

12-1-2008

# Modulation of oxidative stress biomarkers by rooibos in adults at risk of developing coronary heart disease

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## Recommended Citation

Macharia, Muiruri, "Modulation of oxidative stress biomarkers by rooibos in adults at risk of developing coronary heart disease" (2008). *CPUT Theses & Dissertations*. Paper 25.  
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**MODULATION OF OXIDATIVE STRESS BIOMARKERS BY  
ROOIBOS IN ADULTS AT RISK OF DEVELOPING  
CORONARY HEART DISEASE**

by

**MUIRURI MACHARIA**

**Thesis submitted in fulfilment of the requirements for the degree**

**Master of Technology: Biomedical Technology**

**in the Faculty of Health and Wellness Sciences**

**at the Cape Peninsula University of Technology**

**Supervisor: Dr JL Marnewick**

**Co-supervisor: Prof J Esterhuyse**

**Cape Town Campus  
December 2008**

## DECLARATION

I, Muiruri Macharia, 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.

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**Signed**

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**Date**

## ABSTRACT

Observational studies link higher intake of polyphenols from plant based foods and beverages with a lower risk of cardiovascular disease (CVD) which remains the leading cause of death in the world. Although the mechanisms explaining this observation remains unclear, research suggest that polyphenols, with their potent antioxidant properties, may decrease oxidative stress by impairing the oxidation of lipids. Increased oxidation of lipids, particularly low density lipoproteins, is recognised as an important risk factor in coronary heart disease. Attention has focussed on dietary sources with antioxidant potential that are capable of modulating the body's oxidative stress via various modes for example increasing the antioxidant capacity or shifting the antioxidant redox status.

Rooibos (*Aspalathus linearis*) has been shown to possess potent antioxidant, immune-modulating and chemopreventive actions in various *in vitro*, *ex vivo* and animal models and consequently has been suggested to be potentially beneficial to human health. The aim of this study was to investigate the effect of rooibos consumption on parameters of oxidative stress and redox status in blood of humans at risk for coronary heart disease. A fourteen-week dietary intervention study was conducted in which forty volunteers consumed six cups of rooibos tea daily for six weeks which was preceded by a 2-week baseline and 2-week washout period and followed by a 4-week control period when water was consumed. Before and after the intervention, several parameters were measured including indices of antioxidant activity, lipid peroxidation (LPO) antioxidant redox status (ratio of reduced to oxidised glutathione i.e. GSH: GSSG) and antioxidant content. Plasma antioxidant capacity was not affected by the 6-week rooibos intake but total polyphenols were significantly ( $P<0.05$ ) raised by 11 %. Significant ( $P<0.05$ ) decreases were reported in markers of LPO (conjugated dienes and malondialdehyde), total cholesterol, low density lipoprotein-cholesterol as well as oxidized glutathione. Reduced glutathione as well as the ratio GSH:GSSG were both significantly ( $P<0.05$ ) increased by the rooibos intervention compared to the control phase. Rooibos consumption did not cause any adverse effects in the study participants and neither were the serum iron levels altered.

These results suggest that rooibos consumption may impair lipid peroxidation in the body and therefore play a beneficial role in cardiovascular health in humans. In addition, the results confirm the safety of short term consumption of rooibos with regards to liver and kidney function as no toxicity was associated with its intake.

## **ACKNOWLEDGEMENTS**

I would like to express my sincere appreciation and gratitude to the following persons/institutions for their support during my Magister Technologiae research at CPUT:

Cape Peninsula University of Technology and South African Rooibos Council for bursaries and joint funding of this project.

My supervisor, Dr JL Marnewick for introducing me to the world of rooibos and antioxidants. Her guidance, availability to all her graduate students, motivation and a constant reminder that there is no research without thoroughness. For this, I will be forever grateful.

My co-supervisor, Prof J Esterhuysen for reading through my draft and for research support.

Mr Fanie Rautenbach, the Oxidative Stress Research Centre Laboratory Manager for his expert training on all the relevant laboratory assays and selfless sharing of his time.

Dr D Blackhurst at the UCT Lipid laboratory for training on various assays.

Mrs I Venter, Dr Opperman and Dr Wolmarans for their expert involvement in the dietary aspects of the study as well as Mr H Neethling for assistance with chemical pathology assays.

My family, especially my mother and brothers Kaguru and Mbugua: the true yardsticks of selflessness.

Finally to Catherine for precious support, patience and understanding and for the important reminder of the world outside antioxidants.

## **DEDICATION**

**To my family, a bunch of philosophers, for their unfailing love and support  
even when I thought I was undeserving.**

**Philosophers lead sheltered lives.**

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## GLOSSARY OF ABBREVIATIONS

<b>Terms/Acronyms/Abbreviations</b>	<b>Definition/Explanation</b>
A	Absorbance at specified wavelength
ATBC	Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study
$^1\text{O}_2$	Singlet oxygen
AAE	Ascorbic acid equivalent
AAPH	2,2' – Azobis (2-amidinopropane) dihydrochloride
ABTS	2, 2'- Azino-di-3-ethylbenzothiazoline sulphonate (ABTS) assay
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOC	Antioxidant capacity
Apo B	Apolipoprotein B
AST	Aspartate aminotransferase
ATBC	Alpha tocopherol $\beta$ -carotene trial
BMI	Body mass index
BP	Blood pressure
CARET	$\beta$ -Carotene and retinol efficacy trial
CAT	Catalase
CDs	Conjugated dienes
CHAOS	Cambridge heart antioxidant study
CHD	Coronary heart disease
CoQ <sub>10</sub>	Coenzyme Q10
CPUT	Cape peninsula university of technology
CVD	Cardiovascular disease
DMACA	4-Dimethylaminocinnamaldehyde

DNTB	Dithiobis 2- nitrobenzoic acid
DPPH	2,2'-diphenyl-1-picrylhydrazyl
$\epsilon$	Coefficient of extinction ( = $1.54 \times 10^5$ )
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EtOH	Ethanol
F-C	Folin Ciocalteu reagent
FI	Fluorescein
FMD	Flow mediated dilation
FRAP	Ferric reducing antioxidant power assay
GAE	Gallic acid equivalent
GGT	Gamma glutamyl transferase
GISSI	Gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico study
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High density lipoprotein
HOPE	Heart outcome prevention evaluation
HPS	Health professionals study
IL-2	Interleukin-2
iNOS.	Inducible nitric oxide synthase
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
LDLox	Oxidised low density lipoprotein
LPO	Lipid peroxidation
M2VP	1-Methyl-2-vinylpyridiumtrifluoromethane sulphonate
MDAs	Malondialdehyde
MeOH	Methanol
MMP	Matrix metalloproteinases
MONICA	Project to Monitor cardiovascular disease
MPA	Metaphosphoric acid
MRC	Medical research council
Na <sub>2</sub> CO <sub>3</sub>	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NO	Nitric oxide
O <sub>2</sub> <sup>-</sup>	Superoxide radical
O <sub>3</sub>	Ozone
OH <sup>·</sup>	Hydroxyl radical
ORAC	Oxygen radical absorbance capacity assay
PCA	Perchloric acid
PE	Phycoerythrin
ROS	Reactive oxygen species
RSA	Republic of South Africa
Rpm	Revolutions per minute
SD	Standard deviation
SGLT 1	Sodium dependent glucose transporter 1
SOD	Superoxide dismutase
TAC	Total antioxidant capacity

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalent
TG	Triglyceride
TNB	5-Thionitrobenzoic acid
UCT	University of Cape Town
UDP-GT	Uridine diphosphate glucuronyltransferase
USDA	United States Department of Agriculture
USA	United States of America
VEAPS	Vitamin E atherosclerosis prevention study
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

## CONFERENCE AND POSTER PRESENTATIONS

1. Does rooibos modulate parameters of oxidative stress status in humans at risk for coronary heart disease? Macharia, M., Rautenbach, F., Marnewick JL. *The indigenous plant use forum*, Graaff-Reinet, South Africa, July 2008.
2. Impact of rooibos on oxidative stress status in humans at risk for heart disease. Macharia, M., Rautenbach, F., Marnewick JL. *CPUT Research day*, Bellville, November 28<sup>th</sup>, July 2008.

## CHAPTER ONE

### INTRODUCTION

According to the World Health Organization, cardiovascular disease (CVD) is the leading cause of death in the world killing 17 million people annually (WHO, 2008). This translates to one in every three deaths. By 2020 CVD will become the leading cause of both death and disability worldwide, with the number of fatalities projected to exceed 20 million a year and many more incapacitated by stroke and heart attack (WHO, 2008). The burden of CVD is shifting from developed to developing countries with 80% of the projected increase occurring in developing and transitional nations like South Africa. Between 1997 and 2004, South Africa lost 195 people every day to CVD making it the leading cause of death after HIV/AIDS (<http://www.statssa.gov.za/>). A recent report released by the Medical Research Council (MRC) of South Africa estimated that direct and indirect costs of death and disability from CVD to the South African economy exceed R8 billion a year (<http://www.bizcommunity.com/Article/196/154/17763.html>). As the existing CVD prevention and treatment strategies are insufficient, the realization of dietary components with relevant effectiveness offers an exciting prospect to reduce the global burden imposed by CVD. Efforts have intensified to identify potential dietary candidates for antioxidant treatment in order to substantiate whether these components may be realistic complementary treatment or preventive options for individuals with high risk for heart diseases.

Oxidative stress, defined as an excess of oxidants over antioxidant defences in the body has been implicated in the aetiology and pathogenesis of CVD and other chronic diseases (Wilcox *et al.*, 2004). Chronic oxidative stress may lead to oxidative alterations of vital biomolecules e.g. proteins, nucleic acids and lipids. Oxidatively modified low density lipoproteins (LDL)-cholesterol have been reported to markedly increase atherosclerosis — the major cause of CVD (Kaliora *et al.*, 2006). A protective role has been suggested for antioxidants against oxidation of LDL and against onset of atherosclerosis thereby averting the establishment of CVD. Observational studies link high dietary intake of plant foods and beverages with a lower incidence of CVD and other chronic diseases (Wilcox *et al.*, 2004) and suggest the association may be attributed to the polyphenolic antioxidants in these foods. In the French population, for example, CVD is less frequent than expected in spite of a high fat intake. This effect, known as the “French paradox”, has been partly attributed to the consumption of red wine (Renaud & de Lorgeril, 1992). Red wine contains many polyphenols that have been shown to effectively impair oxidation of LDL.



Diets rich in antioxidants such as polyphenols may therefore modulate or prevent CVD and could play an important role in the general health of the South African population.

Polyphenols are a group of compounds ubiquitous in plants and have been reported to exhibit a wide range of biological activities including anti-atherogenic, antibacterial, antiviral, anti-inflammatory, anti-allergic, antioxidant and vasodilatory (Cook & Samman, 1996; Harbone & Williams, 2000; Nijveldt *et al.*, 2001). As they are mostly hydrophilic and do not bind LDL, the mechanisms involved in their anti-atherogenic activity are proposed to be via the scavenging of oxidants such as free radicals, acting as chelating agents, protecting vitamin E,  $\beta$ -carotene and lycopene in the LDL or preserving serum paraoxonase activity thus promoting hydrolysis of lipid peroxides (Kaliora *et al.*, 2006). Beyond these broad activities attributed to polyphenols, research is uncovering more specific properties like their direct impact on enzyme systems that control the expression of inducible nitric oxide synthase (iNOS). This enzyme generates nitric oxide which, although an oxidant, is essential in immune and cardiac health (Madamanchi *et al.*, 2005).

At present no information is available on the possible modulating properties traditional/fermented rooibos (*Aspalathus linearis*) herbal tea may have on CVD risk. Since the phenolic profile of this herbal tea differs from that of the much studied black and green teas (*Camellia sinensis*), the mechanisms of protection could also differ, thus it is necessary to investigate the possible protective properties of rooibos. Previous studies have shown rooibos to contain a rich, complex mixture of unique polyphenols that exhibit good antioxidant activity *in vitro* (reviewed in Joubert *et al.*, 2008) as well as *in vivo* in experimental rats (Marnewick *et al.*, 2003), but the *in vivo* protective effects in humans have not been established yet. Rooibos was also previously shown to exhibit *in vitro* as well as *ex vivo* antimutagenic activity (Marnewick *et al.*, 2000; Marnewick *et al.*, 2004) and to regenerate coenzyme Q10 and subsequently inhibit lipid peroxidation in rat liver (Kucharska *et al.*, 2004).

The present study follows-up on these findings to explore possible *in vivo* effects in humans with the main objective of assessing the possible modulation of oxidative stress by rooibos in adults at risk for developing coronary heart disease (CHD). To achieve this objective, a 14-week pre- and post-measurement single group intervention study was conducted in which volunteers (n = 40) consumed 6 cups of traditional/fermented rooibos daily for 6 weeks preceded by a 2-week baseline period (run-in) and a 2-week washout period and followed by a 4-week control period where volunteers consumed water. Before and after the intervention, several oxidative stress parameters were measured including indices of antioxidant activity, lipid peroxidation (conjugated dienes and malondialdehyde), antioxidant redox status (ratio of reduced to oxidised

glutathione) and antioxidant content (total polyphenols). A secondary objective of the study was to assess the safety of rooibos intake by humans which was achieved by evaluating the participants' reports as well as laboratory assays for indicators of liver and kidney function.

The rationale for the choice of the study population was based on the view that elevated oxidative stress may be required for clear detection of improvements from dietary antioxidant intervention. Hypertension, dyslipidemia and other risk factors for CHD are accepted to be conditions accompanied by elevated oxidative stress (Kaliora & Dedoussis, 2007).

Several limitations should be considered when interpreting the results presented here. The study assessed systemic markers of oxidative stress and antioxidant capacity which may not necessarily reflect levels in tissues. Furthermore, the two methods used for the estimation of lipid peroxidation are limited because of poor specificity (Hwang & Kim, 2007), but still remain the most used and reported on methods for this kind of investigation, simply indicating a lack in the development and substantiation of newer more specific methodologies. Inclusion of more analytes not utilised here (e.g. uric acid and homocysteine) in the estimations can aid in interpretation of results particularly in light of the association of the latter with CVD, but did not fall within the timeframe of this study, and should be included in future analysis. Despite these limitations, the results of the study are expected to add immensely to our present understanding of the health promoting properties of rooibos.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Oxidants, reactive oxygen species and oxidative stress

A free radical is any chemical species capable of independent existence that contains one or more unpaired electrons (Wilcox *et al.*, 2004). The presence of unpaired electrons makes free radicals highly reactive by donating or extracting electrons from non radicals in an attempt to attain stability. This may trigger a chain of reactions each capable of generating new radicals. In a biological setup like the human body, vital macromolecules such as lipids, proteins and nucleic acids may be oxidatively modified resulting in cell or tissue damage, hence the myriad pathologies linked to an excess of free radicals (Wilcox *et al.*, 2004). Not all oxidants in the body, however, are free radicals. 'Reactive oxygen species' (ROS) is a more inclusive term that describes both radical and non-radical oxidants which may be oxygen, halide or nitrogen centred (Table 2.1).

**Table 2.1: Important reactive oxygen species in living systems**

---

Free radicals

Hydroxyl radical	OH·
Superoxide radical	O <sub>2</sub> <sup>·-</sup>
Nitric oxide radical	NO·
Lipid peroxy radical	LOO·

Non radicals

Hydrogen peroxide	H <sub>2</sub> O <sub>2</sub>
Singlet oxygen	<sup>1</sup> O <sub>2</sub>
Hypochlorous acid	HOCl
Ozone	O <sub>3</sub>

---

(Adapted from Wilcox *et al.*, 2004)

In this study, the term ROS will be used to denote reactive species of any kind for example radical / non radical or oxygen / nitrogen centred. Oxidation and production of ROS is an integral part of human living and all cells in the body are therefore constantly exposed to oxidants from both endogenous and exogenous sources (Table 2.2). The generation of ROS, however, is not to be avoided at all costs. In fact, production of super oxide and hypochlorous acid by activated phagocytic cells and leukocytes, respectively, is deliberate and directed to eliminate potentially pathogenic microorganisms (Bahorun *et al.*, 2006). Oxygen radicals are involved in signal transduction, gene transcription and regulation of soluble guanylate cyclase

activity in cells while nitric oxide (NO) is essential in regulation of vascular tone, leukocyte adhesion, platelet aggregation, thrombosis as well as being a potent synaptic neurotransmitter (Bahorun *et al.*, 2006). In addition, the involvement of ROS in cell differentiation and apoptosis has been suggested (Evans & Halliwell, 2001).

**Table 2.2: Sources of reactive oxygen species**

Origin	Source
Internally generated sources	Mitochondria Activated phagocytes Xanthine oxidase Transition metals- mediated reactions Arachidonate pathways Inflammation Ischaemia & reperfusion
Externally induced sources	Diet Cigarette smoke Radiation and ultra violet light Ozone Pesticides Drugs e.g. cyclosporine A

(Adapted from Wilcox *et al.*, 2004)

Reactive oxidative species are therefore important in normal physiology and immune defences but their ability to kill invading organisms mean that they can also damage normal tissues. A balance is therefore necessary between these prooxidant species and the various antioxidant strategies available in the body to neutralise them.

In the human body, this balance exists and is in slight favour towards oxidants to allow the body to complete its metabolic duties. If the generation of ROS overwhelms the available antioxidant defences, oxidative stress – defined as “an imbalance between oxidants and antioxidants in favour of the former”- can arise (Evans & Halliwell, 2001). The antioxidant rationale is based on the assumption that antioxidants can limit the deleterious effects caused by oxidative stress thereby slowing the incidence of disease and the process of ageing.

## 2.2 Oxidative stress and atherosclerotic cardiovascular disease

As mentioned in the previous chapter, CVD remains an important health concern both globally and nationally, hence reducing its mortality and morbidity is of prime significance. Cardiovascular disease is a collective term for a group of diseases that affect the heart and

blood vessels. Coronary heart disease (CHD), the most predominant form of CVD accounting for half of CVD mortality, is principally caused by atherosclerosis (Singh & Jialal, 2006).

The earliest manifestation of atherosclerosis is the progressive development of the fatty lesion prompted by the transport of low density lipoprotein (LDL) into the arterial wall in a concentration-dependent process (Vita, 2005). Like other lipoproteins, LDL is composed of a lipid layer and a protein unit called apoprotein B (apo B). In their metabolic activities, the cells of the arterial wall produce and secrete oxidative products e.g. nitric oxide, which deplete the antioxidants within the vascular wall and since LDL is localized in the sub-endothelial space, it is an easy target for oxidative damage by ROS originating from the vascular cells. The likelihood of oxidation increases with diminishing antioxidant levels (Singh & Jialal, 2006).

Oxidation of LDL proceeds in two phases (Singh & Jialal, 2006). The lipid portion is first oxidized with little alteration to the protein unit producing minimally oxidized LDL which is chemotactic for circulating monocytes. The second phase occurs when the monocytes attracted by the minimally modified LDL enter the vessel wall and differentiate into macrophages ready to endocytose the modified LDL. The macrophages release more oxidants which further oxidize the LDL particle modifying the apo B unit and rendering it unrecognizable by its receptors on the macrophage. Usually, entry of LDL into the macrophage is regulated via a feedback mechanism mediated by these receptors. The oxidized LDL (LDL<sub>ox</sub>) now enters the macrophage through an additional receptor called the scavenger receptor and since this influx is under no feedback regulation, massive accumulation of LDL<sub>ox</sub> occurs within the macrophage (Westhuysen, 1997). Termed "foam cells" because of their sponge-like appearance under the microscope, these lipid-laden macrophages represent the visible manifestation of an atherosclerotic lesion (Chrysohoou *et al.*, 2007). As in any other injury, a fibrous tissue (cap) forms over the lesion which continues to grow and invade the lumen of the vessel as the foam cells accumulate. Thinning of the fibrous cap can lead to rupture of the plaque, leading to thrombus and subsequent stroke or infarction (Vita, 2005).

It is likely that reducing the amount of LDL<sub>ox</sub> generated may limit the damage to the vascular system caused by the above sequence of events. Increased dietary intake of antioxidant rich foods/beverages is accepted as a possible mechanism of supplementing the body's endogenous antioxidant reserves which in turn may inhibit or slow the oxidation of LDL (Rimm, 2002).

### 2.3 Antioxidants

An antioxidant is defined as “any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate” (Halliwell & Gutteridge, 1995). Oxidizable substrates might be proteins, lipids, DNA or any other susceptible biological molecule. These antioxidants could either be from exogenous sources e.g. diet or formed endogenously in the body. Although specific antioxidants have different mechanisms of action as exemplified in table 2.3, the functional hierarchy by which any antioxidant protects against ROS will fall within one of the following three broad categories (Wilcox *et al.*, 2004):

- Primary/preventive – form the first line of defence and suppresses formation of ROS. Examples include enzymes like, Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase.
- Radical scavengers/chain breakers – as second line of defence, these antioxidants trap ROS to inhibit oxidation chain initiation thus breaking the reaction, e.g. polyphenols, carotenoids and vitamins C and E.
- Repair enzymes – act as third line of defence by repairing damaged macromolecules. These include lipases, proteases and transferases.

**Table 2.3: Examples of antioxidants and their mechanisms of action**

Antioxidant/source	Mechanism of action
<b>Endogenous</b>	
Superoxide dismutase (SOD)	Dismutation of $O_2^{\cdot-}$ to $H_2O_2$
Catalase (CAT)	Decompose $H_2O_2$ to molecular oxygen and water
Glutathione (GSH)	Intracellular reducing agent
CoQ <sub>10</sub>	Inhibit lipid peroxidation; reduce mitochondrial oxidative stress
Uric acid	Scavenge peroxy and OH· radicals; chelate transition metal ions
<b>Dietary antioxidants</b>	
Vitamin E	Scavenge $O_2^{\cdot-}$ ; up-regulate antioxidant enzymes; inhibit LPO
Vitamin C	Scavenge $O_2^{\cdot-}$ ; tocopherol regeneration
Lycopene	Trap $^1O_2$
Ellagic acid	Scavenge $H_2O_2$ ; stimulate glutathione-S-transferase
Genistein, quercetin	Scavenge $H_2O_2$
Catechins	Metal chelation; scavenge $O_2^{\cdot-}$ , $H_2O_2$ , OH· and $^1O_2$ tocopherol regeneration.

Adapted from Ratnam *et al.*, 2006; CoQ<sub>10</sub>= coenzymeQ<sub>10</sub>, LPO= lipid peroxidation

### 2.3.1 Interaction between antioxidants

In addition to their individual effects, antioxidants have been shown to interact with each other in a synergistic way. *In vitro* studies have shown interactions between  $\alpha$ -tocopherol and ascorbic acid as well as  $\alpha$ -tocopherol and flavonoids (Bendich *et al.*, 1984; Pedrielli & Skibsted, 2002). The tocopheryl radical formed when vitamin E reacts with ROS accepts a hydrogen ion from ascorbic acid/flavonoid and in the process is regenerated to the active  $\alpha$ -tocopherol. A high intake of vitamin C was shown to increase tissue vitamin E concentration in some but not all experimental animal studies (Bendich *et al.*, 1984; Burton *et al.*, 1990; Igarashi *et al.*, 1991). While interactions of antioxidants in humans are more unclear, it could be possible that combinations of various types of antioxidants may be more effective rather than larger quantities of any one of them (Langseth, 1995).

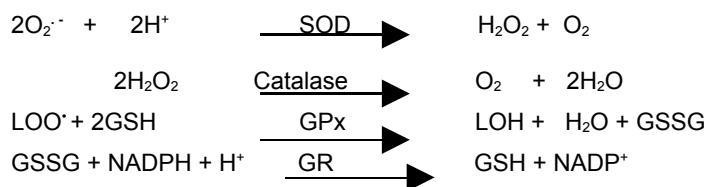
### 2.3.2 Endogenous antioxidants

Antioxidants synthesised in the body include antioxidant enzymes, metal binding proteins and other small molecule antioxidants (Evans & Halliwell, 2001). The enzymes, which include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), catalyse the breakdown of superoxide and peroxides usually in the intracellular environment before they can react with other ions resulting in the formation of more potent ROS (Young & Woodside, 2001). Transition metal-binding proteins (e.g. ceruloplasmin, transferrin, and ferritin) prevent interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide producing the highly reactive hydroxyl radical in what is known as the Fenton reaction (Young & Woodside, 2001).

Reduced glutathione (GSH) is a major antioxidant in human tissues that provides reducing equivalents to the GPx-catalysed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol (Young & Woodside, 2001). In the process, GSH becomes oxidised to GSSG which is recycled back to GSH in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 2.1). When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG will decrease due to GSSG accumulation or reduction in GSH levels. Determination of this ratio is a useful indicator of oxidative stress and can be used to monitor the effectiveness of antioxidant intervention (Young & Woodside, 2001).

Other small molecule antioxidants include albumin, bilirubin and uric acid. Uric acid is an end product of purine metabolism in humans and higher primates and has been shown to possess antioxidant functions as it scavenges peroxy and hydroxyl radicals and binds ions of transition metals which would otherwise generated free radicals (Sevanian *et al.*, 1991). However,

excessive uric acid is associated with gout and CVD in humans and pro-oxidant tendency *in vitro* (Lotito & Frei, 2006).



**Figure 2.1: Enzymatic antioxidant defenses (Adapted from Langseth, 1995)**

Albumin is the main carrier protein in the plasma and its sulphhydryl groups can react with hydrogen peroxide and peroxy radicals while bilirubin is able to scavenge peroxy radicals *in vitro* and is thought to be able to protect albumin-bound fatty acids against oxidative damage (Halliwell, 1998).

### 2.3.3 Dietary antioxidants

The diet, particularly oriental tea, herbal teas, fruits, vegetables, nuts and seeds, provide essential antioxidants such as vitamins C and E as well as antioxidant phytochemicals like carotenoids and polyphenols (Lotito & Frei, 2006).

#### 2.3.3.1 Antioxidant vitamins and carotenoids

Besides polyphenols, carotenoids and vitamins C and E have received much focus as protective dietary components. Vitamin C (ascorbic acid) is essential for protection of humans against scurvy. Its activity lies in its role as an essential co-factor in numerous enzymatic hydroxylation reactions derived from its strong reducing potential. This property makes the vitamin an excellent water soluble, chain-breaking antioxidant that directly reacts with superoxide and hydroxyl radicals as well as singlet oxygen (Jialal *et al.*, 1990). It also acts indirectly as an antioxidant by regenerating the lipophilic vitamin E at the aqueous-lipid interphase (May *et al.*, 1998).

Vitamin E exists in eight forms, namely; –  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ - tocotrienols and  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$  tocopherols (Langseth, 1995). Although all are reported to possess antioxidant activity,  $\alpha$ -tocopherol, at plasma concentrations of 15-40  $\mu\text{mol}$ , is the most abundant form and the most potent lipophilic, chain breaking antioxidant in the human body (Chopra & Bhagavan, 1999). Before the interest shifted to polyphenols, vitamin E was the most extensively investigated antioxidant nutrient, reflected in its present popularity as a nutritional supplement.



Carotenoids are a group of naturally occurring fat-soluble compounds primarily found in plants, algae and photosynthetic bacteria (Palace *et al.*, 1999). Over 600 of these pigments are known to occur naturally and are responsible for many of the red, orange and yellow hue of plant leaves, fruits and flowers as well as the colour of some birds, insects, fish and crustaceans (Palace *et al.*, 1999). Animals appear incapable of synthesizing carotenoids but they incorporate these compounds from their diets to serve various functions. The best understood nutritional role for carotenoids in humans, particularly  $\beta$ -carotene, is their role as precursors to vitamin A (Palace *et al.*, 1999). Fruits and vegetables such as carrots, spinach and sweet potatoes are good sources of  $\beta$ -carotene. Carotenoids are also believed to function as biological antioxidants, protecting cells and tissues from the damaging effects of oxidants. Lutein and zeaxanthin are carotenoids abundant in maize and leafy vegetables and are suggested to act as antioxidants in the macular region of the human retina while lycopene abounds in tomatoes and is an effective quencher of the singlet oxygen radical (Snodderly, 1995). Other important antioxidant carotenoids include  $\alpha$ -carotene and  $\beta$ -cryptoxanthin.

#### **2.3.3.2 Antioxidant Vitamins and $\beta$ -carotene and cardiovascular disease**

Epidemiological evidence has generally shown a relationship between dietary intake of carotenes and vitamins C, E versus reduced morbidity and mortality from heart diseases. The 1991 WHO/MONICA Study provided some of the first evidence for the possible cardioprotective role for antioxidants (Gey *et al.*, 1991). In this study both the blood levels of alpha-tocopherol and tocopherol/cholesterol ratio were inversely correlated with mortality rates. Vitamin C and  $\beta$ -carotene were found not to be protective. Other early studies have reported inverse relationship between plasma  $\alpha$ -tocopherol and/or vitamin C and alpha-tocopherol intake or supplementation and incidence of cardiac events (Riemersma *et al.*, 1991; Gey *et al.*, 1993; Rimm *et al.*, 1993). Several large long-term observational studies linked higher levels of vitamin E to a reduced incidence of CVD suggesting that results are more definitive with prolonged supplementation (Rimm *et al.*, 1993; Stampfer *et al.*, 1993; Knekt *et al.*, 1994; Kushi *et al.*, 1996; Losonczy *et al.*, 1996). Results from intervention trials have been largely inconclusive. The Cambridge heart antioxidant study (CHAOS) study in England, a trial of vitamin E supplementation in 2002 CHD patients demonstrated that vitamin E supplementation (400 IU or 800 IU) significantly decreased the risk of myocardial infarction and CVD-related mortality after 200 days (Stephens *et al.*, 1996). This was one of the few clinical trials to show a positive correlation. Other important studies like the HOPE, VEAPS, GISSI, CARET and ATBC all had no (or just a marginal) positive correlation of vitamins A, C, E and  $\beta$ -carotene with cardiac events (ATBC group, 1994; Omenn *et al.*, 1996; Marchioli, 1999; Yusuf *et al.*, 2000; Hodis *et al.*, 2002). In fact, the relative risk of death from CVD increased in the CARET and ATBC studies and even more

alarming - the latter study was terminated early because of an increased incidence of lung cancer in the treatment group.

Researchers have been puzzled by the discordance between data from epidemiological studies and those from clinical trials. One of the many reasons given as possible explanations is that the complex mixture of antioxidant micronutrients in a diet might be more effective than large doses of individual antioxidant supplements (Langseth, 1995). Vitamin C, for example, despite its known antioxidant potential, has not been consistently associated with decreased oxidative damage when individually supplemented as opposed to when combined with vitamin E (Bendich *et al.*, 1984). It has also been concluded that  $\beta$ -carotene most likely has a beneficial role in coronary heart disease (CHD) but that it probably acts in concert with other antioxidant nutrients (Tavani & La Vecchia, 1999). By using rooibos as prepared domestically, this study took cognisance of the current research endeavours of testing antioxidant effects of wholesome dietary sources as opposed to isolated supplements.

### **2.3.4 Polyphenols**

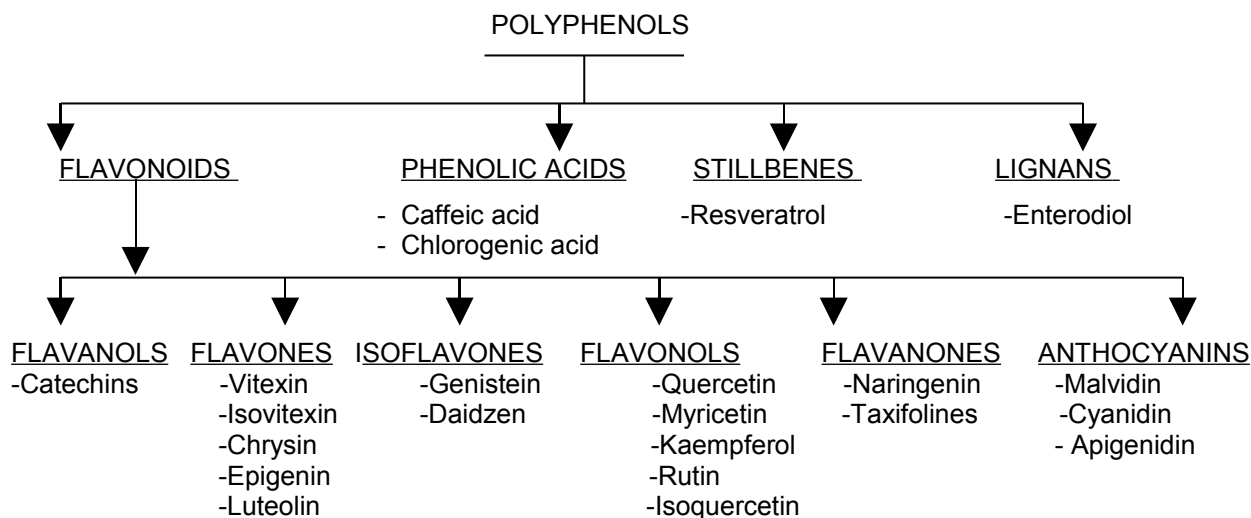
#### **2.3.4.1 Introduction**

Although not considered essential dietary constituents and with no recommended daily allowance set yet, plant phenolic compounds have attracted considerable attention in the past decade because of their antioxidant properties. They are the major antioxidant components of *Aspalathus linearis* (rooibos) and will therefore be discussed in more detail than the other dietary antioxidant components.

Polyphenols are ubiquitous in vascular plants where they serve to protect against parasites, herbivores and oxidative cell damage (Scalbert *et al.*, 2002). In addition, they play a part in pollination by producing colours that attract insects to their food sources, e.g. anthocyanins produce the mauve, blue, red, pink and violet colours of fruits and vegetables (Coulter, 1990). Phenolic compounds such as salicylic acid also serve as signalling molecules while lignin provide mechanical support (Parr & Bolwell, 2000). Over 8000 phenolic structures have been identified and categorized into several classes as shown in figure 2.2 (Cohen *et al.*, 2000).

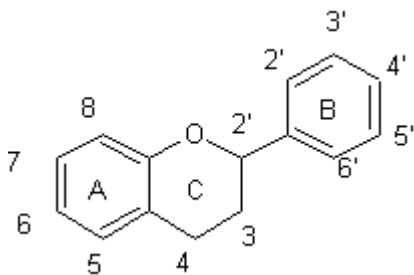
Flavonoids, the most abundant and most extensively studied class, comprise a series of C-15 compounds with two phenolic rings joined by an oxygen-containing pyran ring as shown in

figure 2.3. Based on variations on the C ring, flavonoids are further classified as flavanols (catechins), flavonols, flavones, flavanones, anthocyanins and isoflavonoids as shown in figure 2.2. Their antioxidant activities are suggested to be dependent on the structure especially the degree of hydroxylation of the B ring and the presence of unsaturated double bonds on the C ring (Brown *et al.*, 1998).



**Figure 2.2: General classification of polyphenols with examples (modified from Cook & Samman, 1996)**

Most flavonoids occur naturally as glycosides, with the commonest sugar moieties being D-glucose, L-rhamnose, xylose, glucorhamnose, galactose, lignin and arabinose (Aherne & O'Brien, 2002). The preferred glycosylation site on the flavonoid molecule is the C-3 position and, less frequently, the C-7 position; e.g. quercetin can be linked to the 3-*o*- glycoside rhamnose to yield quercetrin, or glucorhamnose to yield rutin (Aherne & O'Brien, 2002). Only the catechins and proanthocyanidins occur naturally in plants unattached to sugar moieties, known as the aglycone forms.



**Figure 2.3: The flavan structure upon which flavonoids are based showing the numbering of the carbon atoms and the aromatic rings – A, B and C.**

#### 2.3.4.2 Challenges in polyphenol research

Compared to other dietary antioxidants discussed in previous sections, research interest in health properties of polyphenols is relatively recent due to a number of challenges. One of the main barriers is difficulty in accurately measuring the daily intake of polyphenols, partly due to the lack of a complete database for phenolic contents of commonly consumed foods. Currently, there is an evolving United States Department of Agriculture (USDA) database for the content of flavonoids in selected foods, which is a compilation of published data from the scientific literature (<http://www.ars.usda.gov/ba/bhnrc/ndl>). Since the amount of polyphenols produced by plants is influenced by several factors including stress from disease, insects, climate, agricultural conditions and seasonal differences, considerable variability will always exist meaning that the level of intake of polyphenols vary considerably among diets (Stewart *et al.*, 2000). Nonetheless, the availability of a comprehensive database will greatly aid researchers in efforts to compare intakes from various studies. Another challenge is presented by the large number of phenolic compounds found in plant foods yielding different biological activities as shown in several *in vitro* studies (Scalbert *et al.*, 2005). Complicating the matter further is the fact that the ultimate form of phenolic compound to which cells and tissues in the body are exposed are often quite different from the original form present in foods and beverages (Hughes, 2005). Many previous studies have exposed cultured cells to aglycones or phenolic-rich extracts derived from plant material; however, phenolic compounds circulating in plasma are usually present as glucuronate or sulphate conjugates with or without methylation of the catechol functional group (Kroon *et al.*, 2004). The conjugates are very likely to have different biological activities from the starting compounds. Nonetheless, despite these challenges, research into the possible contribution of polyphenols has greatly increased in the last decade.

#### 2.3.4.3 Polyphenols in human diet

Fruits and fruit juices are among the best sources of polyphenols in the human diet because of the high content of these compounds in most fruits and the relatively large servings (100-200 g). A serving of apples, a popular component of the South African diet, provides as much as 400 mg of total phenols (Lotito & Frei, 2004). Vegetables such as onions, spinach and broccoli as well as popular beverages like tea, coffee and red wine, also contribute significant amounts of polyphenols in the human diet. With the current data on food phenolic content, a well-balanced diet with the recommended 5-7 daily servings of fruits and vegetables per day and moderate amounts of chocolate, tea, coffee or wine, has been estimated to provide over 1 g of total phenols per day (Lotito & Frei, 2006).

Accurate determination of dietary intake of polyphenols, however, is difficult as values depend on availability of comprehensive food compositional tables, dietary habits and preferences as well as actual polyphenol content in foods which, in turn, depends on several factors including plant variety, seasonal differences, degree of ripeness and variation in degree of light exposure (Stewart *et al.*, 2000) as previously mentioned. Vegetables grown conventionally in South Africa and Spain, for example, were shown to contain four- to five-fold more flavonols than those in the United Kingdom where greenhouses are used for plant cultivation (Stewart *et al.*, 2000). Rautenbach and Venter (2008) recently compared the oxygen radical absorbance capacity (ORAC) values of selected South African (RSA) fruits and vegetables with reported USDA data. Apple and pear varieties from RSA showed lower ORAC values while bananas, peaches and red grapes as well as vegetables had much higher ORAC values when compared to the international data. The method of processing and culinary preparation also influence the polyphenol content of food sources, e.g. peeling of fruits substantially decreases their phenolic content since these compounds are plentiful in the outer parts (D'Archivio *et al.*, 2007). Boiling (15 min) of onions and tomatoes resulted in a loss of about 75% of the initial quercetin content while frying for the same duration lead to losses of about 30% of the same flavonol demonstrating that the method of cooking influences the phenolic content (D'Archivio *et al.*, 2007). It is therefore not surprising that the reported dietary intake for phenolic compounds is highly variable among population groups studied. Nonetheless, a diet rich in plant foods and beverages will be high in phenolic compounds. Table 2.4 lists the main subgroups of flavonoids and their food sources.

**Table 2.4: Subgroups of flavonoids, specific examples and food sources**

<b>Subgroup</b>	<b>Examples</b>	<b>Food sources</b>
Anthocyanins	Cyanidin, malvidin	Red wine, fruits berries e.g. blackberries
Dihydrochalcones	nothofagin, aspalathin	Rooibos
Flavanols	Catechins, Procyanidins	Tea (green, black), Red wine
	Theaflavins	Black tea
Flavanones	Hesperetin	Citrus fruits and juices e.g. lemon, lime, cumin,
	Naringenin	oranges, grapefruits
Flavones	Luteolin	Parsley, thyme, hot peppers
Flavonols	Quercetin, kaempferol	Onions, apples, broccoli, kale, red wine
Isoflavones	Daidzein, genistein	Legumes especially soybeans

Modified from Lotito and Frei, 2006.

#### **2.3.4.4 Absorption, bioavailability and metabolism of polyphenols**

Ultimately, to support the hypothesis that dietary polyphenols account for the reported biological effects in humans, it is vital to establish their bioavailability after consumption. Bioavailability is the proportion of a chemical compound in a food, nutritional supplement, drug, or other

substance that is absorbed and available in the tissues of the body (D'Archivio *et al.*, 2007). Once ingested, a compound is processed and subsequently absorbed along the gut and eventually transported to the liver where most compounds are metabolized. Some compounds are metabolized so extensively that only a small amount enters the systemic circulation unchanged. When this occurs, the bioavailability is reduced and thus its availability at the target site and subsequent bioactivity are decreased unless the new metabolites are more active (D'Archivio *et al.*, 2007).

Like any other ingested compound, the bioavailability of polyphenols depends on gut absorption. It is now accepted that dietary polyphenols are absorbed along the gut but whether passively by diffusion or actively via the sodium dependent glucose transporter 1 (SGLT1), the exact mechanism involved still remains unresolved (Walle, 2004). Aglycones are generally absorbed from the small intestines. Most polyphenols, however, are not absorbed intact because they occur in food as glycosides, esters or polymers requiring hydrolysis before absorption can occur. Although hydrolysis has now been shown to occur in the small intestines and the oral cavity, the bulk of the process occurs in the colon where it was initially thought to be restricted (Saura-Calixto *et al.*, 2007). Anthocyanins appear to be the only polyphenols absorbed intact as glycosides although only to a limited extent; their positive charge has been suggested to account for this uniqueness given that other flavonoids are largely neutral compounds (Walle, 2004).

Absorption of polyphenols is influenced by several factors for example molecular weight and esterification. The high molecular weight of proanthocyanidins, which are polymers of catechins found in large amounts in fruits and cocoa, explains why they are virtually unabsorbed in the gut in the intact form (Holt *et al.*, 2002). Esterification of catechins with gallic acid and of caffeic acid esterification with quinic acid considerably reduced the absorption of these two phenolic compounds (Scalbert *et al.*, 2002). The food sources and matrices within which phenolics are ingested also influence absorption of these compounds, e.g. absorption of quercetin from onion is four fold greater than that from apples (Hollman *et al.*, 1997). Debate on the effect of milk on absorption, bioavailability and bioactivity of polyphenols has been more intense and protracted. Milk may lead to the formation of polyphenol-milk protein complexes which can decrease the bioavailability and antioxidant potential of polyphenols *in vivo*, thus negating the beneficial attributes of black tea (Siebert *et al.*, 1996; Langley-Evans, 2000; Arts *et al.*, 2002). In contrast, other investigators report no masking effects of milk addition on increases in plasma antioxidant potential and concentrations of flavonoids after the consumption of black tea or cocoa drink (Leenen *et al.*, 2000; Reddy *et al.*, 2005; Keogh *et al.* 2007; Kyle *et al.*, 2007). A recent study in

which 40 g of cocoa powder were dissolved in 250 mL of whole milk found no effect of the milk on the bioavailability of cocoa powder flavonoids in healthy humans (Roura *et al.*, 2008). However, it can be deduced from the findings of Sharma *et al.* (2008) that the apparent influence of milk may differ depending on the assay method used. These authors report that milk markedly reduced the efficacy of black tea in scavenging the DPPH radical but enhanced and stabilized the scavenging activity of the tea when the  $\beta$ -carotene bleach method was used. Different polyphenolic components were considered, using different antioxidant assay systems and targeting varied parameters therefore making interpretation difficult (Sharma *et al.*, 2008). One may speculate that if milk affects polyphenols, then the effect varies depending on the individual polyphenolic components. On the balance of literature, this study assumed no effect by milk on the in vivo antioxidant effect of the rooibos polyphenols.

After absorption, polyphenols are bound to albumin and transported to the liver where they are extensively metabolized by conjugation (glucuronidation, sulphation or methylation) or catabolized to smaller phenolic compounds (Yao *et al.*, 2004). Investigations in animal and human studies indicate that this process takes place fairly quickly and efficiently such that free aglycones are generally either absent or present in trace amounts after consumption of nutritional doses (Yao *et al.*, 2004). Green tea catechins are an exception as the aglycone forms can make up a significant proportion of the total amount in plasma (Van het Hof *et al.*, 1998).

The concentrations of polyphenols in blood reached after ingestion varies according to the nature of the polyphenol and the food source. Table 2.5 shows the bioavailability of selected polyphenols ingested in phenolic-rich foods. For most polyphenols, maximum plasma concentrations are rapidly attained within 1-2 h after ingestion but rarely exceed 1  $\mu$ M and the maintenance of elevated levels in plasma requires repeated ingestion over time (Van het Hof *et al.*, 1999). With such trace levels in plasma, determination of the bioavailability of polyphenols in tissues is important but such data are very scarce. Two studies compared the levels of polyphenols in human prostate tissue. In the first study, significantly lower prostatic level of genistein was reported in men with prostatic cancer than in those with normal prostate while plasma concentrations of the isoflavone were higher in the cancerous group (Hong *et al.*, 2002). In the second study, daily consumption of 1.4 L of green tea for 5 days produced prostatic concentration of the four green tea catechins ranging from 21 - 107 pmol/g tissue (Henning *et al.*, 2006). A different study in women ingesting isoflavones reported higher concentration of equol in breast tissue than in serum whereas genistein and daidzein were more concentrated in serum than in breast tissue (Maubach *et al.*, 2003). Equol is an end product of daidzein metabolism that possesses estrogenic activity and is superior to all other isoflavones in its

antioxidant activity (Setchell *et al.*, 2002). It is clear that plasma concentrations of polyphenols are not directly correlated with concentration in tissues.

In summary, most polyphenols are absorbed to some extent depending on the specific type of polyphenol. The final form of polyphenol predominant in the body usually differs from the original ingested form. The bioavailability of polyphenols is only partial with a large proportion of ingested amounts remaining unabsorbed and the absorbed fraction being extensively metabolized.

**Table 2.5: Bioavailability of selected polyphenols / polyphenol-rich foods**

Polyphenols	Source	Quantity of polyphenol ingested	Maximum concentration in plasma ( $\mu\text{M}$ )
<b>Anthocyanins</b>			
Cyanidine 3-glucoside	Orange juice (1L)	71 mg Cy-3-glc	0.002
Malvidin 3- glucoside	Red wine (0.5L)	68 mg Mal-3-glc	0.001
Malvidin 3- glucoside	Grape juice (0.5 L)	117 mg Mal-3-glc	0.003
<b>Flavanols</b>			
EGCG	Green tea 5 g	105 mg	0.13-0.31
	Red wine 120 mL	34 mg	0.072
	Chocolate 80 g	137 mg	0.26
	Pure compound	50-1600 mg	0.28-7.4
	Green tea extract	110-328 mg	0.26-0.7
	Grape seed extract	18 mg	0.011
<b>Flavanones</b>			
Hesperidin	Orange juice	61 mg	0.48
Naringenin	Grapefruit juice	199 mg	5.99
Naringenin	Pure compound	135 mg	7.4
<b>Flavonols</b>			
Quercetin	Apples	107 mg	0.3
Quercetin	Onion	100 mg	7.6
Quercetin 4 glucoside	Pure compound	100 mg	7.1
Quercetin	Pure rutin	200 mg	1.1
<b>Isoflavones</b>			
Daidzein	Soymilk	108 mg	0.47
Genistein	Soymilk	102 mg	0.41
Daidzein	Pure compound	50 mg	0.76
Genistein	Pure compound	50 mg	1.26
<b>Phenolic acids</b>			
Gallic acid	Pure compound	50 mg	1.8GA + 2.3 MeGA
Gallic acid	Assam black tea	50 mg	1.8GA + 2.3 MeGA
Chlorogenic acid	Coffee (200 mL)	96 mg	0.5 caffeic acid
Caffeic acid	Red wine (200 mL)	1.8 mg	0.06

Adapted from D'Archivio *et al.*, 2007. Abbreviations: GA= gallic acid; MeGA = methylgallic acid; Cy-3-glc = Cyanidine 3-glucoside; Mal-3-glc = Malvidin 3- glucoside



The maximum concentration in plasma rarely exceeds 1  $\mu\text{M}$  after the consumption of 10–100 mg of a single phenol. Clearly, a mechanistic link between the strong antioxidant properties of flavonoids *in vitro* and their biological activity *in vivo* has not yet been accurately established.

#### **2.3.4.5 Polyphenols and cardiovascular disease**

The hypothesis that dietary polyphenols may provide cardio-protective benefits is largely based on their observed ability to act as antioxidants. Evidence from large observational studies has generally supported a beneficial role for polyphenols in cardiovascular health. In the Zutphen Elderly Study for example, a 68% lower risk of mortality from CHD was seen in men who consumed >29 mg flavonoids/day compared to those consuming 0-19 mg flavonoids/day (Keli *et al.*, 1996). Similarly, an inverse relationship was shown between polyphenol intake and incidence of CVD, stroke and myocardial infarction (Knekt *et al.*, 2002). However, researchers now question whether the amount of bioavailable polyphenols is sufficient to account for their attributed bioactivity. Evidence suggests that, besides antioxidant activity, other mechanisms are involved in their cardio-protective activity as will be outlined in this section. These include decreasing platelet aggregation, modulation of eicosanoid production and enhancing vascular endothelial function (Vita, 2005). All these are critical parameters in cardiovascular pathology.

##### **2.3.4.5a Antioxidant mechanism**

Since dietary polyphenols exhibit profound antioxidant properties it has been hypothesized that their intake can influence the risk for CVD by protecting against the oxidative damage associated with atherosclerosis (Kris-Etherton & Keen, 2002). Consequently, considerable research has focused on the possible link between CVD and flavonoid intake especially from red wine, tea and cocoa. A lower than expected incidence of heart disease in France, in the so-called “French paradox”, was attributed to a comparatively higher consumption of red wine whose presumed protective effects could be attributed, at least in part, to the antioxidant properties of polyphenols (Renaud & De Lorgeril, 1992). Consistent with this, Whitehead *et al.* (1995) showed that consumption of 300 mL of red, but not white wine, significantly raised serum antioxidant activity in healthy subjects. They attributed the differences to the higher levels of flavonoids in red wine (2.0 g/L) compared to white wine (0.2 g/L). To test the hypothesis that flavonoids account for the observed increase in serum antioxidants, investigators gave increasing amounts of flavonoid-rich cocoa (0 g, 27 g, 53 g, and 80 g) to adult volunteers (Wang *et al.*, 2000). A dose response relationship was observed between the levels of plasma epicatechin and a trend for increased serum antioxidant capacity and decreased lipid

peroxidation. A review of human clinical trials involving tea consumption indicates an overwhelming majority of the studies reporting improved antioxidant activity (Weisburger, 2003).

Researchers however, are increasingly questioning the *in vivo* efficacy of polyphenol bioactivity including antioxidant activity. A point implied in a previous section of this study and well articulated by Lotito and Frei (2006), is that despite the large polyphenol intakes in the region of 1 g/day, the absorption of flavonoids remains low compared to that of other dietary antioxidants such as vitamins C and E. The authors point out that the maximal plasma concentrations seen in humans after intake of flavonoid-rich meals are between 0.06 - 7.6  $\mu\text{M}$  compared to steady-state concentrations of vitamins E (15 - 40  $\mu\text{M}$ ) and C (30 - 150  $\mu\text{M}$ ). In comparison, the steady state concentrations of quercetin in human plasma are less than 1  $\mu\text{M}$ . Furthermore, the small amounts of polyphenols absorbed are extensively metabolized in the intestines and liver and that chronic intake of flavonoid-rich foods (the strategy in this study) does not result in significant build-up of flavonoids in plasma. Their argument, therefore, appears plausible that flavonoids' ability to act as antioxidants in plasma is limited. Hence to what extent the antioxidant properties of plant foods and beverages contribute to the observed protection against CVD and other diseases, where oxidative damage by ROS are considered central, remains unclear.

Many of the phytochemicals in fruit and vegetables show properties unrelated to antioxidants that interfere with some stages of disease pathogenesis, e.g., elevated plasma homocysteine levels have been confirmed as an independent risk factor in vascular disease (Boushey *et al.*, 1995). Since plants are good sources of folates, the observation that folates can reduce homocysteine levels, implies competing mechanisms in protective effects linked to high dietary intake of plant food (Ward *et al.*, 1997). It is clear that the complex relationship between antioxidant status and disease, though intensively studied, is still poorly understood. The long held concept that polyphenols protect cells by scavenging ROS is now seen as an oversimplified view of their mode of action and focus is turning to their other known biological activities e.g. interaction with enzymes (Azzi *et al.*, 2004; Halliwell, 2008). One proposition is that polyphenols interact directly with receptors/enzymes involved in signal transduction to modify redox balance - well exemplified by the interaction of soy isoflavones with oestrogen receptors to affect endocrine functions (Halliwell *et al.*, 2005; Moskaug *et al.*, 2005). While considerable literature supports a role for oxidative stress in disease development and a contribution of dietary polyphenols to their prevention, an understanding of the molecular events underlying the biological effects is vital for evaluation of the overall impact on pathogenesis.

#### **2.3.4.5b Inhibition of low density lipoprotein oxidation**

Owing to their antioxidant properties, polyphenols are thought capable of interfering with the oxidative modification of LDL thereby preventing or slowing atherosclerosis. Phenolic substances in grapes and red wine were reported to inhibit LDL oxidation *in vitro*, as did quercetin, epicatechin and olive oil (Chopra *et al.*, 2000; Fito *et al.*, 2000). Consumption of purple grape juice by healthy individuals reduced the susceptibility of LDL to oxidation and improved endothelial function while studies investigating effects of green and black teas and red wine, have been unclear (Stein *et al.*, 1999). In one study, the intake of 8 cups/day of green tea for 3 days showed increased bioavailability of polyphenols but failed to enhance resistance of LDL to oxidation *ex vivo* at 60 hours, although it led to measurable increases in urinary 4-O-methylgallic acid – a marker of uptake and metabolism of tea polyphenols (Van het Hof *et al.*, 1999). The authors attributed the lack of effect on LDL oxidation to incorrect timing of blood collection. Since the half life of plasma flavanols is about 2-4 hrs, it was suggested that their fasted blood contained only minor amounts of tea flavanols. However, as demonstrated by Lotito and Fraga (2000), protection may be through other mechanisms such as regeneration of vitamin E in the LDL particle which would require a longer time to observe.

#### **2.3.4.5c Endothelial and platelet function**

The vascular endothelium plays a central role in the regulation of vascular homeostasis by producing factors that act locally in the vessel wall and lumen e.g. nitric oxide (NO), a potent vasodilator (Vita & Keaney, 2002). Nitric oxide has been shown to have a host of other crucial vascular functions e.g. preventing leukocytes and platelet adhesion to endothelial surface, platelet aggregation, expression of adhesion molecules as well as altering expression of non-cellular components that make up the vascular wall matrix, making NO relevant in lesion formation (Vita & Keaney, 2002). The vascular endothelium also produces other molecules that impact on vascular tone (e.g. prostacyclin), fibrinolysis (e.g. plasminogen activator and its inhibitor), coagulation (e.g. heparin), and inflammation (e.g. cytokines) (Widlansky *et al.*, 2003). The profound association of endothelial dysfunction with increased risk of CVD events underlines the importance of the endothelium to cardiovascular health. Many interventions known to reduce CVD risk are able to reverse endothelial dysfunction. Lipid lowering drugs, exercise and cessation of smoking have all been shown to reverse endothelial dysfunction in atherosclerotic patients (Widlansky *et al.*, 2003). The state of the endothelium may therefore be an indicator of vascular health especially with the observation that adverse effects to the

endothelium seem to be a unifying factor of otherwise diverse CVD risk factors from atherosclerosis to diabetes mellitus (Vita 2005).

Endothelial dysfunction can be indicated by the presence of an impaired endothelium-dependent vasodilation which can be measured in the brachial artery in response to reactive hyperemia using flow-mediated dilation (FMD) - a non invasive ultrasound technique (Corretti *et al.*, 2002). The response is impaired in the presence of coronary risk factors and is correlated with abnormal responses in coronary circulation. A few studies have examined whether dietary factors can affect endothelial function. For vitamin C in particular, there is extensive evidence that acute treatment reverses endothelial dysfunction in many conditions including atherosclerosis, diabetes mellitus and congestive heart failure (reviewed in Duffy *et al.*, 1999). Stein *et al.* (1999) investigated the effects of grape juice consumption on endothelial function and LDL oxidation. After a daily intake of 60 mL of the juice for 14 days, FMD significantly increased by  $4.2 \pm 4.4\%$  from an impaired value of  $2.2 \pm 2.9\%$  at baseline. Lag time to LDL oxidation also increased significantly by 35%. The study was the first intervention study to use whole food to investigate the *in vivo* effects of a flavonoid-rich diet on endothelial function in humans. Two studies examined the effects of black tea on endothelial function in healthy subjects as well as in subjects with angiographically proven coronary artery disease (Duffy *et al.*, 2001; Hodgson *et al.*, 2002). Again, both studies reported an improved FMD and no effect from an acute dose of caffeine meaning the caffeine content in tea had no detectable role in the results. Other studies using polyphenol-rich cocoa and red wine have also shown improved FMD after brief consumption (reviewed in Vita 2005).

Often, the first clinical manifestation, and often a fatal event in CVD, is platelet adherence to, and aggregation on, the atherosclerotic vessel wall leading to thrombus formation (Pearson *et al.*, 2005). A beneficial role for polyphenols in platelet activation and function would therefore impact heavily on CVD morbidity. The influence of grape juice on platelet activity was investigated by adding the juice *ex vivo* to platelets and several observations were made including a reduction in platelet aggregation, a decrease in platelet production of the superoxide radical and an increase in production of NO by the platelets (Freedman *et al.*, 2001). Results from three studies involving black tea were not in agreement. One study in humans showed no effect of consumed black tea on platelet function but another indicated benefits comparable to those mentioned above for grape juice (Vita, 2005). P-selectin is one of the specific platelet receptors important for platelet adhesion and aggregation hence the demonstration that daily intake of 5 cups of black tea for 5 weeks reduced plasma concentration of P-selectin is of interest (Hodgson *et al.*, 2001)

The formation and progression of atherosclerosis lesions is characterized by excessive vascular remodelling with increased proliferation of new vessels like adventitial *vasa vasorum* evident in advanced coronary atherosclerosis (Oak *et al.*, 2005). These new vessels have been suggested to contribute to the development and progression of atherosclerosis by providing oxygen and nutrients to the lesion sites. Recent evidence has shown that natural polyphenols from green tea and red wine prevented the development of atherosclerosis by inhibiting the formation of these new blood vessels (Oak *et al.*, 2005). Although the exact mechanisms involved remain unclear, it has been proposed that polyphenols inhibit both vascular endothelial growth factor (VEGF), a major proangiogenic factor as well as matrix metalloproteinases (MMPs) - enzymes involved in destabilization and rupture of atherosclerotic plaques (Oak *et al.*, 2005).

Collectively, these studies indicate possible protective roles of polyphenols in cardiovascular health by involvement in vascular health and platelet function and at the same time offer plausible mechanisms, other than that as antioxidants, by which polyphenols can contribute to cardiovascular health.

#### **2.3.4.6 Can excess polyphenols be harmful?**

The array of health benefits attributed to dietary antioxidants has led to unprecedented appreciation by the increasingly health conscious public. However as is true with many agents/compounds, excessive amounts of polyphenols may not be without risks, even though they are from dietary sources. Some dangers linked to consumption of polyphenols are documented and the spectre of the ATBC study mentioned earlier (ATBC group, 1994) serves to warn us that much is still unknown and that certain dietary supplements of any kind may have effects conflicting what is desired.

The suggested disease-protective effects of polyphenols are often attributed to their powerful antioxidant activities *in vitro*. However, they can also exert pro-oxidant activities *in vitro* and in the oral cavity, but it should be mentioned that no evidence for systemic pro-oxidant effects of polyphenols has been shown in humans yet (Lambert *et al.*, 2007; Halliwell, 2008). Some polyphenols given at high doses to experimental animals were shown to account for chronic neuropathy (quercetin), reduced fertility (isoflavones) and induction of fore-stomach and kidney tumours (caffeic acid) (Setchell *et al.*, 1987; Hagiwara *et al.*, 1991; Dunnick & Hailey, 1992). In line with synthetic antioxidants, isoflavones can inhibit thyroid peroxidase and thus interfere with thyroid function. Vitexin, an isoflavone abundant in millet and genistein from soy, were shown to

increase thyroid weight and decreased plasma levels of thyroid hormones in experimental rats (Doerge & Chang, 2002). Could this be one of the causes of endemic goitre in West Africa, an area where millet is a staple food? Consumption of polyphenols e.g. tannins, may also have anti-nutritional effects exemplified by the inhibition of iron absorption when consumed simultaneously with black tea and therefore of potential risk to individuals in populations with low iron status (Temme & Van Hoydonck, 2002). Furthermore, tannins (abundant in green and black teas but negligible in rooibos herbal tea) have been shown to interact with proteins and inhibit certain enzymes (Mennen *et al.*, 2005). Polyphenols may interfere with drug bioavailability and pharmacokinetics as seen with benzodiazepines and terfenadine which show up to 3-fold increase in bioavailability when taken with grape juice, a rich source of naringenin (Ameer & Weintraub, 1997). Rooibos infusions have been reported to modulate the activity of CYP P450 enzymes by inhibiting (Jang *et al.*, 2004) or enhancing (Matsuda *et al.*, 2007) the *in vivo* activity and expression of CYP2C11 and CYP3A isozymes respectively in rats. Since CYP2C11 is known to metabolize various drugs, its inhibition may conceivably enhance their half lives and possibly result in toxicity. Increased activity of CYP3A enhances the hydroxylation of midazolam, a benzodiazepine derivative and therefore may influence the drug's bioactivity as suggested by the authors. By assessing liver and kidney profiles, a secondary outcome of this study established whether consumption of 6 cups of rooibos herbal tea for 6 weeks has potential risks to the study group.

It is important to note, however, that most of the risks associated with polyphenols have only been shown in animal and *in vitro* studies and it is not clear whether the results can be extrapolated to human situations because of the dose and bioavailability issue. The doses used in these studies are much higher than those from habitual dietary intakes (Mennen *et al.*, 2005). The limited absorption of most of the dietary polyphenols, long regarded as a constraint to bioactivity, may be an apparent protective mechanism. Strategies to boost tissue levels by using supplements or by manipulation of absorption should therefore be implemented with caution. More research in this area is needed and this study calls attention to the possible problems with intake of excessive amounts of supplementary antioxidants beyond what is provided by an antioxidant-rich diet.

#### **2.4 Rooibos: A possible health promoting/protective tool?**

The afore-mentioned interest in health promoting constituents of plant foods has led to the increasing interest in *Aspalathus linearis*, a South African flowering shrub recognised as one of the few economic plants to make the transition from a wild resource to a cultivated crop in the 20<sup>th</sup> century (Rabe *et al.*, 1994). This shrub is used to make a mild tasting herbal beverage rich in polyphenol antioxidants but with no caffeine and very little tannins which is not only enjoyed as a pleasant drink but is also claimed to cure insomnia, allergies and nervous breakdown as well as to improve the appetite (Morton, 1983). Although used for many generations in South Africa, rooibos is relatively new in the international arena and a brief background is appropriate to put everything into perspective.

#### 2.4.1 History and processing

Rooibos (pronounced ROY boss), which is Afrikaans for 'red bush', has been used as a beverage in its native South Africa for over 300 years (<http://www.wesgro.org.za/>). The indigenous Khoi and San were the first to discover that the leaves and stems of the rooibos plant made a sweet, tasty herbal tea. They were the ones who first harvested the wild growing shrubs, chopped them with axes then bruised them with wooden hammers before piling the harvest in heaps to ferment and finally spreading it in the sun to dry (Joubert *et al.*, 2008).

In 1904, Benjamin Ginsberg, a Russian immigrant began trading it from the locals, but it was in the 1930s that rooibos became a cultivated crop when Dr P.F. le Fras Nortier, a local medical doctor, realising its commercial potential, initiated plantation cultivation by farmers (<http://www.rooibosltd.co.za/background/index.html>). The shortage of *Camellia sinensis* (black) tea fuelled by the Second World War prompted a steady growth in demand and production of the herbal tea. However, at the end of the war this boom stopped, forcing the producers to establish the Clanwilliam Tea Cooperative in 1948 in an attempt to salvage the industry (Joubert *et al.*, 2008). In 1954, a ministerial directive established the Rooibos Tea Control Board to replace the cooperative and with the goals of streamlining marketing, stabilising prices and standardising quality. The board was privatised in 1993 and the company, Rooibos Limited, largely owned by rooibos producers, was created (<http://www.rooibosltd.co.za/background/index.html>). According to the Western Cape Investment and Trade Promotion Agency, Rooibos Limited accounts for almost 70% of exported rooibos with the rest going through smaller companies like Cape Natural Tea Products and Khoisan (<http://www.wesgro.org.za/>). Annual output now exceeds 14 000 tons and exports are made to countries worldwide including Germany, the Netherlands, Japan, England, China and the USA

(Joubert *et al.*, 2008). The popularity of this herbal tea has been attributed to the low tannin content and absence of caffeine (Morton, 1983).



Figure 2.4: Map of southern Africa showing the Cedarberg region of South Africa

*Aspalathus linearis* is a short legume native to the Pakhuis pass area in the Northern Cedarberg region of the Western Cape, 250 Km north of Cape Town (Joubert *et al.*, 2008) shown in Figure 2.4. It is a polymorphic species with various wild forms differing in colour and height of growth. According to Joubert *et al.* (2008), the commercially cultivated variant was previously classified as *Aspalathus linearis* (Brum.f) Dahlg. subsp. *linearis* but was later revised and combined with *Aspalathus linearis* subsp. *pinifolia* under *Aspalathus linearis*. In natural state, it is a shrubby bush with a mature height of 1-1.5 m supported by a central stem that divides just above the ground into several delicate branches that bear bright-green, needle-like leaves 10-40 mm in length (Joubert *et al.*, 2008). Figure 2.5 show rooibos growing in the Cedarberg area of South Africa. The plant has adapted to the region's highly acidic, nutrient-poor sandy soils, hot dry summers and winters of scant rainfall. The network of roots just beneath the soil and a taproot of 2 m deep reach for moisture in dry periods and like all legumes, the nitrogen fixing bacteria in its root nodules convert nitrogen dioxide to biologically useful ammonia hence minimizing need for nitrogen-based fertilization of the crop (Muofhe & Dakora, 1999). Planting is done between February and March and first harvesting done 18-24 months later with a further 2-3 more times after which the land is ploughed and left for five years before the next rooibos planting (<http://www.rooibosltd.co.za/background/index.html>). However, other crops are planted in this



period to fertilise the land. Harvesting is done by cutting the leaves- bearing stems 30 cm above the ground by hand using a sickle or by machine.



**Figure 2.5: Rooibos growing in the Cedarberg region of South Africa (courtesy of Rooibos Limited, SA)**

Two types of rooibos herbal teas (figure 2.6) are produced depending on the processing method chosen (Joubert *et al.*, 2008). Traditional or “fermented” rooibos is made by machine-cutting the stems and leaves into fine lengths of 3-5 mm, bruising them between rollers before wetting and piling them in heaps to trigger chemical oxidation also referred to as “fermentation”. It is during this oxidation step, which lasts for 12-24 hrs, that rooibos attains its characteristic aroma and red colour and hence its name. The second type, “green” or unfermented rooibos is made by immediately oven-drying the fresh harvest to prevent oxidation before cutting into pieces and sieving. The green rooibos has higher levels of polyphenol antioxidants than its traditional/fermented counterpart as the fermentation process results in a substantial loss of these molecules (Joubert *et al.*, 2008).



**Figure 2.6: Green (a) and fermented/traditional (b) rooibos**

Green rooibos is a recent entrant into the market and was developed to maximize antioxidant levels in response to recent interest in dietary antioxidants and unlike traditional/fermented rooibos, green rooibos has a yellow/green colour. Both types are available either organic or conventionally grown with an increasing shift towards the former (<http://www.rooibosltd.co.za/background/index.html>). The final product must be certified free of microbial contamination by the Perishable Products Export Control Board of South Africa and to pass these stringent health and safety requirements, steam pasteurization is done as the final step before packing (<http://www.wesgro.org.za/>). Monitoring and certification for organic rooibos is done by various international organisations such as Lacon and Ecocert from Germany.

#### **2.4.2 Phenolic profile**

Several phenolic compounds have been identified in rooibos. These include a rich mix of flavonoids like the flavonols quercetin, isoquercetin and rutin; the flavones luteolin, orientin, isoorientin, vitexin, isovitexin and chrysoeriol and the rare dihydrochalcones, aspalathin and nothofagin (Joubert & Ferreira 1996; Bramati *et al.*, 2003). At present, rooibos is the only known natural source of aspalathin while the closely-resembling nothofagin has only one other known natural source – the red beech tree (*Nothofagus fusca*) native to New Zealand (Joubert & Ferreira, 1996). One analysis found aspalathin, isoorientin orientin and rutin to be the most abundant flavonoids in both traditional/fermented and green/unfermented rooibos as shown in table 2.6. In addition to flavonoids, several phenolic acids that add to the polyphenolic pool have been reported in rooibos. These include protocatechuic, hydroxybenzoic, vanillic, coumaric, caffeic and ferulic acids (Rabe *et al.*, 1994).

**Table 2.6: Flavonoid content in aqueous extracts of traditional and green rooibos**

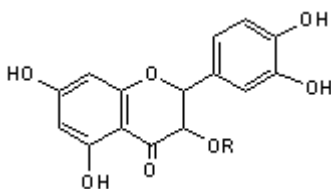
Flavonoid	Traditional/fermented rooibos mg/g $\pm$ SD	Green/unfermented rooibos mg/g $\pm$ SD
chrysoeriol	0.022 $\pm$ 0.001	0.0079 $\pm$ 0.0004
luteolin	0.029 $\pm$ 0.001	0.022 $\pm$ 0.002
quercetin	0.107 $\pm$ 0.002	0.042 $\pm$ 0.006
isovitexin	0.265 $\pm$ 0.002	0.659 $\pm$ 0.005
vitexin	0.330 $\pm$ 0.002	0.504 $\pm$ 0.002
isoquercitrin and hyperoside	0.429 $\pm$ 0.002	0.326 $\pm$ 0.006
isoorientin	0.833 $\pm$ 0.007	3.57 $\pm$ 0.18
orientin	1.003 $\pm$ 0.010	2.336 $\pm$ 0.049
rutin	1.269 $\pm$ 0.006	1.69 $\pm$ 0.14
Aspalathin	1.234 $\pm$ 0.010	49.92 $\pm$ 0.80
Nothofagin*	0.17	1.08
Total polyphenol*	29.69–34.25	35.08–39.30

Note: The extracts were prepared using 1 g of rooibos in 60 mL of hot distilled water, steeped for 10 minutes. After removal of the tea leaves, the solution was cooled and filtered. The table gives the amounts of analytes in mg per g of extract. \*= from Joubert *et al.*, 2008:393. SD not given. All other values: adapted from Bramati *et al.*, 2003).

Significant differences in total polyphenols have been reported between traditional/fermented and green/unfermented rooibos and these have been attributed to the chemical and enzymatic changes that result during fermentation as well as to the choice of drying method (Joubert, 1996; Standley *et al.*, 2001). Aspalathin and nothofagin represent a large percentage of polyphenols in green rooibos yet the two are extensively oxidized to different isoforms like dihydro-iso-orientin in the case of aspalathin during fermentation such that less than 7% of the original dihydrochalcones remain in the fermented rooibos (Joubert, 1996). Figure 2.7 shows the structures of the main polyphenolic compounds found in rooibos.

#### 2.4.2.1 Comparison with black and green teas (*Camellia sinensis*).

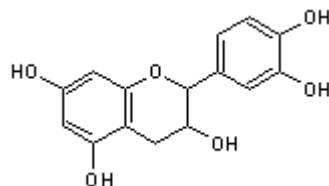
A 200 mL cup of rooibos contains 60-80 mg of total polyphenols largely based on the flavonoids shown in table 2.6 as well as the six phenolic acids (Joubert, 1996). For comparison, a similar cup of the more studied black tea contains a total of 166-193 mg total polyphenols per cup, constituting 24-40 mg catechins, 8-15 mg combined flavonols and flavones, ~85 mg thearubigins and 7-15 mg theaflavins (Hakim *et al.*, 2001). Green tea has higher levels than black tea largely present as catechins (250 - 260 mg) and to a lesser extent flavonols, caffeine, gallic acid and the purines, theobromine and theophylline (Graham, 1992).



## Flavonol

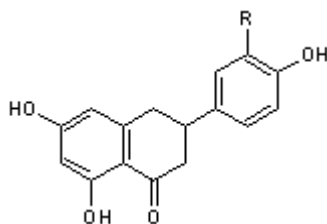
Quercetin: R = H, Rutin: R = rutosyl

Isoquercetin: R = D-glucopyranosyl



## Flavanol

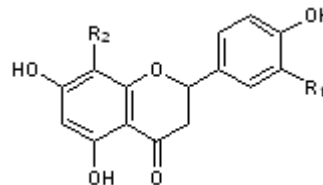
(+)- catechin



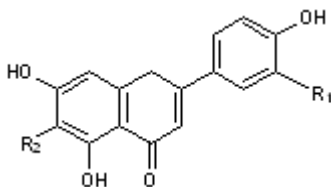
## Flavone

Luteolin: R = OH; Chrysoeriol: R = OCH<sub>3</sub>  
glucopyranosyl

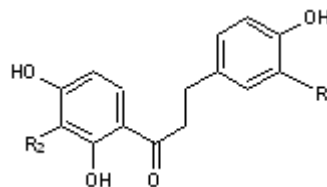
glucopyranosyl



## C-C linked flavone glycoside

iso-orientin: R<sub>1</sub> = OH; R<sub>2</sub> =Iso-vitexin: R<sub>1</sub> = H; R<sub>2</sub> =

## C-C linked flavone glycoside

Orientin: R<sub>1</sub> = OH; R<sub>2</sub> = glucopyranosylVitexin: R<sub>1</sub> = H; R<sub>2</sub> = glucopyranosyl

## Dihydrochalcone

Aspalathin: R<sub>1</sub> = OH; R<sub>2</sub> = glucopyranosylNothofagin: R<sub>1</sub> = H; R<sub>2</sub> = glucopyranosyl**Figure 2.7: Structures of the main polyphenolic constituents of rooibos (Joubert & Ferreira, 1996)**

Clearly, a cup of rooibos has less total polyphenols than a similar serving of green or black tea, but it is important to note that the types of polyphenols in rooibos differ from those found in *C. sinensis*. Thus the total polyphenol content cannot be the sole basis of comparison of their potential health benefits. While rooibos lacks compounds like epigallocatechin gallate (EGCG), a catechin abundant in green/black tea that has shown antioxidant and anticarcinogenic properties, its many other phytochemicals possess not only strong antioxidant activities but many other biological properties as will be discussed below.

### 2.4.3. Biological activities of rooibos

#### 2.4.3.1 Anecdotal

The story of rooibos mentions that in 1968, Annekie Theron, a young South African mother claimed that rooibos eased her infant's colic restlessness, vomiting and stomach cramps (<http://www.rooibosltd.co.za/background/index.html>). Finding no literature on rooibos, she began documenting the effects of rooibos on local infants with colic and allergic conditions concluding that rooibos was indeed beneficial. She published her observations in a book titled *Allergies: An Amazing Discovery*, in 1970, triggering the rooibos health acclaim. Today rooibos is used in South Africa for varied conditions like infant colic, diaper rash, digestive disorders, insomnia, nervous tension, loss of appetite and eczema. More research is needed to establish the accuracy of these claims and to identify the specific substances which might account for any observable benefits.

#### 2.4.3.2 Antioxidant activity and inhibition of lipid peroxidation

Several studies have used different test systems and variables to investigate the antioxidant activity of rooibos *in vitro*. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and beta-carotene bleaching methods were used to compare the antioxidant activities of unfermented, semi-fermented and fermented rooibos herbal teas with equivalents from *Camellia sinensis*, namely; green (unfermented), oolong (semi-fermented) and black (fermented) teas (Von Gadow *et al.*, 1997a). In the DPPH method, the order of inhibition for both rooibos and *C. sinensis* teas was unfermented>fermented>semi fermented. The individual inhibition ranking was green tea (90.8%), unfermented rooibos (86.6%), fermented rooibos (83.4%), and black tea (81.7%). The order in the  $\beta$ -carotene bleaching method was green tea>black tea>fermented rooibos>unfermented rooibos. Two other studies reported a lower activity by rooibos compared to green and black teas (Pazdzioch-Czochra & Widenska, 2002; Schulz *et al.*, 2003). As known for *C. sinensis*, a loss of antioxidant activity accompanies the fermentation process in rooibos mainly affecting the content of the two dihydrochalcones, aspalathin and nothofagin. The bulk of aspalathin is converted to orientin and iso-orientin which are both less efficient radical scavengers *in vitro* (Joubert *et al.*, 2004). Since rooibos is traditionally brewed for longer periods than other true teas (McKay & Blumberg, 2007), the demonstration by Von Gadow *et al.* (1997b) that longer brewing time enhances antioxidant activity of the tea as well as soluble solids could imply relevance to bioactivity of rooibos in

comparison to *C. sinensis*. However, detecting this effect on activity seems to depend on the method used since the Rancimat (and not  $\beta$ -carotene bleach) method demonstrated this effect. Several studies assessed the *in vitro* antioxidant activities of specific components in rooibos (Von Gadow *et al.*, 1997c; Joubert *et al.*, 2004; Snijman, 2007). Quercetin was the most potent scavenger of the DPPH radical with aspalathin, orientin, luteolin and isoquercitrin following closely. Perhaps more importantly, the first two studies included alpha-tocopherol in the evaluation and reported aspalathin to be more effective than the vitamin in scavenging the DPPH radical and the  $O_2^{\cdot -}$  radical. As mentioned earlier, alpha-tocopherol is one of the most powerful and important lipophilic antioxidants in the body. The *in vitro* antioxidant activity of rooibos in cellular systems against various oxidants has also been demonstrated in human polymorphonuclear leukocytes (Yoshikawa *et al.*, 1990) and in mouse leukaemic cells (Ito *et al.*, 1991). A possible role for rooibos in CVD health was implied when the herbal tea was shown to inhibit *in vitro* lipid peroxidation (Marnewick *et al.*, 2005). The effect of (acetone and ethanol – soluble) rooibos extracts on lipid peroxidation (LPO) in rat liver microsomes were determined by assessing the ability of various teas to inhibit the formation of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA). The authors reported a 91% inhibition by green rooibos against 99% for green tea and 65% for fermented rooibos. In another study, purified orientin was also shown to protect against LPO in mice liver (Uma Devi *et al.*, 2000) while rutin appeared to maintain the strength of capillary walls and indirectly improved hypertension by improving relevant factors like varicose veins and lower leg oedema (MacLennan *et al.*, 1994).

A few studies have examined the *ex vivo* antioxidant activity of rooibos but not always with positive correlation. In the same way that diminished production of LPO products implies increased protection against peroxidation, resistance of erythrocyte membrane to ROS damage is often used as an indicator of potential protection by antioxidants. Although long-term consumption of rooibos failed to significantly lessen the vulnerability of Japanese quail erythrocytes to haemolysis-induced by either hydrogen peroxide ( $H_2O_2$ ) or hypotonicity or the production of LPO products measured as TBARS, a decreased *ex vivo* peroxide-induced hemolysis of these red cells was shown (Inanami, 1995; Simon *et al.*, 2000). A 10-week intake study of rooibos by male Fisher 344 rats, although having no impact on the antioxidant capacity (as measured by the ORAC assay) in the liver, significantly increased the GSH/GSSG ratio and enhanced the activities of two phase II enzymes GST-alpha and UDP-GT (Marnewick *et al.*, 2003). Green and black teas did not exhibit such effects in that study. When a xenobiotic compound, for example a potential carcinogen, is encountered in the body, phase I reactions alters the compound by adding a functional group which can be conjugated with endogenous

polar moieties (e.g. amino acids, glutathione and, sulphates) in phase II reactions thereby facilitating elimination of otherwise toxic metabolic end products (Dinkova-Kostova *et al.*, 2002). The mentioned study by Marnewick *et al.* (2003) is important in that it also examined various indices to evaluate the safety of long-term intake of rooibos. Neither traditional nor green rooibos adversely affected body and liver weights, liver and renal profiles or total protein, cholesterol and iron status of the experimental animals– a finding with relevant bearing to the present study. Recently, the effect of rooibos on the antioxidant status in men occupationally exposed to lead was investigated (Nikolova *et al.*, 2007). Consumption of rooibos for 8-week was reported to significantly enhance plasma GSH levels by 48% and to reduce LPO measured as MDA. While the study did not report on the GSH: GSSG ratio, it is notable as the first to report the effect of rooibos on glutathione status and lipid peroxidation in humans.

#### **2.4.3.3 Chemoprevention and antimutagenic activities**

Although cancer studies falls outside the scope of this study, it is important to mention that more studies have investigated the possible involvement of rooibos herbal tea in cancer prevention than in CVD. Rooibos was reported to confer *in vitro* protection against genotoxicity induced by various agents in Chinese hamster ovary cells (Sasaki *et al.*, 1993), Chinese hamster lung fibroblasts (Edenharder *et al.*, 2002), mouse embryo fibroblast cells (Komatsu *et al.*, 1994) and in the *Salmonella typhimurium* assay (Marnewick *et al.*, 2000). *In vitro* studies also linked luteolin and quercetin to apoptosis and reduced proliferation of thyroid and colon cancers respectively (Yin *et al.*, 1999; Mori *et al.*, 2001). However, it is doubtful whether the low amounts of these two flavonols in rooibos can be sufficient to account for such effects in humans. A recent study investigated the antimutagenic activity of the major flavonoids of rooibos and reported flavonoid–mutagen interactions ranging from antimutagenic, co-mutagenic and pro-mutagenic to mutagenic (Snijman *et al.*, 2007). Luteolin and chrysoeriol were reported to be the most potent antimutagens of rooibos.

In experimental animals, rooibos was also shown to afford protection against a range of carcinogens in various studies reviewed by Joubert *et al.* (2008). Since chemoprotective compounds act by various mechanisms including enzyme and signal modulation as well as inhibition of oncogene activation and carcinogen-adduct formation, these compounds can also interfere with normal homeostasis in the body (Lee & Park, 2003). Hence in evaluating potential anticarcinogenic benefits of rooibos and other dietary inputs, their possible toxicological effects should also be considered.

#### 2.4.3.4 Other diverse activities

The anti-allergic effects of rooibos and its possible interference in iron bioavailability were investigated in two of the earliest human studies on this plant. In one of the studies, radio-labelled and elemental iron were administered to 30 healthy young men and the effect of water, rooibos and black teas on the metal's absorption compared (Hesseling *et al.*, 1979). Rooibos (7.25%) did not have a significant effect on iron absorption and was not different from water (9.34%) in this regard. This was in contrast to the black tea (1.70%), which has also been shown in other studies to inhibit bioavailability of non-heme iron by as much as 94% (Hurriel *et al.*, 1999). Although tea intake is unlikely to cause iron deficiencies in healthy individuals on balanced diets, vegetarians and people at risk (expectant women, infants) are advised to drink tea between meals and with milk/lemon added to minimize the chelating of iron by tea polyphenols especially tannins (Farkas & Harding, 1987). An early human study on allergies, reported rooibos to be ineffective as a potential anti-allergenic agent (Hesseling & Joubert 1982). However, a later study indicated that administration of this herbal tea stimulated interleukin-2 (IL-2) generation in splenocytes and restored the production of antigen-specific antibodies in serum (Kunishiro *et al.*, 2001). Studies in rabbits and rats by Khan and Gilani (2006) showed that rooibos possesses smooth muscle relaxing effects mediated possibly through dominant potassium ion ( $K^+$ ) channel activation along with weak calcium ion ( $Ca^{2+}$ ) antagonist mechanisms. The selective bronchodilatory effect of a rooibos extract was shared by one of its known flavonoid compounds, chrysoeriol, while orientin was shown to have inhibitory effect on the gut. The investigators suggested that these observations may explain the medicinal use of rooibos in hyperactive gastrointestinal, respiratory and cardiovascular diseases with the potential to be developed as a remedy for the congestive airway disorders. A recent study has demonstrated antimicrobial activity by rooibos (Almajano *et al.*, 2008) while acid polysaccharide extracts from rooibos were earlier reported to suppress the cytopathic effects of HIV-infected MT-4 cells (Nakano *et al.*, 1997). It was suggested that the extracts possibly inhibited the virus-CD4 receptor binding – an observation of immense importance given the impact of the still cureless HIV caused AIDS. However, it should be noted that consumption of rooibos cannot be claimed to fight the virus since the extract can only be obtained chemically and is therefore unavailable in the beverage.



## 2.5 Summary and significance of literature review

The link between oxidative stress and human diseases particularly CVD has been explored in this review and dietary antioxidants as the reason for the association of diets rich in fruits, vegetables, wine and teas to decreased incidence of CVD. Polyphenols have been highlighted as the principle antioxidants thought to be involved but as the review expounds, it has not been easy to reconcile the apparent benefits of their intake on one hand, and the modest absorption and bioavailability on the other. The review inclines to the view that besides antioxidant activity, many more modes of action, known and probably unknown, possibly account for cardio-protective benefits attributed to polyphenol intake including roles in endothelial function, platelet aggregation and inhibition of LPO. The richness of rooibos herbal tea as a source of common and rare polyphenols has been discussed as well as the anecdotal attributes to the herbal tea and the current scientific knowledge about its bioactivity. Results from *in vitro* and animal studies seem promising enough to warrant further investigations in humans. Very few studies in humans involving rooibos have been undertaken and it is therefore expected that the outcome of this study will significantly contribute to the scientific knowledge about this unique South African herbal tea.

## CHAPTER THREE

### MATERIALS AND METHODS

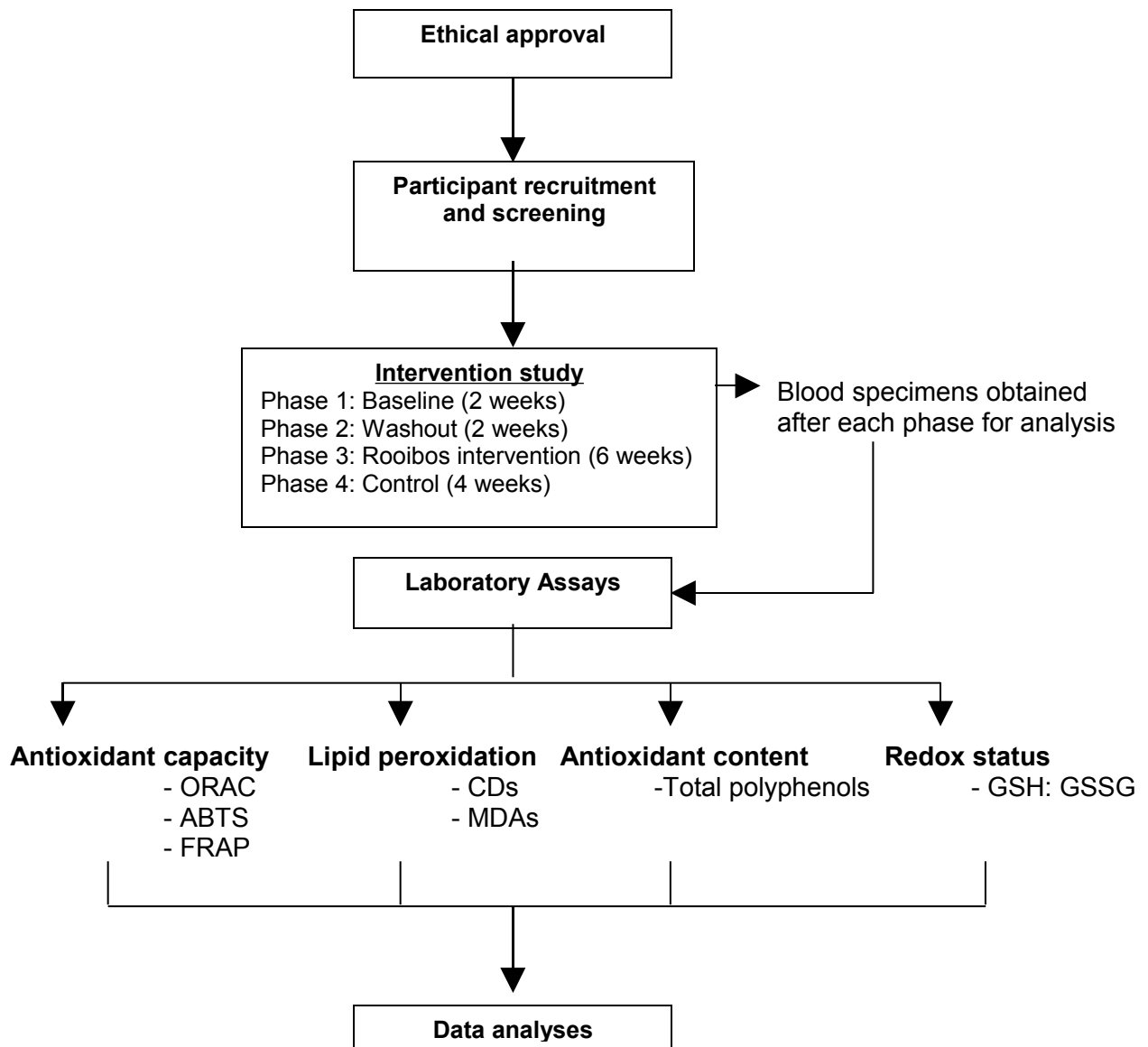
#### 3.1 Introduction

This chapter describes the design as well as the materials and methods used in the study. A pre- and post-measurement single group intervention study design was used to investigate the possible modulation of oxidative stress parameters by rooibos in individuals at risk for CHD. Participants consumed six cups of rooibos daily for six weeks in the intervention phase which was sandwiched between a preceding 2 weeks baseline (or run in) period, 2 weeks wash out period and a final 4- week control period (Figure. 3.1). The design was chosen because it allows participants to serve as their own controls hence maximizing the number of participants in the intervention phase. The Faculty of Health & Wellness Sciences Research Ethics Committee of the Cape Peninsula University of Technology (CPUT) approved the study protocol before commencement of recruitment. Informed written consent was obtained from each participant (Appendix A) after attending two pre-recruitment information sessions.

#### 3.2 Participants

The study was conducted in the Western Cape area of South Africa and participants were recruited via posters on all CPUT campuses as well as via general emails sent to CPUT students, staff and the public (Appendix B). All participants were required to meet the following inclusion criteria: (a) be of good health and stable weight for 6 months prior to the study, (b) not pregnant or lactating, (c) not on unusual diets, medication or undesirable alcohol consumption (>2 drinks per day), (d) free of CVD, diabetes as well as renal, hepatic and endocrine disorders, (e) not on vitamin and/or antioxidant supplementation and (f) be between 30-60 years of age. In addition, recruits were screened to establish each had at least two or more of the following risk factors for CHD: hyperlipidaemia (raised cholesterol - >5.2 mmol/L and triglycerides), smoking, hypertension - 140/90 mm Hg or pre-hypertension – 120-139/80-90 mm Hg, increased body mass index (BMI) of 25 - 30 and also not requiring any medication for these medical conditions (Yusuf *et al.*, 2004; Bash *et al.*, 2008; Tamariz *et al.*, 2008). This was done by filling out anthropometric and blood pressure assessment forms (Appendix C) as well as by blood chemistry assays. The participants' risks for developing heart disease in next 10 years (Appendix D) were calculated using the Framingham risk scoring. The design of the study including the various inclusion and exclusion criteria, were established in consultation with three

experts\*. Forty five participants were recruited and each was randomly assigned a number which served as the reference throughout the study in order to maintain confidentiality.



**Figure 3.1: Overview of the study design**

<sup>M</sup> Physician and heart specialist Prof D Marais, University of Cape Town (UCT), Dr D Blackhurst (Lipid Laboratory, University of Cape Town) and Prof V Mersch-Sundermann (Freiburg University Medical Centre, Germany)

### 3.3 Dietary design

Participants were instructed to restrict and in certain cases refrain from consuming flavonoid-rich foods and beverages for the duration of the study to minimize the potential of confounding effects from dietary flavonoids. For this purpose, they received a list of acceptable/restricted foods and beverages which are known to be low in total polyphenols (Appendix E). Compliance to dietary restrictions was ensured by means of accurate self-administered dietary records completed for 3 consecutive days per week for two weeks in each phase. These records also served to estimate the total daily flavonoid intake of each participant throughout the study. Standard measures and portion sizes (e.g. small, medium, or large) were assigned to each food/beverage category and the details were explained to the participants prior to the distribution of the dietary records. The dietary recording forms and written instructions on how to complete them were given to each participant (Wolmarans, 2003). To facilitate the estimation of portion sizes, food diagrams and models as well as sample containers were presented to the participants in an information session before commencement of the study. A 2 week run in period (baseline phase) preceded the study where participants were required to follow their normal diet and familiarize themselves with the completion of the dietary records. The study dietician and her team were available during the collection of the completed dietary records to address any questions or concerns regarding their completion. Fasting blood samples were drawn after completion of the 2-week run-in period to establish baseline values for each of the participants. Thereafter, the 2-week washout phase where participants had to adhere to the dietary restrictions/recommendations, the 6-week rooibos intervention phase and the 4-week control phase commenced (Table 3.1). After the completion of each phase fasting blood samples were drawn for the analysis of various serum/plasma biomarkers.

**Table 3.1: Phases of the study and their respective durations and dietary protocols**

<b>Study phase*</b>	<b>Time period and dietary protocol</b>
Baseline	Two weeks with no change in diet only dietary record keeping to establish baseline values
Washout	Two weeks following a flavonoid-restricted diet while keeping dietary records
Rooibos intervention	Six weeks following a flavonoid-restricted diet while consuming 6 cups of rooibos daily and keeping dietary records
Control	Four weeks following the same flavonoid-restricted diet while consuming 6 cups of water daily and keeping dietary records

\* Fasting blood samples were collected at the end of each phase

During the rooibos intervention phase, participants were instructed to prepare the rooibos by following a standard recipe provided to them. This recipe entailed adding one rooibos tea bag to 200 mL of freshly boiled water and brewing for 5 minutes to reach full flavour and increase extraction of the polyphenols. The prepared rooibos could then be consumed either by the addition of milk and sugar/sweetener or without, according to individual preference. Alternatively, the brewed rooibos could also be cooled and taken as an “iced tea”. Before the study begun, this preparation method was simulated in the laboratory with/without the addition of 10 mL of milk per 100 mL of rooibos in order to characterise the study drink in terms of total polyphenols, soluble solids extracted and antioxidant activity. The rooibos used in the study was the unflavoured traditional/fermented type rooibos of superior grade generously donated by Mr Redelinghuys (Rooibos Limited, Clanwilliam, South Africa).

### 3.4 Collection and preparation of blood samples

At the end of each phase, fasting peripheral venous blood samples were collected in vacutainer tubes (BD vacutainers, Plymouth, England) containing either ethylenediaminetetraacetic acid (EDTA) or no anticoagulant. Samples were protected from light and stored on ice until processing later that same day. Blood samples were centrifuged ( $3000 \times g$ , 10min,  $4^{\circ}\text{C}$ ) to obtain plasma or serum and stored in 1.5 mL eppendorf tubes (Eppendorf, Germany) at  $-40^{\circ}\text{C}$  until analysis. Whole blood samples for GSH analysis were aliquoted and stored at  $-80^{\circ}\text{C}$ . Samples for GSSG analysis were prepared by adding 10  $\mu\text{L}$  of a 30 mM GSH scavenger (1-methyl -2-vinylpyridinium trifluoromethanesulphonate, M2VP, purchased from Merck, SA) to 100  $\mu\text{L}$  of whole blood and also stored at  $-80^{\circ}\text{C}$ . The M2VP was added to prevent the oxidation of GSH to GSSG causing falsely elevated levels in the sample.

### 3.5 Chemical pathology

*Chemistry:* Serum samples were used for the determination of clinical biochemical parameters including glucose, creatinine, total cholesterol, total iron, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), total bilirubin (T.Bili), unconjugated bilirubin (D.Bili), and total protein. The analyses were done on a Technicon RA 1000 automated analyzer using kits (Kat medical, South Africa).

*Lipid profile:* Cholesterol and triglyceride (TG) levels were determined in plasma by enzymatic methods using the RA 1000 analyzer, while the high density lipoprotein (HDL) levels were obtained after precipitation of apo-B-containing lipoproteins. The cholesterol and triglyceride

contents of the HDL fraction were measured, while the LDL cholesterol was calculated using the Friedewald equation (Equation 3.1).

$$[\text{LDL-cholesterol}] = [\text{Total cholesterol}] - [\text{HDL-cholesterol}] - ([\text{TG}]/5)$$

#### Equation 3.1

(Friedewald *et al.*, 1972)

### 3.6 Antioxidant capacity

The measurement of antioxidant capacity (AOC) takes into account the interactive nature of antioxidants, providing a combined picture of the antioxidant defences in a system and circumventing the need to estimate antioxidants individually (Cao & Prior, 1998). This approach is therefore a functional measure rather than a measure of individual antioxidant concentrations. Several antioxidants such as vitamins C and E, carotenoids, uric acid, polyphenols, bilirubin and some enzymes contribute to the body's antioxidant capacity, but unknown antioxidants may also make a substantial contribution (Cao & Prior, 1998). To measure AOC in biological and food samples, several methods are available which use different radical generators to measure the degree of inhibition of free radical generation by antioxidants in a sample (Cao & Prior, 1998). The measured AOC of a sample depends on which technique and which free radical generator or oxidant is used (Cao & Prior, 1998). The AOC of the rooibos samples prepared with and without milk as well as the blood samples were measured using the oxygen radical absorbance capacity (ORAC), azino-di-3-ethylbenzothiazoline sulphonate (ABTS) and ferric reducing antioxidant power (FRAP) assays, described in the section below.

#### 3.6.1 The oxygen radical absorbance capacity (ORAC) assay

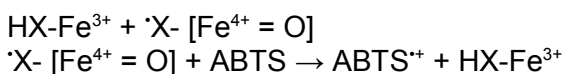
This fluorescence-based method was developed by Glazer (1990) based on the discovery that the fluorescence of phycoerythrin (PE) changes with respect to time, upon damage caused by peroxy or hydroxyl radical attack. The initial method only considered the "flat" phase of the reaction but it has since undergone various modifications. The version employed in this study was described in 2003 and uses a fluorescein salt instead of PE and 2,2' azobis (2-amidinopropane) dihydrochloride (AAPH) as the radical generator (Prior *et al.*, 2003). Considered to have a good specificity using a physiologically relevant radical generator, the method's popularity is growing, especially in the food and beverage industry. It has been used to generate the evolving United States Department of Agriculture (USDA) antioxidant database, currently considered to be the most comprehensive globally (Wu *et al.*, 2004). A distinct

superiority of ORAC over other total antioxidant capacity (TAC) assays is that it takes free radical action to completion and uses an “area-under-the-curve” technique (thereby combining both time and degree of inhibition of radical generation) to quantify AOC (Cao *et al.*, 1995). The use of different extraction techniques enables separate estimation of aqueous and lipid soluble AOC. Since polyphenols - the antioxidants under focus in this study - are water soluble, only the hydrophilic phase was considered. Both rooibos and the biological samples were deproteinated with perchloric acid (PCA) as plasma proteins can significantly affect ORAC estimates, leading to underestimations of other antioxidants (Ghiselli *et al.*, 1994). Chemicals such as trolox, AAPH, fluorescein and PCA were purchased from Sigma-Aldrich (South Africa), while sodium di-hydrogen orthophosphate mono hydrate and di-sodium hydrogen orthophosphate dehydrate, used to prepare the ORAC buffer (75 mM phosphate buffer, pH 7.4) were purchased from Merck (South Africa). Reactions were done and read in black 96-well fluorescence microplates (Sigma–Aldrich, South Africa) using a Fluoroskan Ascent analyzer (Thermo Electron Corporation, Finland). All centrifugation in the study was done using a refrigerated bench top centrifuge (Eppendorf 5810R, Eppendorf, Germany). A fluorescein (FI) stock solution was prepared by dissolving 0.0225 g of FI in 50 mL of a 75 mM phosphate buffer resulting in a 0.0011 solution. Ten microlitres of this solution was then added to 2 mL phosphate buffer to prepare the second stock solution from which 240 µL was subsequently diluted in 15 mL phosphate buffer to prepare the working FI solution. A 500 µM standard stock solution of Trolox was prepared by dissolving 0.00625 g of Trolox in 50 mL phosphate buffer, aliquoted into 1.5 mL eppendorf tubes and frozen at -40°C to ensure the use of a constant stock solution throughout the analysis period. The 500 µM solution was also used to prepare the standard series (5, 10, 15, 20, 25 µM) and generate the calibration curve. The calibration curve was prepared by plotting a graph of the standard series concentrations versus their areas under the curve (AUC). The radical generator was prepared by dissolving 0.125 g of AAPH in 5 mL of the phosphate buffer using a 15 mL screw cap conical centrifuge tube. Addition of the phosphate buffer was made immediately prior to addition of all reagents to the 96 well microplate. Frozen plasma and rooibos samples were thawed at 4°C. To precipitate the proteins, 400 µL of 0.5M PCA was added to 400 µL plasma, mixed for 30 seconds on a vortex and then centrifuged at 4000 × g for 5 minutes. The supernatant was removed and 100 µL was added to 400 µL phosphate buffer to make a total dilution of 1:10. Each reaction well contained 12 µl of each standard and/or sample (done in triplicate), 138 µl of the FI working solution and 50 µL of the freshly prepared AAPH solution. The plate was immediately read in the Fluoroskan reader preset at 37 °C and programmed to record fluorescence every 5 minutes for 2 hrs at excitation and emission wavelengths of 485 and 520 nm, respectively. The ORAC values were obtained using a quadratic regression equation ( $y = ax^2 + bx + c$ ) to calculate the AUC for each respective

well and subtracting the AUC value of the blank well. The values obtained are usually compared to that of Trolox, reporting the results as micromole Trolox equivalents per millilitre ( $\mu\text{mol TE/mL}$ ). Microsoft® Excel 2002 (10.2614.2625) was used for these calculations.

### 3.6.2 The 2, 2'-azino-di-3-ethylbenzothiazoline sulphonate (ABTS) assay

The ABTS radical cation scavenging assay was first described for the determination of the TAC of body fluids (Miller *et al.*, 1993). In this original method, metmyoglobin was activated to its ferryl state by hydrogen peroxide then incubated with ABTS to form a stable, long-lived radical cation,  $\text{ABTS}^{+\cdot}$ , thereby developing the characteristic blue colour (Figure 3.2). Antioxidants present in the sample reaction mixture will suppress the colour development by either scavenging  $\text{ABTS}^{+\cdot}$  formed or interfering in the radical generating process. Measurement of the absorbance at a specific time after addition of antioxidants enables the calculation of a percentage scavenging.



Where:

$\text{HX-Fe}^{3+}$  = metmyoglobin

$\cdot\text{X-} [\text{Fe}^{4+} = \text{O}]$  = ferrylmyoglobin

**Figure 3.2: Generation of 2, 2'-azino-di[3-ethylbenzthiazoline sulphonate] radical cation from metmyoglobin**

However, rapidly-reacting antioxidants may contribute to the reduction of the ferrylmyoglobin radical leading to positive interference (Strube *et al.*, 1997). To circumvent this limitation, modifications of ABTS-based methods were made (Re *et al.*, 1999), generating  $\text{ABTS}^{+\cdot}$  before the addition of the sample by reacting ABTS with potassium peroxodisulphate. This modification was used in the present sample analyses. Decreasing intensity of the blue colour, which reflect increased reduction of the  $\text{ABTS}^{+\cdot}$  by antioxidants, is monitored at 734 nm and indicates the concentration of antioxidants in the sample.

The  $\text{ABTS}^{+\cdot}$  radical was generated by adding 8  $\mu\text{L}$  of a 140 mM potassium peroxodisulphate (Merck South Africa) solution (0.1892 g dissolved in 5 mL distilled water) to 5 mL ABTS solution (0.0192 g of ABTS, Sigma-Aldrich, South Africa, dissolved in 5 mL of distilled water). This solution was thoroughly mixed and left in the dark until use 24 hrs later. The Trolox standard was prepared by dissolving 0.0125 g Trolox in 50 mL absolute ethanol, EtOH (Saarchem) and



aliquoted in 1.5 mL eppendorf tubes. This was used as the stock solution to prepare the standard series (0, 50, 100, 150, 250, 500  $\mu\text{M}$ ) using absolute EtOH as the diluent. The frozen plasma and rooibos samples were thawed at 4°C and preparation done as for ORAC samples. The reaction mixture consisted of 25  $\mu\text{L}$  of each standard and/or sample (done in triplicate) and 300  $\mu\text{L}$  of the ABTS<sup>•+</sup>-solution after the appropriate dilution was made to obtain an absorbance reading of  $2 \pm 0.1$  at 734 nm. The clear 96 well microplates were left to incubate at room temperature for 30 minutes before reading the absorbance in the Multiskan spectrophotometer (Thermo Electron Corporation, Finland) set at 25 °C and 734 nm. Data analyses and calculations were done on Microsoft® Excel 2002 (10.2614.2625) program based on a calibration curve plotted using the standard series and the results expressed as  $\mu\text{mole Trolox equivalents per litre of plasma}$ .

### **3.6.3 The ferric reducing antioxidant power (FRAP) assay**

First described by Benzie and Strain (1996), the FRAP assay measures the ability of a sample to reduce iron from the ferric ( $\text{Fe}^{3+}$ ) to ferrous ( $\text{Fe}^{2+}$ ) state. It is considered an AOC assay since antioxidants are known to donate electrons in the same manner as reductants in a redox reaction. The assay, therefore, does not directly measure the AOC of an antioxidant and it completely differs from and ORAC since no free radicals or oxidants are applied in FRAP (Cao & Prior, 1998). The AOC of a compound against a given oxidant does not always match its ability to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and it is therefore possible for an otherwise strong antioxidant to yield a near zero FRAP value as is the case with thiol group-containing antioxidants like glutathione (Cao & Prior, 1998). Additional complications may arise when antioxidants such as ascorbic acid are assessed. Not only does ascorbate reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , it also reacts with the final indicator,  $\text{Fe}^{2+}$ , generating more free radicals. Despite these limitations, FRAP assay has an advantage of operational simplicity while producing fast, reproducible results with serum/plasma assays of pure or mixed antioxidants (Benzie & Strain, 1996).

The assay measures the change in absorbance at 593 nm due to the reduction of a  $\text{Fe}^{3+}$ -tripirydyltriazine complex at low pH forming a deep-blue coloured ferrous form whose intensity directly reflects the reducing power of the antioxidants in the sample. The sodium acetate and glacial acetic acid used to constitute the 300 mM, pH 3.6 acetate buffer were purchased from Merck (South Africa). Iron (III) chloride ( $\text{FeCl}_3$ ), hydrochloric acid and tripyridyl triazine were also obtained from Merck while the ascorbic acid used to make the standard solution was purchased from Sigma (South Africa). The standard solution was made by dissolving 0.0085 g ascorbic

acid in 50 mL distilled water. This solution was used as the stock solution to prepare the standard series (0, 50, 75, 125, 250, 500  $\mu$ M) using distilled water as the diluent. The frozen rooibos and plasma samples were thawed at 4°C and prepared as for ABTS above. The FRAP reagent was prepared by mixing 30 mL acetate buffer, 3 mL TPTZ solution (10 mM in 40 mM HCl), 3 mL FeCl<sub>3</sub> solution (20mM) and 6.6 mL distilled water to yield a straw coloured solution. The reaction mixture consisted of 10  $\mu$ L of each standard or sample (done in triplicate) and 300  $\mu$ L of the FRAP reagent. The clear 96-well microplates were incubated in the oven at 37 °C for 30 minutes before reading the absorbance in the Multiskan spectrophotometer (Thermo Electron Corporation, Finland) set at 25 °C and 593 nm. Data analyses and calculations were done on Microsoft® Excel 2002 (10.2614.2625) program based on a calibration curve plotted using the standard series and the results expressed as  $\mu$ mole ascorbic acid equivalents per litre of plasma.

### 3.7 Antioxidant content

Antioxidants found in the body are from various sources including vitamins C and E, carotenoids, uric acid, polyphenols, urates, bilirubin and some enzymes as well as from other unknown sources (Cao and Prior, 1998). Ideally, the concentrations of each species would need to be estimated to obtain a level that truly reflects the body's antioxidant content. However, since polyphenols account for the bulk of the antioxidants in rooibos (Joubert & Ferreira, 1996; Bramati *et al.*, 2003) it was hypothesized that any effect of the intervention would impact on the total polyphenol level more than any other group. Estimation of total polyphenols was therefore chosen as the most suitable assay for antioxidant content. The flavonols and flavanols content of the study beverage were also estimated but since the physiological levels are very low, plasma levels were not determined.

#### 3.7.1 Total polyphenols

As mentioned earlier, polyphenols are a diverse group of chemicals produced by plants and are characterized by the presence of more than one phenol ring. Most assays for polyphenols exploit the reactivity of the aromatic group with various reagents. This study used a modified Folin-Ciocalteu (F-C) to avoid plasma interference in the assay and is based on the reaction of the aromatic group with the F-C reagent (Serafini *et al.*, 1998). The reagent, which is a mixture of phosphotungstic and phosphomolybdic acids, is reduced to blue oxides of tungstene and molybdene during phenol oxidation (George *et al.*, 2005). Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) provides

the alkaline environment necessary for this reaction. The blue colour that develops is monitored at 765 nm and indicates the quantity of polyphenols usually expressed as gallic acid equivalent (GAE) or catechin equivalent litre of solute.

Absolute ethanol and F-C reagent were purchased from Merck (South Africa) while  $\text{Na}_2\text{CO}_3$  and gallic acid were obtained from Sigma-Aldrich (South Africa). The working F-C reagent was prepared by combining 1 mL of the reagent with 9 mL of distilled water. Gallic acid was used as the standard and 10% EtOH as the diluent to make up the standard series (0, 20, 50, 100, 250, 500 mg/L). The initial phase of sample preparation was aimed at maximally extracting polyphenols in plasma as well as precipitating the proteins. The conjugated polyphenols were first hydrolysed by adding 250  $\mu\text{L}$  of the thawed plasma to 500  $\mu\text{L}$  of 1.0 M hydrochloric acid (HCl) and vortexing the mixture vigorously for 1 minute before incubating for 30 min at 37 °C. Thereafter, 500  $\mu\text{L}$  of 2M sodium hydroxide (NaOH) in 75% methanol was added, vortexed for 3 minutes and incubated again for 30 minutes at 37°C to free polyphenols linked to lipids. Proteins were then precipitated by adding 750  $\mu\text{L}$  of 0.75 M metaphosphoric acid, mixing then centrifuging for 5 minutes at 3000  $\times$  g. The supernatant containing the extracted polyphenols was separated and stored on ice in the dark until analysis. Later, 25  $\mu\text{L}$  of each extracted sample as well as control and standards were added in appropriate wells of the clear 96-well microplate in triplicate after which 125  $\mu\text{L}$  of the Folin reagent solution was added into each well. Five minutes later, 100  $\mu\text{L}$   $\text{Na}_2\text{CO}_3$  was added in each well to increase the pH. The microplate was left for 2 hours at room temperature before taking the reading in the Multiskan spectrophotometer set at 25°C and 765 nm. Data analyses and calculations were done on Microsoft® Excel 2002 (10.2614.2625) program based on a calibration curve plotted using the standard series and the results expressed as milligram gallic acid equivalents per litre of plasma.

### 3.7.2 Flavanols

The method used in this study for the estimation of flavanols in the prepared rooibos samples was described by Treutter (1989) and makes use of 4-dimethylaminocinnamaldehyde (DMACA) which reacts with flavanols to form a characteristic light blue colour that is measured at 640 nm.

Hydrochloric acid (32%) and absolute methanol (both purchased from Merck South Africa) were mixed to make a 1:3 solution (25 mL acid and 75 mL alcohol). To make the DMACA solution, 0.015 g of the powder (Merck South Africa) was dissolved in 30 mL of the HCl-MeOH solution. The standard solution was freshly made by dissolving 0.0145 g catechin hydrate in 500 mL

MeOH solution. This solution was used as the stock solution to prepare the standard series (0, 5, 10, 25, 50 and 100  $\mu\text{M}$ ) using methanol as the diluent. The frozen rooibos samples were thawed at 4°C and prepared as for ABTS above. Later, 50  $\mu\text{L}$  of each rooibos sample as well as standards were added to appropriate wells of the clear 96-well microplate in triplicates after which 250  $\mu\text{L}$  of the DMACA solution was added into each well using a multi channel pipette. The plate was then incubated for 30 minutes at room temperature before taking the reading in the Multiskan spectrophotometer set at 25°C and 640 nm. Data analyses and calculations were done on Microsoft® Excel 2002 (10.2614.2625) program based on a calibration curve plotted using the standard series and the results expressed as catechin equivalents per litre of tea.

### 3.7.3 Flavonols

The method used in this study for the estimation of flavonols in the prepared rooibos samples was adapted from Mazza *et al.* (1999) and exploits the reaction of HCl with flavonols using quercetin as the standard. Absolute EtOH (Merck, South Africa) was appropriately diluted to make 10% and 95% EtOH solutions. Two different HCl solutions were also prepared: a 2% solution with distilled water and a 0.1% with the 95% EtOH as the diluent. The standard solution was freshly prepared by dissolving 4 mg quercetin (Sigma-Aldrich, South Africa) into 50 mL 95% EtOH. This solution was used as the stock solution to prepare the standard series (0, 75, 125, 250, 500 and 1000  $\mu\text{M}$ ) using 95% EtOH as the diluent. The frozen rooibos samples were thawed at 4°C and prepared as for flavanols above. Later, 12.5  $\mu\text{L}$  of each rooibos sample as well as standards were added in appropriate wells of the clear 96-well microplate in triplicate followed by 12.5  $\mu\text{L}$  of the 0.1% HCl, then finally 225  $\mu\text{L}$  2% HCl was added into each well using a multi channel pipette. The plate was then incubated for 30 minutes at room temperature before taking the reading in the Multiskan spectrophotometer set at 25°C and 360 nm. Data analyses and calculations were done on Microsoft® Excel 2002 (10.2614.2625) program based on a calibration curve plotted using the standard series and the results expressed as milligram quercetin equivalents per litre of tea.

### 3.8 Lipid peroxidation

Measurement of lipid peroxidation (LPO) has been one of the most extensively applied assays in oxidative stress studies due to procedural convenience and relative reliability (Hwang & Kim, 2007). While several markers can be used, lipid peroxidation has traditionally been estimated by the increase in thiobarbituric acid reactive substances (TBARS) which reflect the production of malondialdehyde (MDA), an oxidation product of lipid oxidation (Hwang & Kim, 2007). It has gained popularity due to its low cost and relative ease of execution. However its application to biological fluids and tissue extracts has a serious drawback in that it lacks specificity. Several

substances unrelated to LPO, such as bilirubin, can react with thiobarbituric acid (TBA) to form a chromogen detectable at 530-535 nm (Korchazhkina *et al.*, 2003) and hence the assay is not totally specific to oxidant-driven LPO. Literature reports that hydroperoxides account for up to 27% human plasma TBARS (Arguelles *et al.*, 2004). It is suggested that at least two different assays of LPO should be used if estimates are to be reliable (Hwang & Kim, 2007) and for this purpose, conjugated dienes, another group of markers were also estimated. Conjugated dienes (CDs) are polyunsaturated molecules linked to several steps in lipid peroxide degradation (Yoshida & Niki, 2004). They are unspecific to the type of LPO product formed and only 30%-55% of LPO is detected by their measurement. On the other hand CD products are found in both bacterial lipid metabolism and animal diets and therefore substantial overestimation can result when applied to *in vivo* oxidation processes in biological materials (Hwang & Kim, 2007). Plasma TBARS were measured according to the method of Yagi (1984) which is based on the reaction of thiobarbituric acid with malondialdehyde, one of the aldehyde products of LPO, while the method of Recknagel and Glende (1984) was used for the estimation of CDs.

### 3.8.1 Thiobarbituric acid reactive substances (TBARS) assay for plasma

A solution was made by dissolving 0.00917 g of butylated hydroxytoluene in EtOH upto 10.42 mL and thereafter 6.25  $\mu$ L of this solution was added to 50  $\mu$ L plasma followed by 50  $\mu$ L o-phosphoric acid (Merck South Africa) and vortexed for 10 seconds. Then 6.25  $\mu$ L of 0.11M TBA reagent (Merck South Africa) in 0.1M NaOH was then added and the mixture vortexed again for 10 seconds before heating in a water bath at 90 °C for 45 minutes. The reaction was stopped by transferring the tubes onto ice for 2 minutes and then room temperature for a further 5 minutes. Then 500  $\mu$ L n-butanol and 50  $\mu$ L saturated sodium chloride were added for better separation of phases before vortexing for 10 seconds. The tubes were then microfuged at 12,000 rpm for 2 minutes at 4 °C and the butanol phase obtained for the assay. On a clear 96-well microplate, 300  $\mu$ L of sample was used in each well and the absorbance read at 532 and 572 nm. The TBARS concentrations were calculated from equation 3.2 using Microsoft® Excel 2002 (10.2614.2625) program.

$$\frac{(A_{532} - A_{572})}{\epsilon} \times 33.4 \mu\text{mol/L of plasma}$$

Where A = absorbance at specified wavelength;

$$\epsilon = \text{coefficient of extinction} = 1.54 \times 10^5$$

Quoted  $\epsilon$  is always based on a 1 cm cuvette; since 300  $\mu$ L in a plate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

### Equation 3.2

### 3.8.2 Conjugated dienes determination in plasma

Chloroform and methanol (both obtained from Merck South Africa) were used to make a 2:1 solution of which 405  $\mu\text{L}$  was added to 100  $\mu\text{L}$  of plasma in an eppendorf tube and the mixture vortexed for 60 seconds before centrifuging at 8000 x g for 15 minutes at 4°C. The top aqueous layer was removed and discarded. A glass pasteur pipette was gently plunged along the wall of the tube through the protein layer to carefully collect the lipid layer and transferred into a clean eppendorf tube before drying under liquid nitrogen for 30 minutes. One mL cyclohexane (Merck SA) was then added and the solution vortexed for 30-60 seconds before transferring 300  $\mu\text{L}$  into a clear 96-well plate and reading the absorbance at 234 nm in the Multiskan spectrophotometer. All samples were done in duplicate. The concentrations of CDs were calculated from equation 3.3 using Microsoft® Excel 2002 (10.2614.2625) program.

$$\frac{(A_{234s} - A_{234b})}{\epsilon} \times 10 \text{ nmol CD/mL of plasma}$$

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

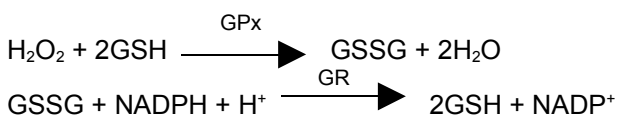
$A_{234b}$  = absorbance of blank at 234 nm  
 $\epsilon$  = coefficient of extinction =  $2.95 \times 10^4$

Quoted  $\epsilon$  is always based on a 1 cm cuvette; since 300  $\mu\text{L}$  in a plate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

#### Equation 3.3

### 3.9 Redox assay

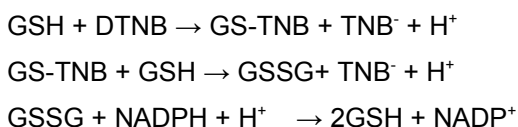
Reduced glutathione (GSH) is an antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of peroxides to water (Figure 3.3). In the process, GSH becomes oxidised to GSSG which is recycled back to GSH by glutathione reductase (GR) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Increased exposure to oxidative stress lowers the GSH:GSSG ratio as the GSSG increases and/or GSH decreases. Estimation of GSSG concentration or determination of GSH:GSSG ratio is therefore an informative indicator of the oxidative stress status and can be used to monitor the efficacy of antioxidant intervention studies.



**Figure 3.3: Oxidation and recycling of glutathione**

(Adapted from Langseth, 1995)

Accurate measurement of GSSG however, is difficult due to its low concentration in tissues and lack of effective means to avoid oxidation of GSH to GSSG during sample preparation. This study used the method described by Asensi *et al.*, (1999) which circumvents this difficulty by using the thiol-scavenging reagent, 1–methyl-2-vinylpyridinium trifluoromethane-sulfonate (M2VP) at a level that rapidly scavenges GSH without interfering with other enzymatic assays. In the method, the thiol reagent, 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB) reacts with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) and a disulfide product, GS-TNB (Figure 3.4). The chromophore is then reduced by GR in the presence of NADPH, releasing a second TNB molecule and recycling the GSH; thus amplifying the response. Any GSSG initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly converted to GSH and is detectable in the assay thereby yielding a measure of total glutathione (GSht).



**Figure 3.4: Principle of the glutathione determination method**

The buffer (pH 7.4) used as diluent for all working solutions in the assay was prepared using sodium di-hydrogen orthophosphate mono hydrate, di-sodium hydrogen orthophosphate dehydrate and EDTA, all purchased from Merck (South Africa). The 0.3 mM 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) purchased from Sigma– Aldrich (South Africa), was used as the chromogen while 1 mM NADPH used to initiate the reaction was purchased from Merck (South Africa). A GSH standard (Merck, South Africa) was used to make a 3  $\mu\text{M}$  stock solution from which the standard series were prepared (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0  $\mu\text{M}$ ). To prepare the assay enzyme, 16  $\mu\text{L}$  of glutathione reductase (168U/mg, Merck, South Africa) was diluted with 984  $\mu\text{L}$  buffer. Determination of the GSH:GSSG ratio requires separate estimation of the concentrations of the individual analytes. Whole blood was used for the estimation of both analytes but storage and processing procedures differ and will therefore be outlined separately. The M2VP-containing whole blood samples were thawed, mixed and incubated at room temperature for 5 minutes. Then 290  $\mu\text{L}$  of cold 5% MPA was added to each tube, mixed and vortexed for 15 seconds before centrifuging at  $1000 \times g$  for 10 minutes. The supernatant was collected and 50  $\mu\text{L}$  was added to 700  $\mu\text{L}$  GSSG buffer and the diluted extract were stored on

ice until analysis. The frozen GSH whole blood samples were thawed and well mixed. Into each tube, 350  $\mu\text{L}$  cold 5% MPA was added, vortexed for 15 seconds and centrifuged for 10 minutes at  $1000 \times g$ . The supernatants were separated and from each, 25  $\mu\text{L}$  was taken and added to 1.5 mL assay buffer with the tubes stored on ice until analysis. To ensure uniformity of test environment and conditions, both assays were done concurrently on the same clear, 96-well microplate with the first two rows (A and B) carrying the standards. The GSH samples were added in rows C-E, while the corresponding GSSG samples filled rows F-H. Thereafter, 50  $\mu\text{L}$  of blank, standard or sample was added to the appropriate wells in triplicate. Then 50  $\mu\text{L}$  of each of the chromogen and enzyme were added to each well before incubating at room temperature for 5 minutes. Hereafter, 50  $\mu\text{L}$  of NADPH was added rapidly to each well with a multi-channel pipette and the plate was read in the Multiskan reader set to read the absorbance every 30 sec for 5 minutes at 412 nm. The concentrations of the analytes were calculated using a 6-point calibration curve constructed from the standard series. The change in absorbance at 412 nm is a linear function of the GSH or GSSG and is described by the following equation (Equation 3.4):

$$A_{412} = \text{slope} \times \text{minutes} + \text{intercept}$$

#### Equation 3.4

In the above equation (3.4), the slope of the regression equation is equal to the rate while the net rate is the difference between the rate at each concentration of the analyte and the blank rate (Asensi *et al.*, 1999). The calibration curves for GSSG and GSH were plotted separately to account for differences in molar concentrations and the lower levels of GSSG in the reaction mixture. The general form of the reaction equation describing the calibration curve is given in equation 3.5.

$$\text{Net rate} = \text{slope} \times \text{analyte} + \text{intercept}$$

#### Equation 3.5

Therefore, the concentration of GSH for example, calculated from the GSH calibration curve is given by equation 3.6:

$$\text{GSHt} = \frac{\text{Net rate} - \text{intercept}}{\text{Slope}} \times \text{dilution factor}$$

#### Equation 3.6



The GSH:GSSG ratios were then calculated by dividing the difference between the GSH and GSSG concentrations by the concentration of GSSG (Equation 3.7). Microsoft® Excel 2002 (10.2614.2625) was used for these calculations.

$$\text{Ratio} = \frac{\text{GSH} - 2\text{GSSG}}{\text{GSSG}}$$

**Equation 3.7**

### **3.10 Data analysis**

All assays were done in triplicate or duplicate as specifically outlined and results are presented as means  $\pm$  SD. Analysis of Variance (ANOVA) was used to determine whether the means of the subgroups differed significantly. When the Anova was positive ( $P < 0.05$ ), a Student-Newman-Keuls test for pairwise comparison of subgroups was performed. Prior to the ANOVA test, Levene's Test for Equality of Variances was performed and if positive ( $P < 0.05$ ), then the variances in the different groups are different (not homogeneous) and a logarithmic transformation to the data was done or a non-parametric statistic was applied, in this case, the Wilcoxon test. In all analyses, a P value of  $< 0.05$  was considered significant.

## CHAPTER FOUR

### RESULTS

#### 4.1 Participant profile and daily flavonoid intake

Forty of the 45 participants that originally started the study completed the 14 week intervention trial. Fourteen were male and 26 were female and the average age was 46.8 years. The main risk factors evaluated included a history of smoking, abnormal lipid profile, elevated blood pressure, elevated body mass index as an indicator of overweight/obesity and fasting plasma glucose level as an indicator of diabetes. A total of 15 participants were either active or former smokers while 25 were non smokers. The average systolic blood pressure and body mass index (BMI) were 133.4 mm Hg and 28.4 kg/m<sup>2</sup> respectively while the mean plasma glucose level was 5.25 mmole/L. The serum HDL, LDL and total cholesterol levels in mmole/L were 1.5, 3.03 and 5.5 respectively. The participants' profiles are shown in Table 4.1.

**Table 4.1: Participant's profile and risk indicators**

Age (years)	46.8 ± 9.7
BMI (kg/m <sup>2</sup> )	28.4 ± 5.5
Total cholesterol (mmole/L)	5.5 ± 1.3
Serum HDL (mmole/L)	1.50 ± 0.34
Serum LDL (mmol/L)	3.03 ± 1.35
Average systolic BP (mm Hg)	133.4 ± 16.9
Fasting glucose (mmole/L)	5.25 ± 0.63
Female	26
Male	14

Values in columns = means ± S.D. Abbreviations: BMI = body mass index; BP= blood pressure; HDL = high density lipoproteins; LDL = low density lipoproteins.

The daily flavonoid intake derived from the dietary records the participants had to complete during each phase is shown in Table 4.2. The daily flavonoid intake was significantly ( $P < 0.001$ ) higher during the rooibos intervention phase (343.18 mg) when compared to the control (27.43 mg) and washout (31.29 mg) phases.

**Table 4.2: Participant/s daily flavonoid intake**

Study phase	Daily flavonoid intake (mg)
Baseline	343.41 ± 106
Washout	31.29 ± 25
Intervention	343.18 ± 90
Control	27.43 ± 24

Values in columns are means ± S.D (n=40).

Hence the total flavonoid intake for the entire intervention period of 6 weeks was 14.41 g per person.

#### 4.2 Antioxidant profile of the rooibos herbal tea

Before the study commenced, the preparation of a cup of rooibos herbal tea, was simulated in the laboratory to characterise the antioxidant profile of the study beverage with/without the addition of milk. As mentioned in chapter two, the effect of milk on antioxidant capacity (AOC) is still an ongoing debate. This pre-study profiling sought to establish the possible impact of milk on the available rooibos polyphenolic compounds and antioxidant activity used in the study for a more appropriate interpretation.

The results of the total polyphenol content, soluble solids, antioxidant content and antioxidant capacity of a cup of rooibos is shown in Table 4.3. With the exception of the total polyphenol content, the level of all other analytes/activities was significantly ( $P < 0.05$ ) higher in the rooibos prepared without milk than when compared with the rooibos where milk was added. A significant ( $P < 0.05$ ) increase in the level of total polyphenols (318.51 mg/L) was shown in the milk-containing rooibos when compared with the beverage without milk (292.64 mg/L). The total flavonol and flavanol content were shown to be significantly ( $P < 0.05$ ) decreased by the addition of milk. The antioxidant capacity of the rooibos assessed using the FRAP, ORAC and ABTS assays showed that the addition of milk significantly ( $P < 0.05$ ) decreased this capacity of the beverage. The comparisons of the three assays with and without milk per 200 mL cup were, FRAP: 260.15 vs 396.68  $\mu$ mole AAE, ABTS: 173.54 vs 237.49  $\mu$ mole TE and ORAC: 1402.09 vs 1729.03  $\mu$ mole TE, respectively. Because of the influence of milk, the amount of soluble solids per cup of the beverage was significantly ( $P < 0.05$ ) higher in the rooibos with milk (1.91 g) when compared to the rooibos where no milk was added (0.66 g).

**Table 4.3: Antioxidant profile of the rooibos herbal tea consumed in the study**

Antioxidant content/ capacity	*One cup with milk	*One cup without milk	#Six cups with milk	#Six cups without milk
Flavanols (mg)	2.11 $\pm$ 0.13	2.91 $\pm$ 0.26	12.66 $\pm$ 0.78	17.46 $\pm$ 1.56
Flavonols (mg)	21.22 $\pm$ 2.11	22.48 $\pm$ 2.20	127.32 $\pm$ 12.66	134.87 $\pm$ 13.20
Total polyphenols (mg)	63.70 $\pm$ 5.22	58.53 $\pm$ 4.86	382.21 $\pm$ 31.32	351.17 $\pm$ 29.16
Soluble solids (g)	1.91 $\pm$ 0.16	0.66 $\pm$ 0.11	11.45 $\pm$ 0.96	3.96 $\pm$ 0.66
FRAP ( $\mu$ mole AAE)	260.15 $\pm$ 24.76	396.68 $\pm$ 30.08	1560.89 $\pm$ 148.56	2380.07 $\pm$ 180.47
ABTS ( $\mu$ mole TE)	173.54 $\pm$ 12.86	237.49 $\pm$ 16.42	1041.24 $\pm$ 77.16	1424.94 $\pm$ 98.52
ORAC ( $\mu$ mole TE)	1402.09 $\pm$ 44.12	1729.03 $\pm$ 48.20	8412.53 $\pm$ 264.72	10374.20 $\pm$ 289.20

Values in columns represent average  $\pm$  S.D, with n=10, \* one cup = 200 mL, # 6 cups = 1.2L, TE = Trolox equivalents, AAE = Ascorbic acid equivalent

### 4.3 Chemical pathology

The effect of the different study phases of this trial on the liver and kidney function enzymes are summarized in Table 4.4. No adverse effects as a result of the intake of six cups of rooibos daily for six weeks were reported by the study participants nor did the liver and kidney function profiles show any. When comparing the rooibos intervention phase with the control phase, there were no significant changes in the levels of serum  $\gamma$ -glutamyl transferase. The AST levels were significantly ( $P < 0.05$ ) decreased (17.76 U/L) in the control phase and a similar trend was also observed in the serum levels of ALT and ALP while the concentration of LDH measured in both the rooibos (180.26 U/L) and control (191.01 U/L) phase did not differ.

Changes in plasma proteins and their main metabolic end product, urea, followed a similar trend. The concentration of the two analytes in plasma were significantly lower ( $P < 0.05$ ) after the rooibos intervention (66.58 g/L and 4.08 mmole/L) compared to baseline (70.57 g/L and 5.34 mmole/L) and washout (82.32 g/L and 4.99 mmole/L), but were not different when compared with the control phase (65.52 g/L and 4.36 mmole/L) respectively. The level of plasma creatinine significantly ( $P < 0.05$ ) decreased from 96.56  $\mu$ mole/L at washout to 76.56  $\mu$ mole/L after intervention and 61.62  $\mu$ mole/L after the control phase. The concentration of direct and total bilirubin were both significantly ( $P < 0.05$ ) elevated from 3.23 and 9.27  $\mu$ mole/L at intervention to 5.02 and 12.25  $\mu$ mole/L, respectively, when comparing the rooibos with the control phase, while the serum iron levels were not significantly different when comparing the intervention (15.66  $\mu$ mole/L) and control (13.89  $\mu$ mole/L) phases. There was also a significant ( $P < 0.001$ ) decrease observed in the plasma glucose levels at the end of the rooibos phase (4.82 mmole/L) when compared to the control phase (5.63 mmole/L).

**Table 4.4: Effect of the various trial phases on selected blood clinical parameters related to liver and kidney function and total iron levels**

Analyte	Baseline	Washout	Intervention	Control
Aspartate aminotransferase (U/L)	24.40 $\pm$ 11.6	21.84 $\pm$ 7.5	24.21 $\pm$ 14.2a	17.76 $\pm$ 8.3b
Alanine aminotransferase (U/L)	21.41 $\pm$ 21.7	21.77 $\pm$ 14.9	18.75 $\pm$ 17.2a	12.93 $\pm$ 8.3b
$\gamma$ -glutamyl transferase (U/L)	32.14 $\pm$ 23.0	30.73 $\pm$ 15.9	30.79 $\pm$ 17.5a	28.08 $\pm$ 13.7a
Alkaline phosphatase (U/L)	80.82 $\pm$ 36.9	82.67 $\pm$ 27.8	79.67 $\pm$ 25.0a	65.66 $\pm$ 20.2b
Lactate dehydrogenase (U/L)	153.92 $\pm$ 58.6	149.45 $\pm$ 31.7	180.26 $\pm$ 44.0a	191.01 $\pm$ 86.3a
Total proteins (g/L)	70.57 $\pm$ 30.3	82.32 $\pm$ 22.2	66.57 $\pm$ 4.6a	65.52 $\pm$ 16.0a
Urea (mmole/L)	5.34 $\pm$ 1.6	4.99 $\pm$ 1.7	4.08 $\pm$ 1.1a	4.35 $\pm$ 1.3a
Creatinine ( $\mu$ mole/L)	93.21 $\pm$ 44.7	96.56 $\pm$ 22.5	76.56 $\pm$ 22.2a	61.62 $\pm$ 22.5b
Conjugated (D) bilirubin ( $\mu$ mole/L)	3.17 $\pm$ 7.0	2.92 $\pm$ 1.5	3.23 $\pm$ 4.0a	5.02 $\pm$ 8.5b
Total bilirubin ( $\mu$ mole/L)	9.52 $\pm$ 6.8	8.90 $\pm$ 5.9	9.27 $\pm$ 4.4a	12.25 $\pm$ 8.9b
Glucose (mmole/L)	5.07 $\pm$ 3.5	5.64 $\pm$ 2.7	4.82 $\pm$ 1.7a	5.63 $\pm$ 1.6b
Iron ( $\mu$ mole/L)	16.10 $\pm$ 5.5	16.82 $\pm$ 4.7	15.66 $\pm$ 4.8a	13.89 $\pm$ 3.1a

Values in columns are means  $\pm$  S.D (n = 40). When comparing the rooibos intervention with the control phase, means followed by the same letter do not differ significantly, but if letters differ then  $P < 0.05$ .

#### 4.4 Blood antioxidant content and activity

Comparing the intervention and control phases, rooibos intake for six weeks was associated with an 11.8% significant ( $P < 0.05$ ) increase in plasma total polyphenols as estimated by the Folin- Ciocalteu method on samples drawn after 12-14 hours overnight fast. At baseline, the blood total polyphenol content was 85.70 mg/L which decreased as expected to 72.30 mg/L after the washout phase. At the end of the rooibos intervention phase, this level increased to 88.38 mg/L which was close to the level recorded in the baseline phase, but then declined again to 79.06 mg/L after completion of the control phase as shown in Table 4.5.

**Table 4.5: Effect of various study phases on plasma levels of total polyphenols**

Phase	Total polyphenols (mg/L)
Baseline	85.70 ± 11.04a
Washout	72.30 ± 10.21b
Intervention	88.38 ± 14.46a
Control	79.06 ± 17.38bc

Values in columns are means ± S.D (n = 40). Means followed by the same letter does not differ significantly. If letters differ, then  $P < 0.05$ .

The blood antioxidant capacities after completion of the four phases are shown in Table 4.6. No significant differences in the plasma AOC as evaluated by ORAC and FRAP and ABTS were observed when the rooibos intervention and control phases were compared.

**Table 4.6: Changes in serum antioxidant capacities after each study phase**

	Baseline	Washout	Intervention	Control
FRAP (µmole AAE/L)	372 ± 90	338 ± 94	350 ± 85a	375 ± 88a
ABTS-TEAC (µmole TE/L)	333 ± 93	325 ± 85	347 ± 95a	371 ± 105a
ORAC (µmole TE/L)	1558 ± 36	1594 ± 41	1402 ± 13a	1383 ± 24a

Values in columns are means ± S.D of 40 participants done in triplicate. Statistical differences are indicated in the table for intervention and control group comparison only. Means followed by the same letter do not differ significantly. If letter differs then  $P < 0.05$ . AAE= ascorbic acid equivalent; TE= trolox equivalent.

#### 4.5 Redox status of participants

Data on the concentrations of circulating GSH and GSSG levels and the GSH/GSSG ratio are presented in Table 4.7. The concentration of GSH in the blood of participants at baseline (513.66 µmole/L) significantly ( $P < 0.05$ ) decreased to 196.14 µmole/L after the washout phase during which dietary flavonoid restriction commenced. Intervention with rooibos significantly ( $P < 0.05$ ) increased this level to 1072.24 µmole/L which was subsequently significantly ( $P < 0.05$ ) decreased to 337.63 µmole/L at the end of the control phase when water replaced rooibos as the fluid taken.

**Table 4.7: Mean concentrations of reduced and oxidized glutathione and the ratios of the two forms over the four phases**

Phase	GSH ( $\mu\text{mole/L}$ )	GSSG ( $\mu\text{mole/L}$ )	GSH:GSSG ratio
Baseline	513.66 $\pm$ 147a	26.90 $\pm$ 4.43a	16.99 $\pm$ 5.78a
Washout	196.14 $\pm$ 100b	11.88 $\pm$ 2.01b	14.29 $\pm$ 8.25a
Intervention	1072.24 $\pm$ 140c	13.09 $\pm$ 2.48c	80.33 $\pm$ 15.24b
Control	337.63 $\pm$ 203d	18.53 $\pm$ 3.96d	15.78 $\pm$ 9.67a

Values in columns are means  $\pm$  S.D (n = 40). Means followed by the same letter do not differ significantly. If letters differ then P<0.05.

Oxidized glutathione (GSSG) was recorded at 26.90  $\mu\text{mole/L}$  at baseline then remained relatively unchanged between washout (11.88  $\mu\text{mole/L}$ ) and intervention (13.09  $\mu\text{mole/L}$ ). However there was a significant (P<0.05) increase to 18.53  $\mu\text{mole/L}$  after the control phase which represents a 29% decrease in GSSG after the rooibos intervention phase was completed when compared to the control phase. Due to the changes in levels of both GSH and GSSG, the participants' average GSH/GSSG ratio was significantly increased (P<0.05) to 80.33 after completion of the rooibos intervention phase when compared to the washout phase (14.29) and control phase (15.78).

#### 4.6 Lipid profile and markers of lipid peroxidation

The participants' lipid profile and changes in lipid peroxidation over the four phases are shown in Table 4.8. Comparing intervention and control phases, rooibos intake was not associated with any significant changes to the participants' HDL or total cholesterol levels but significant (P<0.05) decreases in serum triglyceride levels were noted. Rooibos intake was also associated with an 11% (significant, P<0.05) decrease in the level of serum LDL from 4.66 mmole/L to 4.19 mmole/L when compared with the control phase.

**Table 4.8: Participants' lipid profile**

Analyte	Baseline	Washout	Intervention	Control
Cholesterol (mmole/L)	5.46 $\pm$ 1.8a	5.54 $\pm$ 1.3a	5.48 $\pm$ 0.9a	5.93 $\pm$ 1.1a
HDL (mmole/L)	1.44 $\pm$ 0.5ab	1.07 $\pm$ 0.5ab	1.01 $\pm$ 0.2a	0.92 $\pm$ 0.2b
Triglycerides (mmole/L)	1.56 $\pm$ 1.1a	1.38 $\pm$ 1.3b	1.29 $\pm$ 0.8b	1.78 $\pm$ 1.8a
LDL (mmole/L)	3.58 $\pm$ 1.3a	4.09 $\pm$ 1.0a	4.19 $\pm$ 0.7b	4.66 $\pm$ 0.9c

HDL = high density lipoproteins; LDL = low density lipoprotein. Values in columns are means  $\pm$  S.D (n = 40). Means followed by the same letter do not differ significantly. If letters differ then P<0.05.

Intervention with the herbal tea was shown to affect the two markers of lipid peroxidation used in the study. As shown in Table 4.9 the 6-week rooibos intake significantly (P<0.05) decreased the

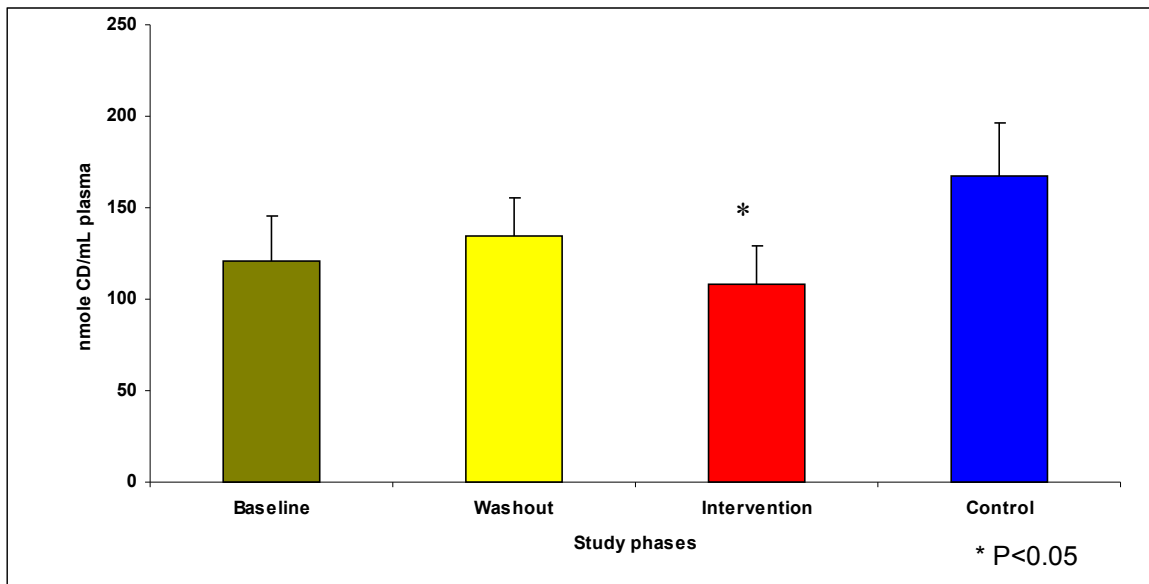
levels of CDs when compared with all the other phases. The change observed from the control (167.33 nmole/mL) to the intervention (108.84 nmole/mL) phase represents a 34.9 % decrease in CDs concentration. A similar trend was observed with regard to the concentration of plasma MDA measured as TBARS. The level after completion of the intervention phase (0.86  $\mu$ mole/L) was significantly ( $P < 0.05$ ) lower compared with that after completion of the washout (1.53  $\mu$ mole/L) and control (1.79  $\mu$ mole/L) phases. Hence intervention with rooibos was associated with a 52% decrease in MDA when compared to the control phase.

**Table 4.9: Change in markers of lipid peroxidation over the four phases**

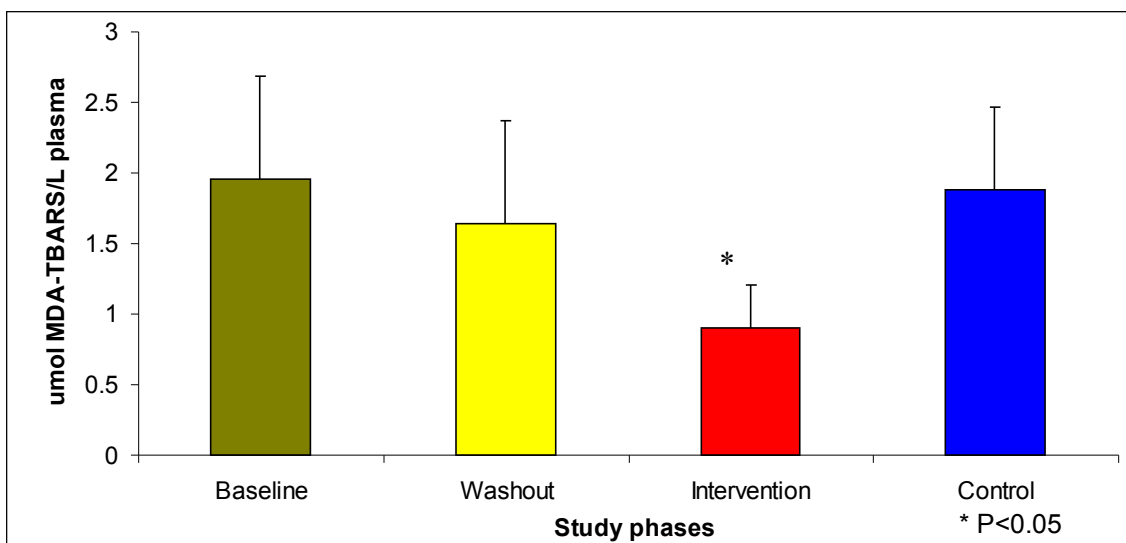
Analyte	Baseline	Washout	Intervention	Control
CDs (nmole/mL)	120.81 $\pm$ 24.31a	134.72 $\pm$ 20.57b	108.84 $\pm$ 20.13c	167.33 $\pm$ 29.45d
TBARS ( $\mu$ mole/L)	1.83 $\pm$ 0.72a	1.53 $\pm$ 0.70a	0.86 $\pm$ 0.26b	1.79 $\pm$ 0.58ac

CD= conjugated dienes; TBARS = thiobarbituric acid reactive substances. Values in columns are means  $\pm$  S.D (n = 40). Means followed by the same letter do not differ significantly. If letters differ the  $P < 0.05$ .

A trend was therefore observed in both CDs and TBARS showing a decrease in both markers of LPO after the completion of the rooibos intervention phase when compared to the control phase where participants were consuming water. This is also depicted in Figures 4.1 and 4.2 below, respectively.



**Figure 4.1 Inhibitory effect of rooibos intervention on plasma levels of the lipid peroxidation product, conjugated dienes**



**Figure 4.2 Inhibitory effect of rooibos intervention on lipid peroxidation as measured by plasma levels of TBARS**



## CHAPTER 5

### DISCUSSION

Certain dietary constituents may influence the incidence of chronic diseases like CHD by improving lipid peroxidation and endothelial function as well as by modulating several genes responsible for cardiovascular health (Kaliora *et al.*, 2006). In this regard, the popularity of herbal teas such as rooibos has increased during the past two decades (Joubert *et al.*, 2008) following advances made in our knowledge on CVD health promoting properties of green and black teas (Yung *et al.*, 2008). Because the phenolic constituents of rooibos differ from green and black teas, it is necessary to investigate the possible impact of this South African herbal tea on oxidative stress indicators relating to cardiovascular health. Very few studies have investigated the possible health promoting effect of rooibos in humans and this study is the first of its kind reporting on the effects rooibos has in humans at risk for developing CHD. All the participants selected had at least two of the risk factors for heart disease outlined in chapters 3 and 4 and included hyperlipidemia, hypertension, history of smoking, raised body mass index and aged over 30 years.

The rooibos beverage used in the study was first simulated in the laboratory with/without the addition of milk prior to the start of the study. As mentioned in chapter two, the effect of milk on antioxidant activity (AOC) is still an ongoing debate. This pre-study profiling sought to establish the possible impact of milk on the rooibos polyphenolic compounds and antioxidant activity used in the study for a more appropriate interpretation. With the exception of the total polyphenol content, the level of all other analytes/activities was higher in the rooibos prepared without milk than when compared with the rooibos where milk was added. A small increase in the level of polyphenols (318.51 mg/L) was measured in the milk-containing rooibos and could possibly be ascribed to interference from various components in milk when using the Folin-Ciocalteu method. The total polyphenol content of a cup of rooibos as measured in this study is in agreement with previously reported findings that a 150-200 mL cup of rooibos contains 60-80 mg of total polyphenols (Joubert, 1996). The AOC of the rooibos assessed using the FRAP, ORAC and ABTS assays show that the addition of milk influences the capacity of the beverage. The comparisons of the three assays with and without milk per 200 mL cup were, FRAP: 260.15 vs 396.68  $\mu\text{mole AAE}$ , ABTS: 173.54 vs 237.49  $\mu\text{mole TE}$  and ORAC: 1402.09 vs 1729.03  $\mu\text{mole TE}$ . Milk proteins may interact with the rooibos phenolics forming covalent or non covalent bonds which may be via either multisite interactions (several phenolics bound to one

protein molecule) or multidentate interactions (one phenolic bound to several protein sites or protein molecules) (Sharma *et al.*, 2008). This binding to proteins may cause the phenolics to lose their antioxidant activity as measured by the various assays as observed in the rooibos containing milk compared to that without milk. On the other hand, the Folin assay used in this study is known to suffer from interference from various compounds for example vitamin C and therefore, the level of polyphenols may still appear elevated despite the possible precipitation. As expected, because of the influence of milk, the amount of soluble solids was higher in the tea with milk (1.91 g) compared to the tea where no milk was added (0.66 g) per cup of the beverage. As mentioned in chapter two, several studies with *C. sinensis* have shown that addition of milk does not impair the bioavailability and activity of polyphenols *in vivo* in humans despite the *in vitro* differences (Leenen *et al.*, 2000; Roura *et al.*, 2007).

The daily flavonoid in the present study, as expected was higher during the intervention phase (343.18 mg) when compared to the control (27.43 mg) and washout (31.29 mg) phases. Hence the total flavonoid intake for the entire intervention period of 6 weeks was 14 grams. Different countries obtain flavonoids from different sources. Chun *et al.* (2007) for example, estimated the mean daily total flavonoid intake in the USA to be 189.7 mg/day mainly obtained from flavan-3-ols (83.5%), followed by flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%). In Australia, an analysis of 24-hour recall data indicated the average intake of an adult is 454 mg/day with 92% being flavan-3-ols (Johannot & Somerset, 2006). More recent studies have estimated individual flavonoid or flavonoid profile intakes in the USA, Denmark, Holland, Finland, France, Greece, Japan and Spain but the dietary intake methodologies vary substantially and the above two studies serve to highlight the variations inherent in such dietary estimates.

The consumption of 6 cups of rooibos per day for 6 weeks did not cause any adverse effects in the study participants and neither were the serum iron levels altered indicating that none of the rooibos constituents interfered with iron uptake. These results are in line with the findings obtained from two previous human studies (Hesseling *et al.*, 1979; Breet *et al.*, 2005) and an experimental study by Marnewick *et al.* (2003) in rats and serves to establish the safety of short term consumption of rooibos with regards to liver and kidney function. The same cannot be said of black and green tea consumption as conflicting evidence have been reported regarding the effect of these *C. sinensis* teas on iron levels in humans (Temme & Van Hoydonck, 2002; Marouani *et al.*, 2007; Mennen *et al.*, 2007; Thankachan *et al.*, 2008). As mentioned in section 2.3.4.6, tannins are known to reduce iron absorption and to interfere with protein metabolism and utilization especially when the iron and tannin sources are ingested together. The low

tannin content of rooibos of 4.4% (Morton, 1983) has led to the view - which was corroborated by the present and referenced studies - that rooibos is free of these anti-nutritive effects.

As expected of a restricted diet, several chemical pathology biomarkers showed a decreasing trend during the intervention and control phases including total proteins (66.58 and 65.52 g/L) and creatinine (76.56 and 61.62  $\mu\text{mole/L}$ ), respectively. However all the pathology biomarkers measured in the study were within the referenced normal ranges (Table 5.1). Serum LDH and total bilirubin measures for all the phases including baseline fell outside the reference ranges suggesting a possible analytical anomaly. As the first human safety data regarding the intake of traditional rooibos, the results presented here therefore indicate that this herbal beverage does not negatively influence any of these indicators of liver and kidney toxicity. Although the level of glucose remained within the reference range (3.6-6.4 mmole/L) the significant decrease observed due to the herbal tea (4.82 mmole/L) compared to the control phase (5.63 mmole/L) is an important finding. Elevated glucose intolerance seems to play a critical role in the progression of cardiovascular disease and is also often associated with hypertriglyceridemia, hypertension, elevated LDL cholesterol, and depressed HDL (Kaliora & Dedoussis, 2007). This finding therefore seems to fit well with the decreased LDL and total cholesterol levels reported in this study and warrants further investigation.

**Table 5.1: Normal reference values for various clinical parameters**

Serum/plasma indicators (units)	Normal values (adults)
Total protein (g/L)	66-87
Urea (mmole/L)	2.5-7.5
Creatinine ( $\mu\text{mole/L}$ )	53-115
Direct Bilirubin ( $\mu\text{mole/L}$ )	0-5.1
Total Bilirubin ( $\mu\text{mole/L}$ )	0-7
Glucose mmole/L)	3.6-6.4
Cholesterol (mmole/L)	3.87-6.71
Triglycerides (mmole/L)	0.68-1.88 (men); 0.63-1.60 (women)
Iron ( $\mu\text{mole/L}$ )	7-30
AST (U/L)	$\leq 46$ (37°C)
ALT (U/L)	$\leq 49$ (37°C)
GGT (U/L)	9-52 (men); 5-32 (women)
ALP (U/L)	39-117
LDH (U/L)	230-460
HDL (mmole/L)	0.78-2.0

Chemistry kit inserts, Kat Medical South Africa <http://www.katmedical.com>

Several intervention studies suggest that a dietary intervention with polyphenol-rich beverage in human subjects can improve plasma AOC and polyphenol content (Lotito & Frei, 2006). Consequently, it was hypothesized in the present study that regular ingestion of rooibos would significantly increase plasma AOC and total phenolic levels higher than when compared to the control phase. Comparing intervention and control phases, rooibos intake for 6 weeks was associated with an 11% significant increase ( $P < 0.05$ ) in plasma total polyphenols as estimated

by the Folin- Ciocalteu method on samples drawn after 12-14 hours overnight fast. However, comparing the two phases, this increase in circulating polyphenols was not associated with any significant changes in plasma AOC when assessed using three methods: FRAP, ABTS and ORAC. As mentioned earlier in section 2.3, the body's antioxidant reserves include phenolics and several other non-phenolic compounds like bilirubin, vitamins C and E, uric acid and other known/unknown metabolites. In light of the restricted diet, it is possible that while the sustained concentration of polyphenols may have increased, that of other antioxidant components may have decreased resulting in no net increases in AOC. Since all assays for AOC lack specificity (Lotito & Frei, 2006), their estimates are therefore not likely to indicate any net changes in AOC. This hypothesis may be supported by the observation that the decline in ORAC values increased with the duration of restriction. The washout, intervention and control phase ORAC estimates were 1595, 1402 and 1384  $\mu\text{mole TE/L}$ , respectively. As a more sensitive AOC assay compared to the other two, ORAC is more likely to detect the subtle changes in the non measured non-phenolic antioxidants. In addition, plasma antioxidant capacity is a fasting measurement and may not represent the active antioxidant pool since the half lives of all the individual compounds including polyphenols (varies from 2-11 hrs) and non polyphenols may fluctuate. Regarding the restricted diet, the 12-14 hour fast may also have a more profound effect on the non phenolic antioxidants such that the AOC remains unchanged or diminished regardless of increased level of polyphenols.

Reduced glutathione is a powerful intracellular antioxidant that plays a vital role in stabilizing various enzymes (Wang & Jiao, 2000) and could also be considered a good marker for tissue AOC (Van Acker *et al.*, 2000). Several clinical conditions are associated with a decrease in cellular GSH levels that may result in a lowered cellular redox potential (Exner *et al.*, 2000). In this study, rooibos consumption was associated with a significant ( $P < 0.05$ ) increase in the level of GSH when the concentration after the intervention (1072.24  $\mu\text{mole/L}$ ) is compared to the level after the control phase (337.63  $\pm$  203). There was a significant ( $P < 0.05$ ) decrease in the level of GSSG (13.09  $\mu\text{mole/L}$ ) after completion of the rooibos intervention phase when compared to the control phase levels of GSSG (18.53  $\mu\text{mole/L}$ ) which may suggest a stabilizing effect of rooibos on glutathione. Due to these changes in levels of both GSH and GSSG, the participants' average GSH/ GSSG ratio was significantly ( $P < 0.05$ ) increased to 80.33 during the intervention phase compared to the control phase ratio of 15.78 and the washout phase ratio of 14.28. The ability of rooibos to enhance the GSH/GSSG ratio was previously shown in experimental rats (Marnewick *et al.*, 2003). In that study, both rooibos and *C. sinensis* teas significantly increased the ratio with significant reduction of GSSG; however, the mechanisms may differ since a notable increase in GSH was seen with rooibos but not with *C. sinensis*. More

recently, Nikolova *et al.* (2007) showed that consumption of rooibos herbal tea for 8 weeks enhanced GSH levels by 48% in humans. The study, which investigated the effect of rooibos on antioxidant status in men occupationally exposed to lead, did not report the GSH/GSSG ratios. In the present study, rooibos enhanced the GSH/GSSG ratio by decreasing GSSG and stabilizing/increasing GSH although the exact mechanisms involved remains to be elucidated. Plasma GSH levels are primarily dependent upon the rates of biosynthesis and utilization in oxidation/reduction reactions. Amounts of the precursor amino acids such as cysteine and the activity of the enzyme glutamate-cysteine ligase are the key factors affecting GSH synthesis (Rebrin *et al.*, 2005). On the other hand, exhaustion of GSH results from its involvement in various reduction reactions including direct quenching of free radicals, enzymatic reduction of hydroperoxides, metabolism of xenobiotics and regeneration of reduced forms of redox pairs such as cysteine/cystine, NADPH/NADP<sup>+</sup>, and thioredoxin<sub>reduced</sub>/thioredoxin<sub>oxidised</sub> (Rebrin *et al.*, 2005). Since rooibos is a poor source of proteins (McKay & Blumberg, 2007), it is unlikely that the observed shift in glutathione redox state was due to *de novo* synthesis from ingested amino acids. Polyphenols from rooibos are suggested to account for these effects in this study. Phytochemicals are known to induce phase II antioxidant enzymes and hence to increase the synthesis of antioxidant and detoxification enzymes and major cellular antioxidants especially glutathione (Rebrin *et al.*, 2005). The substantial increase in the GSH/GSSG ratio in the blood of participants after intervention with rooibos may suggest a decreased oxidative stress or an increased antioxidant capacity in the cell, thereby lowering the risk of oxidative damage. While changes in plasma redox state may not necessarily reflect those occurring in the tissues, it may still be possible that enhancement of plasma GSH reflects its stabilization in critical organs like the liver (Rebrin *et al.*, 2005). This may lead to enhanced detoxification capacity, as glutathione is known to, either directly or via the glutathione S-transferases, interact with reactive toxic metabolites, thus decreasing the risk of oxidative damage to biomolecules (Siess *et al.*, 2000).

In the present study, lipid peroxidation was estimated using two *in vivo* markers – MDA and CDs. Both markers were significantly ( $P < 0.05$ ) reduced by 34.9% for CDs and 52% for MDA after intervention with rooibos when compared to the control phase, respectively. The effects of rooibos consumption on the *in vivo* markers of LPO in humans have to-date not been reported in the scientific literature. Rooibos was previously reported to reduce age-related lipid peroxide accumulation (measured as TBARS) in brains of rats consuming the herbal tea for 21 months and to inhibit MDA formation in rat tissues and liver microsomal preparation (Ulicna *et al.*, 2006; Marnewick *et al.*, 2005). The effects of other phenolic-rich beverages especially *C. sinensis* on LPO have been studied more extensively and several studies support an inhibitory role for green and black tea against LPO. Proposed mechanisms via which this is achieved include

inhibition of lipid absorption and cholesterol synthesis as well as by up-regulation of the LDL receptor (Bursill *et al.*, 2007; Koo & Noh, 2007). The present study is supportive of a LDL - cholesterol lowering effect by rooibos as the beverage significantly ( $P < 0.05$ ) lowered the levels of LDL and triglycerides in the plasma. Total cholesterol was significantly ( $P < 0.012$ ) lowered by 7.56% but only a marginal (non-significant) increase in HDL was observed when intervention (1.01 mmole/L) and control (0.92 mmole/L) phases are compared. However, the baseline level of HDL (1.44 mmole/L) was significantly ( $P < 0.05$ ) higher compared to the other three phases. It was hypothesized that the restricted flavonoid diet may be a contributing factor to the observed decrease in HDL in the final three phases. The herbal tea was associated with a significant 11% decrease in the circulating level of LDL and it remains to be elucidated whether this reduction is achieved by up regulation of the LDL receptor or by other mechanisms. According to the results obtained from this study, rooibos decreased LPO possibly by also decreasing the amount of LDL available for oxidation and therefore a beneficial role of rooibos in the prevention of CHD may be suggested. The observed decrease in plasma LPO could also be a reflection of improved redox state in tissues brought about by the rooibos-induced GSH:GSSG ratio enhancement discussed earlier. The redox state of cells is known to impact profoundly on cellular functions such as the glutathione S-transferase-mediated elimination of electrophilic xenobiotics and some of the end-products of lipid peroxidation (Rebrin *et al.*, 2005). Inami *et al.* (2007) recently reported that green tea catechins lower plasma Ox-LDL concentration without any significant change in plasma LDL concentration. The authors concluded that this reduction in Ox-LDL may account for the beneficial effects of green tea on CHD. Oxidized LDL was not measured in the present study as it fell outside the scope, but it will be analysed at a later stage.

## CHAPTER SIX

### CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to assess the possible modulation of oxidative stress by rooibos in adults at risk for developing CHD which is the main form of CVD. Since oxidative stress has been implicated in the aetiology of CVD, it is often hypothesized that possession of two or more risk factors for CVD leads to situations of elevated oxidative stress. Rooibos has previously been shown to possess good antioxidant properties in *in vitro* and in experimental animal studies and thus the choice of the study population aimed at detecting any effects in humans at risk for developing heart disease. Additionally, the safety and possible anti-nutritive effects of beverages are always of interest given the association of *Camellia sinensis* teas with iron malabsorption. Assessing whether rooibos has similar undesirable effects in human subjects was the secondary objective of this study.

Consumption of 6 cups of this indigenous herbal tea for 6 weeks was shown not to be associated with any adverse effects based on liver and kidney function. Rooibos was found to reduce products of lipid peroxidation measured as conjugated dienes and malondialdehydes suggesting that the herbal tea has an inhibitory role in lipid peroxidation possibly by decreasing the amount of LDL available for oxidation and therefore suggesting a beneficial role in prevention of CHD. Rooibos was also found to positively affect antioxidant redox status largely by enhancing the levels of GSH and to a lesser extent, by lowering the levels of GSSG.

The protection of plasma LPO and enhancement of antioxidant redox by rooibos could be a mechanism for the prevention or delay of pathologies related to ROS damage for example CHD. However, with little previous information on the effect of this beverage on these parameters in humans, this study raises more questions and provides grounds for further work to confirm the physiological implications of these findings. Changes seen on the levels of GSH are particularly intriguing and necessitate more studies to clarify whether, and by which mechanisms, rooibos (or its constituent compounds) could influence enzymes relevant to GSH activity in humans. The reduction of LPO products and increase of total polyphenols in plasma with no accompanying effect on the antioxidant profile may suggest events at cellular level worth examining. Another avenue of investigation would be the possible effect of rooibos on human tissues especially given the results of earlier-mentioned studies investigating the effect on liver and brain tissue of experimental rats consuming rooibos. Studies to assess the bioactivity, bioavailability and excretion of the unique rooibos polyphenols and even the effect of

rooibos intake on inflammation and DNA damage protection/prevention would aptly supplement our current knowledge about this herbal tea. The role of elevated glucose in diabetes and progression of cardiovascular disease is well established hence the decrease in the level of plasma glucose observed in this study also requires further evaluation.

While the objectives of this study were accomplished, several limitations should be considered when interpreting the results presented here. The study assessed systemic markers of oxidative stress and antioxidant capacity which may not necessarily reflect levels in tissues. Furthermore, the two methods used for the estimation of lipid peroxidation are limited because of poor specificity although they still remain the most used and reported on methods for this kind of investigation, which indicates a lack in the development and substantiation of newer more specific methodologies. Inclusion of more analytes not investigated here (e.g. uric acid and homocysteine) would aid in the interpretation of results particularly in light of the association of the latter with CVD, however, this did not fall within the timeframe of this study. In addition, the absence of a placebo group in this study was an important limitation necessitated by the difficulty of producing a placebo to match the taste of rooibos. Despite these limitations, the results of the study are expected to add immensely to our present understanding of the health promoting properties of rooibos as a potential dietary candidate for components that may offer realistic complementary preventive option for individuals with high risk for heart diseases.



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**BIBLIOGRAPHY**

- Aherne, S.A. & O'Brien, N.M. 2002. Dietary flavonols: Chemistry, food content and metabolism. *Nutrition*, 18:75-81
- Almajano, M.P., Carbo, R., Jiménez, J.A.L. & Gordon, M.H. 2008. Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry*, 108:55–63.
- Alpha-tocopherol, Beta-Carotene-Prevention Study Group. 1994. The effects of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*, 330:1029-35.
- Ameer, B., Weinrub, R.A. 1997. Drug interactions with grapefruit juice. *Clinical Pharmacokinetics*. 33(2):103-121.
- Arguelles, S., Garcí'a, S., Maldonado, M., Machado, A. & Ayala, A. 2004. Do the serum oxidative stress biomarkers provide a reasonable index of the general oxidative stress status? *Biochimica & Biophysica Acta*, 1674: 251– 259.
- Arts, M.J., Haenen, G.R., Wilms, L.C., Beetstra, S.A., Heijnen, C.G., Voss, H-P. & Bast, A. 2002. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *Journal of Agricultural and Food Chemistry*, 50: 1184-1187.
- Asensi, M., Sastre, J., Pollardor, V., Lloret, A., Lehner, M., Asuncion, J.G. & Vina, J. 1999. Ratio of reduced to oxidized glutathione as an indicator of oxidative stress status and DNA damage. *Methods in Enzymology*, 299:267-277.
- Azzi, A., Davis K.J.A. & Kelly, F. 2004. Free radical biology: terminology and critical thinking. *Federation of European Biochemical Societies Letters*, 558:3-6.
- Bahorun, T., Soobrattee, M.A., Luximon-Ramma, V. & Aruoma, O.I. 2006. Free radicals and antioxidants in cardiovascular health and disease. *Internet Journal of Medical Update*, Jul – Dec: Vol 1, Issue No. 2:1-17.
- Bash, L.D., Selvin, E., Steffes, M., Coresh, J., Astor, BC. 2008. Poor glycemic control in diabetes and the risk of incident chronic kidney disease even in the absence of albuminuria and retinopathy: The Atherosclerosis Risk in Communities (ARIC) Study. *Archives of Internal Medicine*, 168(22):2440-7.
- Bendich, A., D'Apollito, P., Gabriel, E. & Machlin, L.J. 1984. Interaction of dietary vitamin C and vitamin E on guinea pig immune responses to mitogens. *Nutrition*, 114:1588-93.
- Benzie, I.F.F. & Strain, J.J. 1996. The Ferric reducing ability of plasma as a measure of

“Antioxidant power”: The FRAP assay. *Journal of Analytical Biochemistry*, 239:70-76.

Boushey, C.J., Beresford, S.A., Omenn, G.S. & Motulsky, A.G. 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *Journal of the American Medical Association*, 274 (13):1049- 1057.

Bramati, L., Aquilano, F. & Pietta, P. 2003. Unfermented rooibos tea: Quantitative characterization of flavonoids by HPLC-UV and determination of the total antioxidant activity. *Journal of Agricultural and Food Chemistry*, 51(25):7472-7474.

Breet, P., Kruger, H.S., Jerling, J.C., Oosthuizen, W. 2005. Actions of black tea and rooibos on the iron status of primary school children. *Nutrition Research*, 25: 983–99.

Brown, J.E., Khodr, H., Hider, C.R., & Rice-Evans, C.A. 1998. Structural dependence of flavonoid interactions with Cu<sup>2+</sup> ions: implications for their antioxidant properties. *Biochemical Journal*, 330:1173-1178.

Bursill, C.A., Abbey, M. & Roach, P.D. 2007. A green tea extract lowers plasma cholesterol by inhibiting cholesterol synthesis and up-regulating the LDL receptor in the cholesterol-fed rabbit. *Atherosclerosis*, 193:86–93.

Burton, G.W., Wronska, U., Stone, L., Foster, D.O. & Ingold, K.U. 1990. Biokinetics of dietary RRR-  $\alpha$ -tocopherol in the male guinea pig at three dietary levels of vitamin C and two levels of vitamin E. Evidence that vitamin C does not “spare” vitamin E *in vivo*. *Lipids*, 25:199-210.

Cao, G. & Prior, R.L. 1998. Comparison of different analytical methods for assessing total antioxidant capacity in human serum. *Clinical Chemistry*, 44: 1309-15.

Cao, G., Verdon, C.P., Wu, A.H.B., Wang, H. & Prior, R.L. 1995. Automated oxygen radical absorbance capacity using the COBAS FARA II. *Clinical Chemistry*, 41:1738-44.

Chopra, M., Fitzsimons, P.E.E., Strain, J.J., Thurnham, D.I. & Howard A.N. 2000. Non alcoholic red wine extract and quercetin inhibit LDL oxidation without affecting plasma antioxidant vitamin and carotenoids concentrations. *Clinical Chemistry*, 46:1162-70.

Chopra, R.K. & Bhagavan, H.N. 1999. Relative bioavailabilities of natural and synthetic E formulations containing mixed tocopherols in human subjects. *International Journal for Vitamin and Nutrition Research*, 69(2):92-5.

Chrysohoou, C., Panagiotakos, D.B., Pitsavos, C., Skoumas, J., Economou, M., Papadimitriou, L. & Stefanadis, C. 2007. The association between pre-hypertension status and oxidative stress markers related to atherosclerotic disease: The ATTICA study. *Atherosclerosis*, 192:169-1.

- Chun, O.K., Chung S.J. & Song W.O. 2007. Estimated dietary flavonoid intake and major food sources of U.S. adults. *Nutrition*, 137:1244–1252.
- Cohen, J.H., Kristal, A.R. & Stanford J.L. 2000. Fruit and vegetable intakes and prostate cancer risk. *National Cancer Institute*, 92:61-8.
- Cook, N.C. & Samman, S. 1996. Flavonoids – chemistry, metabolism, cardioprotective effects and dietary sources. *Journal of Nutritional Biochemistry*, 7:66-76.
- Corretti, M.C., Anderson, T.J. Benjamin, E.J., Celermajer, D., Charbonneau, F., Creager, M.A., Deanfield, J. & Vogel, R. 2002. Guidelines for the ultrasound assessment of endothelial – dependent flow –mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *Journal of the American College of Cardiology*, 39:257-65.
- Coultate, T.P. 1990. In: (2nd ed.), *Food: The Chemistry of Its Components*, The Royal Society of Chemistry (1990), 137–149.
- D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C. & Masella, R. 2007. Polyphenols, dietary sources and bioavailability. *Annals of the First Super Sanita*, 43(4):348-361.
- Dinkova-Kostova, A.T., Holtzclaw, W.D., Cole, R.N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M. & Talalay P. 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of USA*, 99:11908 – 11913.
- Doerge, D.R. & Chang, H.C. 2002. Inactivation of thyroid peroxidase by soy isoflavones, *in vitro* and *in vivo*. *Chromatography B*, 777:269–279.
- Duffy, S.J, Vita, J.A & Keaney, J.F. Jr. 1999. Antioxidants and endothelial function. *Heart Failure*, 15:135-52.
- Duffy, S.J., Keaney Jr, J.F., Holbrook, M., Gokce, N., Swerdloff, P.L., Frei, B. & Vita, J.A. 2001. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation*, 104:151-6.
- Dunnick, J.K. & Hailey, J.R. 1992. Toxicity and carcinogenicity studies of quercetin, a natural component of foods. *Fundamental and Applied Toxicology*, 19:423-31.
- Edenharder, R., Sanger, J.W., Glatt, H., Muckel, E. & Platt, K.L. 2002. Protection by beverages, fruits, vegetables, herbs, and flavonoids against genotoxicity of 2-acetylaminofluorene and 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) in

metabolically competent V79 cells. *Mutation Research*, 521:57-72.

Evans, P. & Halliwell, B. 2001. Micronutrients: Oxidant/antioxidant status. *British Journal of Nutrition*, 85:S67-S74.

Exner, R., Wessner, B., Manhart, N. & Roth, E. 2000. Therapeutic potential of glutathione. *Wiener Klinische Wochenschrift*, 112:610-616.

Farkas, C.S. & Harding, L-R.W. 1987. Effect of tea and coffee consumption on non-heme iron absorption: some questions about milk. *Human Nutrition Clinical Nutrition*, 44:161-163.

Fito, M., Covas, M.I., Lamuela-Raventós, R.M., Vila, J., Torrents, J., De La Torre, C. & Marrugat, J. 2000. Protective effects of olive oil and its phenolic compounds against LDL oxidation. *Lipids*, 35 (6):633-8.

Freedman, J.E., Parker, C. III, Li, L., Perlman, J.A., Frei, B., Ivanov, V., Deak, L.R., Folts, J.D. 2001. Select flavonoids and whole juice from purple grapes inhibits platelet function and enhance nitric oxide release. *Circulation*, 103 (23):1182-8.

Friedewald, W.T., Levy R.I. & Fredrickson, D.S. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. *Clinical Chemistry*, 18:499—502.

Georgé, S., Brat, P., Alter, P. & Amiot, M. 2005. Rapid determination of polyphenols and Vitamin C in plant-derived products. *Journal of Agricultural and Food Chemistry*, 53:1370-1373.

Gey, K.F., Puska, P., Jordan, P. & Moser, U.K. 1991. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *American Journal of Clinical Nutrition*, 53:326S-334S.

Gey, K.F., Stahelin, H.B. & Hicholzer, M. 1993. Poor plasma status of carotene and vitamin C is associated with higher mortality from ischaemic heart disease and stroke: Basel prospective study. *Clinical Investigator*, 71:3-6.

Ghiselli, A., Serafini, M. & Ferro-Luzzi, A. 1994. New approaches for measuring plasma or serum antioxidant capacity: a methodological note. *Free Radical Biology and Medicine*, 16: 135-7.

Glazer, A.N. 1990. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods in Enzymology*, 186:161-168.

Graham, H.N. 1992. Green tea composition, consumption and polyphenolic chemistry.

---

*Preventive Medicine*, 21:334-350.

Hagiwara, A., Hirose, M., Takahashi, S., Ogawa, K., Shjrai, T. & Ito, N. 1991. Fore-stomach and kidney carcinogenicity caffeic acid in F344 rats and C57BL/6N× C3H/HeNF<sub>1</sub> mice. *Cancer Research*, 51:5655-60.

Hakim, I.A., Hartz, V., Harris, R.B., Balentine, D., Weisgerber, U.M., Graver, E., Whitacre, R. & Alberts, D. 2001. Reproducibility and relative validity of a questionnaire to assess intake of black tea polyphenols in epidemiological studies. *Cancer Epidemiology Biomarkers and Prevention*, 10:667-78.

Halliwell, B. & Gutteridge J.C. 1995. The definition and measurement of antioxidants in biological systems. *Free Radical Biology and Medicine*, 18:125-6.

Halliwell, B. 1998. Albumin – an important extracellular antioxidant? *Biochemical Pharmacology*, 37:569-71.

Halliwell, B. 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? *Archives of Biochemistry and Biophysics*, 476:107–112.

Halliwell, B., Rafter, J. & Jenner A. 2005. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *American Journal of Clinical Nutrition*, 81:277S-83S.

Harborne, J.B. & Williams C.A. 2000. Advances in flavonoid research since 1992. *Phytochemistry*, 55:481-504.

Henning, S.M., Aronson, W., Niu, Y., Conde, F., Lee, N.H., Seeram, N.P., Lee, R-P. & Heber, D. 2006. Tea polyphenols and theaflavins are present in prostate tissue of humans and mice after green and black tea consumption. *Nutrition*, 136:1839-43.

Hesseling, P.B, Klopper, J.F. & Van Heerden, P.D. 1979. The effects of rooibos tea on iron absorption. *South African Medical Journal*, 55:631-632.

Hesseling, P.B. & Joubert, J.R. 1982. The effect of rooibos tea on the type I allergic reaction. *South African Medical Journal*, 62: 1037-1038.

Hodgson J.M., Puddey, I.B., Mori, T.A., Burke, V., Baker, R.I. & Beilin, L.J. 2001. Effects of regular ingestion of black tea on haemostasis and cell adhesion molecules in humans. *European Journal of Clinical Nutrition*, 55:881-6.

Hodgson, J.M., Puddey, I.B., Burke, V., Watts, G.F. & Beilin, L.J. 2002. Regular ingestion of black tea improves brachial artery vasodilator function. *Clinical Science*, 102:195-201.

- Hodis, H., Mack, W., LuBree, L., Muhrer, P., Sevanian, A., Liu, C., Hwung, J., Selzer, R. & Azen, S. 2002. Alpha tocopherol supplementation in healthy individuals reduces low density lipoprotein oxidation but not atherosclerosis: the Vitamin E Atherosclerosis Prevention Study (VEAPS). *Circulation*, 106:1453-1459.
- Hollman, P.C.H., van Trijp, J.M.P., Buysman, M.C.P., Gaag, M.S., Mengelers, M.J.B, De Vries, J.H.M. & Katan, M.B. 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *Federation of European Biochemical Societies Letters*, 418:152-156.
- Holt, R.R., Lazarus, S.A., Cameron, S.M., Zhu, Q.Y., Schramm, D.D., Hammerstone, J.F., Fraga, C.G. & Keen C.L. 2002. Procyanidin dimer B2 in human plasma after the consumption of a flavanol-rich cocoa. *American Journal of Clinical Nutrition*, 76:798-804.
- Hong, S.J., Kim, S.I., Kwon, S.M., Lee, J.R. & Chung, B.C., 2002. Comparative study of concentration of isoflavones and lignans in plasma and prostatic tissues of normal control and benign prostatic hyperplasia. *Yonsei Medical Journal*, 43:236-41.
- Hughes, D.A. 2005. Plant polyphenols: modifiers of immune function and risk of cardiovascular disease. *Nutrition*, 21:422-423.
- Hurriel, R.F., Reddy, M. & Cook, J.D. 1999. Inhibition of non-heme iron absorption in man by polyphenolic-containing beverages. *British Journal of Nutrition*, 81:521-528.
- Hwang, E-S & Kim G-H. 2007. Biomarkers for oxidative stress status of DNA, lipids and proteins *in vitro* and *in vivo* cancer research. *Toxicology*, 229:1-10.
- Igarashi, O., Yonekawa, Y. & Fujiyama-Fujihara, Y. 1991. Synergistic action of vitamin E and vitamin C *in vivo* using a new mutant of Wistar- strain rats, ODS, unable to synthesize vitamin C. *Journal of Nutrition Science and Vitaminology*, 37:359-69.
- Inami, S., Takano, M., Yamamoto, M., Murakami, D., Tajika, K., Yodogawa, K., Yokoyama, S., Ohno, N., Ohba, T., Sano, J., Ibuki, C., Seino, Y. & Mizuno, K. 2007. Tea catechin consumption reduces circulating oxidized low-density lipoprotein. *International Heart Journal*, 48:725-732.
- Inanami, O. 1995. The suppression of age-related accumulation of lipid peroxides in rat brain by administration of roibos tea (*Aspalathus linearis*). *Neuroscience Letters*, 196:85-88.
- Ito, A., Shinohara, K., Kator, K., 1991. Protective action of roibos tea (*Aspalathus linearis*) extracts against inactivation of L5178Y cells by H<sub>2</sub>O<sub>2</sub>. In: *Proceedings of the International Symposium on Tea Science, The Organizing Committee of ISTS, Shizuoka, Japan*, 381-384.

- Jang, E.-H., Park, Y.-C., Chung, W.-G., 2004. Effects of dietary supplements on the induction and inhibition of cytochrome P450s protein expression in rats. *Food and Chemical Toxicology*, 42:1749–1756.
- Jialal, I., Vega, G.L. & Grundy, S.M. 1990. Physiological levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis*, 82:185-91.
- Schannet, I.* & Somerset, S.M. 2006. Age-related variations in flavonoid intake and sources in the Australian population. *Public Health Nutrition*, 9(8):1045–1054.
- Joubert, E. & Ferreira, D. 1996. Antioxidants of rooibos tea – a possible explanation for its health promoting properties? *South African Journal of Food Science and Nutrition*, 8:79-83.
- Joubert, E., Gelderblom, W.C.A., Louw, A. & de Beer, D. 2008. South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Ethnopharmacology*, 119:376–412.
- Joubert, E. 1996. HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos (*Aspalathus linearis*) as affected by processing. *Food Chemistry*, 55:403-411.
- Joubert, E., Winterton, P., Britz, T.J. & Ferreira, D. 2004. Superoxide anion and alpha, alpha-diphenyl-beta-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts crude phenolic fractions, tannin and flavonoids. *Food Research International*, 37:133-138.
- Kaliora A.C, Dedoussis, G.V.Z. & Schmidt, H. 2006. Dietary antioxidants in preventing atherogenesis. *Atherosclerosis*, 187:1–17.
- Kaliora, A.C. & Dedoussis, G.V.Z. 2007. Natural antioxidant compounds in risk factors for CVD. *Pharmacological Research*, 56:99–109.
- Keli, S.O., Hertog, M.G., Feskens E.J.M. & Kromhout, D. 1996. Dietary flavonoids, antioxidant vitamins and incidence of stroke: the Zutphen study. *Archives of Internal Medicine*, 156:637-642.
- Keogh, J.B., McInerney, J. & Clifton, P.M. 2007. The effect of milk protein on the bioavailability of cocoa polyphenols. *Food Science*, 72 (3): S230-S233.
- Khan A. & Gilani, A.H. 2006. Selective bronchodilatory effect of Rooibos tea (*Aspalathus linearis*) and its flavonoid, chrysoeriol. *European Journal of Nutrition*, 45:463–469.
- Knekt, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliövaara, M., Reunanen, A.,

- Hakulinen, T. & Aromaa, A. 2002. Flavonoid intake and risk for chronic diseases. *American Journal of Clinical Nutrition*, 76:560-568.
- Knekt, P., Reunanen, A., Jarvinen, R., Seppanen, R., Heliövaara, M. & Aromaa, A. 1994. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. *American Journal of Epidemiology*, 139(12):1180-9.
- Komatsu, K., Kator, K., Mitsuda, Y., Mine, M. & Okumura Y. 1994. Inhibitory effects of Rooibos tea, *Aspalathus linearis*, on C3H10T1/2 cell transformation. *Cancer Letters*, 77:33-38.
- Koo, S.I. & Noh, S.K. 2007. Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. *Journal of Nutritional Biochemistry*, 18:179–183.
- Korchazhkina, O., Exley, C. & Spencer, A. 2003. Measurement by reversed-phase HPLC of malondialdehyde in normal human urine following derivatisation with 2, 4-ditrophenylhydrazine. *Chromatography B Analytical Technologies in the Biomedical and Life Sciences*, 794:353-362.
- Kris-Etherton, P. & Keen, C.L. 2002. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Current Opinion in Lipidology*, 13:41-9.
- Kroon, P.A., Clifford, M.N., Crozier, A., Day, A.J., Donovan, J.L., Manach C, Williamson G. 2004. How should we assess the effects of exposure to dietary polyphenols *in vitro*? *American Journal of Clinical Nutrition*, 80:15-21.
- Kucharská, J., Uličná, O., Gvozdjaková, A., Sumbalová, Z., Vancová, O., Bozek, P., Nakano, M. & µGreksák, M. 2004. Regeneration of coenzyme Q9 redox state and inhibition of oxidative stress by Rooibos tea (*Aspalathus linearis*) administration in carbon tetrachloride liver damage. *Physiological Research*, 53 (5):515-521.
- Kunishiro, K., Tai, A. & Yamamoto, I. 2001. Effects of rooibos tea extract on antigen – specific antibody production and cytokine generation *in vitro* and *in vivo*. *Bioscience Biotechnology and Biochemistry*, 65:2137-2145.
- Kushi, L.H, Folsom, A.R., Prineas, R.J., Mink, P.J., Wu, Y. & Bostick, R.M. 1996. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *New England Journal of Medicine*, 334 (18):1156-62.
- Kyle, J.M., Morrice, P.C., McNeill, G., Duthie, G.G. 2007. Effects of infusion time and addition of milk on content and absorption of polyphenols from black tea. *Journal of Agricultural and Food Chemistry*, 55:4889-4894.
- Lambert, J.D., Kwon, S.J., Hong, J. & Yang, C.S. 2007. Salivary hydrogen peroxide



---

produced by holding or chewing green tea in the oral cavity. *Free Radical Research*, 41:850-853.

Langley-Evans, S.C. 2000. Consumption of black tea elicits an increase in plasma antioxidant potential in humans. *International Journal of Food Sciences and Nutrition*, 51, 309-315.

Langseth, L. 1995. Oxidants, antioxidants and disease prevention. *ILSI-Europe concise monogram series*, 1-26.

Lee, B.M. & Park, K-K. 2003. Beneficial and adverse effects of chemopreventive agents. *Mutation Research*, 523:524, 265-278.

Leenen, R., Roodenburg, A.J., Tijburg, L.B.M. & Wiseman S.A. 2000. A single dose of tea with or without milk increases plasma antioxidant activity in humans. *European Journal of Clinical Nutrition*, 54: 87-92.

Losonczy, K.G., Harris, T.B. & Havlik, R. 1996. Vitamin E and vitamin C supplement use and risk of all cause and coronary heart disease mortality in older persons: the established populations for epidemiologic studies of the elderly. *American Journal of Clinical Nutrition*, 64(2):190-6.

Lotito, S.B. & Fraga, C.G. 2000. Catechins delay lipid oxidation and  $\alpha$ -tocopherol and  $\beta$ -carotene depletion following ascorbate depletion in human plasma, *Proceedings of the Society for Experimental Biology and Medicine*, 225:32-38.

Lotito, S.B. & Frei B. 2004. Relevance of apple polyphenols as antioxidants in human plasma: contrasting *in vitro* and *in vivo* effects. *Free Radical Biology and Medicine*, 36:201-211.

Lotito, S.B. & Frei, B. 2006. Consumption of flavonoid rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? *Free Radical Biology and Medicine*, 41:1727-1746.

MacLennan, W.J., Wilson, J., Rattenhuber, V., Dikland, W.J., Vanderdonckt, J. & Moriau M. 1994. Hydroxyethylrutosides in elderly patients with chronic venous insufficiency: its efficacy and tolerability. *Gerontology*, 40(1):45-52.

Madamanchi, N.R., Vendrov, A. & Runge, M.S. 2005. Oxidative stress and vascular disease. *Arteriosclerosis Thrombosis and Vascular Biology*, 25:29-38.

Marchioli, R. 1999. Antioxidant vitamins and prevention of cardiovascular disease: Laboratory, epidemiological and clinical trial data. *Pharmacological Research*, 40(3):227-238.

Marnewick, J.L., Batenburg, W., Swart, P., Joubert, E., Swanevelder, S. & Gelderblom, W.C.A. 2004. *Ex vivo* modulation of chemically induced mutagenesis by subcellular liver fractions of rats treated with rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) tea, as well as green and black (*Camellia sinensis*) teas. *Mutation Research*, 558:145-154.

Marnewick, J.L., Gelderblom, W.C. & Joubert, E. 2000. An investigation on the antimutagenic properties of South African herbal teas. *Mutation Research*, 471:157-166.

Marnewick, J.L., Joubert, E., Swart, P., Van der Westhuizen, F.H. & Gelderblom, W.C. 2003. Modulation of hepatic drug metabolizing enzymes and antioxidant status by rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), green and black (*Camellia sinensis*) teas in rats. *Journal of Agricultural and Food Chemistry*, 51:8113-8119.

Marnewick, J.L., Joubert, E., Joseph, S., Swanevelder, S., Swart, P. & Gelderblom, W.C. 2005. Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas. *Cancer Letters*, 224:193-202.

Marouani, N., Chahed, A., Hédhili, A. & Hamdaoui, M.H. 2007. Both aluminium and polyphenols in green tea decoction (*Camellia sinensis*) affect iron status and hematological parameters in rats. *European Journal of Nutrition*, 46(8):453-459.

Matsuda, K., Nishimura, Y., Kurata, N., Iwase, M., Yasuhara, H., 2007. Effects of continuous ingestion of herbal teas on intestinal CYP3A in the rat. *Journal of Pharmacological Science*, 103:214–221.

Maubach, J., Bracke, M.E., Heyerick, A., Depypere, H.T., Serreyn, R.F., Mareel, M.M. & De Keukeleire, D. 2003. Quantification of soy-derived phytoestrogens in human breast tissue and biological fluids by high performance liquid chromatography. *Chromatography B Analytical Technologies in the Biomedical and Life Sciences*, 784:137-144.

May, J.M., Qu Z.C. & Mendiratta, S. 1998. Protection and recycling of alpha tocopherol in human erythrocytes by intracellular ascorbic acid. *Archives of Biochemistry and Biophysics*, 349:281-9.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B & Ewert, B. 1999. Anthocyanins, phenolics, and color of cabernet franc, merlot, and pinot noir wines from British Columbia. *Journal of Agricultural and Food Chemistry*, 47:4009-4017.

McKay, D.L. & Blumberg J.B. 2007. A review of bioactivity of South African herbal teas: Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). *Phytotherapy Research*, 21:1-16.

Mennen, L., Hirvonen, T., Arnault, N., Bertrais, S., Galan, P. & Hercberg, S. 2007.

- Consumption of black, green and herbal tea and iron status in French adults. *European Journal of Clinical Nutrition*, 61 (10): 1174-1179.
- Mennen, L.I., Walker, R., Bennetau-Pelissero, C. & Scalbert, A. 2005. Risks and safety of polyphenol consumption. *American Journal of Clinical Nutrition*, 81:326S-9S.
- Miller, N.J., Rice-Evans, C.A., Davies, M.J., Gopinathan, V. & Miller, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring antioxidant status in premature neonates. *Clinical Science*, 84:407-412.
- Mori, H., Niwa, K., Zheng, Q., Yamada, Y., Sakata, K. & Yoshimi, N. 2001. Cell proliferation in cancer prevention: effects of preventive agents on estrogen-related endometrial carcinogenesis model and on an *in vitro* model in human colorectal cells. *Mutation Research*, 480-481:201-7.
- Morton, J. 1983. Rooibos tea, *Aspalathus linearis*, a caffeineless, low tannin beverage. *Economic Botany*, 37:164-173.
- Moskaug, J., Carlsen, H., Myhrstad, M.C. & Blomhoff, R. 2005. Polyphenols and glutathione synthesis regulation. *American Journal of Clinical Nutrition*, 81:268S-76S.
- MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high risk individuals: a randomized placebo-controlled trial. 2002. *Lancet*, 360:23-33.
- Muofhe, M.I. & Dakora, F.D. 1999. Nitrogen nutrition in undulated field plants of the shrub tea legume *Aspalathus linearis* assessed using <sup>15</sup>N natural abundance. *Plant and Soil*, 209(2):181-6.
- Nakano, M., Itoh, Y., Mizuno, T. & Nakashima, H. 1997. Polysaccharides from *Aspalathus linearis* with strong anti-HIV activity. *Bioscience Biotechnology and Biochemistry*, 61:267-271.
- Nijveldt, R.J., van Nood, E., van Hoorn, D.E., Boelens, P.G., Van Norren, K. & van Leeuwen, P.A. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *American Journal of Clinical Nutrition*, 74:418-25.
- Nikolova, V., Petrova, S., Petkova, V., Pavlova, S., Michailova, A. & Georgieva, T. 2007. Antioxidant effects of rooibos tea on workers occupationally exposed to lead. *Toxicology Letters*, 172S:S120-1.
- Oak, M-H, Bedoui, J. & Schini-Kerth, V.B. 2005. Antiangiogenic properties of natural polyphenols from red wine and green tea. *Nutritional Biochemistry*, 16:1-8.
- Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A.,

- Keogh, J.P. & Hammar, S. 1996. Effects of a combination of  $\beta$ -carotene and vitamin A on lung cancer and cardiovascular disease. *New England Journal of Medicine*, 334:1150-1155.
- Palace, V.P., Khaper, N., Qin, Q. & Singal, P.K. 1999. Antioxidant potential of vitamin A and carotenoids and their relevance to heart disease. *Free Radical Biology and Medicine*, 26(5-6):746-61.
- Parr, A.J. & Bolwell, G.P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. *Journal of the Science of Food and Agriculture*, 80:985-1012.
- Pazdzioch-Czochra, M. & Widenska A. 2002. Spectrofluorimetric determination of hydrogen scavenging activity. *Analytical Chimica Acta*, 452:177-84.
- Pearson, D.A., Holt, R.R., Rein, D., Paglieroni, T., Schmitz, H.H. & Keen, C.L. 2005. Flavanols and platelet reactivity. *Clinical & Developmental Immunology*, 12(1):1-9.
- Pedrielli, P. & Skibsted, L.H. 2002. Antioxidant synergy and regeneration effect of quercetin, (-)-epicatechin and (+)-catechin on alpha tocopherol in homogeneous solutions of peroxidating methyl linoleate. *Journal of Agricultural and Food Chemistry*, 50:7138-44.
- Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B. & Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (ORACFL) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, 51:3273-3279.
- Rabe, C., Steenkamp, J.A., Joubert, E., Burger, J.F.W. & Ferreira, D. 1994. Phenolic metabolites from rooibos tea (*Aspalathus linearis*). *Phytochemistry*, 35(6):1559-1565.
- Ratnam, D.V., Ankola, D.D., Bhardwaj, V., Sahana, D.K., Kumar, M.N.V.R. 2006. Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. *Journal of Controlled Release*, 113:189-207.
- Rautenbach, F. & Venter I. 2008. Comparison of the total antioxidant capacity of commonly consumed South African fruits and vegetables with international data. Presented at the *Indigenous Plant Use Forum*, Graaff-Reinet, South Africa, July 2008.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26:1231-7.
- Rebrin, I., Zicker, S., Wedekind, K.J., Paetau-Robinson, I., Packer, L. & Sohal, R.S. 2005. Effect of antioxidant-enriched diets on glutathione redox status in tissue homogenates and mitochondria of the senescence-accelerated mouse. *Free Radical Biology and Medicine*,

39:549-557.

Recknagel, R.O. & Glende Jr, E.A. 1984. Spectrophotometric detection of lipid conjugated dienes. *Methods in Enzymology*, 105:331-337.

Reddy, V.C., Vidya, Sagar, G.V., Sreeramulu, D., Venu, L. & Raghunath, M. 2005. Addition of milk does not alter the antioxidant activity of black tea. *Annals of Nutrition and Metabolism*, 49:189-195.

Renaud, S. & De Lorgeril, M. 1992. Wine, alcohol, platelets and the French Paradox for coronary heart disease. *Lancet*, 339:1523-6.

Riemersma, R.A., Rice-Evans, C.A., Tyrrell, R.M., Clifford, M.N. & Lean, M.E.J. 2001. Tea flavonoids and cardiovascular health. *Quarterly Journal of Medicine*, 94:277-282.

Riemersma, R.A., Wood, D.A., Macintyre, C.C.A., Elton, R.A., Gey, K.F. & Oliver, M. 1991. Risk of angina pectoris and plasma concentrations of vitamins A, C and E and carotene. *Lancet*, 337:1-5.

Rimm, E.B. 2002. Fruit and vegetables: building a solid foundation. *American Journal of Clinical Nutrition*, 76:1-2.

Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovannucci, E., Colditz, G.A. & Willett, W.C. 1993. Vitamin E consumption and the risk of coronary heart disease in men. *New England Journal of Medicine*, 328(20):1450-6.

Roura, E., Andrés-Lacueva, C., Estruch, R., Mata-Bilbao, M.L., Izquierdo-Pulido, M., Waterhouse, A.L. & Lamuela-Raventós, R.M. 2007. Milk does not affect the bioavailability of cocoa powder flavonoid in healthy human. *Annals of Nutrition and Metabolism*, 51(6): 493-498.

Sasaki, Y.F., Yamada, H., Shimoi, K., Kator, K. & Kinae, N. 1993. The clastogen-suppressing effects of green tea, Po-lei tea and Rooibos tea in CHO cells and mice. *Mutation Research*, 286:221-232.

Saura-Calixto, F., Serrano, J. & Goni, I. 2007. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chemistry*, 101:492-501.

Scalbert, A., Johnson, I.T, Saltmarsh, M. 2005. Polyphenols: antioxidants and beyond. *American Journal of Clinical Nutrition*, 81(S):215-7.

Scalbert, A., Morand, C, Manach, C. & Rémésy C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomedicine and Pharmacotherapy*, 56:276-

282.

Schulz, H., Joubert, E. & Schutze, W. 2003. Quantification of quality parameters for reliable evaluation of green rooibos (*Aspalathus linearis*). *European Food Research Technology*, 216:539-543.

Serafini, M., Maiani, G. & Ferro-Luzzi, A. 1998. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *Nutrition*, 128:1003-1007.

Setchell, K.D.R, Gosselin, S.J. & Welsh, M.B. 1987. Dietary estrogens: a probable cause of infertility and liver disease in captive cheetahs. *General and Comparative Endocrinology*, 93:225-33.

Setchell, K.D.R., Brown, N.M. & Lydeking-Olsen, E. 2002. The Clinical Importance of the Metabolite Equol—A Clue to the Effectiveness of Soy and Its Isoflavones. *Nutrition*, 132: 3577–3584.

Sevanian, A., Davies, K.J. & Hochstein, P. 1991. Serum urate as an antioxidant for ascorbic acid. *American Journal of Clinical Nutrition*, 54:1129S-1134S.

Sharma, V., Kumar, H.V. & Rao, J.M. 2008. Influence of milk and sugar on antioxidant potential of black tea. *Food Research International*, 41:124-129.

Siebert, K.J., Troukhanova, N.V. & Lynn, P.Y. 1996. Nature of Polyphenol-Protein Interactions. *Journal of Agricultural and Food Chemistry*, 44:80–85.

Siess, M.-H., Le Bon, A.-M., Canivenc-Lavier, M.-C. & Suschetet, M. 2000. Mechanisms involved in the chemoprevention of flavonoids. *BioFactors*, 12: 193-199.

Simon, M., Horovska, L., Greksák, M., Dusinsky, R. & Nakano, M. 2000. Antihemolytic effects of rooibos tea (*Aspalathus linearis*) on red blood cells of Japanese quails. *General Physiology and Biophysics*, 19:365-371.

Singh, U. & Jialal, I. 2006. Oxidative stress and atherosclerosis. *Pathophysiology*, 13:129–142.

Snijman, P.W., Swanevelder, S., Joubert, E., Green, I.R. & Gelderblom, W.C.A. 2007. The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*): Some dose-response effects on mutagen activation-flavonoid interactions. *Mutation Research*, 631:111–123.

Snodderly, D.M. 1995. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *American Journal of Clinical Nutrition*, 62:1448S

-1461S.

Stampfer, M.J., Hennekens, C.H., Manson, J.E., Colditz, G.A., Rosner, B. & Willett, W.C. 1993. Vitamin E consumption and the risk of coronary heart disease in women. *New England Journal of Medicine*, 328(20):1444-9.

Standley, L., Marnewick, J.L., Gelderblom, W.C.A, Joubert, E. & Britz, T.J. 2001. Influence of processing stages on antimutagenic and antioxidant potentials of rooibos tea. *Agricultural and Food Chemistry*, 49:114-117.

Stein, J.H., Keevil, J.G., Wiebe, D.A., Aeschlimann, S., Folts J.D. 1999. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation*, 100(10):1050-55.

Stephens, N.G., Parsons, A., Schofield, P.M., Kelly, F., Cheeseman, K., Mitchinson, M.J & Brown, M.J. 1996. Randomised controlled trial of vitamin E in patients with coronary heart disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet*, 347:781-786.

Stewart, A., Bozonnet, S., Mullen, W., Jenkins, G.I., Lean, M.E.J. & Crozier, A. 2000. Occurrence of flavonols in tomato and tomato-based products. *Journal of Agricultural and Food Chemistry*, 48:2663-9.

Strube, M., Haenen, G.R., Van Den Berg, H., Bast, A. 1997. Pitfalls in a method for assessment of total antioxidant capacity. *Free Radical Research*, 26(6):515 – 21.

Tamariz, L.J., Young, J.H., Pankow, J.S., Yeh, H.C., Schmidt, M.I., Astor, B. & Brancati, F.L. 2008. Blood viscosity and hematocrit as risk factors for type 2 diabetes mellitus: The Atherosclerosis Risk in Communities (ARIC) Study. *American Journal of Epidemiology*, 168(10):1153-60.

Tavani, A. & La Vecchia, C. 1999. Beta-carotene and risk for coronary heart disease *Biomedicine and Pharmacotherapy*, 53(9):409-16.

Temme, E.H.M & Van Hoydonck, P.G. 2002. Tea consumption and iron status. *European Journal of Clinical Nutrition*, 56:379-86.

Thankachan, P., Walczyk, T., Muthayya, S., Kurpad, A.V. & Hurrell, R.F. 2008. Iron absorption in young Indian women: The interaction of iron status with the influence of tea and ascorbic acid. *American Journal of Clinical Nutrition*, 87(4):881-886.

Treutter, D. 1989. Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *Journal of Chromatography*, 467:185-193.

Ulicna, O., Vancova, O., Bozek, P., Carsky, J., Sebekova, K., Boor, P., Nakano, M. &

- Greksak, M. 2006. Rooibos tea (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. *Physiological Research*, 55:157-164.
- Uma Devi P., Ganasoundari, A., Vrinda, B., Srinivasan, K.K., Unnikrishnan, M.K. 2000. Radiation protection by ocimum flavonoids orientin and vicenin: mechanisms of action. *Radiation Research*, 154(4):455-60.
- Van Acker, F., Schouten, O., Haenen, G. R. M. Van der Vijg, W. J. F., Bast, A. 2000. Flavonoids can replace R-tocopherol as antioxidant. *Federation of European Biochemical Societies Letters*, 473:145-148.
- Van het Hof, K.H., Wiseman, S.A., Yang, C.S. & Tijburg, L.B.M. 1999. Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proceedings of the Society of Experimental Biology and Medicine*, 220(4):203-9.
- Van het Hof, K.H., Kivits, G.A.A., Weststrate, J.A. & Tijburg, L.B.M. 1998. Bioavailability of catechins from tea: the effect of milk. *European Journal of Clinical Nutrition*, 52:356-9.
- Vita, J.A. & Keane Jr, J.F. 2002. Endothelial function: a barometer for cardiovascular risk? *Circulation*, 106:640-2.
- Vita, J.A., 2005. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. *American Journal of Clinical Nutrition*, 81(S):292-7.
- Von Gadow, A., Joubert, E. & Hansmann, C.F. 1997a. Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*), with green, oolong and black tea. *Food Chemistry*, 60: 73-77.
- Von Gadow, A., Joubert, E. & Hansmann, C.F. 1997b. Effect of extraction time and additional heating on the antioxidant activity of rooibos tea (*Aspalathus linearis*) extracts. *Journal of Agricultural and Food Chemistry*, 45:1370-74.
- Von Gadow, A., Joubert, E. & Hansmann, C.F. 1997c. Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*), alpha tocopherol, BHT, and BHA. *Agricultural and Food Chemistry*, 45:632-638.
- Walle, T. 2004. Absorption and metabolism of flavonoids. *Free Radical Biology and Medicine*, 36:829-837.
- Wang, J.F., Schramm, D.D., Holt, R.R., Ensunsa, J.L., Fraga, C.G., Schmitz, H.H. & Keen, C.L. 2000. A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. *Nutrition*, 276:945-51.
- Wang, S.Y. & Jiao, H. 2000. Scavenging capacity of berry crops on superoxide radicals,



- hydrogen peroxide, hydroxyl radicals and singlet oxygen. *Journal of Agricultural and Food Chemistry*, 48:677-684.
- Ward, M., McNulty, H., Mcpartlin, J., Strain, J.J., Weir D.G., Scott, J.M. 1997. Plasma homocysteine a risk factor for cardiovascular disease is lowered by physiological doses of folic acid. *Quarterly Journal of Medicine*, 90(8):519-524.
- Weisburger, J. 2003. Prevention of coronary heart disease and cancer by tea: A review. *Environmental Health and Preventive Medicine*, 7:283-88.
- Westhuyzen, J. 1997. The oxidation hypothesis of atherosclerosis: An update. *Annals of Clinical Laboratory Science*, 27:1-10.
- Whitehead, T.P., Robinson, D., Allaway, S., Syms, J. & Hale, A. 1995. Effect of red wine ingestion on the antioxidant capacity of serum. *Clinical Chemistry*, 41(1):32-35.
- Widlansky, M.E., Gokce, Keaney Jr, J.F. & Vita, J.A. 2003. The clinical implications of endothelial dysfunction. *American College of Cardiology*, 42:1149-60.
- Wilcox, J.K., Ash, S.L. & Catignani, G.L. 2004. Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition*, 44:275-295.
- WHO. The global burden of disease 2004 update. 2008. World Health Organisation, Geneva, Switzerland.
- Wolmarans, P. Instructions for keeping a food intake record. Medical Research Council, Parow Valley, Cape Town. (Copyright NIRU 2003).
- Wu, X., Gu, L., Holden, J., Haytowitz, D.B., Gebhardt, S.E., Beecher, G. & Prior, R.L. 2004. Development of a database for total antioxidant capacity in foods: a preliminary study. *Journal of Food Composition and Analysis*, 17:407-422.
- Yagi, K. 1984. Assay for plasma lipid peroxides. *Methods in Enzymology*, 109:328-31.
- Yao, L.H., Jiang, Y.M., Shi, J., Tomás-Barberán, F.A., Datta, N., Singanusong, R. & Chen, S.S. 2004. Flavonoids in food and their health benefits. *Plant Foods for Human Nutrition*, 59:113-122.
- Yin, F., Giuliano, A.E & Van Herle, A.J. 1999. Growth inhibitory effects of flavonoids in human thyroid cancer cell lines. *Thyroid*, 9(4):369-76.
- Yoshida, Y. & Niki, E. 2004. Detection of lipid peroxidation in vivo: total hydroxyoctadecadienoic acid and 7- hydroxycholesterol as oxidative stress marker. *Free*

*Radical Research*, 38:787-794.

Yoshikawa, T., Naito, Y., Oyamada, H., Ueda, S., Tanigawa, T., Takemura, T., Sugino, S. & Kondo, M. 1990. Scavenging effects of *Aspalathus linearis* (rooibos tea) on active oxygen species. *Advances in Experimental Medicine and Biology*, 264:171-174.

Young, I.S. & Woodside, J.V. 2001. Antioxidants in health and disease. *Clinical Pathology*, 54:176-186.

Yung, L.M., Leung, F.P., Wong, W.T., Tian, X.Y., Yung, L.H., Chen, Z.Y., Yao, X.Q. & Huang, Y. 2008. Tea polyphenols benefit vascular function. *Inflammopharmacology* (in press).

Yusuf, C., Dagenais, G., Pogue, J., Bosch, J. & Sleight P. 2000. Vitamin E supplementation in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *New England Journal of Medicine*, 342:154-60.

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## APPENDICES

### Appendix A: Informed consent form



#### INFORMED CONSENT FORM

I, \_\_\_\_\_, agree to participate in a research study entitled "MODULATION OF BLOOD OXIDATIVE STRESS MARKERS AND DNA DAMAGE BY ROOIBOS TEA IN VOLUNTEERS AT RISK FOR CORONARY HEART DISEASE" conducted by the Nutritional Antioxidant Research Group from the Cape Peninsula University of Technology (CPUT) under the direction of Dr. J. L. Marnewick (Room 3.73, Antioxidant Research Group, Sciences Building, Faculty of Health and Wellness Sciences, CPUT, tel 021-4608314, email: marnewickj@cput.ac.za). I understand that my participation is voluntary. I can refuse to participate or stop taking part without giving any reason, and without penalty. I can ask to have all information obtained about me in the study to be returned to me, removed from the research records, or destroyed.

This project forms part of a clinical human intervention study to determine the effect of rooibos tea consumption on antioxidant/oxidative stress measures in the blood of volunteers at risk for heart disease. There are no known risks to participate in this study. If I volunteer to take part in this study, I will be asked to do the following:

- 1) Answer questions about my health, food, nutrition, and physical activity.
- 2) Take part in a study over a 14 (fourteen) week period which includes the completion of a self-administered dietary record, based on habitual dietary intake, for the first 2 (two) weeks. The next 2 weeks is the period prior to the intervention study (known as the wash-out period) and I will be requested to follow a flavonoid-restricted, omit flavonoid-rich beverages from my diet and continue to complete the dietary records. The following 4 (four) weeks is the intervention period in which I will consume either 6 cups of rooibos tea or water daily, follow the same flavonoid-restricted diet and once again complete the dietary food records. I will receive extensive training at the beginning of the study on how to complete the dietary record and food frequency questionnaire.
- 3) If I am willing, a qualified phlebotomist or nursing sister will take samples of my blood on 6 (six) occasions. The first blood samples to be drawn will be for screening

purposes (11-14 June 2007), while the remaining 5 (five) multiple samples will be drawn 2 weeks after the beginning (30 July 2007) of the study, during and at the end of the study (6-7 November 2007). Blood samples will be analysed to determine levels of various enzymes, blood lipids as well as antioxidant/oxidative stress status.

- 4) To supply urine samples on the same time points as the blood samples for antioxidant/oxidative stress analysis.
- 5) Someone from the study may call me to clarify my information.

I understand that these questions and blood tests are not for diagnostic purposes. My blood will not be tested for HIV-AIDS. If I have questions about my test results I should see a physician. No risk is expected but I may experience some discomfort when my blood is drawn or when the researchers ask me questions about health, nutrition, physical activity and smoking habits. The risks of drawing blood from my arm include the unlikely possibilities of a small bruise or localized infection and bleeding. These risks will be reduced by using a qualified phlebotomist or nursing sister to draw the blood.

No individually-identifiable information about me, or provided by me during the research, will be shared with others without my written permission, except if it is necessary to protect my welfare (for example, if I were injured and need physician care) or if required by law. I will be assigned an identifying number and this number will be used on all of the questionnaires I fill out. The investigator will answer any further questions about the research, now or during the course of the project.

**I understand that there will be no laboratory costs to me for participation in this study and that I will not be compensated to participate in the study.**

**I declare that I have read the details of the project or have listened to the oral explanation thereof, and declare that I understand it. I have had the opportunity to discuss relevant aspects with the researcher and declare that I voluntarily participate in the project. I hereby give consent to participate in the project.**

I declare that I take part in this study on my own risk and that the Cape Peninsula University of Technology (CPUT), any of its workers or students are not responsible if anything should happen to me during the course of the experiment.

I also declare not to sue the CPUT for any personal losses or damage that might occur during the project due to negligence from the CPUT, its workers, students or other subjects that take part in this study.

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**Name of Participant**

**Signature**

**Date**

**Telephone:** \_\_\_\_\_

**Email:** \_\_\_\_\_

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**Name of Researcher**

**Signature**

**Date**

Please sign both copies, keep one and return one to the researcher.

## Appendix B: Advertisement call for study participants

Antioxidant Research Laboratory Cape Town Campus, Cape Peninsula University of Technology

### ANTIOXIDANT INTERVENTION STUDY SEEKS VOLUNTEERS

Male and female volunteers are needed to participate in a “first of its kind” antioxidant research study investigating the health benefits of the popular South African herbal tea, ROOIBOS. Tea contains components referred to as polyphenols which are strong antioxidants and have been suggested to lower the risk for certain chronic diseases such as heart disease and certain cancers which are all associated with oxidative stress.

#### Participation requirements (only two of the risk factors below are required)

- Age between 30 and 60
- Elevated cholesterol levels (>5 to 7.5 mmol/L)
- Increased body mass index (>22 to 35 kg/m<sup>2</sup>: Calculation: weight ÷ (height x height)
- Family history of heart disease

In addition to the risk factors all potential volunteers should:

- Be medically stable with no clinical significant abnormalities of the liver, kidneys or blood
- Female participants should not be pregnant (actual or intended)
- Not take any chronic oral medication

**All volunteers interested in this study will be screened beforehand** at no cost to determine if they meet the requirements to partake in the study. Please feel free to contact us even if you do not know or are uncertain about your health status requirements for this study.

#### Benefits

- Participants will learn more about their own health and antioxidant status as results of all tests conducted during this study will be given to them without any costs involved
- This research is conducted at no cost to participants
- Rooibos tea, dietary records, tea cup and measuring utensils will be provided to all participants

In this 14 week study, participants will be required to complete food records, follow their normal diet with some beverage intake restriction and drink 6 cups of rooibos tea per day for 4 weeks thereof. Blood samples will be drawn and urine samples will be collected from participants during the study to monitor important antioxidant biomarkers. Participants will receive no monetary compensation, but will benefit by receiving their blood test results at the end of the study.

#### Study investigator

Dr Jeanine Marnewick (PhD, Biochemistry), Antioxidant Research Group, Faculty of Health and Wellness Sciences, Sciences Building Rm 3.73, Cape Town Campus, CPUT

#### Study dates and contact information:

All interested participants please submit your contact details telephonically or via email to us before 21 June 2007 to set up an appointment

Telephone: 021-4608314 or 4603775

Email: [marnewickj@cput.ac.za](mailto:marnewickj@cput.ac.za) or [rautenbachf@cput.ac.za](mailto:rautenbachf@cput.ac.za)

This study has been approved by the Health and Applied Sciences Research Ethics Committee of CPUT: 11 May 2007

**Appendix C: Anthropometry and blood pressure form**



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<b>Subject number:</b>	<b>Date:</b>
<b>Date of birth:</b>	<b>Age:</b>

	<u>Date:</u>				<u>Date:</u>			
	<u>Baseline:</u>				<u>Intervention completion:</u>			
<b>Measurement:</b>	1	2	3		1	2	3	
<b>Blood pressure (mm Hg)</b>								
<b>Weight (kg)</b>								
<b>Height (cm)</b>								
<b>Body Mass Index (kg/m<sup>2</sup>)</b>								
<b>Waist circumference (cm)</b>								

**Appendix D: Participants calculated risk for developing heart disease in next 10 years  
Using Framingham criteria**

Code	Sex	Age (years)	Tot cholesterol (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	Blood pressure (systolic)	Glucose (mmol/L)	Smoke	Total points	Percentag e risk
2	F	53	6.9	2	0.54	132	5	former	4	<b>4</b>
3	F	58	5	1.4	0.64	128	5.1	no	7	<b>6</b>
4	M	38	7	1.9	0.54	162	5.6	yes	5	<b>8</b>
5	M	60	7.2	2	0.7	128	5.3	former	5	<b>8</b>
6	F	42	4.3	1.2	0.52	104	5.1	smoker	3	<b>3</b>
7	F	50	4.7	1.3	0.48	145	4.6	No	8	<b>7</b>
8	F	39	4.9	1.3	0.48	133	4.9	No	-4	<b>&lt;1</b>
10	F	59	3.4	1	2.32	130	3.4	no	9	<b>8</b>
11	M	66	5.6	1.6	3.84	156	4.8	no	7	<b>13</b>
12	M	29	4.8	1.3	3.02	144	4.8	yes	3	<b>5</b>
14	F	50	5.4	1.5	3.7	122	4.8	no	7	<b>6</b>
15	F	43	6	1.6	4.26	131	5.4	no	-2	<b>1</b>
16	F	51	4.5	1.3	2.96	138	4.8	former	8	<b>7</b>
17	F	48	5.9	1.6	4.06	144	5.2	yes	5	<b>4</b>
19	F	49	5.2	1.5	3.54	132	4.7	yes	5	<b>4</b>
20	M	65	5.5	1.5	3.76	178	6.4	former	9	<b>20</b>
21	F	41	5.3	1.5	3.54	128	4.1	no	1	<b>2</b>
23	F	39	4.2	1.1	2.94	136	5.6	former	-2	<b>1</b>
26	F	45	5.4	1.4	3.88	126	5.6	No	4	<b>4</b>
27	F	54	4.8	1.3	3.04	143	6.6	No	8	<b>7</b>
29	M	46	9	2.2	3.16	105	7.6	No	5	<b>8</b>
30	F	45	5.2	1.4	3.68	110	5.1	No	3	<b>3</b>
31	M	35	4.7	1.2	3.08	134	5.4	No	1	<b>3</b>
32	M	32	3.8	1	2.62	128	5.3	No	-1	<b>2</b>
34	F	60	10	2.8	6.72	125	5.6	No	8	<b>7</b>
35	F	42	4.9	1.4	3.4	113	4.7	No	-3	<b>&lt;1</b>



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36	M	41	5.4	1.5	3.5	148	5.7	No	3	<b>5</b>
37	F	40	6.1	1.8	3.62	110	4.9	No	-5	<b>&lt;1</b>
40	F	48	3.9	1.2	2.54	131	5.2	No	4	<b>4</b>
42	M	59	5	1.3	3.4	136	6.5	No	6	<b>10</b>
44	M	58	4.4	1.2	2.88	161	6	No	7	<b>13</b>
50	M	35	5.2	1.4	3.58	135	5.1	No	1	<b>3</b>
51	M	27	5.2	1.4	3.66	130	5.3	No	0	<b>3</b>
53	F	41	5.4	1.4	3.82	131	5	yes	3	<b>3</b>
56	F	55	5.5	1.5	3.72	169	6.2	yes	13	<b>15</b>
62	M	37	6.6	1.7	4.02	120	5.8	yes	2	<b>4</b>
63	F	43	4.7	1.3	3.12	116	5.2	yes	-1	<b>2</b>
64	F	57	4.7	1.3	3.16	155	4.9	No	9	<b>8</b>
66	F	46	6.1	1.6	4.14	111	16.8	yes	7	<b>6</b>
67	F	45	6.9	1.9	4.72	128	5.7	No	1	<b>2</b>

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## Appendix E: Dietary restrictions for the rooibos intervention study



### Dietary restrictions for the Rooibos Intervention study

July 2007

Study participants are kindly asked to strictly adhere to the following beverage and food intake restrictions during the study, starting after the 2-week baseline period (in other words from 13 August to 6 November), with regards to:

#### 1. Beverages

**Avoid** the intake of the following flavonoid-containing beverages while participating in this study, as it may influence the outcome of the study.

1. Coffee (all brands: Pure and instant; Caffeinated and decaffeinated; Filter, percolated or plunger)
2. Tea (all brands: Black, green, herbal, ice, flavored and unflavored)
3. Cocoa drinks (all brands e.g. Hot chocolate, Milo, Ovaltine)
4. Red wine; Rosé (all labels)
5. Fruit juices – 100% pure juices and blends (all brands of red grape, orange, apple and berry juices along with red Grapetizer and Appletizer)

The following beverages may be taken in **restricted quantities per day**:

1. Soda drinks: Coca cola, Coke Light, Tab, Fanta, Cream Soda, Sprite, Lemon Twist (one 340 mL can per day)
2. Two glasses of white wine (120 mL per glass) or one beer (340 mL) or one spirit drink (1 tot / 25 mL) per day

The following beverages may be taken as **usual/freely per day**:

1. Fruit juices (specific flavors: Litchi, White grape, Hanepoot), fruit nectars ( $\leq$  50% fruit juice), dairy fruit blends and fruit juice and yoghurt blends
2. Diluted base drinks (specific brands: OROS, Jive, Tang, Drink-O-Pop, Sweet-O)
3. Water / Bottled / Mineral (all brands: Still and sparkling; Flavored and unflavored)

#### 2. Fruits

**Restrict** the number of portions consumed **per day** of the following fruits:

1. Apples (one apple per day)
2. Oranges or naartjies (one orange or naartjie per day)
3. Black/Red grapes or berries (one cup per week)

All other fruits (banana, pears, etc.) may be consumed **as usual/freely**.

#### 3. Dark Chocolate

**Restrict** the intake of dark chocolate to a maximum of three 40g (about 6 blocks) portions **per week**.

All other chocolate (milk) may be consumed **as usual/freely**.

#### 4. Dietary supplements

**No** vitamin C **supplementation** or any other antioxidant supplement (capsules or powder) should be taken during the study period as it may influence the outcome of the study.

***NOTE: Study participants are also kindly asked not to change any other aspect regarding their food and beverage intakes during the study period compared to before the study.***