MODULATION OF OXIDATIVE STRESS BY ROOIBOS (ASPALATHUS LINEARIS) HERBAL TEA, CHINESE GREEN (CAMELLIA SINENSIS) TEA AND COMMERCIAL TEA SUPPLEMENTS USING A RODENT MODEL

by

BARTOLOMEU DAVID CANDA

Thesis submitted in fulfillment 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: Prof Jeanine L. Marnewick
 Co-supervisor: Prof Oluwafemi O. Oguntibeju

Bellville Campus
November 2012

CPUT copyright information
The thesis may not be published either in part (in scholarly, scientific or technical journals), or as a whole (as a monograph), unless permission has been obtained from the University
DECLARATION

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

___________________________                                   ______________________
Signed                          Date
ABSTRACT

Human and experimental animal studies have shown that biomarkers of oxidative damage are elevated in subjects with certain diseases or risk factors. Consequently, it is hypothesized that oxidative stress plays an important role in the pathogenesis of these diseases and that dietary intake of, or supplementation with antioxidants may be protective or be useful therapeutic targets. This study was designed to investigate the modulatory effect of *Camellia sinensis* (Chinese green tea), *Aspalathus linearis* (rooibos herbal tea) and the two commercial supplements on the antioxidant status of the liver and kidney of tert-butyl hydroperoxide (t-BHP)-induced oxidative stress male Wistar rats. Rooibos and green tea are beverages well-known for their antioxidant content.

Based on the specific beverage consumed, sixty male Wistar rats were randomly assigned into six groups, i.e. fermented rooibos (FRT), unfermented rooibos (URT), Chinese green tea (CGT), rooibos supplement (RTS), Chinese green tea supplement (GTS) and control (CTL). The animals had free access to the respective beverages and standard diet for 10 weeks, while oxidative stress was induced during the last 2 weeks via intraperitoneal injection of 30 µM of t-BHP per 100 g body weight.

Among all the beverage and/or supplement preparations, the commercial rooibos supplement had the highest total polyphenol content and antioxidant activity while fermented rooibos, as previously shown, had a lower antioxidant content and potency when compared to its unfermented counterpart. The ability of these beverages and/or supplements to modulate the antioxidant status in tissues was organ specific and varied according to the assessment method. When considering the liver, the intake of unfermented rooibos, Chinese green tea and the commercial rooibos supplement significantly (P<0.05) restored the t-BHP-induced reduction and increased the antioxidant status with regards to oxygen radical absorbance capacity and trolox equivalent antioxidant capacity (TEAC) levels. All the beverages and/or supplements also significantly (P<0.05) enhanced the renal antioxidant capacity as assessed by the TEAC assay. In what may be an indication of decreased oxidative stress, all the beverages were associated with a general decline in activities of the antioxidant enzymes which reached significant levels in renal superoxidase dismutase activity. Generally, the beverages did not impact significantly on lipid peroxidation (LPO) although there were differing trends in the two LPO markers assessed. While thiobarbituric
acid reactive substances levels showed a declining trend in both tissues, the conjugated dienes were generally elevated.

In conclusion, this study confirms *Camellia sinensis* and *Aspalathus linearis* as well as their two supplements as good sources of dietary antioxidants and results demonstrated that rooibos and green tea improved the liver and kidney antioxidant capacity of oxidative stress-induced rats. Their impact on antioxidant status in rats was shown to vary between organs and according to the method of assessment. Hence multi-method, multi-organ assessment may be a more informative approach in *in vivo* antioxidant studies.
ACKNOWLEDGEMENTS

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

Cape Peninsula University of Technology for the bursary and opportunity to be part of this institution.

My supervisor, Prof, J. L. Marnewick for her support, encouragement and guidance; always combining “smile and strictness”, you have provided the tools to achieve this goal. For this I will be forever grateful.

My co-supervisor, Prof, O. O. Oguntibeju for his inputs, motivation and belief that it is possible if you want to. Your words “that research is a mix of happiness and disappointments that one should learn from”. For this will be forever grateful.

Mr F. Rautenbach: Oxidative Stress Research Centre Laboratory Manager for his expert training and support on all the relevant laboratory assays and selfless sharing of his knowledge.

Mr H. Neethling, Miss B. Alinde and Mr I. Francisco for their great assistance in the course of my training.

To Mr D. Awoniyi, for sharing his knowledge on the topic with me.

My family for their belief, psychological support and uplifting encouragement when the future was grey. Mrs Sancha Canda without you I would not be writing this thesis.

Mr M. Macharia, the angel that God brought along the way, I will be forever grateful.

The completion of this thesis was indirectly supported by many people who could not be mentioned here. You boosted my belief that “it is possible” and helped make this a reality. To all of you I say thank you and may God bless you.
DEDICATION

To Sancha, Hannelor, Yussara, Roney, Claver, Natercia and my mother for their sacrifice, patience and endless love when I was physically and psychologically away.

To God for His abundant love and blessings

“Whatever you do, work at it with all your heart”

Colossians, 3: 23
# TABLE OF CONTENTS

Declaration ii  
Abstract iii  
Acknowledgement v  
Dedication vi  
Glossary of abbreviations xii

## CHAPTER ONE: INTRODUCTION 1

## CHAPTER TWO: LITERATURE REVIEW 4

2.1 Oxidants and oxidative stress 4  
2.2 Pathological conditions associated with oxidative stress 6  
2.2.1 Cancer and oxidative stress 7  
2.2.2 Chronic kidney disease and oxidative stress 9  
2.2.3 Coronary heart disease and oxidative stress 11  
2.2.4 Liver disorders and oxidative stress 12  
2.2.4.1 Non alcoholic fatty liver disease 13  
2.2.4.2 Hepatitis C viral infection (HCVI) 13  
2.3 The use of biomarkers and antioxidant levels to estimate oxidative stress 15  
2.3.1 Lipid biomarkers 16  
2.3.2 DNA damage 18  
2.3.3 Protein biomarkers 20  
2.4 Antioxidant defence system 22  
2.4.1 Endogenous antioxidant defence system and oxidative stress 22  
2.4.2 Exogenous antioxidant defence system and oxidative stress 23
2.4.2.1 Carotenoids, vitamins and selenium
2.4.2.2 Polyphenolic compounds
2.4.2.3 Polyphenol in the diet
2.4.2.4 Absorption and metabolism of polyphenol
2.4.2.5 Mechanism of action of polyphenol
2.4.3 Camellia sinensis (Chinese green tea)
  2.4.3.1 Biochemical composition of Camellia sinensis
  2.4.3.2 Bioavailability and bioactivity of Camellia sinensis
  2.4.3.3 Toxicity
2.4.4 Aspalathus linearis (Rooibos herbal tea)
  2.4.4.1 Biochemical composition of Aspalathus linearis
  2.4.4.2 Bioavailability and mechanism of action of Aspalathus linearis
  2.4.4.3 Toxicity
2.4.5 Combination of antioxidant supplements

CHAPTER THREE: MATERIAL AND METHODS

3.1 Study design and ethical approval
3.2 Preparation of beverages and supplements
3.3 Experimental animals and diet
3.4 Analytical methods
  3.4.1 Quantification of total polyphenol and flavanoid content of the aqueous herbal tea and tea supplements
  3.4.1.1 Total polyphenol content
  3.4.1.2 Flavanol content
  3.4.1.3 Flavonol content
  3.4.2 Determination of the antioxidant capacity of the various tea and supplements samples
  3.4.2.1 Ferric reducing antioxidant power (FRAP) determination
  3.4.2.2 Oxygen radical absorbance capacity (ORAC) determination
  3.4.2.3 Trolox equivalent antioxidant capacity (TEAC) determination
3.4.3 Antioxidant enzymes determination 47
3.4.3.1 Catalase (CAT) 47
3.4.3.2 Glutathione peroxidase (GPx) 48
3.4.3.3 Superoxide dismutase (SOD) 49
3.4.4 Determination of non-enzymatic antioxidants 49
3.4.4.1 Glutathione (GSH) 49
3.4.5 Determination of lipid peroxidation (LPO) 50
3.4.5.1 Conjugate dienes (CDs) determination 51
3.4.5.2 Thiobarbituric acid reactive substances (TBARS) determination 51
3.4.6 Protein determination 52
3.5 Statistical analysis 53

CHAPTER FOUR: RESULTS 54

4.1 Antioxidant profile of the beverages 54
4.2 Daily beverage intakes and body weight gains 54
4.3 Effects of beverage intervention on the oxidative stress status of the liver and kidney in the experimental animals 55
4.3.1 Antioxidant capacity 55
4.3.2 Antioxidant enzymes 57
4.3.3 Protein content and glutathione levels 58
4.3.4 Lipid peroxidation 59

CHAPTER FIVE: DISCUSSION AND CONCLUSIONS 61

CHAPTER SIX: REFERENCES 67
LIST OF FIGURES

Figure 2.1: Mechanism of oxidative damage 15
Figure 2.2: The flavan structure upon which flavonoid are based 25
Figure 2.3: Classification of polyphenols 26
Figure 4.1: Oxygen radical absorbance capacity activities in liver and kidney of rats consuming rooibos, green tea and commercial tea supplements after $t$-BHP-induced oxidative stress 57

LIST OF TABLES

Table 2.1: Free radicals, non radical oxidants and non radical thiol-reactive species 5
Table 2.2: Sources of oxidants 5
Table 2.3: Components of the antioxidants defence system 6
Table 2.4: Diseases and conditions associated with oxidative stress 7
Table 2.5: Oxidative modifications of proteins 21
Table 2.6: Classes and dietary sources of flavanoids 27
Table 2.7: Activities of flavanoids and the species involved 29
Table 2.8: Composition (%) of green and black teas 30
Table 2.9: Phenolic profile of fermented and unfermented rooibos 34
Table 4.1: Polyphenol content and antioxidant activity of the beverages and supplements 54
Table 4.2: Body weight gains daily intakes of $t$-BHP-induced oxidative stress rats 55
Table 4.3: Antioxidant capacity in the liver of rats consuming RT, GT and commercial tea supplements after $t$-BHP-induced oxidative stress 56
Table 4.4: Antioxidant capacity in the kidney of rats consuming RT, GT and commercial tea supplements after $t$-BHP-induced oxidative stress 56
Table 4.5: Antioxidant enzyme activity in the liver tissue of oxidatively stressed rats consuming the various antioxidant beverages and supplements 57
Table 4.6: Antioxidant enzyme activity in the kidney tissue of oxidatively stressed rats consuming the various antioxidant beverages and supplements

Table 4.7: Protein content and glutathione levels in the liver tissue of oxidatively stressed rats consuming the various antioxidant beverages and supplements

Table 4.8: Protein content and glutathione levels in kidney tissue of oxidatively stressed rats consuming the various antioxidant beverages and supplements

Table 4.9: Lipid peroxidation levels in the liver tissue of oxidatively stressed rats consuming the various beverages and supplements

Table 4.10: Lipid peroxidation levels in the kidney tissue oxidatively stressed rats consuming the various beverages and supplements

ADDENDUM

Ethics certificate
<table>
<thead>
<tr>
<th>Abbreviation/Acronyms/Term</th>
<th>Definition/Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>6-HD</td>
<td>6- hydroxidopamine</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACP</td>
<td>Acid phosphates</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AOPPs</td>
<td>Advanced oxidative protein products</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Pierce bicinchoninic acid</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetra chloride</td>
</tr>
<tr>
<td>CD</td>
<td>Conjugated diene</td>
</tr>
<tr>
<td>CGT</td>
<td>Chinese green tea</td>
</tr>
<tr>
<td>GTS</td>
<td>Green tea supplement</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DETAPAC</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DMACA</td>
<td>4-dimethylaminocinnamaldehyde</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>DNPH</td>
<td>Dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl radical</td>
</tr>
<tr>
<td>EC</td>
<td>Epicatechin</td>
</tr>
<tr>
<td>ECG</td>
<td>Epicatechin-3-gallate</td>
</tr>
<tr>
<td>EE</td>
<td>Energy expenditure</td>
</tr>
<tr>
<td>EGC</td>
<td>Epigallocatechin</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>F2-Iso</td>
<td>F2-isoprostanes</td>
</tr>
<tr>
<td>FC</td>
<td>Folin-Ciocalteu</td>
</tr>
<tr>
<td>FCR</td>
<td>Folin Ciocalteu reagent</td>
</tr>
<tr>
<td>FR</td>
<td>Free radicals</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>FRT</td>
<td>Fermented rooibos</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-glutamyl transferase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidation</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione–S-transferase</td>
</tr>
<tr>
<td>GTE</td>
<td>Green tea extract</td>
</tr>
<tr>
<td>GTP</td>
<td>Green tea polyphenol</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LMWA</td>
<td>Low molecular weight antioxidants</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MDA-TBA</td>
<td>Malondialdehyde- thiobarbituric acid</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metaloproteinase-9</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>Nox2</td>
<td>NADPH Oxidase 2 protein</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised LDL</td>
</tr>
<tr>
<td>PUFA</td>
<td>Poly unsaturated fatty acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RS</td>
<td>Reactive species</td>
</tr>
<tr>
<td>RTS</td>
<td>Rooibos supplement</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>t-BHP</td>
<td>Tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thionitrobenzoic acid</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tri [2-pyridyl]-s-triazine</td>
</tr>
<tr>
<td>TRX</td>
<td>L-arginine, thioredoxin</td>
</tr>
<tr>
<td>UDP-GT</td>
<td>Uridine diphosphate glucuronyltransferase</td>
</tr>
<tr>
<td>UL</td>
<td>Upper level</td>
</tr>
<tr>
<td>URT</td>
<td>Unfermented rooibos</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet light</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

The importance of oxidative stress (OS) has been widely reported in many studies and is generally defined as “a disturbance in the redox balance in cells in favour of oxidants, with the imbalance resulting in oxidative damage to cellular components” (Powers & Jackson, 2008). These oxidants/reactive intermediate species (i.e. free radicals) may be generated intra cellularly or extra cellularly through a variety of processes either from exogenous or endogenous sources as a result of exposure to some environmental insults. The most important source of reactive species results from metabolic reactions during energy production (ATP production) in mitochondria. Although moderate accumulation produces signalling for physiological function, overproduction may lead to serious damage to important cellular components (Nohl et al., 2005). However, this overproduction is counteracted by antioxidant systems either endogenously or exogenously in order to maintain homeostasis. When this homeostasis is compromised, important molecules in cellular components such as proteins, lipids and nucleic acids (DNA/RNA) may be damaged, which could lead to the development of many diseases in a variety of organs (Jabs, 1999).

Numerous types of free radicals are formed in the course of metabolism. The most important reactants are free radical oxygen and its radical derivatives (also referred to as reactive oxygen species – ROS), superoxide, hydroxyl radical and hydrogen peroxide produced via the electron transport chain (Cheeseman & Slater, 1993; Agarwal et al., 2005). Equally important are nitrogen radicals (also referred to as reactive nitrogen species – RNS) such as nitric oxide (NO) and peroxinitryte which are formed during the conversion of L-arginine to L-citrulline (Agarwal et al., 2005). Other highly reactive molecules include hypochlorous acid and transition metals. However, it should be noted that these molecules also play a role in other physiological functions, i.e. enzyme reactions, electron transport in mitochondria, signal transduction and gene expression (activation of nuclear transcription factors and cell differentiation), immunity, defence against micro-organisms, regulation of vascular tone, and the aging process (Cadenas, 1997; Evans & Halliwell, 2001; Georgieva, 2005). Nevertheless, the presence of OS in biological tissues is not only a matter of imbalance between reactive species production and antioxidant action, but a combination of various factors such as, type of reactive species, time of exposure, nature of tissue individual ability to provide and release endogenous antioxidants and intake of exogenous antioxidants (Salganik, 2001; Finkel, 2003; Wang & Kim, 2007).
CHAPTER ONE

INTRODUCTION

For homeostasis, living systems should prevent the production of excessive reactive species while simultaneously maintaining an adequate level. This can be done through exogenous (compounds present in the diet) and endogenous (physiological compounds) antioxidants which may result in protection against the formation of oxidants (ROS/RNS), interception of damaging species and amelioration of resultant damage (Sies, 1997). Antioxidants are loosely divided into two groups (i) enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase heme oxygenase-1, eosinophil peroxidase, metallothionein and glutathione reductase and (ii) non-enzymatic antioxidants which include dietary supplements or synthetic antioxidants including vitamin A (retinol), vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), selenium, zinc, taurine, hypotaurine, glutathione, β-carotene, carotene, polyamines, melatonin, NAPH, adenosine, urate, coenzyme Q-10, polyphenols, phyto-estrogens, cystein, homocysteine, methionine, nitroxides (Yamauchi, 1997; Matés & Sánchez-Jiménez, 2000; Agarwal et al., 2005).

Experimental evidence indicate that ROS are involved in the pathology of various diseases e.g. kidney disease where studies suggest that uraemia induces tubulointerstitial damage (Ichikawa et al., 1994; Guo et al., 2008) and heart failure, where ROS is involved in deterioration of cardiac hypertrophy and vascular dysfunction (Blum, 2009). In male albino rats, supplementation with bread fortified with green tea greatly improved renal function in a chronic renal failure model (El-Megeid et al., 2009).

Our diet may therefore play an important role in the improvement of many diseases/conditions. Human diet is a potential source of numerous oxidant and antioxidant compounds that are important for normal metabolism. Health professionals, nutritionists and dieticians generally agree that fruits, vegetables and beverages such as teas and herbal teas have health promoting properties and that regular consumption of these products reduces the risk of diseases such as heart disease and cancer (Papas, 1999). The role of our diet as a source of antioxidants or pro-oxidants depends on its components. Some factors can also affect the antioxidant status, i.e. food processing and storage, food additives and nutritional supplements, absorption and bioavailability (Papas, 1999). Tea has been used since ancient times in traditional medicine and is also the most popular beverage consumed worldwide. The most significant and widely investigated components with pharmacological/antioxidant relevance in tea are polyphenols. Their levels vary depending on the genetic makeup of the plant (Camellia sinensis), processing of the plant material (leaves are processed differently to produce green, black and oolong tea) and environmental factors (Kuroda & Hara, 1999; Khan & Mukhtar, 2007). Epidemiology data partly credit the
longevity of communities in countries such as Japan, China and India to the regular consumption of tea (Cabrera et al., 2006; Zhou et al., 2010).

Determination of the antioxidant status and oxidative damage is important when assessing the oxidative stress status. Many reports have described a number of assays to assess both the antioxidant capacity and oxidative damage but no clear guidelines exists for the selection and interpretation of specific methods to investigate the impact of antioxidants on the redox status of various tissues. Current assessment methods are challenging due to various influencing factors, e.g. the short biological half-lives of most of the biomarkers measured; molecules measured are also not homogenously distributed among the various organs/tissues and poor bioavailability of the active compound. It is well known that organs in the same animal may react differently when exposed to the same environment, due to differences in tissue and enzyme specificities within these organs. The recommendation therefore is that a battery of assays should be done when assessing the oxidant-antioxidant balance (Trevisan et al., 2001; Gedik et al., 2002; Blumberg, 2004; Collins, 2004; Powers & Jackson, 2008).

In the scientific environment, little evidence exists regarding the potential modulation of oxidative stress by the indigenous South-African herbal tea, rooibos (Aspalathus linearis) when compared with green tea (Camellia sinensis) and supplements in important organs involved in the metabolic processes. In this study the impact of various beverages on oxidative status in liver and kidney tissues of Wistar rats will be investigated. These beverages will define the various experimental groups to be included viz., fermented rooibos (FRT), unfermented rooibos (URT), Chinese green tea (CGT), rooibos supplement (RTS), green tea supplement (GTS) and water.

Chapter one introduces the concept of oxidative stress, its implications in disease and the possible amelioration by dietary intervention. These points are discussed in more depth in chapter two as well as a selection of relevant diseases where oxidative stress is strongly implicated. In the same chapter, the use of biomarkers as evidence for oxidative damage and the importance dietary antioxidants or supplementation are discussed. Chapter three describes the animals, beverages and methods used in the study while the results are presented in chapter four. In chapter five the results are discussed and conclusions were drawn with future research directions suggested.
CHAPTER TWO
LITERATURE REVIEW

2.1 Oxidants and oxidative stress

The term “oxidative stress” was introduced and defined by Helmut Sies as a disturbance in the oxidant-antioxidant equilibrium in favour of the former leading to damage (Sies, 1991). It has recently been redefined simply as a “disruption of redox signalling and control” to account for the observation that oxidative stress includes individual signalling and control process occurring via discrete redox pathways rather than via mechanisms directly attributed to a systematic balance (Azzi, 2007; Jones, 2008). Oxidative stress results from decreased antioxidant levels and an elevated release of reactive species (oxidants).

“Reactive species” (RS) is used here as a collective term describing both free radicals and non-radical reactive derivatives. Free radicals (FRs) are chemical species capable of independent existence that contain one or more unpaired electrons (Willcox et al., 2004). The presence of unpaired electrons makes FRs unstable and very reactive towards other molecules in an attempt to gain stability. Reactive species (RS) can be categorised as oxygen- (ROS) or non oxygen centered where the central atom can be nitrogen (RNS), sulfur, or halide. Major ROS radicals include superoxide (O$_2^-$), hydroxyl (OH$^+$) and peroxyl (RO$_2^-$) radicals, while hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (1$^1$O$_2$) are examples of non-radical ROS (Table 2.1, page 5). Nitric oxide (NO$^-$) and nitrogen dioxide (NO$_2^-$) are common RNS radicals and peroxynitrite (ONOO$^-$) is an example of a non radical RNS, while epoxides, malondialdehydes and metal ions are examples of non radical thiol-reactive chemicals (Jones, 2008).

Exposure of humans and other aerobic organisms to reactive species (RS) is unavoidable since the mitochondrial production of energy (as ATP) from molecular oxygen requires and generates O$_2^-$ which leaks into the cytosol. These species may cause damage to important biomolecules (lipids, proteins and DNA) by initiating chain reactions in which FRs are passed from one molecule to another or they may directly damage the biomolecules while forming various bioactive oxidation products.
Table 2.1: Free radicals, non radical oxidants and non radical thiol-reactive species

<table>
<thead>
<tr>
<th>Free radicals</th>
<th>Non radical oxidants</th>
<th>Non radical thiol-reactive chemical species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion radical</td>
<td>Hydrogen peroxide</td>
<td>Conjugated aldehydes, e.g., acrolein</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Hydroperoxyfatty acids</td>
<td>4-hydroxynonenal, malondialdehyde</td>
</tr>
<tr>
<td>Trichloromethyl radical</td>
<td>Aldehydes</td>
<td>Quinones</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>Quinones</td>
<td>Epoxides</td>
</tr>
<tr>
<td></td>
<td>Peroxynitrite</td>
<td>Zinc ions</td>
</tr>
<tr>
<td></td>
<td>Disulfides</td>
<td>Mercuric ions</td>
</tr>
</tbody>
</table>

Source: (Jones, 2008; Halliwell, 1987)

Besides mitochondrial RS production, aerobic organisms are constantly exposed to oxidants from both endogenous and exogenous sources (Table 2.2). The generation of RS, however, is not always deleterious as they are essential in various physiological processes. For example, activated phagocytic cells and leukocytes deliberately produce superoxide and hypochlorous acid respectively which are directed towards eliminating pathogenic microorganisms (Bahorun et al., 2006). Reactive species are also involved in cell differentiation and signaling, neurotransmission and gene transcription, as well as endothelial function and apoptosis (Evans & Halliwell, 2001; Bahorun et al., 2006).

Table 2.2: Sources of oxidants

<table>
<thead>
<tr>
<th>Exogenous</th>
<th>Endogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>Fenton and Herber-Weiss reactions</td>
</tr>
<tr>
<td>Drugs</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>Tobacco smoke</td>
<td>Cell metabolism (ATP production)</td>
</tr>
<tr>
<td>Poor quality diet</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>Irradiation e.g. UV and X-rays</td>
<td>Oxidation of hemoglobin</td>
</tr>
<tr>
<td>Environmental pollutants e.g. heavy metals, N-containing compounds</td>
<td>Enzyme activity including cytochrome P450 system</td>
</tr>
<tr>
<td>Ozone</td>
<td></td>
</tr>
<tr>
<td>Physical and psychological stress</td>
<td></td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from: (Kohen & Nyska, 2002; Georgieva, 2005)

This paradoxical need for a toxic molecule is characteristic of aerobic organisms which must therefore continually strive to neutralize the damaging effects of the reactive species.
The neutralization is achieved by a complex antioxidant defense system made of various endogenous and exogenous components (Table 2.3). If the oxidative damage/products formation exceeds the capacity of the defense system (antioxidant system- Table 2.3), it may initiate and/or hasten disease processes of e.g. cardiovascular disease (CVD), cancer, diabetes as well as neurological, immune and ocular diseases (Table 2.4, on page 7). Increased oxidation of low density lipoprotein (LDL), for example, is suggested as a mechanism linking oxidative stress with CVD (Willcox et al., 2004; Vogiatzi et al., 2009), while direct damage to DNA, suppression of apoptosis by RS and oxidative damage to DNA repair enzymes may contribute to carcinogenesis (Halliwell, 2007; Oka et al., 2008).

### Table 2.3: Components of the antioxidants defence system

<table>
<thead>
<tr>
<th>Endogenous</th>
<th>Exogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non enzymatic</strong></td>
<td>Carotenoids e.g. β-carotene</td>
</tr>
<tr>
<td>Thiols (glutathione, lipoic acid, N-acetyl cysteine)</td>
<td>Vitamin C</td>
</tr>
<tr>
<td>Ubiquinones (coenzyme Q10)</td>
<td>Vitamin E</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Plant phenols (flavonoids and other phenols)</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Metals (copper, manganese, selenium and Zinc)</td>
</tr>
<tr>
<td>Tranferrin</td>
<td>Albumin</td>
</tr>
</tbody>
</table>

*Adapted from: (Willcox et al., 2004; Valko et al., 2007; Halliwell, 2009)*

### 2.2 Pathological conditions associated with oxidative stress

Elevated oxidative damage has been associated with numerous pathological conditions as shown in Table 2.4 on page 7, (Halliwell, 1987; Haddad, 2002; Harrison et al., 2003; Willcox et al., 2004; Han et al., 2006; Valko et al., 2007; Wood & Granger, 2007). Although a clear correlation between disease and oxidative stress has not been proven for most of these conditions, this link appears plausible in several conditions. A few of those relevant to the current study are briefly discussed here.
Table 2.4: Diseases and conditions associated with oxidative stress

<table>
<thead>
<tr>
<th>Condition</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aging</td>
<td>Malnutrition</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Arthritis and inflammatory diseases</td>
<td>Neonatal lipoprotein oxidation</td>
</tr>
<tr>
<td>Cancer</td>
<td>Neurodegenerative diseases</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td>Pancreatitis</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Pulmonary dysfunction</td>
</tr>
<tr>
<td>Eclampsia</td>
<td>Renal diseases</td>
</tr>
<tr>
<td>Eye diseases (cataracts)</td>
<td>Shock and ischaemia</td>
</tr>
<tr>
<td>Liver dysfunction (non alcoholic fatty liver diseases)</td>
<td>Sickle-cell anaemia</td>
</tr>
<tr>
<td></td>
<td>Skin lesion</td>
</tr>
</tbody>
</table>

2.2.1 Cancer and oxidative stress

Cancer is a collective term for diseases in which a group of cells derived from one cell grow in an uncontrolled manner due to the loss of control in the normal cell growth mechanism and/or development (Gallagher et al., 2009; American Cancer Society, 2011). Cancer is thought to have a genetic basis where changes introduced by various carcinogens (e.g. tobacco, sunlight and infections) may produce mutations that affect normal cell growth and division (Brash & Cairns, 2009; American Cancer Society, 2011).

According to the World Health Organization (WHO), cancer is the world’s second leading cause of death after CVD (WHO, 2007). About 40% of all cancer deaths may be avoided by taking preventive measures such as immunization against hepatitis B and human papilloma virus and by choosing better lifestyle habits such as eating a healthy diet, exercising regularly and eliminating exposure to carcinogens such as cigarette smoking and smoke.

Cancer progression is a stepwise process where the initiated cells evolve further and become progressively more malignant. Oxidative stress is widely believed to play an important role in the various stages of cancer including initiation, promotion and progression. Although the exact mechanism remains unclear, human studies support the hypothesis that oxidative DNA damage is an important mutagenic and carcinogenic trigger (Giustarini, et al., 2009). The division of cells with oxidatively damaged DNA can result in mutations which, if mutagenic, can lead to cancer. Although all four bases can be modified by RS, mutations are
usually associated with changes in guanine (G) cytosine (C) pair while that of adenine (A) thymine (T) seldom result in mutations (De Bont & Van Larebeke, 2004). The most frequent mutations are base pair substitutions, while deletions and insertions are less common. Evidence for RS involvement in the initiation of cancer in humans is further reinforced by the presence of oxidative DNA modifications in cancer tissue (De Bont & Van Larebeke, 2004).

Cigarette smoking is by far the main contributor to the development lung cancer which is the most common cancer globally in terms of both incidence and mortality (Rudin et al., 2009). Cigarette smoke, a rich source of RS and known carcinogens (e.g. nitrosamines and polycyclic aromatic hydrocarbons), has been consistently associated with increased accumulation of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative stress, in lungs and urine which may be partly induced by FRs (Waris & Ahsan, 2006). Increased RS-mediated DNA base damage has also been reported in breast cancer thus suggesting an involvement of oxidative damages in the pathologies of these cancers. Similarly, 8-OHdG has also been shown to accumulate in liver cancer (Waris & Ahsan, 2006). This cancer is often linked to chronic infection with hepatitis B or C viruses or ingestion of aflatoxins and the oxidative stress induced by these viruses and/or agents is thought to be one of the intracellular carcinogenic triggers (Wild & Hall, 2000). G→T transition has been shown to be one of the more common types of mutations produced by aflatoxin lesion and RS damage to DNA (Waris & Ahsan, 2006).

Measuring markers of oxidative status is useful for monitoring cancer development. Several studies have consistently reported a strong correlation between cancer and levels of biomarkers of oxidative stress. For example, Manimaran & Rajneesh (2009) reported low levels of catalase (CAT), superoxide dismutase (SOD), vitamin C and vitamin E in blood of ovarian cancer patients. Another study found that malondialdehyde (MDA) levels were higher in hepatocytes with oxidative damage (Lu et al., 2008). Other studies, however, did not find a correlation between markers of oxidative stress and cancer. In a study assessing the relation of oxidative stress to the risk breast cancer, urinary excretion of isoprostanes did not differ between cases and controls, although, among overweight women, levels of isoprostanes were positively associated with this type of cancer risk (Dai et al., 2009).

As there is no cure for cancer, prevention and control programmes including immunization, reducing alcohol consumption/tobacco use and improving diet, are important measures to decrease the burden. It is estimated that one third of human cancers are attributed to dietary habits, and therefore dietary consideration is recognized as a plausible preventive strategy.
against cancer (De Mejia et al., 2009). Moreover, chemotherapy and radiotherapy have numerous side effects, which may impact on the quality of life.

According to the WHO (2007), cancer remains one of the leading causes of morbidity and mortality worldwide. It is predicted that by 2020, the number of new cases of cancer in the world will increase to more than 15 million, with deaths increasing to 12 million; much of this burden will occur in the developing world (WHO, 2007). It is therefore imperative to devise policies to halt the burden of chronic diseases including cancer. Such policies will include lifestyle changes and modification of diet if there is to be any success in the global fight against cancer as a public health problem (WHO, 2007).

Epidemiological data show an inverse correlation between tea consumption and cancer prevalence. In one study evaluating the involvement of green tea compounds in the course of prostatic cancer development, Bettuzzi and co-workers (2006) enrolled 60 men aged between 45-70 years old with high-grade prostate intraepithelial neoplasia and reported a positive effect on low urinary tract symptoms, suggesting potential benefits with administration of tea polyphenols. One meta-analysis concluded that consumption of green tea also protects against the development of breast cancer (Sun et al., 2006). Rooibos is another herbal tea with reported antimutagenic properties. A study investigating the modulating effect of rooibos and honeybush herbal tea on tumour promotion in mouse skin reported a reduction of the mean number of tumors per mouse in the processed and unprocessed rooibos groups, suggesting protective effect of this herbal tea (Marnewick et al., 2005). Similar results were found in a study comparing the antimutagenic properties of aqueous extracts of Aspalathus linearis (rooibos tea) and Camellia sinensis (green tea) (Van der Merwe et al., 2006).

2.2.2 Chronic kidney disease and oxidative stress

Chronic kidney disease (CKD) is now a major cause of cardiovascular mortality and morbidity globally (Beaglehole & Yach, 2003; Atkins, 2005; Vanholder et al., 2005). In South Africa and other developing countries, the rise of CKD prevalence parallels that of diabetes and hypertension and is expected to replace infectious diseases in the list of leading causes of mortality and morbidity (Atkins, 2005).

There is ample evidence for increased oxidative stress in uremia - one of the key features of CKD. In uremic patients, there is increased plasma protein oxidation (thiol residue oxidation
and carbonyl formation) and decreased circulating and intracellular antioxidants (Coppo et al., 2010; KDOQI, 2000). Experimental data suggest that uremia induces tubulointerstitial damage, while experimental proteinuric models such as puromycin-induced nephrosis have been associated with advanced oxidation protein products (AOPPs) (Guo et al., 2008).

AOPPs induce intracellular superoxide generation in podocytes via mechanisms involving NADPH oxidase (Coppo et al., 2010). Moreover, LDL from uremic patients was shown to be more susceptible to oxidation in vivo than LDL from healthy volunteers (Maggi et al., 1994). The oxidative stress in uraemia may result from the typical features of the CKD patient such as advanced age, diabetes, chronic inflammation, malnutrition or as a consequence of dialysis treatment. Several studies have shown that various indicators of oxidative stress e.g. lipid peroxidation, AOPPs, 8-OHdG and F2-isoprostones are increased in CKD (Salomon et al., 2000; Targh et al., 2000; Handelman et al., 2001; Ikizler et al., 2002). Increased oxidative stress may contribute to the excessive CVD incidence and mortality that accompany CKD.

An association between AOPP and carotid arteriosclerosis was reported in CKD patients, a finding that was supported by a study evaluating AOPP as an independent risk factor for coronary artery disease (CAD) in the general population (Kaneda et al., 2002). Furthermore, oxLDL and plasmalogen were associated with increased cardiovascular mortality in patients with advanced chronic kidney disease (CKD) (Bayes et al., 2003; Stenvinkel et al., 2004). Although increased oxidative stress is associated with many complications of CKD (such as amyloidosis, anaemia, hypertension and malnutrition), no large prospective epidemiological studies have yet demonstrated a link between oxidative stress and patient outcome.

Several studies have investigated the potential benefits of tea beverages against kidney disease. Uličná et al. (2006) reported a reduction of MDA levels in liver tissues, plasma and lens of diabetic rats after administration of aqueous and alkaline extract of rooibos tea while green tea catechins reduced haemodialysis-induced production of hydrogen peroxide and hypochlorus acid (Hsu et al., 2007). Additionally, feeding male albino rats with green tea-fortified bread improved kidney function against CKD induced by excessive dietary arginine (El-Megeid et al., 2009). Green tea extract was also shown to stabilize gentamicin-induced oxidative stress and histopathological abnormalities in rats (Abdel-Raheem et al., 2009). Collectively, these findings suggest that rooibos herbal tea and green tea may be beneficial dietary components with regard to renal health which therefore merits further investigations.
2.2.3 Coronary heart disease and oxidative stress

Cardiovascular disease (CVD), the leading cause of death globally, is a group of disorders affecting the heart and blood vessels and includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. Coronary heart disease (CHD), the most predominant form of CVD accounting for 42.1% of CVD mortality (WHO, 2009), results from atherosclerosis.

Oxidative stress is now believed to play an important role in both initiation and progression of the atherosclerotic process by enhancing the release of factors which lead to the attraction, adhesion to the arterial wall and the ultimate differentiation of monocytes to macrophage (Traverso, 2001; Vogiatzi et al., 2009). These factors include monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule molecule-1 (VCAM-1) and macrophage colony-stimulating factor (M-CSF). Firstly, the production of free radicals from a variety of sources may trigger LDL oxidation which increases macrophage mobilization which in turn releases superoxide as a defence mechanism. The release of superoxide by macrophages aggravates LDL oxidation and consume NO by formation of peroxynitrite (NOO-) which may lead to endothelial dysfunction via various processes (Vogiatzi et al., 2009). Further exposure to superoxide from other sources, may activate the nuclear factor kB (NF- kB) regulatory complex and activate the transcription of atherosclerosis-related genes, namely tumour necrosis factor, VCAM-1, intracellular adhesion molecules-1 (ICAM-1), MCP-1, matrix metaloproteinase-9 (MMP-9) and pro-coagulant tissue factor (Tardif, 2005; Vogiatzi et al., 2009), this series of events results in the aggregation of macrophages into the arterial wall which further incorporates oxidized LDL (Tardif, 2005).

A study investigating biomarkers of oxidative stress in patients between 45-60 years with evidence of acute myocardial infarction reported an increase in levels of ceruloplasmin and MDA, while levels of SOD, catalase, glutathione peroxidase, glutathione, vitamin C, vitamin E and β-carotene were significantly decreased when compared to control subjects (Bansilar et al., 2007; Pasupathi et al., 2009).

Like cancer, the prevalence and incidence of CVD is now a global phenomenon and not limited to industrialised countries due to globalization and technological advancement (Singh et al., 2002; WHO, 2009). According to the WHO (2009), approximately 80% of premature CHD events can be prevented by modifying diet and lifestyle. The low CHD incidence in Japan, China, Switzerland, Spain, and France is attributed to the diets consumed in these
countries (Stampfer et al., 2000). Epidemiological data have suggested that large intake levels of vitamin E and vitamin C in a diet can lower the risk of coronary artery disease (Shaikh & Suryakar, 2009). Generally, however, clinical trials have failed to demonstrate any beneficial effect of the isolated use of vitamin E in primary or secondary prevention of CVD in the general population (Gaziano, 2004; Lee et al., 2005; Farbstein et al., 2010). One reason for this failure may be the fact that as is true for many pharmaceutical agents, vitamin E supplementation would be predicted to show benefit only in those individuals in which it is needed. This highlights the need for proper patient selection as was demonstrated in a study investigating the cardio-protective effect of vitamin E supplementation in angiographically-proven CVD patients. This study reported a lower incidence of the combined end-point (non-fatal myocardial infarction and cardiovascular death) in patients taking 400 or 800 IU of vitamin E per day (Nuttall et al., 1999). In contrast, a large trial with healthy women age 45 years or older with no previous history of cardio or cerebrovascular disease and no use of aspirin or non-steroidal antinflammatory) failed to show any effect on CVD mortality (Lee et al., 2005). These women were taking 600 IU of vitamin E from natural sources.

Plant polyphenols have attracted significant attention as cardio-beneficial agents. A study investigating the protective effect of tea polyphenols in hypertensive rats reported a protective effect against hypertension in the groups taking green and black tea (Negishi et al., 2004). Persson and co-workers (2010) concluded that green herbal tea and rooibos tea may have cardio-protective effects through the inhibition of angiotensin-converting enzyme (ACE) activity as an oral intake of a single dose of rooibos significantly inhibited ACE activity after 30 min and 60 minutes in healthy volunteers. When consumed for a longer period of time by adults at risk for developing CVD, rooibos was recently reported to significantly improve the lipid profile and redox status, both relevant to heart disease (Marnewick et al., 2011). Similarly, green tea (daily consumption of 4 cups of green tea beverage or extracts) was related to the reduction of plasma amyloid alpha, an independent CVD risk factor in metabolic syndrome (Basu et al., 2011).

2.2.4 Liver disorders and oxidative stress

The liver is one of the most important organs in the body where it has numerous important functions, including storage (e.g. glycogen), excretion and decomposition (e.g. erythrocytes) synthesis (e.g. hormones, plasma proteins and cholesterol) and detoxification.

There are many kinds of liver disorders with different etiologies including infection, exposure to toxic compounds, autoimmune processes, genetic defects or cancerous pathology.
Lifestyle factors (e.g. alcohol abuse, cigarette smoking) have contributed to the increase in liver diseases in general. In 2008, more than 43,000 people died of liver disease in the United States of America (American Liver Foundation, 2008). In developing countries, figures may be underestimated due to limitations in screening and diagnosis/data collection capabilities (Das et al., 2010). Although, the molecular pathways differ amongst the various diseases, experimental and clinical evidences suggest a central role of oxidative stress in the pathogenesis of many of them. The mechanisms underlying two main disorders will be highlighted.

2.2.4.1 Non alcoholic fatty liver diseases (NAFLD)

NAFLD encompasses a range of conditions varying from simple steatosis to non-alcoholic steatohepatitis (NASH), the extreme form of NAFLD. The mechanisms involved in the development and pathogenesis of NAFLD are not completely understood but oxidative stress and insulin resistance (IR) are believed to play a major role (Hijona et al., 2010). Insulin resistance is a key feature of NAFLD as it leads to elevated serum free fatty acids which provide fuel for accelerated triglyceride synthesis (Sanyal et al., 2001; Hijona et al., 2010). Accumulation of triglyceride in the liver enhances oxidative stress in the hepatocytes as has been shown in several animal models (Browning & Horton, 2004). Subsequently, a strong association between severity of NASH and degree of oxidative stress has been shown in animal models and human studies (Sanyal et al., 2001; Chalasani et al., 2004; Yesilova et al., 2005). In the livers of NAFLD patients, for example, the level of 3-nitrotyrosine, a marker of hepatic protein nitration, was found to be elevated when compared to controls (Sanyal et al., 2001).

2.2.4.2 Hepatitis C viral infection (HCVI)

Hepatitis C virus (HCV) is a major cause of viral hepatitis which affects approximately 130–170 million people globally with approximately 350,000 deaths occurring per year due to HCV-related liver disease (Shepard et al., 2005). Chronic HCVI often leads to severe liver diseases including liver cirrhosis and liver cancer. The mechanism of the pathogenesis of HCVI is unclear but as in NAFLD, oxidative stress has been strongly implicated as the infection is characterized by increased markers of oxidative stress including lipid (MDA and 4-Hydroxynonenal [HNE]) and DNA (8-OHdG) damage products as well as decreased antioxidant enzymes [SOD] (Li et al., 2004; Maki et al., 2007).
The elevated oxidative stress in HCV infection may be due to chronic inflammation which enhances and sustains a continuous generation of RS via NAD(P)H oxidase 2 (NOX 2) protein-mediated disruption of hepatic phagocyte function. This may also enhance systemic oxidative stress since a damaged liver will not effectively export the key antioxidant—GSH—to other tissues. Additionally, the excess iron deposits which have been shown to accompany HCV infections are likely to promote further generation of free radicals via the Fenton reaction. Finally, it has been shown that HCV can directly induce oxidative stress in hepatocytes when its core protein associates with the outer mitochondrial membrane via its COOH-terminal region. This association results in mitochondrial dysfunction by facilitating Ca^{2+} accumulation which then impairs electron transport and promotes ROS production at complex I of the electron transport chain (Choi & Ou, 2006).

The management strategies for liver disease range from prevention where this is possible (hepatitis and alcoholic liver disease) to transplantation when the long term-consequences of ongoing liver damage leave no other option. Prevention includes vaccination as well as lifestyle and dietary changes (Riley III & Bhatti, 2001; WHO, 2008). Several studies evaluating the hepato-protective effect of various beverages suggest a beneficial potential for Aspalathus linearis and Camellia sinensis. A long term administration of green tea stabilized activity of lactate dehydrogenate (LDH), gamma-glutamyl transferase (GGT), acid phosphates (ACP), alkaline phosphatase (ALP) and bilirubin and simultaneously decreased lipid peroxidation in adult Wistar rats (Hamden et al., 2009). Indeed, several previous studies had earlier reported on the hepato-protective effect of epigallocatechin-3-gallate (EGCG) – a component of green tea (Alessio et al., 2002; Bose et al., 2008). A recent phase I clinical trial, however, concluded that a high dose of green tea polyphenol (GTP) did not alter liver and kidney functions (Frank et al., 2009). Furthermore, two unpublished cases of green tea hepatotoxicity were reported in a recent systematic review although these were suggested to result from concurrent medication with diclofenac and progestogens (Mazzanti et al., 2009).

Similar to green tea, rooibos herbal tea was also shown to enhance activities of important phase II enzymes, glutathione-S-transferase—α and uridine 5’-diphosphoglucuronosyltransferase, (Marnewick et al., 2003) and additionally, to sustain levels of the endogenous antioxidant, coenzyme Q9 (Kucharská et al., 2004). More recently, Uličná and co-workers (2008) showed that drinking rooibos tea prevented liver from large scale fibrosis.
Although, as stated earlier, a clear connection between disease and oxidative stress remains to be established, the link appears probable in several conditions like the four discussed above. Figure 2.1 summarises some of the mechanism of the above discussed diseases.

**Figure 2.1:** Mechanism of oxidative damage (where, TID = tubule interstitial damage, NAFLD = non alcoholic fatty liver diseases, HCV = hepatitis C viral, SFFA = serum free fatty acid, NHA = neurohumoral activation, PIC = proinflammatory cytokine, endot dysf = endothelial dysfunction)

### 2.3 The use of biomarkers and antioxidant levels to estimate oxidative stress

The degree of oxidative stress can be estimated by measuring relatively stable end products of oxidative processes (biomarkers). For a product to qualify as a reliable biomarker of oxidative stress it must be a stable oxidation product that can accumulate to detectable levels. Its source and oxidative pathway should be unequivocal and it should correlate with the severity of the disease/condition that it is alleged to reflect (Dalle-Donne et al., 2006).

As highlighted above, oxidative stress can also be investigated by evaluating the antioxidant defence *in vitro* which can reveal the extent to which RS are quenched by antioxidants present in the samples. Such measurements, however, have limitations as there are
significant differences between the physiological environment and the in vitro assay conditions; in addition, antioxidants present in the in vitro sample are metabolized in the body which might alter their antioxidative potency.

Three categories of biomarkers—lipid, DNA and protein—will be discussed briefly in the following section.

2.3.1 Lipid biomarkers

Lipids are biomolecules characterized by solubility in organic solvents but not in water. They function in cellular metabolism, energy storage and signal transduction and as structural components of cell membranes (Sherwood, 2010). In the body, the key lipid-rich points are lipoproteins and cell membranes. Most lipids have an arrangement of fatty acids that are composed of a chain of methyl groups with a carboxyl group at one end. Lipid peroxidation (LPO) starts with the removal of a hydrogen atom from a methylene group next to a carbon double bond producing a carbon-centred radical which is stabilised by a rearrangement of the double bond to form a conjugated diene (CD) (Halliwell & Chirico, 1993). Oxygen, which is abundant in the cell membranes, can easily react with the carbon-centred radical forming the peroxyl radical which reacts further with another phospholipid/triglyceride-linked fatty acid forming a hydroperoxy group and a new carbon-centred radical. Further reactions by the lipid hydroperoxide will produce cyclic peroxide, cyclic endoperoxide and finally aldehydes e.g. malondialdehyde (MDA). This sequence of reactions yields other important products including aldehydes (MDA and 4-hydroxy-2-nonenal [4-HNE]), hydrocarbons (pentane and ethane), transconjugated dienes, isoprostanes and cholesteroloxides (Esterbauer et al., 1989; Abuja & Albertini, 2001; Hwang & Kim, 2007). In vivo, MDA, 4-HNE and other aldehydes can form cross-linkages with DNA and proteins which alters the function of these biomolecules. The aldehydes diffuse more readily than free radicals thereby transferring the damage to distant parts of the body. The three most commonly assessed LPO products (MDA, CDs and isoprostanes) will be discussed in this section.

As indicated above, the oxidation of polyunsaturated lipids releases several end products including MDA. The measurement of MDA as thiobarbituric acid-reactive substances (TBARS) is one of the most frequently utilized methods to assess LPO. In this assay, MDA is heated with thiobarbituric acid (TBA) under acidic conditions and the resultant reaction yields a relatively stable product that can be quantified by spectrophotometry (at 532 nm) or HPLC (Halliwell & Chirico, 1993; Hwang & Kim, 2007). This method is easy and fast to perform but it has attracted immense criticism for several reasons. First, it is not specific as many
products other than MDA (e.g. biliverdin, acetaldehyde, sucrose, reducing sugars and other aldehydes) may react with TBA to yield products similar to the MDA-TBA adduct even in peak absorbance. Secondly, there is no guarantee that the MDA measured is from oxidative processes since degradation of fatty acids can occur in the heating step of the analysis. Thirdly, MDA levels vary greatly even in blood samples from the same individual depending on the anticoagulant used. Finally, it is difficult to compare results from the literature since the exact intensity of the colour formed during the reaction depends on the type and strength of the acid that is used yet different laboratories use different TBA assays. It is not surprising therefore that the assay’s popularity is waning and is increasingly modified to include high-performance liquid chromatography (HPLC) methodology where the MDA-TBA adducts is separated from interfering chromophores thus improving specificity (Hwang & Kim, 2007). Still, since lipid hydroperoxides and aldehydes are obtained from the diet and then excreted in urine, measurements of MDA in plasma or urine is unreliable as an index of whole-body LPO unless the diet is strictly controlled.

Conjugated dienes (CDs) are primary products of LPO which result from rearrangement of the double bond when a hydrogen atom is abstracted from a fatty acyl methylene group (Gümüşlü et al., 1997; Kohen & Nyska, 2002). CDs are measured spectrophotometrically at 230–235 nm which in itself is a notable drawback since many other physiological substances (e.g. poly unsaturated fatty acid-PUFAs) absorb in the same UV-range. Furthermore, there is no suitable reference material available yet as this is essential to account for the PUFA absorption. As in the case of MDAs, the diet should be strictly controlled since plasma CDs content is >90% derived from 9, 11 diene-conjugated linoleic acid from dietary dairy products (Romieu et al., 2008).

Isoprostanes (IsoP) are free radical oxidation products of arachidonic acid, esterified to lipids then cleaved and released into the circulation by phospholipases. They are considered the most consistent markers of LPO in vivo (Morrow, 2005). Up to 64 F2-isoprostanes can be formed in vivo but 8-iso-IPF2a (15- F2-IsoP) is one of the more abundant and to-date one of the most comprehensively studied biomarkers of oxidative stress (Lawson et al., 1999). At present, F2-IsoPs analysis is commonly done by hyphenated chromatography as well as enzyme- and radio immunooassays. Normal plasma levels are low as only a small proportion of isoprostanes exist freely in plasma while the rest is esterified to lipids. Levels are nonetheless elevated in situations like atherosclerosis where lipid peroxidation plays an important role (Morrow, 2005). In plasma, however, isoprostanes have a short half life (about 18 minutes) and are excreted rapidly which poses an analytical dilemma as it is not possible to measure them over a period of time. To measure isoprostanes formed over a given time
period, urine samples must be used although local kidney peroxidation presents a problem. This is in turn can be overcome by measuring urinary levels of 2, 3-dinor 8-iso PGF1, the metabolite of iso-PFG2a (Morrow et al., 1999). Like in the MDA and CDs assays, diet has a confounding effect in the analysis of isoprostanes but this is circumvented by correcting for levels of arachidonic acid (Gopaul et al., 2000).

2.3.2 DNA damage

Deoxyribonucleic acid (DNA) is a molecule often described as an organism’s blueprint as it contains all the information used to manage the organism’s function, behaviour and development. Despite relative stability, DNA may be attacked by RS producing damage which, if not efficiently cleared/repaired, may lead to permanent modification that serves as the first step in the path to mutagenicity (Cecarini et al., 2007). Oxidative damage to DNA is mainly affected by the highly reactive hydroxyl radical (•OH) by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and C-H bonds of 2-deoxyribose. The damage generates a range of DNA lesions, including strand breaks and modified bases, many of which have potential to breach the integrity of the genome (Dizdaroglu, 1991).

One of the most abundant and extensively studied modified bases is 8-oxo-7, 8-dihydro-2’-deoxyguanosine (8-oxo-dG) which results from the oxidation of guanine. It pairs preferentially with adenine thus generating guanine/cytosine (GC) to thymine/adenine (TA) mutations after replication. This damage is usually repaired (mainly by base excision repair [BER] enzymes) before the cell reaches replication phase (Jaruga & Dizdaroglu, 1996). Variations in repair capability is thought to affect the amount of oxidative DNA damage as several human diseases characterized by defects in DNA repair mechanisms (e.g. xeroderma pigmentosum, ataxia telangiectasia, Bloom's syndrome and Fanconi's anaemia) are also characterized by an elevated amount of 8-oxo-dG (Degan et al., 1995; Evans et al., 2000). Other abundant oxidatively modified bases include formamidopyrimidine adducts of adenine and guanine, e.g., 4,6-diamino- 5-formamidopyrimidine (FapyAdenine) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGuanine) (Burgdorf & Carell, 2002) which is readily formed in the absence of oxygen by ionizing radiation and other RS producing agents (Ono et al., 1995). 5-Hydroxyuracil and uracil glycol are oxidative deamination products of deoxycytosine (dC) and are detected in comparable levels to 8-oxo-dG in human DNA (Burcham, 1999). The thymine analogue (thymine glycol) pairs with adenosine yielding a C to T transition which is weakly mutagenic and it blocks transcription and replication (Basu et
al., 1989; Dianov et al., 2000; Marnett & Plastaras, 2001). Important pyrimidine-derived lesions include 5-hydroxyuracil (5-OH-Ura) and 5-hydroxycytosine (5-OH-Cyt) the latter of which is thought to be the most mutagenic product of oxidative DNA damage (Feig et al., 1994). The hydroxyl radical (•OH) that is oxidized to produce these adducts plays a major role in various DNA-protein interactions including the binding of transcription factors to DNA which is therefore disrupted in this process (Rogstad et al., 2002). If not effectively cleared, this non-mutagenic lesion can trigger apoptosis as a consequence of chromosomal breakage thus indirectly leading to deletion mutations (Rogstad et al., 2002).

Several methods are available for the estimation of DNA damage including immunological (Santella, 1999), adducts measurement by chromatography e.g. high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GCMS) (Phillips et al., 2000) and comet assay (Gedik et al., 2002). All these may be easily done in various samples including urine, plasma as well as tissue homogenates but the high cost of measuring DNA adducts has limited the routine use of this approach (Phillips et al., 2000).

Immunoassays to detect DNA damage evolved from the hypothesis that DNA could become immunogenic if linked to a carrier molecule. Several antibodies are now available commercially for measurement of DNA adducts in urine but 8-oxo-dGuo is the target of most assays since its presence is independent of dietary influence (Kadiiska et al., 2005). The immunogen is fixed to the microplate wells and 8-oxo-dGuo competes with the solid-phase antigen for the antibody. Free antibody is then bound to a peroxidase-labelled secondary antibody thus, the lower the final absorbance the higher the concentration in the urine. The assay has comparable sensitivity to HPLC of 8-oxo-dGuo (Phillips et al., 2000; Gedik et al., 2002).

High-performance liquid chromatography (HPLC) is widely used for the measurement of 8-oxo-dGuo in tissues or lymphocytes. Using appropriate non-ionic detergents and or detergents, cells are lysed (or tissue homogenised), nuclei separated by centrifugation and DNA finally released by adding sodium dodecyl sulphate followed by digestion of RNA and proteins and ethanol precipitation. Samples are then separated on the column. Samples can be stored frozen for many months but with caution to preserve viability especially in liquid nitrogen or at -80 °C or, preferably, freezing after DNA extraction. Oxidation of guanine, during any stage of sample preparation/storage, is a key concern but this can be minimized by the use of antioxidants. The method is limited in that it does not definitely identify individual adducts in a mixture (Phillips et al., 2000; Dotan et al., 2004).
The single–cell gel electrophoresis assay, also referred to as the comet assay, has emerged as the standard method to measure DNA damage due to its robustness, simplicity, sensitivity, cost effectiveness and safety as no labeling with radioisotopes and other hazardous markers is necessary (Collins, 2004). It is essentially a method for detecting DNA strand breaks but can be adapted to measure oxidized bases by incorporating a step to recognize and remove damaged bases in the DNA (Collins, 2004). Besides the use in basic DNA damage and repair research, the assay has found wide applications in genotoxicity testing, nutritional studies, molecular epidemiology and ecogenotoxicology (Collins, 2004). The assay’s main drawback has been the concern that the damage detected in leukocytes, the preferred sample material for the assay, may not reflect the damage in the target tissues (Collins, 2004). Nonetheless, the assay has a huge advantage over other assays requiring extensive sample preparation in that there is little chance for spurious oxidation of guanine to occur. Lymphocytes frozen according to protocols have a viability of at least one year without significant increase in damage (Gedik et al., 1992; Singh, 2000; Collins, 2004). Lymphocytes are embedded in agarose, lysed in a detergent then subjected to an alkaline electrophoresis. Negatively charged DNA is attracted to the anode, but only the loops of DNA possessing a break are free to migrate, resulting in an image appearing as a comet when seen under the fluorescence microscope after suitable staining. As it is not possible to include an internal standard, a standard lymphocyte sample should be run alongside the experimental samples which can then be used for quantification especially in population studies (Gedik et al., 1992; Singh, 2000; Collins, 2004; Kumari et al., 2008).

2.3.3 Protein biomarkers

Protein oxidation implies covalent modification induced either directly by RS or indirectly by reaction with a by-product of oxidative stress. Such damage can impact on numerous protein functions such as enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity (Shacter, 2000). Although cysteine and methionine are the most susceptible to oxidative attack due to their vulnerable sulfur atoms, all amino acid side chains can be modified via one or more of the many protein oxidation mechanisms yielding numerous products as shown in Table 2.5 (Hu, 1994; Vogt, 1995).
### Table 2.5: Oxidative modifications of proteins

<table>
<thead>
<tr>
<th>Modification</th>
<th>Amino acids involved</th>
<th>Oxidizing source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulfides, glutathiolation</td>
<td>Cysteine (Cys)</td>
<td>All, peroxinitrite (ONOO⁻)</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>Methionine (Met)</td>
<td>All, ONOO⁺</td>
</tr>
<tr>
<td>Carboxyls (aldehydes, ketones)</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>Oxo-histidine</td>
<td>Histidine (His)</td>
<td>radiation, MCO, (^1 \text{O}_2)</td>
</tr>
<tr>
<td>Dityrosine</td>
<td>Tyrosine (Tyr)</td>
<td>Radiation -Ray, MCO, (^1 \text{O}_2)</td>
</tr>
<tr>
<td>Chlorotyrosine</td>
<td>Tyroso (Tyr)</td>
<td>HOCI</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>Tyroso (Tyr)</td>
<td>ONOO⁻</td>
</tr>
<tr>
<td>Tryptophanyl modifications</td>
<td>Tryptophan (Trp)</td>
<td>gamma-rays</td>
</tr>
<tr>
<td>Hydro(pero)xy derivatives</td>
<td>Val, Leu, Tyr, Trp</td>
<td>gamma-rays</td>
</tr>
<tr>
<td>Chloramines, deamination</td>
<td>Lysine (Lys)</td>
<td>HOCI</td>
</tr>
<tr>
<td>Lipid peroxidation adducts</td>
<td>Lys, Cys, His</td>
<td>gamma-rays, MCO</td>
</tr>
<tr>
<td>(MDA, HNE, acrolein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid oxidation adducts</td>
<td>Lys, Cys, His</td>
<td>Hypochlorous acid (HOCI)</td>
</tr>
<tr>
<td>Glycoxidation adducts</td>
<td>Lys</td>
<td>Glucose</td>
</tr>
</tbody>
</table>

(Adapted from Shacter, 2000). MCO = metal catalyzed oxidation; All = gamma-rays, MCO, ozone, \(^1 \text{O}_2\); Lys = lysine; Val = valine; Leu = leucine; MDA = malondialdehyde; HNE = 4-hydroxyl-2-trans-nonenal.

As shown in table 2.5, carboxyls are the most ubiquitous products of protein oxidation and can be generated in response to a wide variety of oxidizing species including alkoxy and peroxy radicals. Not surprising therefore, they are the most commonly measured products of protein oxidation in biological samples and are highlighted here as the representative group. Once formed, carboxyls are chemically stable which is relevant for their detection and storage. They are present in most protein preparations at very low levels of approximately 1 nmol/mg protein which allows for the detection of physiologically elevated levels (Shacter, 2000). Significantly elevated levels have been reported in numerous conditions of elevated oxidative stress such as acute pancreatitis (Winterbourn et al., 2003), ocular pseudoexfoliation (Yagcı et al., 2008), diabetes (Rattan, 2008) and myeloid leukaemia (Ahmad et al., 2008).

Several methods are available for the detection of protein carboxyls ranging from immunodetection by enzyme-linked immunosorbent assay (ELISA) or Western blot to analytical HPLC. In most of the methods, the carboxyl group is first coupled with dinitrophenylhydrazine (DNPH) forming a stable dinitrophenyl hydrazone product which can be detected by various means (Shacter, 2000). The spectrophotometric DNPH assay can be linked to protein fractionation by high performance liquid chromatography (HPLC) to improve the sensitivity and specificity given by measuring total carboxyls in a protein mixture (Levine
et al., 1990). The hydrazine carbonyl product can also be detected semi-quantitatively with antibodies as has been done in ELISA and Western blot analysis which has the advantages of high sensitivity, small sample volume, reproducibility and large sample throughput. Samples have been shown to be viable for 3 months at – 80 °C (Dalle-Done et al., 2003).

2.4 Antioxidant defence system

In order to counteract the damaging effects of RS, living organisms have evolved a complex network of antioxidants. An antioxidant is defined as any substance that delays, prevents or removes oxidative damage to a substrate (Halliwell, 2007). Numerous substances have been shown to have antioxidant properties in vitro which, however, do not necessarily translate to in vivo activity as it may be lost in the course of metabolism. The antioxidant potential of a particular substance is usually influenced by factors related to its uniqueness including its antioxidant mechanism, substrate, site of action (extra- or intra-cellular) and the concentration required for its activity (Halliwell, 2007).

Whether synthesized in the body (endogenous) or originating exogenously from the diet (Table 2.3, page 6), antioxidants in the body form a synergistic defence system against reactive species operating at three main levels (Rotilio et al., 1995; Willcox, 2004; Barkin & Hersh, 2008):

(i) Preventive antioxidants that suppress the formation of reactive species forming other components (e.g., reducing hydroperoxide to water).

(ii) Interceptors/scavengers that act against free radicals either physically or chemically before they can damage cellular molecules (e.g., carotenoids scavenging singlet oxygen; SOD convert superoxide to hydrogen peroxide and phenolic/aromatic amines scavenging free radicals).

(iii) Those that repair damaged biomolecules e.g. transferases, lipases, proteases and DNA repair enzymes.

2.4.1 Endogenous antioxidant defence system and oxidative stress

This category of antioxidants comprise of enzymes and non enzymatic (metal binding proteins and small molecule) species as previously shown in Table 2.3, page 6. The enzymes include catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) family, while the non enzymatic antioxidants (low molecular weight antioxidants [LMWA]) include lipoic acid, glutathione (GSH), L-arginine, thioredoxin (TRX), coenzyme...
Q10, melatonin, uric acid, bilirubin, transferrin, ceruloplasmin, albumin and lactoferrin (Percival, 1998; Pham-Huy et al., 2008). The antioxidant enzymes intercept RS before they can react with biomolecules or other RS to generate more stable, long-acting oxidants. The LMWA especially the metal binding proteins such as transferrin and ceruloplasmin, prevent the occurrence of the Fenton reaction in which transition metals (e.g. iron) react with RS producing the highly reactive hydroxyl radical (Young & Woodside, 2001). Glutathione, another LMWA, is a tripeptide present in all eukaryote and prokaryote cells, synthesized by enzymes, glutamate-cysteine ligase and glutathione synthetase. Glutathione plays a role in a variety of physiological functions, including acting as a core member of the antioxidant system and acting as a cofactor for GPx, a defence mechanism against peroxides forming glutathione disulfide-oxidased glutathione (GSSG) that is further transformed to reduced glutathione (GSH) in the presence of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and glutathione reductase (GR) (Livingstone & Davis, 2007). In homeostatic conditions, the cellular ratio between GSH/GSSG is 100:1; however, during oxidative stress this ratio may shift to 4:1, which is utilised as a biomarker of oxidative stress (Han et al., 2006). Glutathione has numerous roles including scavenging FRs in an aqueous milieu, acting as a cofactor in enzyme reactions, maintenance of intercellular communication, preventing oxidation in –SH, chelating copper ions and participating in protein folding degradation and cross-linking (Niki, 1987; Kohen & Nyska, 2002; Pham-Huy et al., 2008).

2.4.2 Exogenous antioxidant defence system and oxidative stress

Exogenous (dietary) antioxidants are important components of the antioxidant system as they complement the endogenous members. The most studied are α-tocopherol (vitamin E), ascorbic acid (vitamin C), pro-vitamin A (β-carotene), flavonoids and trace minerals (see table 2.3). Their mechanisms of action are varied but synergy between them is well recognised as exemplified by the interactions between catechins, vitamin E and vitamin C. The tocopheryl radical formed when vitamin E neutralises a free radical, accepts a hydrogen ion from catechins or vitamin C and is restored back to the active α–tocopherol form (Kohen & Nyska, 2002; Bouayed & Bohn, 2010). Several studies have reported elevated circulating levels of vitamin E after a high dietary intake (Bendich et al., 1984; Burton et al., 1990; Igarashi et al., 1991).
2.4.2.1 Carotenoids, vitamins and selenium

Carotenoids are a group of over 600 pigmented compounds principally found in plants where they give the bright colours to leaves, fruits and flowers as well as the hue of some birds, insects, fish and crustaceans (Palace et al., 1999). Animals cannot synthesize carotenoids in vivo, but they ingest carotenoids in their diets where they may have various uses. Of the approximately 40 carotenoids that form part of the human diet, lycopene and, especially, β-carotene are the best studied. β-carotene, converted to retinol which is important for vision, is proposed as an effective peroxyl radical scavenger (Paiva & Russell, 1999; Pham-Huy et al., 2008). A daily recommended intake between 2500 and 5000 IU has been suggested (Meyer, 2011; USDA, 2011) but the routine use as a supplement has raised some concerns due to the relative increased risk of heart diseases (Pham-Huy et al., 2008). Rich dietary sources of lycopene and β-carotene include tomatoes, carrots, fruits, spinach, grain and oils (Paiva & Russell, 1999; Pham-Huy et al., 2008, Meyer, 2011; USDA, 2011).

Another antioxidant vitamin is vitamin C. The first report of the benefits of vitamin C was in 1747 when James Lind conducted a “clinical trial” using lemon and oranges for treatment of scurvy (Bartholomew, 2002). It is considered a first line of defense as the major water soluble antioxidant and acts synergistically with α-tocopherol by reducing α-tocopheroxyl radicals rapidly to regenerate α-tocopherol and possibly inhibits α-tocopheroxyl-mediated radical propagation (Niki et al., 1995). Sources of vitamin C include fresh fruits, such as lemon, orange, grape fruit, watermelon, papaya, mangos and many vegetables. In addition, it is the most widely used vitamin supplement with a recommended intake range from 75-90 mg/day (Naidu, 2003).

The fat-soluble vitamin E exists in eight forms, namely; α-, β-, γ- and δ- tocotrienols and α-, β-, γ- and δ tocopherols (Niki, 1995; Pham-Huy, 2008). Alpha-tocopherol is the most bioactive of the isomers and has received much attention due to its radical-scavenging capability in the lipophilic phase and alleged beneficial association with several diseases such as cancer, CVD as well as ocular and inflammatory diseases (Pham-Huy et al., 2008; Niki, 2010). Good dietary sources include vegetable oils, whole grain, fruits, grain, and meat but the final content may be affected by cooking and storage conditions (Pham-Huy, 2008). The daily reference intake ranges from 9-15 IU (7-10 mg) while the tolerable upper level (UL) is about 1000 mg (Hathcock, 2005; Meyer, 2011; USDA, 2011).

Selenium is a mineral that plays key roles in physiological reactions as a co-factor for many enzymes, including glutathione peroxidase and superoxide dismutases, hence enhancing
antioxidant activity. It exerts its effects via seleno-proteins and has been reported to have roles as an anticarcinogen and immunomodulator and to improve male fertility (Barciela et al., 2008; Fujii et al., 2003). Rich dietary sources include onion, garlic, nuts, sea food, meat and liver and the daily recommended intake is 55-70 µg (Barciela et al., 2008; Pham-Huy et al., 2008, USDA, 2011).

2.4.2.2 Polyphenolic compounds

Polyphenols are plant secondary metabolites characterized by the presence of one or more aromatic rings to which one or more hydroxyl groups are attached (Figure 2.2). Over 8000 phenols are known and are well distributed in nearly all vascular plants where they have well established roles including attracting pollination agents, molecular signaling, mechanical support and protection against parasites, herbivores and oxidative cell damage (Scalbert et al., 2005).

Figure 2.2: The flavan structure upon which flavonoids are based (where A & B=aromatic rings and C=oxygenated heterocyclic ring) (Bravo, 1998; Manach et al., 2004)

In the past two decades, polyphenols have attracted immense interest mainly due to their antioxidant properties, their great abundance in the human diet and their possible role in the prevention of several oxidative stress-related diseases such as cancer, CVD and neurodegenerative diseases (Scalbert et al., 2005; Pandey & Rizvi, 2009). Polyphenols are grouped into several classes as shown in Figure 2.3 on page 26.
The most extensively studied and widely distributed polyphenols are the flavanoids whose basic flavan skeleton consists of two aromatic rings linked through a cyclic 3-carbon bridge (Figure 2.2 on page 25). Variations in the C-ring are used to define flavonoid subclasses whose antioxidant activity is largely dependent on the degree of hydroxylation of the B-ring and the number of C-C double bonds on the C-ring (Bravo, 1998; Manach et al., 2004). Flavanoids, like all polyphenols, are able to react with electrons on oxidants, interact with metal ions, and interact strongly with multifunctional groups for selective and unselective binding to biological molecules such as proteins (Rice-Evans & Parker, 2003; Jaganath & Crozier, 2010).

**2.4.2.3 Polyphenols in the diet**

Fruits, vegetables and beverages, e.g., coffee, wine and herbal teas are the main dietary sources of polyphenols (Aherne & O’Brien, 2002), see Table 2.6 on page 27. Apples provide as much as 400 mg of total phenols while vegetables such as onions, spinach and broccoli as well as popular beverages like tea, coffee and red wine, also contribute significant amounts of polyphenols to the human diet. 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). This is, however, a highly variable estimate since actual phenolic content of food products vary greatly under influence of numerous factors including cultivar and variety, seasonal and climatic variations, growth conditions, stage of harvesting and conditions of storage (Stewart et al., 2000). Vegetables grown in greenhouses in the UK, for example, were shown to have 4-5 fold less flavonols than those grown in the open in Spain and South Africa (Stewart et al., 2000). Processing and culinary preparation also influence the polyphenol content of foods, e.g. canned fruits have a substantially lower polyphenol content since these compounds are lost when the fruits are peeled for the cooking process. Frying onions for 15 minutes also contribute to a 30% loss of quercetin while boiling for the same duration loses 75% of this flavonol, clearly showing that the method of cooking impacts on the phenolic content (D’Archivio et al., 2007).

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Selected compound</th>
<th>Dietary source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Quercetin</td>
<td>onion, apple, asparagus, berries caulifower, grape, moringa, nectarines, tomatoes strawberry, peaches, peepal, spinach, tea</td>
</tr>
<tr>
<td></td>
<td>Kaempferol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myricetin</td>
<td></td>
</tr>
<tr>
<td>Flavones</td>
<td>Apigenin, Luteolin,</td>
<td>citrus species, lettuce, lemongrass rooibos</td>
</tr>
<tr>
<td></td>
<td>Oriental, Nobiletin</td>
<td></td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td>(+)-catechin</td>
<td>apples, apricots, barley, black berries, cherries, Camellia sinensis, dark chocolate, grapes, peaches, pistachios, red wine, peanuts, nectarines</td>
</tr>
<tr>
<td></td>
<td>(-)-epicatechin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proanthocyanidins</td>
<td></td>
</tr>
<tr>
<td>Flavanones &amp; Chalcones</td>
<td>Narigenin, Eriodicytol</td>
<td>bananas, grape fruits, lemon, kiwi, orange juice</td>
</tr>
<tr>
<td></td>
<td>Hesperetin, Aspalathin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nothofagin</td>
<td>rooibos, tomato</td>
</tr>
<tr>
<td>Isoflavanones</td>
<td>Genistein, Daidzein</td>
<td>black beans, green pear, kudzu,</td>
</tr>
<tr>
<td></td>
<td>Glycitein</td>
<td>red glover, soya, perennial wine</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Cyanidin, Delphinidin</td>
<td>berries, cherries, Lollo Rosso, lettuce, peaches, plums, red grapes, red onion</td>
</tr>
<tr>
<td></td>
<td>Malvidin, Pelargonidin</td>
<td></td>
</tr>
</tbody>
</table>

Source: Jaganath & Crozier (2010)

### 2.4.2.4 Absorption and metabolism of polyphenols

Polyphenols are now known to be absorbed along the gut but the exact mechanism is not established. Both passive (diffusion) and active (sodium dependent glucose transporter 1) mechanisms have been suggested. Polyphenols occur as esters or bound to sugar moieties.
(glycosides) and must first, with the exception of anthocyanins, be hydrolysed for absorption to occur. Anthocyanins’ positive charge is thought to facilitate their direct absorption (Walle, 2004). The hydrolysis occurs chiefly in the colon and, to a much less extent, in the small intestines and the oral cavity (Saura-Calixto et al., 2007).

The absorption of polyphenols is influenced by several factors including the molecular weight and the extent and type of esterification. Proanthocyanidins in fruits and cocoa, for example, are hardly absorbed in the gut due to their large molecular sizes while the extent of catechin absorption decreases with increasing esterification with gallic acid (Holt et al., 2002; Scalbert et al., 2002). The absorption of quercetin from onion is four fold greater than that from apples which highlights the matrix effect on absorption (Hollman et al., 1997). Some studies have reported the negating matrix effect of milk while others have refuted such claims asserting that the observed influence may depend on the assay method used to estimate the antioxidant effect (Arts et al., 2002; Roura et al., 2008; Sharma et al., 2008).

Once absorbed, polyphenols are bound to albumin and transported to the liver where they are extensively metabolized after conjugation (glucuronidation, sulphation or methylation) or catabolized to smaller phenolic compounds before excretion in urine or bile (Cabrera et al., 2006; Mejia et al., 2009). These processes seem to vary with the type of polyphenols; the flavonol EGCG, for example, is more likely to go through biliary excretion with a plasma half-life of about 2-3 hours, while the flavanones, naringenin and hesperetin are mainly excreted via urine (Mejia et al., 2009; Krogholm et al., 2010).

### 2.4.2.5 Mechanism of action of polyphenols

Table 2.7 on page 29, summarizes the proposed mechanisms via which polyphenols function as antioxidants (Nijveldt et al., 2001; Rice-Evans & Parker, 2003; Manach, 2004; Patel, 2008). They include:

1. **direct radical scavenging of free radicals.** The hydroxyl group in the B ring allow polyphenols to react with superoxide or with nitric-oxide resulting in a less reactive radical or its inactivation;
2. **interference with inducible nitric-oxide synthase activity;**
3. **inhibition of xanthine oxidase activity;** flavonols pathway is a xanthine oxidase, and xantine oxidase and xantine dehydrogenase play a cardinal role in a transformation of xanthine to uric acid;
(iv) leukocyte immobilization; The physiologic response to inflammatory process or ischemia is to release endothelium mediator factors which attract leukocytes to the vessels wall followed by neutrophil degranulation resulting in release of oxidants and inflammatory mediators leading to tissue damage. Polyphenols decrease the number of leukocytes immobilized and inhibit neutrophil degranulation by modulating receptor-directed Ca\(^{2+}\) channel;

(v) interaction with enzymes.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Mechanism of action</th>
<th>Involved powerful flavonoids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant</td>
<td>inhibit oxidation and protect cells from reactive species damages</td>
<td>Epicatechin and rutin, quercetin, kaempferol, myricetin</td>
<td>Cabrera et al., (2006)</td>
</tr>
<tr>
<td>Anti-atherosclerotic activities</td>
<td>suppress oxidation of amino acid bound to LDL</td>
<td>Quercetin, rutin, epicatechin</td>
<td>Gross (2004); Ou et al., (2010)</td>
</tr>
<tr>
<td>Anti-platelet and anti thrombogenic inhibition of thromboxane A(_2)</td>
<td>Quercetin, kaempferol, myricetin</td>
<td>Jochmann et al., (2008)</td>
<td></td>
</tr>
<tr>
<td>Antiviral effect</td>
<td>flavonoids inhibit envelope formation</td>
<td>Quercetin, flavonoids in glycone form</td>
<td>Choi et al., (2009)</td>
</tr>
<tr>
<td>Anti-inflammatory effect</td>
<td>inhibit neutrophils degranulation and inhibit cyclooxygenase parthway which reduce release of arachidonic acid</td>
<td>Quercetin</td>
<td>Baba et al., (2009)</td>
</tr>
<tr>
<td>Antidiarrheal effects</td>
<td>molecular mechanisms unclear.</td>
<td>Cyanidine-3-O-glucoside, myricetin, quercetin, deoxyhexoside</td>
<td>Yao et al., (2011)</td>
</tr>
</tbody>
</table>

2.4.3 *Camellia sinensis* (Chinese green tea)

In recent years, green tea has become a focus of attention due to the numerous health benefits attributed to its consumption. The plant, *Camellia sinensis*, is believed to have been
originally discovered and grown in Southeast Asia and its consumption can be traced back to 2737 B.C in China where it was used as tea for the first time (Cabrera et al., 2006; Zhou et al., 2010). It is now one of the most popular beverages worldwide and is consumed in different parts of the world, depending on the processing method, as unfermented (green tea), semi-fermented (oolong tea) and fermented (black and red tea) (Cabrera et al., 2006).

2.4.3.1 Biochemical composition of Camellia sinensis

The chemical composition of C. sinensis is complex and ranges from organic substances to minerals (Table 2.8). These include proteins, amino acids, fiber, lipids, pigments (chlorophyll, carotenoids), carbohydrates and phenolic compounds (Chacko et al., 2010; Mohanpuria et al., 2010). Polyphenols may account for up to 30% of the dry weight and include flavanols, flavanones, flavonols, and phenolic acids. The main class of polyphenols is flavonols (catechins) which are more plentiful in green tea than in black or oolong tea, as these compounds are substantially influenced by the degree of processing, growth conditions, variety and origin of the plant as well as the manner in which the final infusion is prepared (e.g., amount of the tea used, brew time, temperature) (Chacko et al., 2010). There are four kinds of catechins in green tea: epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin gallate (EGCG) (Mohanpuria et al., 2010).

Table 2.8: Composition (%) of green and black teas

<table>
<thead>
<tr>
<th>Compound</th>
<th>Green tea</th>
<th>Black tea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Amino acid</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Fiber</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Other carbohydrates</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Lipids</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Pigments</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Minerals</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Oxidized phenolic compounds</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

From Chacko et al., 2010
2.4.3.2 Bioavailability and bioactivity of *Camellia sinensis*

The potential health effects of green tea depend not only on the amount of tea consumed but also on the bioavailability of its bioactive constituents. The four main green tea catechins have been detected in rat portal vein after oral ingestion indicating that tea catechins are absorbed from the intestine (Okushio *et al.*, 1996). In rats given 0.6% green tea polyphenols (GTP) in their drinking water for 4 weeks, plasma levels of EGCG were much lower than those of EGC or EC. But in mice given the same extract, plasma levels of EGCG were much higher than those of EGC and EC. These findings suggest that there may be species differences in the bioavailability of EGCG compared to the other catechins (Kim *et al.*, 2000).

In humans, catechin levels in plasma reach their peak 2 to 4 h after ingestion but there is little published data regarding their distribution in tissues. In the study mentioned earlier where rats were given 0.6% GTP, significant levels of EGC and EC were found in the esophagus, large intestine, kidney, bladder, lung, and prostate. In contrast, EGC and EC levels were comparatively low in liver, spleen, heart, and thyroid; EGCG levels were higher in the esophagus and large intestine, but lower in other organs, perhaps as a result of poor systemic absorption of EGCG. Studies in rats have shown that EGCG is mainly excreted through the bile, while EGC and EC are excreted via urine and bile. Evaluation of the actual bioavailability of metabolites in tissues may be more crucial than simply evaluating their levels in plasma, but data are still very limited even in animals. Although many health benefits have been attributed to green tea consumption since ancient times, scientific investigations on this beverage and its constituents have only intensified in the last three decades (McKay & Blumberg, 2007). Many studies have indicated GTP possesses antioxidant, antimutagenic, antidiabetic, antibacterial, anti-inflammatory, and hypocholesterolemic properties (Cabrera *et al.*, 2006).

Antioxidant activity is one of the key attributes of green tea. Green tea is rich in polyphenols as well as other antioxidant components such as minerals. These compounds may increase the tea’s antioxidant potential. Green tea contributes antioxidant activity *in vitro* by scavenging oxidant molecules and chelating transition metal ions thus preventing their participation in Fenton and Haber-Weiss reactions (McKay & Blumberg, 2007). They may also function indirectly as antioxidants via several mechanisms such as inhibiting redoxsensitive transcription factors, inhibiting of ‘pro-oxidant’ enzymes, such as inducible nitric oxide synthase, and finally by induction of antioxidant enzymes, such as SOD (Cabrera *et al.*, 2006). In various human studies, intake of green tea has been associated with increased antioxidant status and reduced oxidative damage in biomolecules. These reports suggest that green tea contribute to defenses against oxidative damage (Wu & Wei, 2002).
Animal models and cell culture studies support a protective role for green tea against cancer (Chung et al., 2003). There are reports that green tea inhibit carcinogenesis in various organs including skin, lung, oral cavity, esophagus, stomach, liver, kidney and prostate (Bianchi et al., 2000; Lambert & Yang, 2003). The antimutagenic effects of green tea are hypothesized to be mediated by several factors including its antioxidant action, the specific induction of detoxifying enzymes, its molecular regulatory functions on cellular signaling, selective improvement in the function of the intestinal bacteria flora and breaking the carcinogen-DNA links that lead to mutations (Cabrera et al., 2006). In women, green tea drinkers showed a significantly reduced risk of breast and ovarian cancers (Zhang et al., 2002; Wu et al., 2003). Similarly in men Jian et al. (2004) showed that the risk for prostate cancer declined with increasing frequency, duration and quantity of green tea consumption suggesting that the tea is protective against prostate cancer. Not all studies have been supportive of green tea’s anti-cancer ability. Hoshiyama et al. (2004) and Koizumi et al. (2003) found no association between green tea consumption and the risk of stomach cancer and instead suggested implicated other factors such as age, smoking, socioeconomic status, Helicobacter pylori infection, history of pectic ulcer, and family history of stomach cancer along with certain dietary components. Clearly, more work is needed to clarify the anti-mutagenic properties of green tea.

An inverse association of green tea with CVD has been shown in many epidemiological studies but a mechanistic explanation for this observation has been firmly established. One of the most widely considered explanation is that green tea may affect the cardiovascular function by reducing the oxidation of LDL-cholesterol and related events such as foam cell formation, endothelial cytotoxicity and induction of proinflammatory cytokines (McKay & Blumberg, 2007). Others suggested mechanisms include increasing HDL-cholesterol levels as well as reduction of plasma cholesterol levels and the rate of cholesterol absorption by interfering with the micellar solubilization of cholesterol in the digestive tract (Raederstoff et al., 2003). Green tea has been reported to have many other health roles including antibacterial and antiviral activity, increasing bone mineral density (hence protecting against fractures), anti-fibrotic properties on the skin and on the arteries (which is closely related to the progression of liver fibrosis in chronic liver diseases), anti-inflammatory properties as well as the preservation of the lens’s antioxidant system thus preventing progression of lens opacity (Cabrera et al., 2006).
2.4.3.3 Toxicity

Green tea is generally considered a safe, non-toxic beverage and consumption is usually without side effects. Several isolated cases, however, have been reported, which may be related to caffeine and aluminum content of green tea and the interaction between polyphenols and iron (Mennen et al., 2005; Mohanpuria et al., 2010). The average cup of green tea contains from 10-50 mg of caffeine, and over-consumption may cause irritability, insomnia, nervousness, and tachycardia. A study investigating the toxicity of green tea extracts and their constituents in rat hepatocytes in primary culture associated high level of green tea extract with hepatotoxicity attributed to low bioavailability of EGCG (Schmidt et al., 2005). Ingestion of green tea on an empty stomach has also been associated with increased activity of liver enzymes (Sarma et al., 2008).

2.4.4 Aspalathus linearis (Rooibos herbal tea)

Rooibos (Aspalathus linearis also known as a Aspalathus contaminata, Aspalathus corymbosus, Borbonia pinifolia and Psoralea linearis) (Mckay & Blumberg, 2007) is a South Africa indigenous shrub native to the Cedarberg Mountain and neighbouring areas in the Western Cape province. It is characterized by a strong two metre-long taproot, reddish-brown branches and bright green, needle-shaped leaves (Mckay & Blumberg, 2007; Van Nierkerk & Viljoen, 2008). The native Khoi-khoi people were the first to use it as a beverage three centuries ago due to the agreeable taste and aroma. Today, rooibos is marketed globally for its suggested health benefits (Joubert et al., 2008; Van Nierkerk & Viljoen, 2008; Marnewick, 2009). Two types of the herbal tea are produced depending on the processing method chosen, fermented (red) and unfermented (green) rooibos. Fermented rooibos is made by cutting the stems and leaves into fine pieces, wetting and piling them in heaps for 12-24 hrs to trigger chemical oxidation during which rooibos attains its characteristic aroma and red colour. The oxidation/fermentation process may lead to a loss of important components such as aspalathin and nothofagin which impacts on the overall antioxidant capacity (Joubert, 1996; Mckay & Blumberg, 2007). “Green” or unfermented rooibos is made by immediately oven-drying the fresh harvest to prevent oxidation before cutting into pieces and sieving.
2.4.4.1 Biochemical composition of *Aspalathus linearis*

Several phenolic compounds have been isolated from rooibos including flavonols flavones and dihydrochalcones as well as several phenolic acids including protocatechuic, hydroxybenzoic, vanillic, coumaric, caffeic and ferulic acids (Joubert & Ferreira, 1996; Bramati *et al.*, 2003). Aspalathin, isoorientin, orientin and rutin are the most abundant polyphenols in both the fermented and unfermented type of the herbal tea as shown in table 2.9. It is clear from the table that processing has a definite impact on the levels of these polyphenolic compounds.

### Table 2.9: Phenolic profile of fermented and unfermented rooibos

<table>
<thead>
<tr>
<th>Compound</th>
<th>Traditional/fermented rooibos</th>
<th>Green/unfermented rooibos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g ± SD</td>
<td>mg/g ± SD</td>
</tr>
<tr>
<td>Chrysoeriol</td>
<td>0.022 ± 0.001</td>
<td>0.0079 ± 0.0004</td>
</tr>
<tr>
<td>Luteolin</td>
<td>0.029 ± 0.001</td>
<td>0.022 ± 0.002</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.107 ± 0.002</td>
<td>0.042 ± 0.006</td>
</tr>
<tr>
<td>Isovivexin</td>
<td>0.265 ± 0.002</td>
<td>0.659 ± 0.005</td>
</tr>
<tr>
<td>Vinitexin</td>
<td>0.330 ± 0.002</td>
<td>0.504 ± 0.002</td>
</tr>
<tr>
<td>Isoquercitrin and hyperoside</td>
<td>0.429 ± 0.002</td>
<td>0.326 ± 0.006</td>
</tr>
<tr>
<td>Isoorientin</td>
<td>0.833 ± 0.007</td>
<td>3.57 ± 0.18</td>
</tr>
<tr>
<td>Orientin</td>
<td>1.003 ± 0.010</td>
<td>2.336 ± 0.049</td>
</tr>
<tr>
<td>Rutin</td>
<td>1.269 ± 0.006</td>
<td>1.69 ± 0.14</td>
</tr>
<tr>
<td>Aspalathin</td>
<td>1.234 ± 0.010</td>
<td>49.92 ± 0.80</td>
</tr>
<tr>
<td>Nothofagin*</td>
<td>0.17</td>
<td>1.08</td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>5.521 ± 0.055</td>
<td>59.08 ± 0.59</td>
</tr>
</tbody>
</table>

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), S.D not given. Other values: adapted from Bramati *et al.* (2003).

2.4.4.2 Bioavailability and mechanism of action of *Aspalathus linearis*

Consumed in its native South Africa for many years, rooibos tea has in recent years become an increasingly popular health drink worldwide due to its low tannin content and caffeine free status; subsequently, there has been a parallel increase in studies to substantiate the health benefits attributed to this herbal beverage (Morton, 1983; Joubert *et al.*, 2008). Recent studies have shown rooibos tea to have anti-mutagenic, anti-carcinogenic, anti-inflammatory and antiviral properties all of which are mainly attributed to the anti-oxidative properties of its
polyphenols which have a strong ability to quench radicals generated in the water phase and confer high thermal stability against deep frying (Fukasawa et al., 2009).

The main polyphenol, aspalathin has only been found in rooibos. Similar to other phenolic compounds, aspalathin is absorbed in the gut and undergoes bacterial action in the intestines forming free aglycones. Recent studies investigating in vivo aspalathin metabolites following oral administration of rooibos, found methylated aspalathin in the urine, but not in plasma, 7 and 11 days after intake (Kreuz et al., 2008).

This finding was later corroborated by Stalmach and co-workers (2009) who demonstrated O-linked methyl sulphate, glucuronide metabolites, O-methyl-aspalathin-O-glucuronide (unfermented tea) and an eriodictyol-O-sulphate (following fermented tea) in human urine. The principal mechanism of biotransformation includes glucuronidation, sulfation or methylation of phenolic hydroxyl groups and the heterogeneity of metabolites may be due to the restriction in bioavailability of some rooibos compounds as a result of chemical variability (Bravo, 1998). More recently Breiter et al. (2011) have confirmed the bioavailability of rooibos compounds in humans and identified methylated aspalathin as the main excreted metabolite. In their study, seven metabolites of aspalathin and nothofagin were identified in urine samples, as well as intact aspalathin and nothofagin. Sulphated, glucuronidated, methylated, both glucuronidated and methylated aspalathin, and glucuronidates of the aglycones of aspalathin and nothofagin were detected.

Similar to green tea, the fermentation process lowers the antioxidant potency of rooibos due to the transformation of the two main 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). Subsequently, higher antioxidant activities have been shown in unfermented rooibos and green Camellia sinensis teas compared to their fermented equivalents (Von Gadow et al., 1997a, Pazdzioch-Czochra & Widenska, 2002; Schulz et al., 2003). Using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical test system, the in vitro scavenging effectiveness of several key rooibos constituents was shown (Von Gadow et al., 1997b; Joubert et al., 2004; Snijman, 2007). These included quercetin, aspalathin, orientin, luteolin and isoquercitrin. Notably, aspalathin was more effective than α-tocopherol, which is one of the most potent and important lipophilic antioxidants in the body.

A possible anti-atherosclerotic role for rooibos was suggested by the demonstration of a protective effect of rooibos against lipid peroxidation using a mouse liver microsomal model (Marnewick et al., 2005). Both “green” and fermented rooibos decreased MDA formation.
substantially (91% and 65%, respectively) and also significantly increased the ratio of reduced to oxidized glutathione (GSH: GSSG). Lipid peroxidation in mice liver was also inhibited by purified orientin (Uma Devi et al., 2000) while rutin appeared to maintain the strength of capillary walls (MacLennan et al., 1994). These findings were recently confirmed in humans when Marnewick and colleagues (2011) reported that the consumption of fermented rooibos significantly improved the lipid profile as well as redox status in adults at risk for developing CHD. In the study, a significant increase in plasma total polyphenols levels and GSH:GSSG ratio and a significant decrease in plasma markers of lipid peroxidation after a daily consumption of six cups of rooibos for six weeks were observed. Recently, Villano et al. (2010) assessed the effect of rooibos herbal tea intake on total antioxidant capacity, lipid triacylglycerols, and cholesterol and glycaemia plasma levels in humans. Although they did not find any change on the lipid profile, they were among the first to show that both fermented and unfermented rooibos teas are able to boost plasma antioxidant defences in humans.

Interestingly, a 10-week intake of rooibos by male Fisher 344 rats had no impact on the total antioxidant capacity in the liver, but it significantly increased the GSH: GSSG ratio and enhanced the activities of two xenobiotic metabolizing enzymes, GST-alpha and Uridine Diphosphate glucuronyltransferase (UDP-GT) (Marnewick et al., 2003). These effects were not shared by green and black teas. Later, histological analysis of livers from rats exposed to the potent pro-oxidant, carbon tetra chloride (CCl₄), showed that rooibos intake diminished CCl₄–induced hepatic steatosis and cirrhosis and decreased the production of hepatic MDA, triacylglycerols and cholesterol as well as plasma aminotransferase, alkaline phosphatase and bilirubin (Uličná, et al., 2003). In one of the first reports mentioning the effect of rooibos on glutathione status and lipid peroxidation in humans, Nikolova et al. (2007) investigated the effect of rooibos on the antioxidant status in men occupationally exposed to lead. Consumption of rooibos for 8-weeks was reported to significantly enhance plasma GSH levels by 48% and to reduce LPO measured as MDA. In a recent study, Persson et al. (2010) added another mechanism by which rooibos may benefit cardiovascular health. In the study where healthy adults consumed 400 mL of various beverages, rooibos herbal tea was found to inhibit angiotensin converting enzyme (ACE). Inhibitors of ACE are used as treatment for hypertension and congestive heart failure as they decrease tension of blood vessels and blood volume. An imbalance in adrenocorticosteroids is associated with numerous CVD risk conditions including hypertension, metabolic syndrome, insulin resistance and type 2 diabetes. In a very recent study, the inhibitory effects of rooibos, aspalathin and nothofagin on adrenal steroidogenesis were investigated (Schloms et al., 2012). Under both basal and stimulated conditions, the total amount of steroids produced in
H295R cells, a human adrenal carcinoma cell line was significantly decreased in the presence of rooibos, aspalathin and nothofagin. These results suggest that rooibos and its two main constituents may modulate adrenal steroids biosynthesis thus possibly contributing to the alleviation of negative effects resulting from an elevation of these hormones.

The anticancer activities of rooibos have been well investigated and the beverage has been shown 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). An assessment of the mutagenic activity of the major flavonoids of rooibos reported luteolin and chrysoeriol as the most potent antimutagens of rooibos (Snijman et al., 2007). Luteolin and quercetin have also been linked to in vitro induced-apoptosis and reduced proliferation of thyroid and colon cancers respectively although it is doubtful whether these activities can be extrapolated in humans due to the low amounts of these two flavonols in rooibos (Yin et al., 1999; Mori et al., 2001). Rooibos extracts were shown to inhibit tumour promotion in mouse skin and to protect against fumonisin B1 promotion in rat liver model utilizing diethylnitrosamine as the cancer initiator (Marnewick et al., 2005; Marnewick et al., 2009). More recently the chemoprotective potential of rooibos was evaluated in esophageal cancer and the herbal tea was shown to reduce the mean total papilloma size by 87%. The reduction correlated with the total polyphenol (r = 0.79; P < 0.02) and fermentation of herbal beverages reduced the inhibitory effects on papilloma development associated with a reduction in the polyphenolic constituents (Sissing et al., 2011).

Rooibos has also been cited as an anti-hypertensive, anti-stress, anti diabetic, immune stimulant, laxative and sedative agent although these claims are in various stages of substantiation (Kawano et al., 2009; Villano et al., 2010; Marnewick et al., 2011; Sissing et al., 2011; Schloms et al., 2012).

2.4.4.3 Toxicity

Although rooibos research has intensified in the last decade, most of the studies are in vitro or animal models in design. A single case reports Salmonella contamination from rooibos which was nonetheless suggested to be from lizard origin (Swanepoel, 1987). Generally, the plant appears to have a low toxicity profile, and the herbal tea is a good option for those who wish to drink a mild, non-stimulating or sedating tea.
Data from animal and human studies attest to the plant’s safety (Marnewick et al., 2003; Marnewick et al., 2004; Marnewick et al., 2005; Khan & Gilani, 2006; Kreuz et al., 2008; Marnewick et al., 2011). It should be noted, however, that these studies did not look at the possible interaction with drugs likely to produce hepatotoxicity or nephrotoxicity which is a potential scenario among patients of chronic or infectious diseases. An isolated but puzzling case of hepatotoxicity was recently reported in a 42-year-old woman with Waldenstrom’s macroglobulinemia, a low-grade B-cell malignancy (Sinisalo et al., 2010). For maintenance, she was on rituximab every 3 months and daily prednisolone and she was also taking cotrimoxazole for pneumonia, alendronic acid and calcium carbonate with vitamin D as prophylaxis for osteoporosis and daily supplements of potassium chloride. Two weeks prior to a scheduled clinic visit she started drinking rooibos one litre per day. Routine evaluation revealed grossly elevated liver enzymes which were restored by withdrawing rooibos and the treatments. According to the authors, it is unknown whether this side effect was due to the combination with one or several drugs or it was a reflection of the patient’s genetically predisposed vulnerability to the drug or herbal tea or even that the rooibos used by the patient was contaminated by some hepatotoxic compound, such as a pyrrolizidine alkaloid (Sinisalo et al., 2010). This case clearly illustrates the need for more human research studies on rooibos, and indeed all herbs, to firmly ascertain their toxicity profiles.

2.4.5 Combination of antioxidant supplements

The diet plays important roles in the pathophysiology of numerous diseases and there have been many attempts to understand the precise mechanisms involved. Effective and informative nutritional studies are difficult to conduct due to the qualitative and quantitative complexity in food and food products, heterogeneity of study population as well as differences in individuals’ dietary habits (Graham, 1992; Cabrera et al., 2006; Dewettinck et al., 2008). In addition, it is often very difficult to identify the specific compound responsible for any benefit seen in a given pathophysiological condition. The isolation of the individual compounds may facilitate the assessment of the direct cause- and dose-effect relationship; however, the absorption and bioavailability of the isolated compounds may be different in situ. Indeed, the metabolites that reach tissues and cells are usually chemically and/or functionally distinct from the dietary form (Kroom et al., 2004). The situation can be further complicated if, as is often the case, the apparent benefit result from the synergistic interaction between two or more compounds. Two well documented synergistic interactions involve α–tocopherol and ascorbic acid as well as α–tocopherol and flavonoids (Pedrielli &
Skibsted, 2002). The tocopheryl radical (formed when vitamin E reacts with radicals) accepts a hydrogen ion from vitamin C or flavonoids and in the process is regenerated to the active vitamin E (Kadoma et al., 2006; Jacobs et al., 2010). Tantcheva et al., (2003) has reported an improved protection against influenza virus infection when vitamins C and E are administered as a combination in experimental animals.

Various antioxidant combinations have been formulated and studied in different situations and populations. One study investigated the efficacy of caffeine and catechin-rich green tea extract (GTE) in energy expenditure (EE) and fat oxidation in humans. The supplementation resulted in a significant increase in EE and 24-hours urinary nor-epinephrine excretion as well as a decrease in respiratory quotient (RQ). When caffeine equivalent to the amount in the supplement was given alone, there was no change in the EE or RQ suggesting an interaction between the two components (Dulloo et al., 1999). In a more recent study, the anti-inflammatory effect of a dietary mix of resveratrol, GTE, tomato extract, α-tocopherol, vitamin C and n-3 (omega-3) polyunsaturated fatty acid was investigated in overweight men. Plasma levels of adiponectin, an anti-inflammatory and anti-diabetic hormone secreted by adipocytes were increased by 7% while C-reactive protein was unaffected. Oxidative stress biomarkers (isoprostanes and uric acid) were decreased and endothelial function was improved (Bekker et al., 2010). Similarly, in a double-blinded trial enrolling 1078 HIV-infected pregnant women, multivitamin supplements were able to delay the progression of the infection as well as the mortality (Fawzi et al., 2004).

Collectively, these data suggest an increased effectiveness of combined antioxidants. Such data has led to the steady rise in the use of dietary supplements in the modern, health-conscious society. In the USA for example, over one-half of the adult population take one or more supplements everyday (Gahche et al., 2011). However, the toxicity of supplements and isolated compounds has always been a concern and results from several studies caution against their excessive usage. Vitamin C, for example, may lead to lung cancer in smokers and people with asbestosis at intakes above 33 000 UI/day or it may lead to yellowing of the skin, diarrhoea, arthralgia and gastric upset at intake of 2 000 mg/day (Wooltorton, 2003). The hypothesis that moderate levels of free radicals induce an endogenous response which protects against exogenous radicals (and possibly other toxic compounds) and prolong the life span has recently been shown in Caenorhabditis elegans (Tapia, 2006; Schulz et al., 2007; Ristow & Zarse, 2010). The paradoxical implication here is that excessive intake of antioxidants may prevent life-extending and other health-promoting effects.
The supplements (refers to page 41 for composition) used in this study are new products thus there is no data available regarding their beneficial or harmful effects. Data regarding these supplements will be a useful output from this study and will compare the supplements against two relatively well investigated beverages.
3.1 Study design and ethical approval

This was an experimental intervention study organised in two phases; which included
1. quantification of polyphenols and antioxidant activity of the various beverages used in the study. The beverages included rooibos (Aspalathus linearis), Chinese green tea (Camellia sinensis) and two tea supplements,
2. assessment of the effects of the beverages and supplements on t-butyl hydroperoxide-induced-oxidative stress in the liver and kidney of male Wister rats.

Ethical approval was granted by the Faculty of Health & Wellness Sciences Research Ethics Committee, Cape Peninsula University of Technology (Project number: CPUT/HWS-REC 2010/A001, Appendix A) as an extension of the previously approved project which results are published in Awoniyi et al. (2011).

3.2 Preparation of beverages and supplements

Aqueous extracts of the various plant materials which included fermented rooibos (“oxidised”), “green” rooibos (“unoxidised”), Chinese green tea, were freshly prepared by adding 2 g of herbal tea to 100 ml of boiled tap water. The two commercial tea supplements (one containing a green tea extract and the other containing a rooibos extract) were crushed using a pestle and mortar, and prepared at a concentration of 2 g/100 mL. The concentration of the rooibos and Chinese green tea used in this study were identical to the ones used in previous studies (Marnewick et al., 2009; Sissing et al., 2011). The rooibos and Chinese green teas were allowed to stand for 30 minutes at room temperature before filtering (Whatman N 4), cooling and serving it to the experimental animals. Aliquots of the freshly prepared rooibos, Camellia sinensis tea and supplements were also stored at -20°C for quantitative antioxidant analyses. Fermented and “green” rooibos (superior grade) were a general gift from Rooibos Ltd ( Clanwilliam, South Africa), while the Chinese green tea as well as the two commercial tea supplements were purchased from a local drug store (Cape Town, South Africa). Each green tea capsule contained 100 mg of epigallocatechin gallate (EGCG), while each rooibos tablet supplement contained: 175 mg of 20% aspalathin-rich extract, 500 µg vitamin A, 150 mg vitamin C, 5 mg Vitamin E and 25 µg selenium.
3.3 Experimental animals and diet

Sixty male Wistar rats obtained from the Animal Unit of the University of Cape Town (South Africa) weighing 120-150 g were randomly allocated to one of the six study groups. Each group consisted of ten rats and were maintained as described by Awoniyi (2010). Briefly, animals were housed in Perspex houses with stainless steel wire-bottomed cages in a controlled environment of 24-25°C with 12 hours light-dark cycles and 50% humidity for 1 week. The rats had free access to diet (standard rat chow) and to the various beverages respectively (fermented and “green” rooibos, and Chinese green tea and tea supplement) and tap water for the control group for 10 weeks as a sole source of drinking fluid. Body weights were monitored twice a week till the end of the experiment. Oxidative stress was induced in all intervention and positive control rats via an intraperitoneal (i.p) injection of 30 μM of t-butyl hydperoxide per 100 g body weight in the final two weeks of the study. At the end of the intervention, the animals (non-fasted) were sacrificed using pentobarbital anaesthesia injected i.p. at 0.4 mL/kg body weight. Thereafter, livers and kidneys were excised and immediately snap frozen in nitrogen and then kept at -80°C. Prior to analysis, the various organs were minced, randomly weighed and homogenised according to the specific assay protocol described below.

3.4 Analytical methods

3.4.1 Quantification of total polyphenol and flavonoid content of the aqueous herbal tea and tea extracts and supplements

3.4.1.1 Total polyphenol content

The most frequently used method for total polyphenol determination is the Folin-Ciocalteu (FC) assay which is based on the reaction of the aromatic group in polyphenols with Folin Ciocalteu reagent (FCR) yielding a blue-coloured product that maximally absorbs at 765 nm (Waterhouse, 2002). In alkaline conditions, phenol groups ionize completely to their phenolate form which can be readily oxidized by the FCR changing colour from yellow to blue. This colour change can be monitored with a spectrophotometer at 765 nm and quantified using a standard. Several modifications have been made to the original Singleton and Rossi (1965) method. This study used the method described by Waterhouse (2002). Briefly, the frozen tea beverage and supplements samples were thawed and then diluted ten-fold with distilled water. An 800 mg/L gallic acid stock standard solution was prepared by dissolving 0.040 g of gallic acid (Sigma, SA) in 50 mL of 10% ethanol. This was used to
prepare the standard series of 0, 20, 50, 100, 250 µg/L, and 500 µg/L using distilled water as a diluent. Folin working solution was prepared by diluting 1 mL of FCR reagent (Merck, SA) with 9 mL of distilled water. The reaction was done in a clear 96 well microplate, and the mixture in each microplate well consisted of 25 µL of standard or sample, 125 µL FCR and 100 µL of Na₂CO₃ (7.5%) added after 5 minutes. The microplate was then incubated for 2 hours at room temperature before reading it in a Multiskan spectrum (Thermo Electron Corporation, USA) set at 25°C and 765 nm. Data processing and calculations were done on Microsoft® Excel 2003 based on a calibration curve plotted using the standard series and absorbance readings. Values of samples were calculated using the regression equation (Y=mX+C) between gallic acid concentration and absorbance, where X=unknown sample concentration; m = slope, Y = absorbance of sample and C = Y-intercept. Results are expressed as mg gallic acid equivalents per litre of beverage or supplement (mg GAE/L).

3.4.1.2 Flavanol content

The flavanols or flavan-3-ols are the most abundant family of bioactive compound present in wine, green tea, red grapes, apples and nuts (Rice-Evans & Packer, 2003; Jaganath & Croizier, 2010). In this study, they were estimated using the method described by Treutter (1989) which is based on the reaction of 4-dimethylaminocinnamaldehyde (DMACA) with flavanols to form a characteristic blue colour that is measured at 640 nm. Hydrochloric acid 32% (25ml) and absolute methanol 75% (HCl-MeOH), both from, Merck, SA, were used (1:4); to prepare the DMACA solution 0.015 g of DMACA in 30 mL of HCl-MeOH solution. The catechin standard was prepared by dissolving 0.0145 g of catechin hydrate in 50 mL MeOH solution and then used as a stock solution to prepare the standard series (0, 1, 2, 5, 10 µg/L and 20µg/L). The various beverage samples were thawed and diluted ten-fold for the assay which was done in triplicate. Each clear microplate well contained 50 µL of sample/standard and 250 µL of DMACA solution and the plate was then incubated for 30 minutes at room temperature before taking the reading in the Multiskan spectrum (Thermo Electron Corporation, USA) set at 25 °C and 640 nm. Data capturing and calculations were done on Microsoft® Excel 2003 based on a calibration curve plotted using the standard series and absorbance readings. Values of samples were calculated using the regression equation (Y=mX+C) between catechin concentration and absorbance, where X = unknown sample concentration; m =slope, Y = absorbance of sample and C = Y-intercept. Results are expressed as mg catechin equivalents per litre of beverage or supplement (mg CE/L).
3.4.1.3 Flavonol content

The estimation of the flavonol content in this study was done by the method described by Mazza & co-workers (1999), which exploit the reaction of HCl with flavonols using quercetin as a standard. Absolute ethanol (Merck, SA) was diluted to make 10% and 95% ethanol solutions. Two different HCl concentrations (2% with distilled water and 0.1% with 95% ethanol) were prepared. The standard series of 0, 5, 10, 20, 40, and 80 µg/L were freshly prepared from stoke solution containing 4 mg quercetin (Sigma-Aldrich, SA) dissolved in 50 mL 95% ethanol. Beverage samples were diluted ten-fold with distilled water. In designated wells of a clear 96 well microplate, 12.5 µL of the quercetin standard series and/or diluted beverages were added to each well in triplicate, followed by 12.5 µL of 0.1% HCl in 95% ethanol. Thereafter, 225 µL of 2% HCl was added and the plate incubated for 30 minutes at room temperature before measuring the absorbance at 360 nm (25°C) on Multiskan spectrum plate reader (Thermo Electron Corporation, USA). Data capturing and calculations were done on Microsoft® Excel 2003 based on a calibration curve plotted using the standard series and absorbance readings. Values of samples were calculated using the regression equation \( Y = mX + C \) between quercetin concentration and absorbance, where \( X = \) quercetin concentration; \( m = \) slope, \( Y = \) absorbance and \( C = \) unknown concentration. Results are expressed as mg quercetin equivalents per litre of beverage or supplement (mg QE/L).

3.4.2 Determination of the antioxidant capacity of the various tea and supplement samples

The most frequently used methods to assess antioxidant capacity include ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC).

3.4.2.1 Ferric reducing antioxidant power (FRAP) determination

This assay was first described by Benzie & Strain in 1996 and assesses the ability of a sample to reduce the 2,4,6-tri [2-pyridyl]-s-triazine (Fe\(^{3+}\)) to the ferrous (Fe\(^{2+}\)-TPTZ complex) state at low pH forming an intense blue-coloured complex with optimal absorbance at 593 nm. The reaction is non-specific and any half-reaction with less-positive redox potential under the reaction conditions than the Fe\(^{3+}/Fe^{2+}\)-TPTZ half-reaction, will drive Fe\(^{3+}\) reduction (Benzi & Strain, 1996). Nevertheless, it is a simple measurement that gives fast, reproducible results with plasma or tissue samples. Changes in absorbance are directly
related to the reducing power of the electron-donating antioxidants present in the reaction mixture. In the present study, a ten-fold dilution of the thawed beverage and supplement samples was used, while 300 mg of the various tissues was homogenised (high-speed blender from OMNI International TH, SA) with 1.2 mL of freshly prepared (75 mM phosphate buffer, pH 7.0) and centrifuged (Eppendorf 5810R, Eppendorf, Germany) for 30 minutes, 4000 rpm (4°C). The buffer was constituted using sodium acetate and glacial acetic acid (Merck, SA). To prepare the stock standard solution, 0.0088 g ascorbic acid (Sigma Aldrich, SA) was dissolved in 50 mL distilled water and was used to prepare the standard series (0, 50, 100, 200, 500, 1000 µM) using distilled water as the diluent. The FRAP reagent was prepared by mixing 30 mL of 300 mM acetate buffer, 3 mL of TPTZ solution (10 mM in 40 mM of HCl), 3 mL of ferric chloride (Merck, SA) solution (20 mM) and 6.6 mL of distilled water to yield a straw coloured solution. Beverages, supplement and tissue samples were diluted ten-fold. Each reaction well in a clear 96 well plate consisted of 10 µL of standard/sample and 300 µL of FRAP reagent. The assay was done in triplicate. The plate was then incubated at 37°C for 30 min before reading in Multiskan spectrum (Thermo Electron Corporation, USA) set at 25°C and 593 nm. Data capture and calculations were done in Microsoft® Excel 2003 based on calibration curve plotted using the standard series. Values of samples were calculated using the regression equation (Y=mX+C) between ascorbic acid concentration and absorbance, where X = unknown sample concentration; m = slope, Y = absorbance of sample and C=Y-intercept. Results are expressed as a µmole ascorbic acid equivalent per L of beverage, supplement or mg of tissue.

3.4.2.2 Oxygen radical absorbance capacity (ORAC) determination

The ORAC assay was first described in 1989 by Glazer based on the discovery that fluorescence of phycoerythrin changes over time upon inhibition caused by peroxyl-radical attack. Cao & co-workers (1998) modified the method to include the advantageous attribute of driving the reaction to completion thus differing from Glazer’s method that only used “the flat period”. In 2001, the method was further modified by Ou et al. to replace phycoerythrin fluorescein as the fluorescent probe

Beverage and supplement samples were thawed and diluted twenty-fold (kidney ten-fold) with phosphate buffer (75 mM, pH 7.4), while tissue samples (300 mg) were homogenised (high-speed blender homogeniser from OMNI International TH, SA) in phosphate buffer (75mM, pH 7.0) (ratio of 1:4 g/ml) and then centrifuged (Eppendorf 5810R, Eppendorf, Germany) at 12 000 rpm, 10 min and 4°C, followed by deproteinization with 5% PCA (1:1)
and centrifugation at 14 000 rpm, 4°C for 15 min. Di-sodium hydrogen orthophosphate dehydrate and di-hydrogen orthophosphate-1-hydrate was obtained from Merck (SA), fluorescein (FI) sodium salt, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) and trolox were all obtained from Sigma-Aldrich (SA). A fluorescein stock solution was prepared by dissolving 0.00225 g of the salt in 50 mL of 75 mM of phosphate buffer to yield a 0.0011 M solution. Thereafter, 10 µL of this solution was added to 2 000 µL of phosphate buffer (75 mM, pH 7.4) and finally 240 µL from that solution was added to 15 mL of phosphate buffer to make the working fluorescein solution. A standard stock solution of trolox was prepared by dissolving 0.00625 g of trolox in 50 mL of phosphate buffer, aliquoted into 2 mL eppendorf tubes and then frozen at –40°C to be used throughout the assays. A fresh aliquot was used to prepare the standard series (5, 10, 15, 20, 25 µM) needed to generate the calibration curve. The AAPH used as a radical generator was prepared by dissolving 0.125 g of AAPH in 5 mL of phosphate buffer using a 15 mL screw cap conical tube. Beverages, supplement and tissue samples were diluted twenty-fold (kidney ten-fold). The assay was done in triplicate in a black 96-well microplate with each reaction well containing 12 µL of sample or standard, 138 µL of FI and 50 µL of AAPH solution. The plate was immediately read in a fluoroskan ascent (Thermo Electron Corporation, USA) reader at 37°C and programmed to record every 5 minutes for a period of 2 hours. Data capture and analyses were done in Microsoft® Excel 2003 considering standard curve obtained by plotting the different concentration of trolox standard and the area under the curve (AUC), and the value of sample was calculated using regression equation; results expressed as micromole trolox equivalent per millilitre of beverage or supplement (µmol TE/mL) or milligram or µmol TE/mg) tissue, respectively.

### 3.4.2.3 Trolox equivalent antioxidant capacity (TEAC) determination

The original version of this method was described by Miller et al. in 1993 and was based on the ability of antioxidant molecules to quench the long-lived 2-2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS**) radical, a blue–green chromophore with absorption at 734 nm. The addition of an antioxidant reduces the preformed radical cation to ABTS leading to its decolourization (Pellegrini et al., 2003). This has been criticized because the faster reacting antioxidants might also reduce the ferryl myoglobin radical and the added hydrogen peroxide could oxidize antioxidants before the measurement. To circumvent this limitation Re & co-workers (1999) improved the technique by mixing ABTS with potassium perssulphate such that the radical is generated directly in a stable form prior to the reaction with the antioxidant. This stable stock solution of ABTS** was produced by adding 88 µl of
140 mM potassium peroxodisulphate (Merck, SA) to an ABTS solution (0.0192 g dissolved in 5 mL distilled water) and allowing the mixture to stand in the dark at room temperature for 24 hours before use. Beverage and supplement samples were thawed at room temperature and a ten-fold dilution used. For the tissues, 300 mg was homogenised (high-speed blender from OMNI International TH, SA) in 1.2 mL of a freshly prepared phosphate buffer (75mM, pH 7.0) and centrifuged (Eppendorf 5810R, Eppendorf, Germany) for 30 minutes, 4000 rpm and 4°C. To prepare the trolox standard solution, 0.0125 g trolox was dissolved in 50 mL absolute ethanol and used as a stock solution to prepare a standard series (0, 50, 100, 150, 250, 500 µM) using absolute ethanol as a diluent. The reaction mixture consisted of 25 µL of each standard or sample (done in triplicate) and 300 µL of the ABTS mixture solution. The clear 96 well microplates were left to incubate at room temperature for 30 minutes before reading the absorbance in the Multiskan spectrum (Thermo Electron Corporation, USA) set up at 25°C and 734 nm. Data analyses was done in Microsoft® Excel 2003 based on a calibration curve plotted using standard series and the results expressed as µmole of trolox per millilitre beverage or supplement sample or mg of tissue sample.

3.4.3 Antioxidant enzymes determination

3.4.3.1 Catalase (CAT)

Catalase mediates the removal of H₂O₂ from the cell by catalyzing the conversion of two molecules of hydrogen peroxide to molecular oxygen and two molecules of water. The estimation of catalase activity was assessed spectrophotometrically by the modified method of Ellerby & Bredesen (2000) which is based on the reaction of catalase in the presence of an optimal concentration of H₂O₂. The reaction mixture consisted of freshly prepared phosphate buffer (50 mM Na-Pi, 0.5%, and Triton X-100, pH 7.5, both from Sigma, SA). Hydrogen peroxide stock solution was made by mixing 10 mL potassium dihydrogen orthophosphate (50 mM, pH 7.0) and 34 µL of 30 % hydrogen peroxide (Sigma, SA). Tissues sample were prepared by homogenising (high-speed blender from OMNI International TH, SA) 0.3 g of the sample in 2 mL of phosphate buffer. The homogenates were then centrifuged (Eppendorf 5810R, Eppendorf, Germany) at 15 000 g for 10 minutes at 4°C and the supernatant thereafter transferred to newly marked tubes and stored at -70°C. A clear 96-well plate was used and each reaction well contained 170 µL of phosphate buffer, 75 µL of hydrogen peroxide stock solution and 5 µL of sample (liver hundred-fold dilution; kidney twenty-fold dilution) and then read in a Multiskan spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation, USA) at 25°C and 240 nm for two
minutes. Data analysis was done in Microsoft® Excel 2007 and catalase activity in the sample was calculated based on the rate of decomposition of hydrogen peroxide which is proportional to the reduction of absorbance at 240 nm wavelength and results expressed as a H₂O₂ μmole/min/μg protein.

3.4.3.2 Glutathione peroxidase (GPx)

Using glutathione (GSH) as a reducing agent, GPx catalyzes the reduction of hydrogen peroxide and organic peroxides (R-OOH) to water and the corresponding stable alcohol thus inhibiting the formation of free radicals. The glutathione peroxidase activity was determined by the method of Flohé & Günzler (1984) by indirectly coupling the reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) produced upon the reduction of hydroperoxide by GPx, is recycled to its reduced state, GSH by GR with the addition of NADPH. The consumption of NADPH is directly proportional to the enzyme activity, which, in a sample corresponds to a decrease in optical density which can be monitored at 340 nm. The assay was freshly prepared using sodium phosphate (50 mM) and Triton X-100 (0.5%) at pH 7.5 (all from Sigma, SA). The GPx assay buffer was constituted with sodium phosphate buffer 50 mM and 1 mM EDTA, pH 7.0 (both from Sigma, SA). GSH solution (Boehringer Mannheim, Germany) was made up by dissolving 30.7 mg GSH per ml of distilled water; 187 U/mL glutathione reductase (Sigma, SA) where after 1.7 μL of this mixture was diluted in 998 μL of assay buffer for a final solution of 0.1U/mL. Subsequently, 12.5 mg/ml of β-NADPH was dissolved in phosphate buffer above described; hydrogen peroxide solution (1.5mM), from a 30% commercial solution; sodium azide 0.0065 g, 100 mM (Sigma, SA) was dissolved in 480 μL distilled water. Tissues were homogenized as described in the catalase assay above. The GPx assay was done in triplicate in a clear 96-well microplate and each reaction cocktail consisted of 210 μL buffer, 2.5 μL GSH, 2.5 μL NADPH, 2.5 μL sodium azide, 2.5 μL glutathione reductase and 2.5 μL of sample (twenty- and two-fold dilution for liver and kidney respectively). The plate was first read in the Multiskan spectrum (Multiskan, Thermo Electron Corporation, USA) 340 nm (25°C) for 3 minutes and a second reading after adding 50 μL hydrogen peroxide for 2 minutes. Data analysis was done on Microsoft® Excel 2003 and glutathione peroxidase activity in the sample calculated based on the rate of decrease in absorbance at 340 nm wavelength over the time using the molar extinction coefficient of glutathione peroxidase and corrected for pathlength.
3.4.3.3 Superoxide dismutase (SOD)

This metallo-enzyme converts the highly reactive superoxide to the less reactive peroxide. The rate of the reduction with O₂ is linear to the xanthine oxidase (XO) activity and is inhibited by SOD. The IC₅₀ (50% inhibition activity of SOD or SOD-like material) can be determined by colorimetric method. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the rate of cytochrome C reduction (Flohé & Günzler, 1984). The method used in this study was described by Ellerby & Bredesen (1984). The phosphate buffer and the preparation of tissues were as earlier described in the catalase assay. The SOD assay buffer was prepared using 50 mM NaH₂PO₄ and 1 mM EDTA, pH 7.0 (both from Sigma, SA). The 6-hydroxydopamine, 1.6 mM (6-HD) (Sigma, SA) was freshly prepared by dissolving 4g in 10 mL of water containing 50µl H₂O₂. The 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) (Sigma, SA) was prepared by dissolving 0.4 mg in 10 mL SOD assay buffer. The reaction mixture done in duplicate consisted of 6 µL of sample (liver hundred and fifty-fold dilution; kidney hundred-fold dilution), 85 µL DETAPAC and 15 µL 6-HD. The plate was incubated for 5 min then read in the Multiskan spectrum (Thermo Electron Corporation, USA) set at 25°C and 490 nm for 12 minutes at 30 seconds interval. Data analysis was done in Microsoft® Excel 2007 using a standard curve with SOD at different concentrations and results expressed as a SOD units/min/ µg protein.

3.4.4 Determination of non-enzymatic antioxidants

3.4.4.1 Glutathione (GSH)

In living cell, glutathione is an antioxidant eliminating a variety of toxins (Kohen & Nyska, 2002). Many studies have found GSH alteration in acute and chronic diseases and therefore a good predictive marker of oxidative stress. Despite being the most predominant intracellular antioxidant, its level varies among tissues. It oxidises rapidly at alkaline pH, thus levels may differ depending on the type of tissue, processing and storage of samples (Jones et al., 1998). This study used the spectrophotometric method described by Asensi et al., (1999). In this method, determination of GSH as well as glutathione disulfide (GSSG) levels include the glutathione reductase (GR) method that use 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) in a recycling assay. Glutathione disulfide is measured by reduction to GSH in the presence of GR and NADPH. The difference between the total and initial GSH values is used to calculate GSSG concentration in each sample. The thiol scavenging reagent, 1-
methyl-2-vinylpyridinium (M2VP) is used to scavenge GSH (masking agent for GSH) without interfering with other enzymatic assays (Ates et al., 2008). In the assay, the thiol reagent DTNB reacts with GSH to form the chromophore, 5-thionitrobenzoic acid (TNB) and a disulfide product, GS-TNB, which is reduced by GR in the presence of NADPH releasing the second TNB molecule and recycling the GSH. The level of GSSG initially formed in the mixture is converted to GSH, therefore measured as a total glutathione level (GSHt). The 50 mM phosphate buffer, pH 7.5, containing 1 mM ethylenetriaminetetraacetic acid (EDTA) used as diluent for all working solutions in the assay (buffer A) was prepared using sodium di-hydrogen orthophosphate mono hydrate, di-sodium hydrogen orthophosphate dehydrate and EDTA, all purchased from Merck (SA). The 0.3 mM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) purchased from Sigma–Aldrich (SA), was used as the chromogen while 1 mM NADPH used to initiate the reaction was purchased from Merck (SA). A GSH standard (Merck, SA) was used to make a 3 µM stock solution from which the standard series were prepared (0, 1.5 and 3.0 µM). To prepare the assay enzyme, 32 µl of GR (168U/mg, Merck, SA) was diluted with 1968 µL buffer A, while 30 mM M2VP solution in 0.1 N HCl was used as the scavenger.

Tissue samples were prepared by homogenising (higher spider blender from OMNI International TH, SA) 200 mg with 15% trichloro acetic acid (TCA) containing 1 mM EDTA (1:10), while 200 mg sample for GSSG was homogenised in 6% perchloric acid (PCA) containing 3 mM M2VP and 1 mM EDTA. The samples were then centrifuged (Eppendorf 5810R, Germany) at 15 000 g, 4°C for five minutes and the supernatant stored at -70°C. Assays were done in triplicate and each well of 96 microplate consisted of 50 µL of standard or sample (liver 300-fold dilution), 50 µL of DTNB and 50 µL of GR. This mixture was incubated at room temperature for five minutes and then 50 µL of NDPH was added to start the reaction. This was immediately read in a Multiskan spectrum (Thermo Electron Corporation, USA) set at 25°C and 412 nm for 5 minutes at 30 second intervals. Data analyses were done in Microsoft® Excel 2007 and GSH concentration was calculated using a 3-point calibration curve of GSH at different concentration, and results expressed as a GSH concentration activity µmole/ µg tissue.

3.4.5 Determination of lipid peroxidation (LPO)

The two markers of LPO used in this study were (i) conjugated dienes and (ii) MDA measured as TBARS.
3.4.5.1 Conjugated dienes (CDs) determination

Conjugated dienes result from the rearrangement of double bonds in lipid hydroperoxides, the primary products of lipid peroxidation and are therefore an important indicator of the LPO process (Kohen & Nyska, 2002). Several methods are available for their estimation; in this study, they were estimated using the method described by Recknagel & Glende (1984). Briefly, the minced tissue (liver and kidney) where defrost for homogenate preparation with homogeniser (higher spider blender from OMNI International TH, SA), which consisted in addition of (10% w/v) phosphate-buffered saline 0.1 M, pH 7.4. Thereafter, chloroform and methanol (2:1) (both obtained from Merck, SA) were added, vortexed and then centrifuged (Eppendorf 5810R, Germany) at 3000 rpm for ten minutes, 4°C for first extraction; the chloroform layer was removed and the pellet underwent second extraction to remove large particles to which final extra -aliquot was rapidly stored at -70°C until needed. Using oxygen-free nitrogen, thawed homogenate aliquots (25 µL) were then evaporated in reaction tubes in a light protected environment. This dried sample was dissolved in 400 µL cyclohexane (Merck, SA), 150 µL transferred onto a clear 96-well plate and the absorbance read at 234 nm against a cyclohexane blank in the Multiskan specturm (Thermo Electron Corporation, USA). All samples were done in duplicate and concentration of CDs formation was calculated based on the difference of absorbance of sample and blank at 234 nm wavelength, where appropriated factoring value was adjusted for a volume of 150 µL, expressed as µmol/mL of extracted tissue.

3.4.5.2 Thiobarbituric acid reactive substances (TBARS) determination

Thiobarbituric acid reactive substances (TBARS) reflect the production of malondialdehyde (MDA), an oxidation product of lipid oxidation (Hwang & Kim, 2007). The TBARS assay has gained popularity due to its low cost and relative ease of execution but it lacks specificity as other substances unrelated to LPO can also be detected in the analysis. The use of HPLC with fluorescence detection-based method was done in this study, due to its improved specificity. Samples were prepared as done in the CD determination described above, and the thawed aliquots (250 µL) were pipetted into 1.5 mL reaction tubes containing 50 µL, 6 M sodium hydroxide then incubated at 60°C for thirty minutes for protein hydrolysis. Thereafter, 125 µL of 35% perchloric acid was added to precipitate proteins and the mixture was spun at 4°C, 2800 rpm for ten min. Samples (100 µL) were pipetted into new reaction tubes with 250 µL 40 mM TBA and 750 µL 0.2 M orthophosphoric acid. This mixture was boiled at 100°C for 1 hour, cooled on ice and centrifuged (Eppendorf AG 5810R, Germany) at 14 000 rpm for
four minutes. The MDA standard was prepared by dissolving 25 µL 1,1,3,3, tetraethoxypropane (TEP) in 100 mL water to give a 1 mM stock solution. Working standards were prepared by mixing 1 mL TEP stock solution in 50 mL 1% sulfuric acid incubated for 2 hours at room temperature. The resulting MDA standard of 20 µmol/L was further diluted with 1% sulphuric acid for the final concentration of (0.5, 1, 2, 5, and 10 µM) to draw a standard curve for the estimation of MDA. The reaction mixture (20 µL) was injected into the Agilent Technology 1200 Series HPLC (Germany) system for separation MDA-TBA complex. The mobile phases used were, A: 100% methanol and B KH₂PO₄ buffer (50 mM, 5.8 pH) in a ratio (A/B) 60%/ 40%. A flow rate of 0.8 mL /min and fluorescent detection with excitation wavelength set at 532 nm and emission wavelength set at 552 nm were used. The series of standard solution against the area was used to construct the standard curve and linear regression used to calculate the concentration of MDA formation, expressed as nmol/mL in the tissue extract sample.

3.4.6 Protein determination

Proteins were determined using the Pierce bicinchoninic acid (BCA) protein Assay which is based on the reaction between copper ions and peptides to produce a purple end product. This reagent is relatively stable under alkaline condition (Stoscheck, 1990). The purple-coloured reaction produced by the chelating of two molecules of BCA with one cuprous ion, and exhibits a strong absorbance monitored at 562 nm. Because the reaction is an end point the incubation time allows that a large number of samples can be assayed. Phosphate buffer 50 mM and tissues samples were prepared as previously done for the enzyme assays. A stock solution of BSA (2 mg/ml) was used to make the standard series of 125, 500, 1000, and 2000 µg/ml. The working reagent was prepared by mixing BCA reagent A (1000 mL containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with reagent B ( 25 mL, containing 4% cupric sulphate) in a 50:1 ratio (all reagents from Sigma, SA). The reaction mixture in each microplate well consisted of 25 µL of standard/sample (kidney twenty-fold dilution and liver five-fold dilution), 200 µL of working reagent, all in triplicate, then incubated at 37°C for thirty minutes before taking the reading in the Multiskan spectrum plate reader (Thermo Electron Corporation, USA) set at 25°C and 562 nm. Data analyses and calculations was done in Microsoft® Excel 2003 based on calibration curve plotted using a regression equation between standard series and absorbance of sample, and the results are expressed as µg protein/mL extracted tissue.
Statistical analyses
Results were expressed as a means ± standard deviation (S.D) for each group. Two way analysis of variance (ANOVA); Duncan Bonferroni Alpha for multiple comparison and Spearman’s Rank Correlation Coefficient tests were used to compare the groups and assess the strength of their associations. In all analyses, the level of significance was considered if P< 0.05.
The results in this study focused on the following:

I. The antioxidant profile of the five beverages.

II. Effects of the beverages on the antioxidant status in liver and kidney tissues of experimental animals oxidatively stressed with tert-butyl hydroperoxide.

4.1 Antioxidant profile of the beverages

Results of the polyphenol content and antioxidant capacity of the beverages used in the study are shown in Table 4.1. The commercial rooibos supplement (RTS) had significantly (P<0.05) and substantially higher levels of flavonols and total polyphenols when compared to the green tea and green tea supplements. In contrast, Chinese green tea (CGT) and the green tea supplement contained significantly (P<0.05) higher levels of flavanols, while the commercial rooibos supplement had the least amount when compared to the rooibos herbal tea. The highest antioxidant activity was recorded for the commercial rooibos supplement as well as the Chinese green tea when assessed by oxygen radical absorbance capacity.

Table 4.1: Polyphenol content and antioxidant activity of the beverages and supplements

<table>
<thead>
<tr>
<th>Aqueous formulations</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polyphenols (mg GAE/L)</td>
<td>981 ± 118a</td>
<td>1354 ± 62b</td>
<td>2723 ± 204c</td>
<td>4836 ± 244d</td>
<td>1920 ± 196e</td>
</tr>
<tr>
<td>Flavanols (mg CE/L)</td>
<td>39 ± 8a</td>
<td>92 ± 3b</td>
<td>896 ± 25c</td>
<td>18 ± 10a</td>
<td>823 ± 38a</td>
</tr>
<tr>
<td>Flavonols (mg QE/L)</td>
<td>299 ± 49a</td>
<td>247 ± 19b</td>
<td>109 ± 16c</td>
<td>181 ± 22d</td>
<td>93 ± 10c</td>
</tr>
<tr>
<td>ORAC (μmoleTE/L)</td>
<td>14557 ± 905a</td>
<td>20889 ± 1281b</td>
<td>33351 ± 312c</td>
<td>33768 ± 2322d</td>
<td>9755 ± 645a</td>
</tr>
</tbody>
</table>

Values in columns represent mean of three measurements for each aqueous sample ± SD. TE = Trolox equivalents, AAE = Ascorbic acid equivalents; CE = catechin equivalents; QE = quercetin equivalents, GAE = Gallic acid equivalents. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05.

4.2 Daily beverage intakes and body weight gains

None of the rooibos, green tea or commercial supplements had any adverse effect on the body weight gains of the respective experimental animals (Table 4.2). Rats consuming the Chinese
green tea and two commercial supplements showed a significantly (P<0.05) lower daily fluid intake, but this did not impact significantly on their body weight gains. The highest daily polyphenol intake were achieved by rats consuming the Chinese green tea and rooibos supplement, followed by rats consuming the unfermented rooibos, while the intakes were comparable for the fermented rooibos and green tea supplemented groups, which had the lowest polyphenol intake. Rats consuming the green tea and green tea supplement had the highest intake of flavanols, while rats consuming the rooibos beverages had the highest flavonol intakes.

### Table 4.2: Body weight gains and daily intakes of tert-butyl hydroperoxide-induced oxidative stress rats

<table>
<thead>
<tr>
<th>Supplement groups</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>112 ± 34a</td>
<td>135 ± 36a</td>
<td>92 ± 26a</td>
<td>123 ± 36a</td>
<td>101 ± 38a</td>
<td>125 ± 25a</td>
</tr>
<tr>
<td>Daily fluid intake (mL/day)</td>
<td>74 ± 13a</td>
<td>69 ± 15a</td>
<td>55 ± 13b</td>
<td>35 ± 4c</td>
<td>39 ± 4c</td>
<td>69 ± 9a</td>
</tr>
<tr>
<td>Total polyphenol intake (mg/day)</td>
<td>73.1 ± 8.7a</td>
<td>93.4 ± 4.3b</td>
<td>149.8 ± 11c</td>
<td>169 ± 8.5d</td>
<td>75 ± 7.6a</td>
<td>ND</td>
</tr>
<tr>
<td>Flavonol intake (mg/day)</td>
<td>2.9 ± 0.6a</td>
<td>6.3 ± 0.21b</td>
<td>49.3 ± 1.4c</td>
<td>0.6 ± 0.35d</td>
<td>32.1 ± 1.5a</td>
<td>ND</td>
</tr>
<tr>
<td>ORAC intake (umole/day)</td>
<td>21.8 ± 3.6a</td>
<td>17.0 ± 1.3a</td>
<td>6.6 ± 0.3b</td>
<td>6.3 ± 0.8b</td>
<td>3.6 ± 0.4c</td>
<td>ND</td>
</tr>
<tr>
<td>TEAC intake (umole/day)</td>
<td>1084 ± 67a</td>
<td>1441 ± 88b</td>
<td>1834 ± 17c</td>
<td>1182 ± 81d</td>
<td>380 ± 25d</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. The daily intakes are based on the respective beverage profiles provided in Table 4.1, ND = not done.

### 4.3 Effects of beverage intervention on the oxidative stress status of the liver and kidney in the experimental animals

#### 4.3.1 Antioxidant capacity

Results of the tissue antioxidant capacities (AOC) in tert-butyl hydroperoxide-induced oxidative stress rats after supplementation with the various beverages and/or commercials supplements are shown in Tables 4.3 (liver) and 4.4 (kidney). With the exception of FRAP activity in the unfermented rooibos group, there was a significant (P<0.05) increase in the AOC in liver tissues of most of the intervention groups when compared to the water control group. These increases were significant (P<0.05) when considering ORAC (unfermented rooibos, green tea and the rooibos supplement groups) and TEAC (rooibos supplement and green tea supplement groups), but not with the FRAP assay. In the green tea supplement group, there was a strong correlation between ORAC and TEAC values (r² = 0.70, P<0.05).
Table 4.3: Antioxidant capacity in the liver of rats consuming rooibos, green tea and commercial tea supplements after tert-butyl hydroperoxide-induced oxidative stress

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>Supplement groups</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAP (μmoleAAE/g)</td>
<td></td>
<td>5.36 ± 0.5a</td>
<td>4.60 ± 0.5a</td>
<td>5.24 ± 1.6a</td>
<td>5.49 ± 2.2a</td>
<td>5.44 ± 1.3a</td>
<td>5.01 ± 0.7a</td>
</tr>
<tr>
<td>ORAC (μmoleTE/g)</td>
<td></td>
<td>4.55 ± 1.4a</td>
<td>6.53 ± 2.1b</td>
<td>6.03 ± 0.6b</td>
<td>6.38 ± 1.2b</td>
<td>5.85 ± 0.8a</td>
<td>4.00 ± 2.1a</td>
</tr>
<tr>
<td>TEAC (μmoleTE/g)</td>
<td></td>
<td>41.3 ± 2.0a</td>
<td>38.9 ± 2.4a</td>
<td>40.1 ± 1.8a</td>
<td>41.9 ± 1.3b</td>
<td>41.7 ± 1.7b</td>
<td>37.4 ± 5.4a</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. TE = Trolox equivalents, AAE = Ascorbic acid equivalents.

In kidney tissue, the intervention did not significantly influence the antioxidant capacity as evaluated by the Ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays, but with the trolox equivalent antioxidant capacity (TEAC) method, significant (P<0.05) increases were noted in all the groups (Table 4.4).

Table 4.4: Antioxidant capacity in the kidney of rats consuming rooibos, green tea and commercial tea supplements after tert-butyl hydroperoxide-induced oxidative stress

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>Supplement groups</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRAP (μmoleAAE/g)</td>
<td></td>
<td>2.83 ± 0.8a</td>
<td>2.92 ± 0.6a</td>
<td>3.46 ± 1.0a</td>
<td>4.99 ± 1.0a</td>
<td>5.26 ± 1.0a</td>
<td>4.10 ± 2.0a</td>
</tr>
<tr>
<td>ORAC (μmoleTE/g)</td>
<td></td>
<td>6.89 ± 1.7a</td>
<td>6.43 ± 1.9a</td>
<td>4.94 ± 1.4a</td>
<td>6.89 ± 0.7a</td>
<td>6.50 ± 2.1a</td>
<td>6.43 ± 0.5a</td>
</tr>
<tr>
<td>TEAC (μmoleTE/g)</td>
<td></td>
<td>37.2 ± 2.1b</td>
<td>39.3 ± 2.6b</td>
<td>39.6 ± 3.0b</td>
<td>37.4 ± 1.5b</td>
<td>42.0 ± 2.0b</td>
<td>30.7 ± 4.5a</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. TE = Trolox equivalents, AAE = Ascorbic acid equivalents.

Comparing the antioxidant capacities of the liver and kidney tissues, ORAC levels were higher in the kidney tissue of all the animal groups except in the Chinese green tea group where activity was higher in the hepatic tissue (Fig 4.1). In contrast, FRAP activities were generally higher in liver tissue in all animal groups while TEAC activities were similar in both tissues types (Tables 4.3 and 4.4).
4.3.2. Antioxidant enzymes

Data on the activities of antioxidant enzymes in the liver of rats consuming the various beverages are presented in Table 4.5. It is known that treatment with tert-butyl hydroperoxide significantly reduces glutathione-related enzyme activities (Yen et al., 2004). When compared to the positive control group, none of the antioxidant treatment groups showed any further significant decreases in the hepatic catalase and glutathione peroxidase activities. When considering the superoxide dismutase activity, the group consuming the fermented rooibos, showed a significant (P<0.05) decrease in the hepatic activity when compared to the control group consuming water.

Table 4.5: Antioxidant enzyme activity in the liver tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Enzyme activity (μmol/min/μg protein)</th>
<th>Supplement groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fermented rooibos</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.153 ± 0.06a</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>0.240 ± 0.06a</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>0.019 ± 0.01b</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide.
In renal tissue, there were modest (P>0.05) increases in catalase (0.061 vs 0.058 µmol/min/µg protein) and glutathione peroxidase (0.079 vs 0.051 µmol/min/µg protein) in rats consuming the green tea supplement, but none of the beverages were associated with significant differences in activities of the two enzymes (Table 4.6). In contrast, the activity of superoxide dismutase (SOD) was uniformly decreased in all groups and there was a significant (P<0.05) decrease associated with green tea consumption. The SOD measure was well correlated with thiobarbituric acid reactive substances ($r^2 = .683; P<0.05$), proteins ($r^2 = .648; P<0.05$) and glutathione peroxidase ($r^2 = .630; P<0.05$).

Table 4.6: Antioxidant enzyme activity in the kidney tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Enzyme activity (µmole/min/µg protein)</th>
<th>Supplement groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fermented rooibos</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.055 ± 0.01a</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>0.048 ± 0.01a</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>0.018 ± 0.01a</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide

Activities of the three enzymes were markedly higher in hepatic tissue compared to renal tissue although this difference was least pronounced for SOD (Tables 4.5 and 4.6).

4.3.3. Protein content and glutathione levels

Results of tissue protein and glutathione levels after supplementation with the various beverages and supplements are shown in Tables 4.7 (liver) and 4.8 (kidney). Overall protein levels in liver and kidney tissues as well as glutathione levels in liver tissue were not significantly influenced by the various beverages and supplements. No glutathione levels could be detected in the various kidney samples analyzed. The level of proteins, however, differed significantly (P<0.05) between the two tissues.
CHAPTER FOUR
RESULTS

Table 4.7: Protein content and glutathione levels in the liver tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Supplement groups</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins (mg/g tissue)</td>
<td>45.87 ± 14.4*</td>
<td>48.58 ± 19.8*</td>
<td>35.79 ± 7.9*</td>
<td>41.82 ± 7.7*</td>
<td>36.20 ± 15.4*</td>
<td>40.65 ± 22.9*</td>
</tr>
<tr>
<td>Glutathione (µg/g tissue)</td>
<td>4.30 ± 1.2*</td>
<td>4.13 ± 1.0*</td>
<td>5.22 ± 1.0*</td>
<td>4.19 ± 1.1*</td>
<td>4.52 ± 1.2*</td>
<td>4.46 ± 1.3*</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide

Table 4.8: Protein content and glutathione levels in the kidney tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Supplement groups</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins (mg/g tissue)</td>
<td>25.76 ± 2.4*</td>
<td>25.07 ± 4.3*</td>
<td>25.31 ± 2.1*</td>
<td>27.91 ± 3.9*</td>
<td>24.12 ± 2.2*</td>
<td>27.38 ± 2.1*</td>
</tr>
<tr>
<td>Glutathione (µg/g tissue)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group, ND = not detected. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide

4.3.4. Lipid peroxidation

The effect of the antioxidant interventions on t-BHP-induced oxidative damage using two markers of lipid peroxidation is shown in Tables 4.9 and 4.10 for liver and kidney tissues, respectively. Consumption of fermented as well as unfermented rooibos resulted in significant (P<0.05) further increases in hepatic CD levels (2.73 and 2.53 µmole/g, respectively) when compared to the t-BHP control (1.72 µmole/g) while the rooibos supplement and green tea groups were rather associated with non-significant decreases in this particular LPO marker. In the rooibos supplement consuming group, the level of CDs correlated well with that of SOD (r² = .762, P<0.05). Although not significant, the levels of hepatic TBARS were shown to be lower in all the antioxidant-supplemented groups when compared to the control.
Table 4.9: Lipid peroxidation levels in the liver tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Supplement group</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated dienes (μmole/g)</td>
<td>2.73 ± 0.5b</td>
<td>2.53 ± 0.5b</td>
<td>1.69 ± 0.6a</td>
<td>1.43 ± 0.2a</td>
<td>2.07 ± 0.2a</td>
<td>1.72 ± 0.3a</td>
</tr>
<tr>
<td>TBARS (nmole/mg)</td>
<td>135 ± 27a</td>
<td>144 ± 21a</td>
<td>146 ± 29a</td>
<td>121 ± 22a</td>
<td>124 ± 16a</td>
<td>153 ± 34a</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide.

In renal tissue, the fermented rooibos also resulted in a further significant (P<0.05) increase of CD’s while the two commercial supplements, rooibos supplement and green tea supplement, non-significantly decreased this marker of lipid peroxidation. No significant changes in thiobarbituric acid reactive substances levels were shown.

Table 4.10: Lipid peroxidation levels in the kidney tissue of oxidatively stressed* rats consuming the various beverages and supplements

<table>
<thead>
<tr>
<th>Supplement group</th>
<th>Fermented rooibos</th>
<th>Unfermented rooibos</th>
<th>Chinese green tea</th>
<th>Rooibos supplement</th>
<th>Green tea supplement</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated dienes (μmole/g)</td>
<td>2.62 ± 0.4b</td>
<td>1.90 ± 0.3a</td>
<td>1.61 ± 0.3a</td>
<td>1.34 ± 0.3a</td>
<td>1.30 ± 0.3a</td>
<td>1.53 ± 0.3a</td>
</tr>
<tr>
<td>TBARS (nmole/mg)</td>
<td>25 ± 2.6a</td>
<td>23 ± 2.7a</td>
<td>21 ± 2.6a</td>
<td>21 ± 3.7a</td>
<td>22 ± 4.2a</td>
<td>23 ± 3.1a</td>
</tr>
</tbody>
</table>

Values in columns represent mean ± S.D of 10 ± 2 rats per group. Similar letters in columns denote lack of significant difference, but if letters differ then P<0.05. * Oxidative stress was induced with tert-butyl hydroperoxide.
There is substantial evidence from human and experimental animal studies that biomarkers of oxidative damage in plasma, urine and cells are increased in subjects with certain diseases or risk factors. The DNA oxidation markers 8-oxo-2'-deoxyguanosine (8-oxodG) and 8-oxo-guanine, for example, are elevated in smokers and patients with certain types of cancer, autoimmune diseases, hepatitis and cystic fibrosis (McCall & Frei, 1999; Bartsch & Nair, 2006; Obtulowicz et al., 2010). The in vivo markers of lipid peroxidation, F₂-isoprostanes and their metabolites, are increased in liver, neurological disorders and in individuals with CVD risk factors, such as cigarette smoking, diabetes mellitus, obesity and hypercholesterolemia (Roberts II & Morrow, 2002; Khadem-Ansari et al., 2010; Davies & Roberts II, 2011). In addition, plasma and tissue levels of protein carbonyls, which reflect protein oxidation, are increased in smokers, rheumatoid arthritis and various neurodegenerative diseases (McCall & Frei, 1999; Greilberger et al., 2008; Mitsumoto et al., 2008). Though unproven, it is therefore possible that these diseases result from oxidative damage to vital biological macromolecules and that dietary intake of, or supplementation with, antioxidants may reduce the risk for disease or be useful therapeutic targets. Numerous experimental studies have shown that dietary antioxidants, such as vitamin C, vitamin E, β-carotene and polyphenols are effective antioxidants in biological systems such as plasma and cultured cells (Tantcheva et al., 2003; Dewettinck et al., 2008; Sissing et al., 2011).

The current study reports on the impact of various antioxidant beverages and commercial supplements on the oxidative status in the liver and kidney tissues of t-BHP-induced oxidative stressed Wistar rats. These beverages, which also defined the various experimental groups, included fermented rooibos (FRT), unfermented rooibos (URT), Chinese green tea (CGT), a commercial rooibos supplement (RTS) and a commercial green tea supplement (GTS).

The antioxidant profile of the five beverages was first assessed. As expected, the fermented rooibos showed a lower total polyphenol content when compared to its unfermented counterpart, since the fermentation process is known to decrease the total polyphenol levels due to chemical and enzymatic changes (Joubert, 1996). The Chinese green tea was shown to have higher total polyphenol content when compared to the rooibos herbal teas and is in accordance with previously published studies (Marnewick et al., 2003; Almajano et al., 2008; Yoo et al., 2008). The flavonol contents were substantially higher in the rooibos herbal teas.
(as well as the commercial rooibos supplement) when compared to the green tea (and commercial green tea supplement), while the flavanol content was again highest in the green tea when compared to the rooibos herbal teas, all in agreement with previous reports (Marnewick et al., 2009; Awoniyi et al., 2012; Pantsi et al., 2011). When comparing the two commercial supplements, it was the rooibos supplement showing the highest polyphenol content and antioxidant capacity (ORAC). The green tea supplement (GTS) only contain one polyphenol component i.e. EGCG and showed, as expected, a low level of antioxidant capacity as measured by ORAC assay since synergistic interactions may contribute to an increased antioxidant capacity. At the time of the study no commercial supplement was available containing only the major rooibos polyphenolic compound, i.e. aspalathin, while a commercial supplement could be found containing the major green tea polyphenolic compound, i.e. EGCG (100 mg EGCG). The rooibos supplement used in the current study contained 175 mg aspalathin, 150 mg vitamin C, 5 mg vitamin E, 500 µg vitamin A and 25 µg selenium. This mixture of different antioxidants may explain this supplement’s higher antioxidant profile since antioxidants are known to interact with each other synergistically through overlapping or complementary effects (Bendich et al., 1984; Pedrielli & Skibsted, 2002; Jain et al., 2011).

The modulation of the antioxidant status in liver and kidney tissues of the t-BHP-induced oxidative stress experimental animals was evaluated using three different antioxidant capacity assays, i.e. ORAC, FRAP and TEAC, as these assays lack specificity (Lotito & Frei, 2006). Previous authors have recommended a battery of assays rather than a single assay approach, keeping in mind the specific research question to be addressed (Griffiths et al., 2002; Collins, 2005; Dilis & Trichopoulou, 2010). In the liver tissue, none of the antioxidant beverages/supplements significantly influenced the FRAP measures. However, the intake of unfermented rooibos, Chinese green tea and the rooibos supplement significantly restored the t-BHP-induced reduction (Kumar & Muralidhara, 2002; Hwang et al., 2005) and increased the antioxidant status with regards to ORAC and TEAC levels. A previous study by Marnewick et al. (2009) also showed unfermented rooibos to restore and enhance the injured liver’s ORAC in rats exposed to diethylnitrosamine. In the current study, it would be difficult to deduce exactly which group of compounds were responsible for this enhanced antioxidant capacity, but it could be suggested that the polyphenolic compounds did contribute to this effect. Further studies will need to be conducted to ascribe this effect to one particular or combination of compounds. In kidney tissue, the intervention did not significantly influence the AOC as evaluated by FRAP and ORAC assays, but with the TEAC method, significant (P<0.05) increases were noted in all the groups when compared to the oxidative stress-induced control group. Differences between the tissues may reflect organ-related metabolic
differences which can influence various factors such as bioavailability at tissue and cellular level (Chen et al., 1997; Scalbert et al., 2002). This also underlies the recommendation to use at least two methods when assessing the antioxidant capacity. Although not significant, the groups consuming the rooibos herbal teas and the Chinese green tea were all associated with lower FRAP activity than the control group in the kidneys, which may be a consequence of FRAP’s inability to measure the SH-group-containing antioxidants such as glutathione (Prior & Cao, 1999).

Antioxidant enzymes are part of the endogenous defense system and protect against oxidative stress via mechanisms discussed earlier. In this study, the enzymatic activity was assessed by measuring catalase, glutathione peroxidase and superoxide dismutase activity. Previous studies have associated Camellia sinensis teas with increased tissue and plasma levels of these enzymes when using models of oxidative stress (El-Behbishy, 2005; Marnewick et al., 2009). In this study however, neither the green tea nor green tea supplement were associated with increased enzyme levels in tissues from both organs, but the results of Marnewick et al. (2009) were confirmed by the current study results in that none of the rooibos herbal teas affected any of the antioxidant enzymes measured in the study. When considering SOD, a decreasing trend was noted, which was underlined by a significant (P<0.05) decrease in renal SOD activity in the Chinese green tea group. An increase in enzymatic activity may be prompted by increased oxidative stress in a negative feedback-like relation. Conversely, decreased enzyme activity may reflect a favorable oxidative status balance; i.e. enzyme levels decrease as the degree of oxidative stress (requiring neutralization) diminishes. This relation was supported by the positive and relatively strong correlation between the SOD activity and TBARS (r² = 0.683; P<0.05). Between the three enzymes, the relation may be most profound with regard to SOD since it is the very first line of defense against oxidative stress.

The enzyme activity results parallel, to some extent, the protein and GSH levels in the current study. While also not significantly influenced by any of the interventions, the protein levels in kidney tissues showed a decreasing trend except in the group consuming the rooibos supplement. As in the case of the SOD and the other antioxidant enzymes, GSH levels may be reflective of the oxidative stress situation. The enzymes and GSH are components of the tissue proteins and their collective decrease will affect the level of proteins. Sohn et al. (1994) and Marnewick et al. (2003) previously reported a lack of influence on GSH levels by Chinese green and black teas as well as rooibos and honeybush herbal teas using experimental animal models. More recently, Marnewick et al. (2011) have
reported on a significant increase in the levels of reduced glutathione in adults at risk for developing heart disease, consuming 6 cups of fermented rooibos daily for 6 weeks, in agreement with results from another human study reporting a ~46% increase in the levels of GSH after consumption of rooibos (Nikolova et al., 2007). The current study also reports that no GSH (below limit of detection of the assay) could be detected in the kidney tissue of the experimental animals, and could have been as a result of the high levels of gamma-glutamyl transpeptidase in this type of tissue, which cleaves GSH as the tissue is thawed (Anderson, 1985). It is recommended that future studies using kidney tissue should process this type of tissue immediately without prior freezing.

Markers of lipid peroxidation in liver and kidney tissues of the rats were assessed by the CDs and TBARS assays which have been widely used for determining oxidative stress in mammalian tissues. Many studies have associated intake of tea and other dietary antioxidants with decreased LPO in animal tissues (Hamden et al., 2009; Raneva & Shimasaki, 2005). In this study the beverages did not impact significantly on lipid peroxidation, although, there were differing trends in the two LPO markers assessed. While TBARS levels showed a declining trend in both tissues, the CDs were generally elevated reaching significance in the fermented rooibos group in both tissues. As discussed in the previous chapter, CDs and TBARS measures different phases of lipid oxidation; CDs are intermediate products while MDA are accumulated end products. This may account for the apparent increase in CDs in contrast to the decreased TBARS. It may be possible that the beverages affect these LPO products differently.

Antioxidants in tissues act as free radical scavengers against lipid free radicals thus countering the initiation and propagation of lipid peroxidation. Levels of antioxidant enzymes are therefore important determinants of tissue antioxidant status, however, increased tissue LPO products are able to inactivate certain antioxidant enzymes, mainly glutathione peroxidase, and inhibit the protective action of glutathione as has been previously reported (Bosch-Morell et al., 1999; Reneva & Shimasaki, 2005). However, the impact of dietary intervention may vary in different tissues as was shown in a study evaluating the effect of dietary restriction on antioxidant status (Dubnov et al., 2000).

Results discussed in this thesis are part of a broader study doing a comprehensive evaluation of the influence of Chinese tea and rooibos herbal teas on various aspects in oxidatively stressed rats. Results already published indicate that both the rooibos herbal teas, the Chinese green tea and the commercial rooibos supplement restored and enhanced
the t-BHP-induced decrease in glutathione and certain antioxidant enzymes while reducing lipid peroxidation (TBARS) in rat testicular tissue and sperm (Awoniyi et al., 2011; Awoniyi et al., 2012) which again points towards tissue/organ variability in responses. A possible source of this variability may be selective toxicity resulting from the agent used to induce the oxidative stress. Oh et al. (2012) have recently showed a time and dose dependent hepatotoxicity in rats treated with t-BHP which was used in this study. This agent may therefore not be the ideal model for multi-organ induced oxidative stress models, an important fact to keep in mind when planning future studies.

It can be concluded from this study that:

- The commercial rooibos supplement had the highest polyphenol content and antioxidant capacity which was likely due to its rich mixture of antioxidant compounds.
- Fermented rooibos, as expected, had lower total polyphenol content and antioxidant potency compared to its unfermented counterpart. Contrary to expectation, however, unfermented rooibos had more polyphenols and higher antioxidant capacity than the Chinese green tea used in this study, but again this could be as a result of species differences, seasonal differences and different agricultural practices, all influencing factors on the quality of the end product.
- Organ and methodological differences were observed in the antioxidant measurements. In the liver tissue, supplementation with unfermented rooibos and the commercial rooibos supplement was associated with significant increase in ORAC levels but not in the other two antioxidant capacity assays. All the beverages were associated with significant antioxidant boost in renal tissue by the TEAC assay but not the other two methods. This underscores the caveat to use at least two methods for these measurements.
- All the beverages were associated with a general decline in activities of the antioxidant enzymes; this trend was underlined by a significant decrease in SOD activity in kidney (Chinese green tea) tissues. As discussed earlier, this may be an indication of decreased oxidative stress challenge.
- Generally, the beverages did not impact significantly on lipid peroxidation although there were differing trends in the two LPO markers assessed. While TBARS levels showed a declining trend in both tissues, the CDs were generally elevated reaching significance in the fermented rooibos group in both tissues.
Strengths and limitations

A key strength in this study is the use of several methods to assess antioxidant capacity and lipid peroxidation. This approach displays results which would otherwise have been missed if only single assays were used. Another innovative strategy was the use of a multi-antioxidant supplement containing a broad combination of antioxidants aiming to resemble the spectrum of antioxidants commonly consumed in a daily intake of fruit and vegetables.

An important missing link in the current study is plasma and/or erythrocyte levels of antioxidants and LPO products. This could have provided a basis to compare tissue levels to the circulating (systemic) situation which could reveal whether the increasing trend of CDs in tissues is only a reflection of enhanced clearance from the circulation. It is possible that proteins or DNA may have been damaged more severely in this model; unfortunately none of these samples were made available for analyses in this study, as they were utilized elsewhere. It is also worth noting that these results represent one point in time and future studies are recommended to include a time-course evaluation. Although this intervention was conducted in experimental rats, it is hoped that these results may have relevance in humans since results for many studies (such as those examining the effects of cholesterol lowering drugs) that were originally done in rodents have subsequently been extended to humans. Despite these limitations, we were able to show the impact of these beverages on important oxidative status aspects in liver and kidney tissues of experimental animals. It is highly recommended that these be confirmed in a series of clinical human intervention studies.
REFERENCES


Marnewick, J. L., Van der Westhuizen, F. H., Joubert, E., Swanevelder, S., Swart, P., Gelderblom, W. C. A. 2009. Chemoprotective properties of rooibos tea (Aspalathus linearis), honeybush (Cyclopia intermedia) herbal and green and black (Camellia sinesis) teas against cancer promotion induced by fumosis B₁ in rat liver. Food and Chemical Toxicology, 47:220-229.


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

At a meeting of the Health and Wellness Sciences-REC on 15 February 2010 the addendum to a previously approved study entitled: The role of indigenous herbal tea antioxidants and commercially available antioxidant supplements on rat testicular and epididymal function, approval was granted to Bartolomeu David Cana pending information and amendments now received. This approval is for research activities related to an MTech Biomedical Technology at this institution.

This certificate should be viewed with the certificate:
18 November 2009 Ref: CPUT/EW-REC 2009/016A

TITLE:
Modulation of oxidative stress by rooibos (Aspalathus linearis) herbal tea, Chinese green (Camellia sinensis) tea and commercial tea supplements using a rodent model

INTERNAL SUPERVISOR: Prof J Mareswick

INTERNAL CO-SUPERVISOR: Dr O Ogunsittieju

Comment:
Research activities are restricted to those detailed in the in the proposal and ethics application submitted 29 January 2010

Approval will not extend beyond 2 March 2011. An extension must be applied for should data collection for this study continue beyond this date.

Prof PENELIQUE ENGEL-HILLS
CHAIR, HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE

e-mail: engelhills@cpot.ac.za
OFFICE OF THE CHAIRPERSON:
HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)

At the meeting of the Health and Wellness Sciences-REC on the 24 October 2008, approval was granted to Dolapo Olaitan AWONIYI, for research activities related to the M Tech Biomedical Technology at the Cape Peninsula University of Technology.

TITLE:
The role of indigenous herbal tea antioxidants and commercially available antioxidant supplements on rat testicular and epididymal function.

Comment:
Research activities are restricted the animal study detailed in the proposal and the ethics application submitted for review in October 2008.

This ethics approval is granted to 17 November 2009. An extension must be applied for should the study continue beyond this date.

Prof Penelope ENGEL-HILLS
CHAIR: Health and Wellness Sciences Research Ethics Committee

e-mail: engelhillsp@cput.ac.za
Addendum to approved study entitled: “The role of indigenous herbal tea antioxidants and commercially available antioxidant supplements on rat testicular and epididymal function”

Ethical approval ref: CPUT/HW-REC 2008/016A

Main Study
In short, the main study (referred to above) entailed an animal experiment, using male, Wistar rats (10 per group) that consumed either 2% (w/v) fermented rooibos or 2% (w/v) unfermented rooibos or 2% (w/v) green tea or commercially available antioxidant tea supplements (rooibos or green) daily for 10 weeks. A positive control group consuming only water was also included. Oxidative stress was induced in all of these animal via daily sub lethal injections of hydroperoxide during the last 2 weeks of the study period. Hereafter the animals were sacrificed and the blood and testis used for the main study. At the same time the kidneys and livers of all the animals were also harvested for analyses at a later stage.

A second study has now evolved from the main study entitled “MODULATION OF OXIDATIVE STRESS BY ROOIBOS (ASPALATHUS LINEARIS) HERBAL TEA, CHINESE GREEN (CAMELLIA SINENIS) TEA AND COMMERCIAL TEA SUPPLEMENTS USING A RODENT MODEL” where the livers and kidneys harvested (main study) are requested to be used for the oxidative stress analysis. The new set of results will be used for and compiled into a MTech thesis. The second study has exactly the same treatment regimes of the main study, with the only difference being the organ type in which the oxidative stress analysis will be determined. The second study will also be a comparative study and results will make a very relevant contribution to the existing data available on 1) rooibos and the modulation of oxidative stress, 2) the comparison of rooibos with green tea as well as 3) the comparison with commercial antioxidant tea supplements (novel). In order to minimise the number of animals used for experimental work, it is requested that approval be given for the use of the already harvested organs (livers and kidneys) to continue with the second study.

Below please find two additional co-workers to be added to the main study because of their involvement in the second study. Also find a new ethical application for the second study with description of the assays to be added.

5c. Co-workers
Please list in the table below the names, qualifications and duties of co-workers involved in this project. The names of persons responsible for performing the surgery and administering the anaesthetic should be clearly indicated.

<table>
<thead>
<tr>
<th>Name</th>
<th>Organisati on</th>
<th>Qualification</th>
<th>Specific Duties</th>
<th>Tel No.</th>
<th>Registration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H Neethling</td>
<td>CPUT</td>
<td>BTech (Chemistry)</td>
<td>Chemical pathology</td>
<td>021-953419</td>
<td></td>
</tr>
<tr>
<td>F Rautenbach</td>
<td>CPUT</td>
<td>MSc (Biochemistry)</td>
<td>Antioxidant analysis</td>
<td>021-953418</td>
<td></td>
</tr>
</tbody>
</table>