 2010). LPS-induced lipid peroxidation is an index of oxidative stress, and several previous studies have reported enhanced lipid peroxidation in many tissues (including liver, heart, brain, small intestine and stomach) of rats (Sener *et al*., 2005; Kaur *et al*., 2006a, 2006b; Sebai *et al*., 2009; 2010; 2011; Depboylu *et al*., 2012). Under conditions of oxidative stress, reactive oxygen and nitrogen species (RONS) attack the polyunsaturated fatty acids (PUFAs) of cell membranes causing destabilization, disintegration and alteration in membrane fluidity and permeability, all events which increase the rate of protein degradation and eventually leads to cell lysis (Bharrhan *et al*., 2010). Decomposition products of lipid hydroperoxides such as MDA and 4-HNE can also cause chaotic cross-linkage with protein and nucleic acids, leading to oxidative protein and DNA damage (Pari and Shagirtha, 2012). In this study plasma and hepatic CD and MDA, as markers of lipid peroxidation, were measured and elevated levels of CD (liver) and MDA (plasma and liver) in the LPS-challenged rats were observed. Pre-feeding rooibos, RPO and/or their combination for 4 weeks in the LPS-challenged rats, inhibited the formation of CD (liver) and MDA (plasma and liver). A large number of *in vitro* studies have established the excellent free radical scavenging ability of rooibos, and this has been confirmed in many *in vivo* studies. The ability of rooibos to lower lipid peroxidation and modulate oxidative stress has been demonstrated in rat brain (Inanami *et al*., 1995), rat tissues and liver microsomal preparations (Ulicna *et al*., 2003; 2006; Marnewick *et al*., 2005) as well as in humans (Nikolova *et al*., 2007; Marnewick *et al*., 2011). Since rooibos is a polyphenol-rich herbal tea, its polyphenolic compounds may be able to bind RONS directly and scavenge them or act as sacrificial antioxidants to inhibit the lipid peroxidation cascade as seen in this study. More recent evidence has shown that RPO provide a strong cellular antioxidant protection against damaging effects of isoproterenol (Narang *et al*., 2005), Cd (Eriyamremu *et al*., 2008), Pb (Ajuwon *et al*., 2010) and streptozotocin (Budin *et al*., 2009; 2011). Vitamin E and carotenes found in RPO are lipid soluble, chain breaking antioxidants and thus, we propose a polyunsaturated fatty acid (PUFA)-radical suppressing property, as being responsible for the ability of RPO to inhibit lipid peroxidation in the LPS-challenged animals. More interesting in this study is that MDA was lowered in the LPS group receiving the combination of rooibos and RPO, significantly more than in the negative control group, indicating that rooibos and RPO may be interacting synergistically to prevent lipid peroxidation.

The impairment of the antioxidant defense system is a critical step in LPS-induced hepatic injury. Evidence has shown that a LPS insult is characterized by the depletion of tissue and circulating antioxidant enzymes, as well as non-enzymatic antioxidants, including GSH, as was seen in this study (Kaur *et al*., 2006a; 2006b; Bharran *et al*., 2010; Abdel-Salam *et al*., 2012). Results from this study further showed that feeding rooibos, RPO or their combination

prior to the LPS challenge, was not effective in augmenting the decrease observed in the activities of GPx and GR in the plasma while In the liver, feeding rooibos alone or together with RPO for 4 weeks prior to the LPS challenge reversed the changes observed in the activities of CAT, SOD and GPx whereas feeding RPO alone was only effective with CAT and GPx activities. The decreased activities of the antioxidant enzymes (SOD, GPx and GR) observed in the LPS-challenged rats is an indication of their inactivation and failure of the antioxidant enzymes to overcome the influx of RONS after LPS exposure (Kono and Fridovich, 1982; Pigeolet *at al*., 1990; Ilaiyaraja and Khanum, 2011). The modulation of the antioxidant enzymes observed in the LPS-challenged rats consuming rooibos and/or RPO could be ascribed to the direct quenching of RONS generated by LPS since antioxidant components of rooibos and RPO are established free radical scavengers. Furthermore, the up-regulation and/or down-regulation of the gene expression of the antioxidant enzymes may be an additional mechanism. Reduced glutathione (GSH) is the major non-protein thiol in plant and animal cells and it is essential for the regulation of a variety of cellular functions (Morimoto *et al*., 2008; Naik *et al*., 2011). It plays an important role in intracellular protection against ROS and other free radicals. Because of its sulphydryl (–SH) group, it can function as a nucleophile, forming conjugates with many xenobiotics and/or their metabolites and also serve as a reductant in the metabolism of hydrogen peroxide and other organic peroxides (Suntres, 2002; 2011). During interaction with free radicals, the –SH group of GSH becomes oxidized, leading to the formation of corresponding disulfide compound (GSSG). Thus, a depletion of GSH is usually associated with an increase in GSSG concentration and a lowered GSH/GSSG ratio during condition of oxidative stress (Dickinson and Forman, 2002; Marnewick *et al*., 2009). Results from the current study revealed a decrease in GSH (plasma) and an increase in GSSG (liver) in rats challenged with LPS. These events invariably resulted in a decrease in the GSH/GSSG ratio in both tissues of LPS-challenged rats. Feeding rooibos, RPO and/or their combination to LPS-treated rats, restored the GSH/GSSG ratio to values comparable to those found in the negative control animals, indicating that both extracts are able to protect against LPS-induced redox imbalance. The protection shown by rooibos in this study is in agreement with previous studies where rooibos has been reported to improve the GSH/GSSG ratio in rat hearts subjected to ischaemia/reperfusion injury (Pantsi *et al*., 2011) and human populations at the risk for cardiovascular diseases (Marnewick *et al*., 2011). Additionally, vitamin E, β-carotene as well as α- and γ-tocopherol, all important antioxidants of RPO, have been shown attenuate LPS-induced oxidative stress (including GSH depletion) and inflammatory related responses in rat (Kheir-Eldin *et al*., 2001; Bharran *et al*., 2010) and mouse models (Chung *et al*., 2010). The ability to improve the GSH/GSSG ratio via increasing GSH (plasma) and/or decreasing GSSG (liver), shown by rooibos and RPO can be adduced to the ability of their individual

antioxidant components to quench free radicals and up-regulate the synthesis of GSH as suggested in some previous studies (Moskaug *et al*., 2005; Masaki and Sakurai, 2007; Takeda *et al*., 2008; Katsuura *et al*., 2009; Rimbach *et al*., 2010).

In summary, this study provides the first evidence of an anti-inflammatory effect of rooibos and RPO in LPS-induced hepatic endotoxemia in rats. LPS-induced cytokine secretion has been shown to require the production of reactive oxygen and nitrogen species *in vitro* and *in vivo*, as demonstrated in this study. Rooibos and RPO contain important phytochemical constituents with excellent antioxidant properties which may in part, explain this observed anti-inflammatory activity. Also, results from this study demonstrate that rooibos, RPO and/or their combination, similarly reduced LPS-triggered oxidative stress and the inflammatory response, by attenuating liver damage, lipid peroxidation, redox imbalance and cytokine secretion, in a Wistar rat model. This suggest that rooibos and RPO may be of benefit in the prophylactic management of LPS-induced liver injury, however, future studies are necessary to fully examine the specific mechanisms underlying the protective effects shown by these two plant extracts.

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CHAPTER SIX

General discussion and conclusions

General discussion

Oxidative stress and chronic (low-grade) inflammation are two central factors that have been linked to the pathophysiology of most disease states. Aggressive generation of reactive oxygen and nitrogen species during oxidative stress may impact on lipids, proteins, enzymes, carbohydrates, nucleic acids and other molecules within the cell, resulting in cellular damage. Chronic inflammation and oxidative stress are inseparably interconnected, since inflammatory processes induced RONS generation and depletes cellular antioxidant capacity. Epidemiological and laboratory studies have shown that the consequences of oxidative stress are the molecular basis for the onset and progression of several disease states. Advances in the field of natural products and medicinal plants chemistry in the past decades are yielding information globally concerning the positive impacts of non-nutritive components from fruits, vegetables, teas and fruits on human health. The non-nutritive components responsible for these observed health effects include ascorbate, tocopherols, tocotrienols, carotenoids and polyphenolic phytochemicals. The health benefits of these bioactive components are mainly attributed to their antioxidant properties and their ability to scavenge ROS, but recent evidence has shown that other properties including anti-inflammatory, anti-atherogenic, vasodilatory, antimutagenic and anti-tumourigenic effects may contribute to their health promoting activities.

Rooibos is a caffeine-free, low tannin (when compared to the *Camelia sinensis* teas) herbal tea prepared from the leaves and stems of the legume *Aspalathus linearis*, and it is unique to South Africa. It has been consumed for its medicinal purposes by the local inhabitants for about 300 years and anecdotal evidence has linked its consumption to numerous health promoting properties. A number of scientific studies, especially *in vitro* studies have demonstrated some biological properties of rooibos, but *in vivo* studies have been limited. Properties that have been demonstrated for fermented/traditional and unfermented/"green" rooibos include antioxidant and oxidative stress modulating effects, antimutagenic, and anti-tumourigenic effects. Red palm oil (the other plant product used in this study) is obtained from the African oil palm plant (*Elaeis guineensis)*. It is a unique oil, consisting of saturated, monounsaturated and polyunsaturated fatty acids and is rich in antioxidants such as tocopherols, tocotrienols, carotenes (mostly β-carotene), coenzyme Q10, squalene and other phytosterols. Many health properties have been demonstrated for RPO, including hypocholesterolemic, cardioprotective, anti-carcinogenic, antioxidant and oxidative stress modulation effects. Recent discoveries suggested that the health benefits observed for fruits, vegetables, teas and spices can be attributed to combined performance of individual phytochemicals in these plants, either as an additive, synergistic or inhibitory interaction. These interactions may occur between phytochemicals within the same plants or from

different plants. Evidence has even shown that polyphenolic phytochemicals and antioxidant vitamins may interact synergistically to modulate lipid peroxidation and oxidative stress. The current study was conducted to investigate possible modulating properties of rooibos and RPO, supplemented either alone or in combination, on the endogenous antioxidant system and inflammatory responses using three Wistar rat models. Thus a possible additive or synergistic interaction between rooibos and RPO in their observed effect was also investigated. The study consisted of 3 phases and at the beginning of each study phase, the phytochemical constitution and *in vitro* antioxidant capacity of the rooibos and RPO were quantified. HPLC quantification of the rooibos extract confirmed the flavonoid profile of rooibos and indicated that the dihydrochalcone, aspalathin was the most abundant flavonoid in the rooibos extract. Others were the flavones orientin, iso-orientin, vitexin, iso-vitexin and hyperoside/rutin. The vitamin E and carotene composition and the relative abundance of their different isoforms were also confirmed by HPLC quantification of the RPO in this study.

In the first study, the modulatory role of chronic feeding of rooibos, RPO or their combination on the endogenous antioxidant system was investigated. An increase in the serum levels of aminotransferases, lactate dehydrogenase, alkaline phosphatase, as well as creatinine, blood urea nitrogen and uric acid have been used widely in literature, as an indication of toxicity of plant extracts to the liver and the kidney, and in extension as a surrogate marker of safety of these extracts. Chronic feeding of the rooibos extract, RPO or their combination for the 22 weeks of the study did not induce any deleterious effects in the liver and kidneys of the experimental rats. This confirmed the safety of rooibos and RPO, even after long-term or prolonged consumption. The histopathological analyses of liver corroborated the absence of toxicity, thus establishing the basis for a further much needed study concerning the safety evaluation of rooibos and red palm oil in humans with longer exposure times. The antioxidant/oxidant (redox) balance was not modified significantly by chronic feeding of RPO alone in the rats. Feeding rooibos alone for 22 weeks was able to positively modulate the endogenous antioxidant system by decreasing lipid peroxidation (shown by inhibition of MDA formation) in the blood and increasing antioxidant enzyme activities (CAT and GR), as well as increase the GSH levels in the liver. The rooibos extract used in this study showed a higher total polyphenolic content than RPO, therefore the lack of effect by RPO alone suggests that the ability to modulate the antioxidant/oxidative balance in this study, may be due to the antioxidant and other biological properties of the polyphenolic constituents specifically. Feeding the combination of rooibos extract and RPO for 22 weeks resulted in a better outcome than when supplementing with rooibos only, especially with regards to an increased plasma total polyphenol content, plasma TAC, whole blood GSH level, GSH/GSSG ratio and decreased MDA formation. These results suggest a positive

interaction, which may be additive or synergistic, between rooibos and RPO in the modulation of the endogenous antioxidant system. It is important to note that this is the first scientific evidence reporting such interactions between these two plant extracts.

Following this first study, it was imperative to establish whether consumption of these plant extracts or their combination will be able to protect against a pathological or an induced oxidative stress state. Thus, the next short-term study was conducted to evaluate the possible protective effects of an aqueous rooibos extract, RPO or their combination on oxidative hepatotoxicity induced by *t*-BHP in male Wistar rats. Hepatic damage induced by *t*-BHP was successfully reversed by the feeding of the extracts alone, or in combination for 8 weeks. Furthermore, *t*-BHP induced lipid peroxidation as shown by the elevation in plasma and hepatic levels of CDs and MDA, with an impairment of the glutathione redox status. Feeding of rooibos, RPO or their combination positively modulated this disturbance in the antioxidant/oxidant balance by inhibiting/decreasing CD and MDA formation in the liver, reversing the changes in antioxidant enzyme activities and improving the hepatic GSH redox status. In the 22-week feeding study a lack of modulating the antioxidant/oxidant balance by RPO alone was observed, but in the oxidative stress-induced study, RPO was able to protect against oxidative hepatic damage induced by *t*-BHP, suggesting that the ability of antioxidants to induce protective effects differ in the healthy and diseased states. Furthermore, results from this study also indicated that the mechanisms of protection by the plant extracts against *t*-BHP-induced oxidative hepatotoxicity may involve one or more of several different antioxidant properties in the extracts, such as (i) inhibition of lipid peroxidation by scavenging ROS, (ii) inhibiting cytochrome P450-mediated metabolism of *t*-BHP to toxic radicals and (iii) up-regulating the synthesis of GSH by activating γ-glutamylcysteine synthetase, the rate limiting enzyme in the biosynthesis of GSH, as previously reported for some other phytochemicals.

It is known that other properties, apart from the antioxidant effects may be involved in the health promoting effects of whole plant extracts and other phytochemicals. Therefore, the ability of rooibos and RPO to modulate inflammatory responses was investigated by monitoring the protective effects of these plant extracts in a LPS-induced hepatic endotoxemic model. Feeding the plant extracts alone to rats for 4 weeks before exposure to LPS, did not prevent the hepatic damage induced by LPS significantly. However, the extracts interacted positively when fed together to prevent hepatic dysfunction induced by LPS significantly. Increased levels of pro-inflammatory cytokines from neutrophils in the liver have been associated with hepatic damage induced by LPS. An elevation in the hepatic levels of TNF-α, IL-1β and IL-6 in this study was noted. Feeding rooibos, RPO or their combination for

4 weeks was able to induce an inhibition of TNF-α and IL-6 in the liver, while IL-1β was not affected. Phytochemicals such as luteolin, rutin and quercetin found in rooibos and vitamin E and its isoforms, such as α- and γ-tocopherol are known to reduce LPS-induced expression of cytokines. Therefore, the inhibition of synthesis of hepatic TNF-α and IL-6 observed in this study may involve the antioxidant phytochemicals present in rooibos and RPO's ability to inhibit the activation of Kupffer cells by LPS, leading to inhibition of TNF-α synthesis and subsequently synthesis of IL-6. Hepatic damage by LPS is usually accompanied by enhanced lipid peroxidation, depletion of endogenous antioxidant enzymes and impairment of the glutathione redox status, as observed in this study. The feeding of rooibos, RPO or their combination was able to reverse the observed increases in hepatic CDs, MDA, GSSG, as well as the decrease in the GSH/GSSG ratio, suggesting that the ability of the extracts to modulate inflammatory responses observed in this study, may be related to their antioxidant and oxidative stress modulating properties.

Conclusions

Results from these three studies substantiated the anecdotal claims of the two plant extracts, rooibos and red palm oil, and generated new knowledge which will be a valuable addition to the literature available and enrich the pool of knowledge regarding the health promoting properties of rooibos and RPO. Results indicated that rooibos and RPO are safe to consume, even after prolonged consumption (22 weeks), while providing rich and unique sources of antioxidants and that their consumption may play important roles in improving the overall health status. These results also suggest that rooibos and RPO can be developed as nutraceuticals, which may be of benefit in the prophylactic management of oxidant-induced liver injury.

Further studies will be necessary to fully maximise the hepatoprotective properties of rooibos and RPO. Possible mechanism(s) of the protective effects must be fully elucidated and future studies including investigating the protective effects of these two plant extracts against pathological conditions in which chronic inflammation play an important role, such as liver cancer will be necessary. A clinical trial to validate the biological properties proposed is also desirable.

ADDENDUMS

ADDENDUM 1: RESEARCH OUTPUT

LOCAL AND INTERNATIONAL CONFERENCE PRESENTATIONS

1. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation of tert-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats. **Poster presentation** at the 19th Annual Meeting of the Society for Free radical Biology and Medicine (November 14-18 2012), San Diego CA, USA.

2. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation of tert-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats. **Oral presentation and Best Paper Winner** (Faculty of Health and Wellness Sciences) at the First CPUT Postgraduate Research Conference (September 7, 2012). Bellville, South Africa.

3. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation of tert-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats. **Poster presentation** at the CPUT Research Day (November 30, 2012). Cape Town, South Africa.

4. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. The effect of rooibos (*Aspalathus linearis*) supplementation on tert-butyl hydroperoxide-induced oxidative damage in liver and kidneys of rats. **Poster presentation** at the 18th Annual Meeting of the Society for Free Radical Biology and Medicine (November 16-20 2011), Atlanta GA, USA.

5. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. The effect of rooibos (*Aspalathus linearis*) supplementation on tert-butyl hydroperoxide-induced oxidative damage in liver and kidneys of rats. **Poster presentation** at the CPUT Research Day (December 2, 2011). Cape Town, South Africa.

PUBLISHED ABSTRACT

1. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL. 2012. Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation of tert-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats. Free radical Biology and Medicine 53 (sup. 2): S102.

2. Ajuwon OR, Katengua-Thamane E, Van Rooyen J, Oguntibeju OO and Marnewick JL (2011). The effect of rooibos (*Aspalathus linearis*) supplementation on tert-butyl hydroperoxide-induced oxidative damage in liver and kidneys of rats. Free Radical Biology and Medicine 51(sup.1): *pp. S*82.

ADDENDUM 2: ETHICS APPROVAL OBTAINED FOR THE USE OF WISTAR RATS IN EXPERIMENTATION.

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14 July 2010
CPUT/HAS-REC 2010/A003

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OFFICE OF THE CHAIRPERSON:
HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

At a meeting of the Health and Wellness Sciences-REC on 4 June 2010 ethics approval was granted to Olawale Razaq Ajuwon, This approval is for research activities related to a DTech: Biomedical Technology at this institution.

TITLE:

Effects of rooibos and red palm oil supplementation on biomarkers of oxidative stress, apoptosis and inflammation in wistar rats.

INTERNAL SUPERVISOR: Prof J Marnewick

Comment:
Research activities are restricted to those detailed in the proposal and ethics application submitted in May 2010.

Approval will not extend beyond 13 July 2011. An extension must be applied for should data collection for this study continue beyond this date.

Prof PENELOPE ENGEL-HILLS
CHAIR: HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE

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ADDENDUM 3: ETHICS APPROVAL EXTENSION FOR THE USE OF WISTAR RATS IN EXPERIMENTATION

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HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
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Approval is hereby granted to Ajuwon Olawale Razaq, for an extension period for the study with no amendments. This approval is for research activities related to a DTech: Biomedical Technology at this institution. This decision will be recorded on the minutes of the meeting of the Health & Wellness Sciences Research Ethics Committee meeting on 22 June 2011.

TITLE:

Effects of rooibos and red palm oil supplementation on biomarkers of oxidative stress, apoptosis and inflammation in wistar rats.

INTERNAL SUPERVISOR: **Prof J Marnewick**

Comment:

Research activities are restricted to those detailed in the proposal and application submitted in March 2011.

Approval will not extend beyond 18 March 2012. An extension must be applied for should data collection for this study continue beyond this date.

Prof PENELOPE ENGEL-HILLS
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ADDENDUM 4: EFFECTS OF ROOIBOS AND/OR RPO SUPPLEMENTATION ON LIVER FUNCTION MARKER ENZYMES IN RATS NOT CHALLENGED WITH LPS

Treatments	ALT (U/L)	AST (U/L)	LDH (U/L)
Control	105.69 ± 28.93	88.98 ± 12.53	234.92 ± 74.55
RTE	109.31 ± 12.10	82.91 ± 11.43	219.22 ± 58.16
RPO	110.55 ± 24.64	129.69 ± 29.71*	263.00 ± 50.32
RTE + RPO	111.65 ± 21.59	103.31 ± 13.46	238.17 ± 24.02

Values are mean ± SD (n=7-9) *Significantly different from control (P<0.05). ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), LPS (lipopolysaccharide), RTE (aqueous rooibos extract), RPO (red palm oil).