EXPOSURE TO POLYPHENOL- ENRICHED ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.) EXTRACTS: IMPLICATIONS OF METABOLISM FOR THE OXIDATIVE STATUS IN RAT LIVER

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DECLARATION

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ABSTRACT

Potential beneficial and/or adverse modulatory effects of polyphenol-enriched extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) on the antioxidant homeostasis in the liver were investigated. Phase II metabolism of aspalathin and mangiferin, the major polyphenols of rooibos and honeybush respectively, was assessed for potential glucuronidation and sulphation. Glucuronidation resulted in a loss of antioxidant activity for aspalathin and mangiferin in post-column HPLC-DAD-DPPH$^{\bullet}$ and HPLC-DAD-ABTS$^{\bullet+}$ assays and also a decreased activity of iron chelating properties of mangiferin in the FRAP assay. Two independent studies for 28 and 90 days with polyphenol-enriched extracts (PEEs) of unfermented rooibos [*Aspalathus linearis* (PER)] and honeybush [*Cyclopia. subternata* (PECsub) and *C. genistoides* (PECgen)] in male Fischer rats were conducted to assess possible beneficial and/or adverse biological effects. PECgen was only included in the 28 day study. PEEs were characterised by *in vitro* antioxidant assays and HPLC analysis. The importance of detailed chemical characterization of rooibos and honeybush when investigating biological effects *in vivo* is clear as distinctive biological effects and major differences in compositions were evident. Biological parameters included were serum chemical parameters, activities of selected antioxidant enzymes, levels of glutathione and the modulation of expression of oxidative stress and antioxidant defense related genes in the liver. Sub-chronic (90 days) exposure of rats to PER and PECsub both adversely affected iron absorption significantly ($P<0.05$) and significantly ($P<0.05$) and markedly lowered levels of reduced glutathione (GSH) in the liver. The high levels of polyphenol intake were implicated and interaction with glutathione was postulated to occur via catechol *o*-quinone conjugations with GSH. This was also implicated in the significantly ($P<0.05$) increased activity of glutathione reductase (GR) following 28 days. These findings suggest that PEEs from rooibos and honeybush have the potential to alter the glutathione homeostasis, which could contribute to oxidative status in the liver. PECsub resulted in alterations in the liver biliary system which was manifested as significantly ($P<0.05$) increased serum total bilirubin (Tbili) and alkaline phosphatase (ALP), depending on the age of the rats, level of total polyphenols and duration of exposure. The expression of a number of oxidative stress and antioxidant defense related genes were differentially altered by the PEEs of rooibos and honeybush in rat liver and further indicated potential oxidative stress. Modulatory effects of PEEs on expression of 84 of these genes in rat liver were assessed with a quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) array and provided additional insights into the possible adverse and protective effects of rooibos and honeybush. Further investigation on total polyphenol dose levels and time of exposure in the application of PEEs of rooibos and honeybush as dietary

supplements and functional foods is recommended and will also be of value in anticipated regulatory requirements for future substantiation of safety and efficacy.

UITTREKSEL

Die moontlike voordelige en/of nadelige modulerende effekte van polifenol-verrykde ekstrakte van rooibos (*Aspalathus linearis*) en heuningbos (*Cyclopia* spp.) op die antioksidant homeostasis in die lewer is ondersoek. Die fase II metaboliete van aspalatien en mangiferin, die hoof verbindings in rooibos en heuningbos onderskeidelik, is ondersoek t.o.v. glukuronidering en sulfonering. Glukuronidasie het gelei tot n verlies in antioksidant aktiwiteit van aspalatien en mangiferin soos bepaal in post-kolom HPLC-DAD-DPPH$^{\bullet}$ en HPLC-DAD-ABTS$^{\bullet+}$ toetse, asook verminderde interaksie met yster van mangiferin in die FRAP toets. Twee onafhanklike studies van 28 en 90 dae is onderneem met polifenol-verrykde ekstrakte (PVEs) van ongefermenteerde rooibos [*Aspalathus linearis* (PVR)] en heuningbos [*Cyclopia. subternata* (PVCsub) and *C. genistoides* (PVCgen)] in manlike Fisher rotte om die moontlike voordelige en/of nadelige biologiese effekte te ondersoek. PECgen was slegs in die 28 dae studie ingesluit. PVEs is gekarrakteriseer deur *in vitro* antioksidant en HPLC analises. Die belang van chemiese karaktirisering van rooibos en heuningbos tydens ondersoeke na biologiese aktiwiteit is duidelik aangesien verskeie en variërende biologiese aktiwiteite en verskille in die komposisie in die huidige studie gesien is. Die biologiese parameters wat ondersoek is om die effek van die PVEs te bepaal het serum kliniese parameters, aktiwiteit van geselekteerde ensieme, glutatioon en evaluering van die ekspressie van oksidatiewe en antioksidant verwante gene in die lewer, ingesluit. Sub-kroniese (90 dae) blootstelling van rotte aan PVR en PVCgen het yster absorpsie negatief beïnvloed. Die beduidende ($P<0.05$) verlaagde vlak van gereduseerde glutatioon in die lewer was toegeskryf aan die hoë vlakke van polifenole ingeneem tydens die studie en word moontlik veroorsaak deur n spesifieke katekol *o*-konjugasie van GSH. Hierdie interaksie was ook moontlik die oorsaak van n beduidende ($P<0.05$) toename in die aktiwiteit van glutatioon reduktase. Dié bevindinge het moontlike implikasies t.o.v die glutatioon homeostase en is n moontlike indikase dat PVEs van rooibos kan bydra tot oksidatiewe stres. PVCsub het veranderinge in die lewer gal-sisteem tot gevolg gehad aangesien daar n beduidende ($P<0.05$) verhoging in die serum totale bilirubin en alkalien fosfaat was. Hierdie veranderinge is beïnvloed deur die ouderdom, vlakke van die totale polifenole en die periode van blootstelling. Die uitdrukking van oksidatiewe en antioksidant verwante gene is op verskillende maniere beïnvloed deur die PVEs van rooibos en heuningbos in rot lewer and dien as n verdere indikasie van onderliggende oksidatiewe stres. Die modulerende effekte van PVEs op geenuitdrukking het gelei tot additionele insig aangaande die moontlike skadelike of beskermende eienskappe van PVEs vir gebruik as kruie produkte of dieet aanvullings. Die indikasies van moontlike oksidatiewe stres was duidelik van biologiese parameters en modulering van geenuitdrukking in die lewer, en vereis verdere ondersoek na die polifenool

dosis en periode van toediening voordat PVEs van rooibos en heuningbos as funksionele voedsel produkte gebruik word. Hierdie ondersoek sowel as toekomstige ondersoeke in hierdie verband sal van waarde wees vir regulatoriese vereistes omtrent die veiligheid en effektiwiteit van rooibos en heuningbos kruie produkte.

For Prieur and our Max:

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x

ABBREVIATIONS

ABTS	2,2′-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
ARC	Agricultural Research Council
BHT	Butylated hydroxytirisole
BSA	Bovine serum albumin
bw	Body weight
CD	Conjugated Dienes
CAM	Complimetary and alternative medicine
CAT	Catalase
COMT	Catechol-O-methyltransferase
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DETAPAC	Diethylenetriaminepentaacetic acid
EDTA	Ethylene diamine tetra-acetic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin gallate
EU	European Union
FDA	Food and Drug Administration
FRAP	Ferric ion reducing antioxidant parameter
GAE	Gallic acid equivalents
GPx	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HPLC	High pressure liquid chromatography
HPLC-DAD	High pressure liquid chromatography-diode array detection
i.d.	inner diameter
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LPH	Lactase phlorizin hydrolase
MDA	Malondialdehyde
MRC	Medical Research Council
MRP2	Multi drug resistance protein 2

M2VP	1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate
NADPH	β-nicotinamide adenine dinucleotide phosphate
NIRU	Nutritional Intervention Unit
ORAC	Oxygen Radical Absorbance Capacity
PER	Polyphenol enriched unfermented rooibos (*A. linearis*) extract
PECsub	Polyphenol enriched unfermented *C. subternata* extract
PECgen	Polyphenol enriched unfermented *C. genistoides* extract
PEE	Polyphenol enriched extract
ss	Soluble solids
PAPS	3′-phosphoadenosine-5′-phosphosulfate
qPCR	quantitative Polymerase Chain Reaction
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
RIN	RNA integrity number
RNA	Ribonucleic acid
Rpm	revolutions per minute
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGLT1	Sodium dependent glucose transporter 1
SULT	Sulfotransferases
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TCM	Traditional Chinese Medicine
TE	Trolox equivalents
TCA	Trichloroacetic acid
TBARS	Thiobarbituric acid reacting substances
TP	Total Polyphenol
TPTZ	Tripyridyl-*s*-triazine
UDPGA	uridine 5′-diphosphoglucuronic acid
UGT	uridine-5'diphosphate glucuronosyl-transferases
US	United States

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The language and style of this thesis is in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Herbal treatments have never been trendier and the use of complementary and alternative medicines (CAM) is typical in the United Kingdom, European Union (EU), United States of America (USA) and Australia (Raynor *et al.*, 2011). In the EU tightened regulations (European Commission, 2004) for some herbal products came into effect in April 2011 and include requirements to provide safety information. However, herbal products not classified as medicinal will escape these regulations. In South Africa CAMs are not incorporated into the Medicines Act (Act No. 59 of 2002) or regulated under the current labeling regulations (R.146/2010) in the Foodstuffs, Cosmetics and Disinfectants Act (Act No. 54 of 1972), resulting in no requirements with regards to proving the quality, safety or therapeutic efficacy of these products. The Department of Health in South Africa has stated that the numerous unregistered products in this category currently pose a serious public health risk. In the ongoing debate and controversies in the classification, control and regulation of herbal products, the objective of global regulations is, however, clear and aims to protect the consumers from adverse health effects and being mislead. Studies on the efficacy and safety are therefore central to future research strategies regarding the use of herbal products, specifically herbal products aimed at being used in functional foods, nutraceuticals or other therapeutic applications.

The beneficial health effects associated with consumption of herbal products are generally attributed to the phytochemicals, polyphenols (Liu *et al.*, 2008) that have been referred to as "magic bullets" (Duthie *et al.*, 2003). Consequently, research on the health properties of polyphenols has increased tremendously with an explosion of studies aiming to confirm their health benefits (Boudet, 2007). The unique South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), are implicated as dietary sources of polyphenol antioxidants (Joubert *et al.*, 2009; Joubert & De Beer, 2011; Joubert *et al.*, 2011). Research investigating the health properties of these herbal teas have mostly focused on rooibos (Joubert *et al.*, 2008), with major recent advances including clinical studies on the bioavailability of the major polyphenols (Courts & Williamson, 2009; Stalmach *et al.*, 2009; Villaño *et al.*, 2010; Breiter *et al.*, 2011) and the protective effects against cardiovascular disease (CVD) (Marnewick *et al.*, 2011). Health properties of rooibos researched in animal models, comprise the protective effects on testicular tissue of oxidative stress induced rats (Awoniyi *et al.*, 2011), anti-hyperlipidaemic activity in hyperlipidamic male LDLr-/- mice (Beltrán-Debón *et al.*, 2011), hypoglycaemic activity in animals (Joubert *et al.*, 2010) and anticancer properties utilising different cancer models in rats and mice (Marnewick *et al.*, 2005; Marnewick *et al.*, 2009; Sissing *et al.*, 2011). Recent studies regarding the health properties of honeybush (*Cyclopia* spp.) include investigations on

antidiabetic properties (Muller *et al.*, 2011), potential as a phytoestrogen for the nutraceutical market (Mfenyana *et al.*, 2008), antioxidant and anticancer activities (van der Merwe *et al.*, 2006; Joubert *et al.*, 2008) and bioavailability studies in pigs (Bock *et al.*, 2008; Bock *et al.*, 2010). *In vitro* and *in vivo* studies on the health promoting properties of rooibos and honeybush (*Cyclopia* spp.) have been extensively reviewed recently (Joubert *et al.*, 2008; Joubert *et al.*, 2009; Joubert *et al.*, 2011). Patent protection with regards to the anti-diabetic potential has been obtained for extracts produced from rooibos (Mose Larsen *et al.*, 2008a) and the patent for honeybush is in the application stage (*Cyclopia* spp.) (Mose Larsen *et al.*, 2008b).

Aspalathin and nothofagin, two *C*-linked dihydrochalcone glucosides, are the major flavonoid constituents of unfermented rooibos (Koeppen & Roux, 1966; Joubert & Schulz, 2006) and aspalathin is one of the main components of fermented rooibos tea (Rabe *et al.*, 1994; Joubert, 1996). Other major phenolic compounds in rooibos include flavones, isoorientin, orientin and flavonols, quercetin-glucosides (Koeppen *et al.*, 1962, Marais *et al.*, 1996; Rabe *et al.*, 1994; Shimamura *et al.*, 2006; Joubert & De Beer, 2012). As aspalathin is novel to rooibos it has been utilised as a potential quality control marker of rooibos herbal products (Joubert *et al.*, 2008, Joubert & De Beer, 2012). Detailed studies with regards to the biological properties, including *in vitro* antioxidant (Joubert *et al.*, 2008; Snijman, 2009) and antimutagenic activity (van der Merwe *et al.*, 2006) of this compound have been conducted. The xanthone mangiferin is the major antioxidant of *Cyclopia* species (De Beer & Joubert, 2010) and has been implicated in the chemopreventive properties of *Cyclopia intermedia* (Marnewick *et al.*, 2005; Marnewick *et al.*, 2009; Sissing *et al.*, 2011). Another xanthone, isomangiferin, and the flavanone hesperidin are other major phenolic compounds present in *Cyclopia* spp. (De Beer & Joubert, 2010).

 Key determinants of the potential health effects of polyphenols relate to their metabolism and bioavailibity resulting in the formation of conjugated metabolites aimed at facilitating their excretion. Knowledge regarding the circulating conjugated metabolites has been obtained from bioavailability studies with phenolic compounds (Manach *et* al., 2005; Williamson & Manach, 2005; Crozier *et al.*, 2009; Crozier *et al.*, 2010). However, limited investigations have been conducted on their antioxidant and other biologically relevant properties (Manach *et al.*, 1998; Harada *et al.*, 1999; Lu *et al.*, 2003; Villaño *et al.*, 2010; Lotito *et al.*, 2011). Glucuronides of (+)-catechin and (-)-epicatechin displayed similar activity in scavenging superoxide generated by xanthine oxidase (Harada *et al.*, 1999) and also retained some anti-inflammatory activity following biotransformation of quercetin and epigallocatechin gallate (Lotito *et al.*, 2011).

Safety concerns about herbal foodstuffs in conjunction with reports on pro-oxidant activity of popular products such as green tea (unfermented *Camellia sinensis*) (Lambert *et al.*, 2010) and green tea derived products (Isbrucker *et al.*, 2006) have led to scientific assessment of the

safety of some traditional herbal products. At high dose levels polyphenols have the potential to act as mutagens, pro-oxidants that generate free radicals, and may also alter the activity of key metabolizing enzymes. Excessive exposure to polyphenols may overwhelm the antioxidant defense mechanisms in the cell, resulting in the over-production of reactive oxygen intermediates (Skibola & Smith, 2000). Therefore, the beneficial antioxidant properties can be outweighed by adverse effects, which is a concern with regards to the unregulated use of commercially available polyphenol-enriched foods or supplements/nutraceuticals. Due to the potential use of rooibos and honeybush as value-added products, food supplements and/or pharmaceutical/nutraceutical products, it is essential to assess possible adverse biological effects.

Metabolism, bioavailability and current challenges in research on health properties of polyphenols is critically reviewed in Chapter 2 and were considered in the study design and experimental approach in the present dissertation.   The effect of biotransformation, specifically glucuronidation on antioxidant activity of aspalathin, the major monomeric phenolic compound present in rooibos (Chapter 3, van der Merwe *et al.*, 2010) and mangiferin, the major monomeric phenolic compound present in honeybush (Chapter 4, van der Merwe *et al.*, 2012) were assessed and evaluated. These studies were aimed at gaining knowledge on the main metabolic pathways involved and the effect thereof on the antioxidant activity of the major phenolic compounds to elucidate their potential role in altering cellular oxidative status *in vivo*.

Short-term and sub-chronic feeding studies were conducted in male Fisher rats (Chapter 5) and serves as a first step toward safety evaluation of value added products of rooibos and honeybush. In addition, the study intended to establish whether the well-characterized polyphenol-enriched rooibos and honeybush extracts adversely affect the liver of rats as a function of the polyphenol dose and the duration of exposure. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) technology was applied to assess effects of the administrated dose on oxidative stress and antioxidant defense related gene expression in rat liver after consumption of polyphenol-enriched rooibos and honeybush for a short term period of 28 days (Chapter 6). This information on the modulation of gene expression was used to provide new insights into the observations with conventional biomarkers, i.e. serum parameters, antioxidant enzymes and glutathione in rat liver.

The key findings of the present study were placed in context (Chapter 7) by comparing the dose and human equivalent dose (HED) in the current study with doses, HEDs and biological effects of previous studies with rooibos and honeybush. Associations and contradictions apparent from comparison of the oxidative stress parameters and the gene expression were discussed. Implication of the modulatory effects of PEEs from rooibos and honeybush on gene

expression was postulated for disease conditions including cancer, cardiovascular disease and cancer.

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CHAPTER 2

LITRATURE REVIEW

"Are these flavonoids and polyphenols as effective as people believe?" (Hu, 2007)

CONTENTS

INTRODUCTION

There has been a significant increase in the use of botanical supplements (Dietz & Bolton, 2011) and despite advances in conventional medicine, interest in phytomedicine and herbal remedies for professionals and the lay public is soaring (Stickel & Schuppan, 2007). The majority of commercially available herbal supplements are not being rigorously tested for safety and efficacy, yet the perception that "natural" products equal "safe" products is increasing their market share (Molinari *et al.*, 2006). Herbal medicines can be defined as a category of products derived from plants and plant material and used for therapeutic purposes (Calapai, 2008). Beneficial effects of herbal medicine are generally contributed to the phenolic compounds contained in these preparations and their associated antioxidant activity (Liu *et al.*, 2008). However, research suggests that bioactivities of these compounds are also manifested through modulation of several cellular signaling pathways involved in cell apoptosis, proliferation, survival and inflammatory responses (Zhang *et al.*, 2007). A number of human intervention studies indicated that polyphenols are at least partly absorbed with resultant biological effects (Kroon *et al.*, 2004; Manach *et al.*, 2005; Williamson & Manach, 2005). Recent reports on metabolites resulting from polyphenol ingestion recommend that emphasis should shift towards research on the activity of these metabolites for better predicting the outcome of dietary polyphenol consumption in health (Walle, 2004; Spencer *et al.*, 2004; Crozier *et al.*, 2009; Rio *et al.*, 2010; Kay, 2010).

This review provides a brief summary of the mechanisms involved in metabolism and bioavailability of dietary polyphenols. This is followed by a discussion on some of the current challenges in investigating the health and adverse effects of these compounds, including assessing bioavailability, different biomarkers used to study biological effects, experimental concentrations applied, dose translation and the effect of metabolism on the biological activity. The potential adverse effects of dietary polyphenols are discussed in terms of pro-oxidant activity, mutagenicity/carcinogenicity and the modulating effect of polyphenols on enzymes leading to potential herb-drug interactions. Global trends in polyphenol marketing and relevant regulations are highlighted and this forms the basis for a discussion on the background, current marketing trends, chemical composition, bioavailability and the safety and potential adverse effects of the South African herbal teas, rooibos and honeybush.

DIETARY POLYPHENOLS: METABOLISM, BIOAVAILABILITY AND CURRENT CHALLENGES IN ASSESSING HEALTH AND POTENTIAL ADVERSE EFFECTS

Phytochemicals are secondary metabolites in plants (Duthie *et al.*, 2003) and are viewed as essential for health and well-being in adulthood and in the elderly population (Holst & Williamson, 2008). However, it is generally regarded as non-essential dietary components for short-term well-being in the human body (Crozier *et al.*, 2009). The cell treats these compounds as non-nutrient xenobiotics and consequently their metabolism is aimed at efficient elimination (Holst & Williamson, 2008). Phytochemicals, which include flavonoids, phenolic and polyphenolic compounds are currently a major focus of functional food development.

Polyphenols are the most abundant antioxidants in the diet with their total dietary intake estimated as high as 1 g per day, being much higher than for other classes of phytochemicals and known dietary antioxidants (Scalbert *et al.*, 2005a). Major dietary sources of polyphenols include fruits, vegetables and plant-derived beverages such as fruit juices, tea, coffee and red wine (Manach *et al.*, 2004; Scalbert *et al.*, 2005a). Research on polyphenols in disease prevention has only become prominent in the last 20 years due to their ambiguous nature in the diet together with diversity and complex chemical structures (Scalbert *et al.*, 2005a). Limitations on the insight from epidemiological studies with phytochemicals and specifically dietary polyphenols include incomplete knowledge on the phenolic content of the dietary sources, lack of information relating to changes in content and character with processing, seasonable variation and the physiological consequences of bioavailability and metabolism (Crozier *et al.*, 2009).

In spite of many challenges in this research field, considerable evidence support a role of polyphenols from various dietary sources in the prevention of different diseases and has been extensively reviewed (Hollman & Katan, 1997; Hollman & Katan, 1999; Middleton *et al.*, 2000; Havsteen, 2002; Tapiero *et al.*, 2002; Higdon & Frei, 2003; Karakaya, 2004; Manach *et al.*, 2004; Scalbert *et al.*, 2005b; Khan & Mukhtar, 2007; Crozier *et al.*, 2009; Prasain *et al.*, 2010; Williamson & Carughi, 2010; Rodrigo *et al.*, 2011, Wang *et al.*, 2011, Quideau *et al.*, 2011). There are numerous reports on their preventive or potential protection in cardiovascular diseases (Hertog *et al.,* 1993; Hollman *et al.*, 1999; Weinreb *et al.*, 2004; Holt *et al.,* 2009; Hodgson & Croft, 2010) and cancer (Mukhtar & Ahmad, 2000; Yang & Landau, 2000; Yang *et al.*, 2001; Galati & O'Brien, 2004; Thomasset *et al.*, 2006; Yang *et al.*, 2007). Polyphenols have also been investigated to play a role in prevention of osteoporosis (Hagiwara *et al.*, 2011), neurodegenerative diseases (Mandel & Youdim, 2004; Singh *et al.* 2008; Chen *et al.,* 2009),

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diabetes mellitus (Scalbert *et al.,* 2005b; Ong *et al.*, 2011) and show potential as phytoestrogens (Duncan *et al.*, 2003).

In light thereof this review will not repeat a summary of the health benefits of polyhenols, but will rather focus on the current issues in research on the health properties of polyphenols and the impact and concerns of exposure to herbal products. Shortfalls in this area includes controversy over the methods used to assess bioavailability, the experimental concentrations used in mechanistic studies, dose translation from animal to human studies and consideration of the effect of metabolism on bioavailability. Different approaches have been developed, but many of the excisting measurements are flawed in not being a constant and accurate indication of what is aimed at being determined. A brief overview of metabolism and bioavailability follows, as an understanding thereof is pivotal to researching health and potential adverse effects of dietary polyphenols.

A brief overview of mechanisms involved in metabolism and bioavailablity of dietary polyphenols

Bioavailability of polyphenols is a concept that incorporates their bioaccessiblity, absorption, metabolism, tissue distribution and bioactivity (Stahl *et al.*, 2002). Bioaccessiblity refers to the amount of an ingested compound that becomes available for absorption in the gastrointestinal tract (Stahl *et al.*, 2002; Scholz & Williamson, 2007) and is also referred to as 'liberation' (Holst & Williamson, 2008)*.* The term bioavailability is aimed at describing the concentration of a given compound or its metabolite at the target organ, but it has become a challenge to find a definition incorporating the intricate nature of the term. Other variables integrated in this concept includes intestinal absorption, excretion of conjugates toward the intestinal lumen, metabolism by the microflora, intestinal and hepatic metabolism, plasma kinetics, the nature of circulating metabolites, binding to albumin, cellular uptake, intracellular metabolism, accumulation in tissues and biliary and urinary excretion (Manach *et al.*, 2004).

The pharmacological definition of bioavailability is often presented under the acronym LADME which refers to the following integrated processes: liberation, absorption, distribution, metabolism and excretion (Holst & Williamson*,* 2008)*.* These terms are defined below and indicated in the diagrammatic presentation of the potential routes of dietary polyphenols in the human body after consumption (Fig 1).

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L = Liberation: processes involved in the release of a compound from its matrix.

A = Absorption: the diffusion or transport of a compound from the site of administration into the systemic circulation.

D = Distribution: the diffusion or transportation of a compound from the intravascular (systemic circulation) to the extra-vascular space (body tissues).

M = Metabolism, the biochemical conversion or biotransformation of a compound.

E = Excretion, the elimination of a compound, or its metabolite, from the body via renal-, biliary- or pulmonary processes.

Exogenous factors affecting bioavailability of a compound are the complexity and composition of the food matrix, the dose and the chemical form of the compound as well as competition and interaction with co-ingested compounds (Scholz & Williamson, 2007). Endogenous factors include the mucosal mass, intestinal transit time, rate of gastric emptying, metabolism and extent of conjugation, and protein-binding in blood and tissues (Holst & Williamson, 2008). These factors result in large inter- and intra-individual variations in bioavailability.

The absorption of flavonoids from the diet was initially considered to be negligible, based on the fact that most flavonoids occurring in food as glycosides and the theory that only aglycones could be absorbed into the blood stream via the intestines (Ross & Kasum, 2002). The metabolism and absorption of flavonoid glycosides has been a controversial issue (Walle, 2004). For instance, glycosides have been considered too hydrophilic for absorption by passive diffusion in the small intestine (Hollman, 2004) implicating that polyphenol *O*-glycosides needs to be deglycosylated in the small intestine (Walgren *et al.*, 1998; Zhang *et al.*, 2007) by β-glucosidases, the broad-specificity cytosolic β-glucosidase (CBG) and lactase phlorizin hydrolase (LPH) (Day *et al.*, 2000a), prior to absorption (Kroon *et al.*, 2004). LPH, present in the brush-border of the mammalian small intestine, is capable of hydrolyzing various flavonol and isoflavone glucosides (Day *et al.*, 2000a). Following deglycosylation, the flavonoid aglycone passively diffuses across the intestinal epithelium as a result of the increase in structural lipophilicity (Walgren *et al.*, 1998). Another important factor in flavonoid glycosyl movement across the apical side of the intestinal epithelium is interaction of the intact glucose moiety with active transporter proteins (Day *et al.*, 1998). This occurs through sodium dependent glucose transporter 1 (SGLT1) transporting from the lumen to the enterocyte cytosol followed by CBG deglycosylation and multi drug resistance protein 2 (MRP2) facilitating efflux from the enterocyte cytosol to the intestinal lumen (Day *et al.*, 1998; Walgren *et al.*, 2000).

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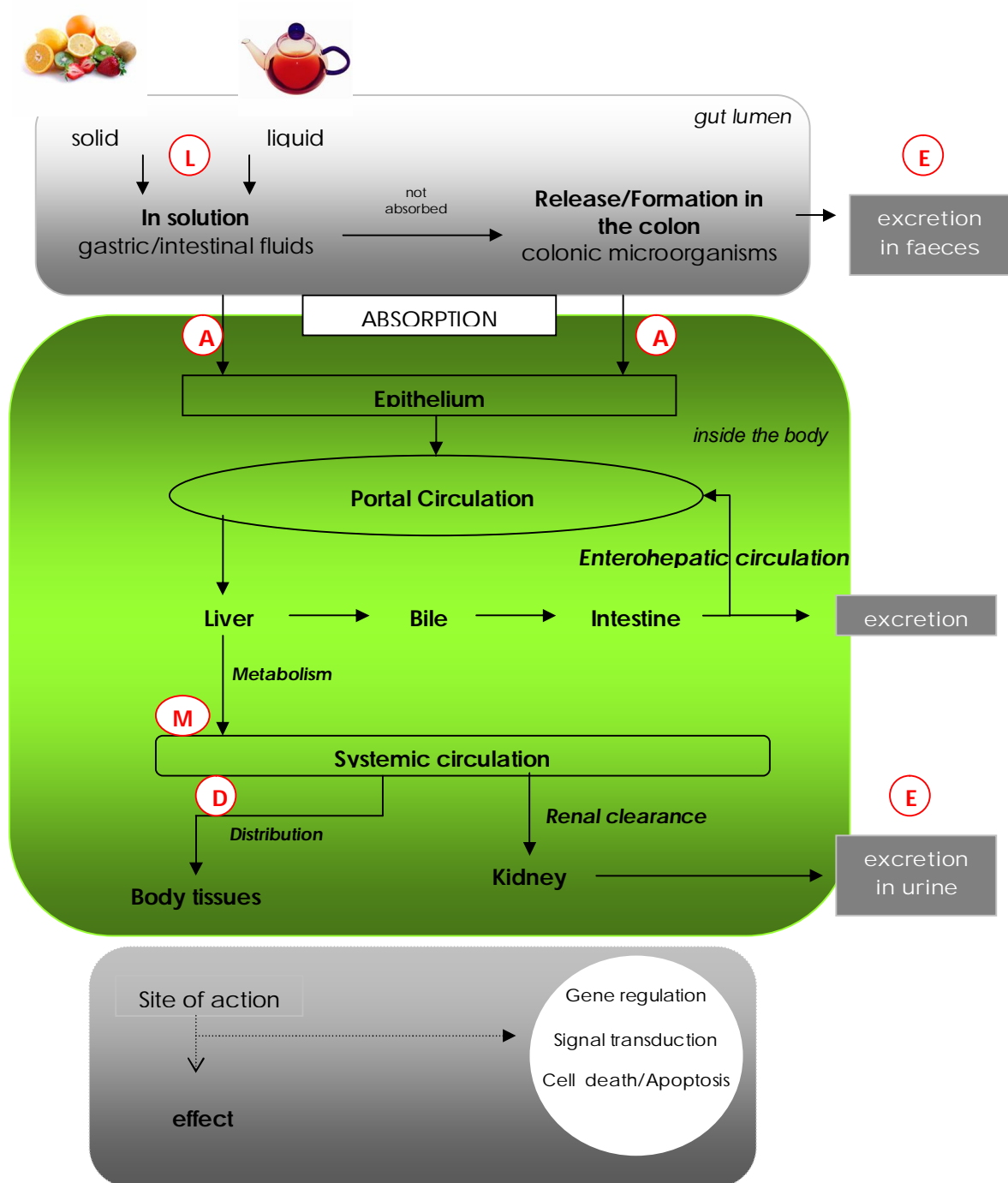
Figure 1   Diagram presenting the potential fate of dietary polyphenols in the human body according to the LADME principle (adapted from Stahl *et al.*, 2002; Holst & Williamson, 2008).

On the other hand, the absorption of intact flavonoid glycosides in the small intestine has also been proposed based on indirect evidence (Hollman *et al.*, 1995). However, a number of studies support the view that quercetin glycosides are not absorbed intact in humans and are not able to reach the systemic circulation (Erlund *et al.*, 2000; Moon *et al.*, 2000; Walle *et al.*, 2004).

C-glycosides are more resistant toward acid, alkaline and enzymatic treatment than corresponding *O*-glycosides *(*Harborne, 1965), resulting in aromatic *C*-glycosides gaining increasing interest as drug candidates due to their stability and biological activities (Braune & Blaut, 2011). Deglycosylation is not necessarily a prerequisite for *C*-glycosyl flavonoid absorption, since intact unhydrolysed metabolites and parent compounds have been reported in the urine with bioavailability studies in animals and humans (Kreuz *et al.*, 2008; Courts & Williamson, 2009). It was hypothesized that this is consequent to passive transcellular movement of the intact molecule across the intestinal epithelium (Huang *et al.*, 2008). This movement is attributed to SGLT1 on the apical side of the enterocyte and implies basolateral active transporter specificity toward intact glycosylflavonoids (Courts & Williamson, 2009). Paracellular diffusion has also been implicated as a potential mechanism of absorption.

Following absorption in the small intestine polyphenols are metabolized by phase I or phase II enzymes (Williamson *et al.*, 2000). Cytochrome P450 (CYP450) enzymes are phase I monooxygenases fundamental in metabolism of drugs and other foreign compounds (Moon *et al.*, 2006) and therefore thought to mediate flavonoid metabolism (Walle, 2004). CYP450-mediated metabolism of different flavonoids has been demonstrated with mouse, rat and human liver microsomes as well as membranes isolated from *Escherichia coli* (Silva *et al.*, 1997; Nielsen *et al.*, 1998; Breinholt *et al.*, 2002; Otake & Walle, 2002a. The phase I metabolism of flavonoids has, however, never been shown to be important *in vivo* or in intact cells, where conjugative metabolism may be expected to compete with CYP450-mediated metabolism (Walle *et al.*, 2004). This was demonstrated in a study with galangin as substrate and a S9 fraction to include oxidation, glucuronidation and sulphation (Otake *et al.*, 2002b). The efficiency of glucuronidation was relatively high followed by sulphation, with a very minor contribution by CYP-mediated oxidation.

Metabolism by the phase II drug-metabolising enzymes includes uridine-5'diphosphate glucuronosyl-transferases (UGT), sulfotransferases (SULT) and catechol-O-methyltransferase (COMT), leading to mono- or multiple glucuronidated, sulphated and methylated conjugates (Kroon *et al.*, 2004; Zhang *et al.*, 2007). Following the metabolism in the small intestine, the predominant metabolites in the hepatic portal vein include glucuronides and possibly methylated glucuronides (Kroon *et al.*, 2004). These conjugates may enter hepatocytes and be further

glucuronidated, sulphated and methylated (O'Leary *et al.*, 2003) with the type of conjugation varying according to the nature of the substrate and the dose ingested (Manach *et al.*, 2004).

Glucuronidation is considered as one of the most important metabolic pathways in the liver and intestine (Zhang *et al.*, 2007). Glucuronidation is mediated by different UGTs and their contribution to the first-pass glucuronidation has been demonstrated in a number of human studies (Zhang *et al.*, 2007). UGTs are membrane-bound enzymes located in the endoplasmic reticulum in many tissues and responsible for catalyzing transfer of a glucuronic acid from UDP-glucuronic acid to polyphenols and numerous dietary consitutents, but also steroids, bile acids and xenobiotics (Manach *et al.*, 2004). Most UGT isoforms are present in the liver of which the isoforms regarded as most important in drug glucuronidation include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 (Miners *et al.*, 2004). Isoforms that have also been found in the intestine include UGT 1A1, 1A3, 1A4, 1A6, 2B15 and 2B4, while UGTA7, 1A8 and 1A10 has been found only expressed in the intestine but not in the liver.

Catechol-*O*-methyl transferase is present in the liver, kidney and small intestine and generally functions to eliminate the potentially active or toxic catechol structures of endogenous and exogenous compounds (Manach *et al.*, 2004). Sulfotransferases occur mainly in the liver and catalyse the transfer of a sulphate moiety from 3'-phosphoadenosine-5'-phosphosulphate to a hydroxyl group on various substrates, including steroids, bile acids and polyphenols. Sulphation is generally a higher affinity, lower-capacity pathway than glucuronidation, resulting in a shift from sulphation toward glucuronidation when the ingested dose increases (Koster et al., 1981). Inhibition of methylation in HepG2 cells also leads to a shift in metabolim of quercetin glucuronides toward sulphation (O'Leary *et al.*, 2003). Irrespective of the contribution of the individual pathways, the capacity for conjugation is regarded as high and the concentration of free aglycones in the plasma is usually very low after intake (Manach *et al.*, 2004). Circulating polyphenols are mostly conjugated derivatives extensively bound to albumin.

Polyphenols that are not absorbed in the small intestine progress to the colon (large intestine) and are considerably modified in terms of chemical structure by the microflora (Rio *et al.*, 2010). Human gut bacteria have been shown to cleave *C*-glucoside derivatives of flavones (Hattori *et al.*, 1988) and xanthones (Hattori *et al.*, 1989; Sanugul *et al.*, 2005a; Sanugul *et al.*, 2005b). Colonic-derived catabolites are absorbed into the bloodstream and can be circulated through the body before excretion in the urine (Crozier *et al.*, 2010). There is a growing interest in these catabolites due to their importantance in the bioavailability and biological activities of polyphenols (Rechner *et al.*, 2002; Crozier *et al.*, 2010).

Assessment of bioavailability of dietary polyphenols

The methods used to assess bioavailability of dietary polyphenols discussed include: total antioxidant capacity (TAC) in blood plasma, pharmacokinetic studies and concentration in the plasma and urine, biomarkers of exposure and effect as well as determination of tissue distribution.

(i) Total antioxidant capacity (TAC) in blood plasma

An increase in the antioxidant capacity of plasma following consumption of polyphenol-rich foods has been proposed to serve as indirect evidence of their absorption through the gut barrier (Scalbert & Williamson, 2000; Tapiero *et al.*, 2002). Increased antioxidant capacity has for instance been reported in human studies after consumption of green tea (*Camellia sinensis*) (Camargo *et al.*, 2006; Khan & Mukhtar, 2007), the herbal tea rooibos (*Aspalathus linearis*) (Villaño *et al.*, 2010), red wine (Fernández-Mar, 2012) and different types of fruit and vegetables (Wang *et al.*, 2011). However, when considering the extensive metabolism of dietary polyphenols, an observed increase of the total antioxidant capacity in plasma following the consumption of polyphenold-rich foods is often challenged as an effective means of measurement (Spencer *et al.*, 2008).

Determination of the total antioxidant capacity (TAC) is a non-compositional assay that does not examine the composition in terms of analytes and provides information in the form of integrated parameters (Sies, 2010). This differs from customary practice in clinical research on medicine where measurements include individual chemical entities. Foods contain many macro and micronutrients in addition to polyphenols that may directly or through their metabolism affect the TAC of plasma and it was recommended that direct assays of urate, ascorbate and tocophorol should be included in determination of the TAC in human plasma to gain more insight (Sies, 2007). Increased TAC in the plasma of humans following consumption of flavonoid-rich foods was attributed to increased uric acid levels (Lotito & Frei, 2006). The latest guidance by the European Food Safety Authority (EFSA), stipulates that an overall antioxidant capacity of plasma is not established to exert a beneficial physiological effect in humans as required by the current European Regulations (EC) No 1924/2006 (EFSA, 2011).

(ii) Pharmacokinetic studies and concentration in plasma and urine

A more direct measurement and evidence of bioavailability is obtained by assessing the plasma and urine concentration of phenolic compounds following consumption or administration of individual phenolics or foodstuffs of which the phenolic content has been characterised (Scalbert & Williamson, 2000; Tapiero *et al.*, 2002). The metabolism of a number of

polyphenols and flavonoids has been established and the metabolites characterized (Kroon *et al.*, 2004).

Manach *et al.* (2005) reviewed 97 bioavailability studies that investigated the kinetics and extent of polyphenol absorption in adults for 18 major polyphenols after ingestion of a single dose of polyphenol as pure compounds, as part of a plant extract or whole food/beverage. The findings indicate that bioavailability varies widely among polyphenols. The total concentration of metabolites in the plasma ranged from 0 to 4 µmol/L following intake of 50 mg aglycone equivalents. Isoflavones and gallic acid, followed by catechins, flavanones and quercetin glucosides are relatively well absorbed in humans, while proanthocyanidins and anthocyanins are poorly absorped. Plasma kinetics differed among the polyphenol classes, with the maximum plasma concentration (C_{max}) reached after ~1.5 h and ~5.5 h for others, with the site of intestinal absorption playing a pivotal role (Manach *et al.*, 2005).

Bioavailability studies in humans have demonstrated great variability in the quantities of polyphenols found intact in urine (Scalbert & Williamson, 2000). A more recent review (Crozier *et al.*, 2010) summarises the identification of metabolites, catabolites and parent compounds in plasma, urine and ileal fluid determined with mass spectrometry after consumption of dietary flavonoids. Glucuronide, sulphate and methylated metabolites are the predominant form of compounds in circulation after absorption in the small intestine, and are quickly removed from the bloodstream. Therefore, plasma analysis is an effective measure of identity and pharmacokinetic profiles of circulating metabolites, but is not necessarily an accurate quantitative determination of uptake from the gastrointestinal tract. Although analysis of the urine provides a more realistic indication, it does not incorporate the possibility of metabolites absorbed into tissue and therefore also poses an underestimate of absorption.

Limited information is available on the accuracy of the measurements of polyphenols in plasma and urine. Hydrolysis with β-glucuronidase and sulphatase or a mixture containing both enzymes are often used as a step in sample-preparation for quantification of polyphenols in physiological fluids (bile, plasma, serum or urine) (Prasain *et al.*, 2004). This method of sample preparation may aid in identification of compounds, but may introduce a varying, unmeasured error factor due to variation in the efficacy of the enzymes used (Crozier *et al.*, 2010). Enzyme hydrolysis, for instance, results in the underestimation of isoflavone metabolites (Gu *et al.*, 2005).

A study reviewing the urinary metabolites as biomarkers of polyphenol intake in humans, included 162 controlled intervention studies and the mean recovery yield and correlations with dose ingested for 40 polyphenols (Pérez-Jiménez *et al.*, 2010). Polyphenols with a high recovery yield (12–37%) and a high correlation with the dose included daidzein, genistein,

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glycitein, enterolactone and hydroxytyrosol, showing good sensitivity as biomarkers of intake throughout the different studies. Hesperidin, naringenin, (-)-epicatechin, (-)-epigallocatechin, quercetin and three microbial metabolites of isoflavones (dihydrodiadzein, equol and *O*-desmethylangolensin) displayed weaker correlations with dose and are therefore currently less suitable as biomarkers of intake.

(iii) Tissue uptake and distribution

Data on the bioavailability of polyphenol metabolites in tissues is very scarce, but is regarded to be more important than data on plasma concentrations (Manach *et al.*, 2004). The presence of polyphenols in a wide range of tissues of rats and mice including the brain (Datla *et al.*, 2001; Abd El Mohsen *et al.*, 2002), endothelial cells (Youdim *et al.*, 2000), heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone and skin (Suganuma *et al.*, 1998; Chang *et al.*, 2000; Kim *et al.*, 2000; Coldham & Sauer, 2000), have been confirmed. Concentrations range from 30 to 3000 ng aglycone equivalents/g tissue depending on the dose and tissue. It is estimated that the time of sampling will have a great influence on the concentration, due to the kinetics of penetration and elimination of polyphenols from the tissue (Manach *et al.*, 2004).

Biomarkers for investigating health and potential adverse effects of dietary polyphenols
(i) Biomarkers of exposure and effect following dietary consumption of polyphenols

Biomarkers have been defined as: "Observable properties of an organism that indicate variation in cellular or biochemical components, structure or function and that can be measured in biological systems or samples" (Bearer, 1998). More recently, it has also been defined as: "Biometric measurements that provide information about the biological condition of the subject being tested" (Amacher *et al.*, 2010). Irrespective of the exact definition, a valid biomarker is expected to link the specific exposure of a dietary compound to a health outcome and is also an early signal of biological effects (Holst & Williamson, 2008). The diagram in Fig. 2 presents a prospective overview of biomarkers and a description of the pathway from exposure to health/disease outcome. Biomarkers for disease prevention and intervention may be measured anywhere along this pathway. Earlier markers of disease are often regarded as more useful, since it can be used to prevent disease, but are associated with less certainty than later markers, which are more closely related to disease but offer fewer chances for dietary intervention. A major challenge in applying biomarkers to nutrition research is a lack of understanding bioavailability and mode of action of bioactive food components (Holst & Williamson, 2008). Polyphenols have been shown to improve the status of different oxidative

stress biomarkers (Williamson & Manach, 2005), but the relevance of the biomarkers used as predictors of disease risk and the suitability of the different methods applied are not always definite (Collins, 2005). Epidemiologic studies have clearly indicated the protective effects of polyphenol consumption against cardiovascular risk (Arts & Hollman, 2005).   The evidence with regards to cancer and neurodegenerative diseases are less clear, but mostly based on animal and *in vitro* studies (Lambert *et al.*, 2005). When considering scientific substantiation of health claims and evidence-based nutrition, research in nutrition is entering a new stage where more diverse methodologies are required (Biesalski *et al.*, 2011).

(ii) Biomarkers of toxicity and oxidative stress

Requirements with regards to safety and toxicity testing of nutraceuticals and products advertised to contain phytochemicals are currently not clear and therefore strict application of toxicology measures used for drug development may not be applicable.   However, the methodologies, guidelines and biomarkers used in drug development may offer good guidelines for toxicity assessment of value-added herbal products.

When considering the preclinical biomarkers for liver toxicity, several approaches may be used and include measuring the depletion of antioxidant reserves, changes in activities of antioxidant enzymes, free radical production and the presence of free radical adducts to protein, lipids and DNA (Amacher *et al.*, 2010). End products of free radical attack are regarded as reliable determination of the occurrence of oxidative stress, while enzyme activities and cellular oxidants may display transient changes. Evaluation of serum biochemical parameters to assess liver damage is conventionally used in basic toxicology research and in preclinical toxicity testing (Ramaiah, 2007).

exposure

internal dose

Biomarker of exposure

target tissue dose

biologically effective dose

Biomarker of susceptibility (predisposition and lifestyle)

Biomarker of effect

early biologic effects

altered structure /function

Health versus Disease
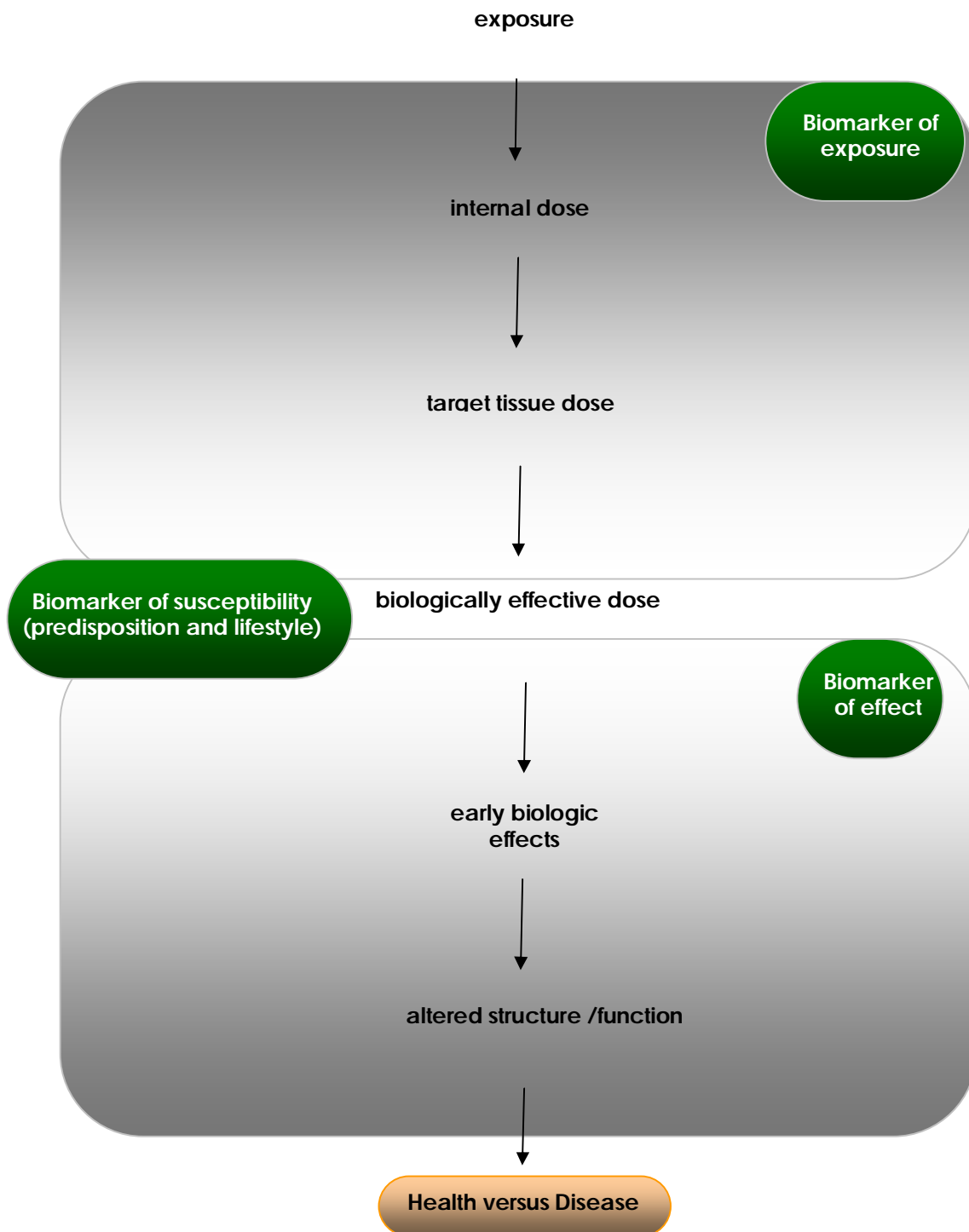
Figure 2 Classes of biomarkers describing the pathway from exposure to health/disease outcome (Holst & Williamson, 2008).

a) Serum biochemical parameters (clinical pathology) used as indicators of toxicology

Liver related biochemical parameters commonly employed in toxicity testing using animal models such as rats, dogs and non-human primates can be classified as indicated in Table 1, as (i) hepatocyte membrane leakage enzymes (ii) cholestatic-induction parameters and those related to (iii) liver function deficits (Ramaiah, 2007).

The majority of the biochemical parameters classified as "leakage" enzymes are cytotosolic and leak out of the cytoplasm by reversible (membrane blebs) or irreversible (mitochondrial membrane damage) hepatic injury (Gores *et al.*, 1990; Van Hoof *et al.*, 1997). Injury or metabolic disturbances results in a release of soluble cytosolic enzymes and causes elevation in the blood. The activity of one of these enzymes, alanine aminotransferase (ALT), is often regarded as the clinical chemistry gold standard for detection of liver injury (Ozer *et al.*, 2008). Cholestatic-induction enzymes are increased in the blood by impaired bile flow (cholestasis), drug treatment or endogenous corticosteroid excess (Sanecki *et al.*, 1987), but the mechanisms are not clearly known (Ramaiah, 2007). Cholestasis also results in increased serum bilirubin levels.

Table 1 Commonly employed hepatic biochemical parameters used to detect liver toxicity in animal models (rats, dogs and primates) and their classification (Ramaiah, 2007).

Biochemical tests	Hepatic "leakage" enzymes		Cholestatic-induction tests	Hepatic function tests
	Cytosol	**Mitochondria**		
Alanine aminotransferase (ALT)	X			
Aspartate aminotransferase (AST)	X	X		
Sorbital dehydrogenase (SDH)	X			
Glutamate dehydrogenase (GLDH)	X	X		
Lactate dehydrogenase (LDH)	X			
Alkaline phosphatase (ALP)			X	
Gamma glutamyltransferase (GGT)			X	
51-Nucleotidase (51-NT)			X	
Total Bilirubin			X	X
Total Bile acids			X	X
Amonia				X
Albumin				X
Coagulation times (APTT and PT times)				X

[a] Classification is indicated with an "x"

In spite of newer methods such as genomics and proteomics offering "novel" predictive markers to detect liver pathology before alterations in clinical and histopahylogy parameters occur, it is argued that serum biochemical parameters (clinical pathology) can provide very valuable information in assessing the extent, severity and type (membrane injury versus cholestasis and hepatic function) of liver damage (Ramaiah, 2007).

b) Liver enzymes used as indication of oxidative stress

Enzymes responsible for detoxifying free radicals are measured by *in vitro* activity assays and frequently used as an indication of the level of stress experienced in a cell or tissue (Amacher *et al.*, 2010). Measuring oxidative stress is complicated by endogenous systems correcting and repairing by reducing stress and consequently limits the ability of testing methods to detect a change (Blumberg, 2004). These systems are induced by elevated oxidative stress and include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidise (GPx). Investigation of antioxidant depletion as a biomarker of oxidative stress could involve assessment of decreases in antioxidant enzyme concentrations. It should therefore be noted that these changes may not be a true reflection of oxidative status or of clinical significance, since it may simply be an indication that the antioxidant defense system is functioning.

Conventional hepatic safety biomarkers are regarded as confimation of organ toxicity after pathological damage has occurred (Amacher *et al.*, 2010). These biomarkers are regarded as suitable for *in vivo* preclinical regulatory studies, but not ideal for short-term predictive screening assays of new chemical entities, especially if limited quantities are available. Development of reliable and early predictive toxicity biomarkers has therefore become an important and novel focus of regulatory agencies and pharmaceutical industries. The potential of such biomarkers are currently being researched in recently advances technologies of toxicoproteomics and targeted, quantitative mass spectrometry.

c) System biology tools for prediction of efficacy and safety of herbal product

With regards to herbal medicine, system biology tools such as functional genomics, transcriptomics, proteomics, metabolomics, pharmacogenomics and toxicogenomics are predicted to become a new translational platform between western medicine and traditional Chinese herbal medicine (TCM) (Lo *et al.*, 2011). DNA microarray analysis has been applied in studies with Chinese herbal medicine since 1998, with the main focus on elucidating the mechanisms involved in biological effects of herbs. A bioactivity database with large-scale gene

expression profiles of quality control herbs has been applied for prediction of therapeutic potential, safety and drug development (Lo *et al.*, 2011). Furthermore, comparison of gene expression signatures indicated alterations in the expression levels of cytochrome P450, glutathione S-transferase and UDPglycosyltransferase genes, implicating potential herb-drug interactions. Gene expression profiling in chemoprevention and cancer risk assessment studies is in the developmental phase (Sikkema *et al.*, 2012). Genome and transcriptome studies have lead to the identification of abnormal proteins or expression profiles for numerous malignancies, but the effects of these changes in gene expression on the cellular signalling network has not been clarified. Regarding drug discovery, direct modeling of toxic endpoints has been deceiving and prevents wide acceptance of computer predictions (Merlot, 2010). Therefore simpler predictions focused on the mechanism of action together with *in vitro* and *in vivo* assays are regarded as more appropriate.

Experimental concentrations applied in mechanistic studies with dietary polyphenols

Associations of *in vitro* results with effects *in vivo* are complicated by the effective concentrations when studying the biological activities of dietary polyphenols (Yang *et al.*, 2008). In this regard concentrations reported in the plasma and tissue only reach low micromolar levels, while the effective concentrations reported in *in vitro* studies are generally much higher, in the range of 100−1000 micromolar (Yang *et al.*, 2008). *In vitro* investigations are often conducted at concentrations that cannot realistically be attained in the body (Manach *et al.*, 2004; Rio *et al.*, 2010). It has been argued that prolonged exposure as a consequence of continual ingestion of e.g. tea catechins will lead to cells being consistently exposed to epigallocatechin gallate (EGCG) or other catechins, which may result in a significant effect even if the concentration is low (Yang *et al.*, 2008). There is, however, no quantitative model that provides an understanding for correlation of the effective concentration *in vitro* to the plasma and tissue concentrations observed in animals and humans.

Another consideration is that the dose of polyphenols administered will affect the type of conjugates, since the dose could influence the primary site of metabolism (Karakaya *et al.*, 2004). Although the metabolic pathways of drugs and food polyphenols are similar, drugs are generally administrated in a fairly large concentrated doses (mg) leading to saturation of the metabolic pathways, while polyphenols are consumed at much lower doses. When polyphenols are administrated at pharmacological doses, they are present in the free form in the blood (Scalbert & Williamson, 2000). Large doses are mainly metabolized in the liver, while smaller doses are metabolized in the intestinal mucosa. An increase in the ingested dose may also result in a shift from sulphation toward the glucuronidation metabolic pathway (Koster *et al.*,

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1981).   These factors may lead to measurements following the administration of single compounds at high doses in animal and human studies, not being representative of the absorption and metabolism of compounds at the level they are present in herbal preparations.

Dose translation from animal to human studies

Development of functional foods or dietary supplements from herbal extracts should consider similar guidelines to that used in drug development with regards to safety. The production of a commercially available drug includes, among others, the following steps: investigation of the mechanism of action, pharmacokinetics, determination of the mechanisms of disposition, metabolism and excretion (ADME), safety testing and assessment of efficacy in clinical trails (Anon, 2005). The calculated dose will be influenced by all these factors and in addition by the drug formulation (Hawk & Leary, 1995). Extrapolation and translation of the drug dose from animal species to humans have become essential for new drug development (Reagan-Shaw, 2008). Challenges, however, exist with regards to the appropriate method for allometric dose translations, especially when starting new animal or clinical studies. It is recommended that the normalization of body surface area (BSA) method should be used for the calculations of starting doses in humans as extrapolated from animals. This method was originally used to derive a safe starting dose for phase I studies of anticancer drugs from preclinical animal toxicology data.   Conversions by simply calculating according to body weight alone may result in inappropriate comparisons between studies.   An example of such a case would be the antioxidant resveratrol (Baur *et al.* 2006; Lagouge *et al.*, 2006) receiving bad press in the public media since the dose used in rats was equivalent to hundreds or thousands of litres of wine per day in human equivalent doses (Wade, 2006).

Effect of metabolism and bioavailability of dietary polyphenols on bioactivity

The most abundant phenolic compound in the diet is not necessarily responsible for observed activity within the body (Manach *et al.*, 2004). Poor absorption, a high level of metabolism and/or rapid elimination may result in significantly altered biological activity of polyphenols after ingestion. Several studies have indicated that aglycones are not significant metabolites in blood, attributed to extensive intestinal and hepatic conjugation (Manach *et al.*, 2005).

Regardless, many *in vitro* studies aimed at elucidating mechanisms of action of polyphenols still incorrectly focus on aglycones or glycosides rather than the identified metabolites and should reconsider their focus, since the biological and antioxidant properties of metabolites of flavonoids mostly differ from the parent compound (Manach *et al.*, 2004; Rio *et al.*, 2010; Lotito *et al.*, 2011).   The relevance of the vast amount of data generated on the activity of the

aglycones *in vitro* is therefore questionable (Kroon *et al.*, 2004).   Although a profound effect on activity is expected after flavonoids have been metabolised, knowledge on the biological properties of the conjugated derivatives in plasma and tissue is limited due to lack of precise identification and availability of commercial standards (Rio *et al.*, 2010).   Due to their intermediate hydrophobicity, phenolic compounds are expected to act at water-lipid interfaces and possibly to be involved in oxidation regeneration pathways with vitamins C and E. However, glucuronidation and sulphation increases hydrophilicity and modifies the possibility of electron delocalization and consequently affects the site of action, interaction with other antioxidants and therefore their bioactivity (Manach *et al.*, 1998; Rio *et al.*, 2010). Investigations on metabolism and activity of metabolites of the major dietary polyphenols have focussed on quercetin, tea flavan-3-ols, the flavanones naringenin and hesperetin, the grape stilbene resveratrol and catechins (Manach *et al.*, 1998; Harada *et al.*, 1999; Day *et al.*, 2000a; Lu *et al.*, 2003; Justino *et al.*, 2004; Dueñas *et al.*, 2010; Lotito *et al.,* 2011). Results of these studies are summarised in Table 2.

Conjugation of quercetin (Figure 3) resulted in decreased activity when comparing the protection against Cu^{2+}-induced oxidation of human low density lipoprotein (LDL) (Manach *et al.*, 1998).   However, the conjugates, specifically 3′-*O*-methylquercetin, still demonstrated significantly protective effects. Glucuronidation of (+)-catechin and (-)-epicatechin did not affect their antioxidant activity (determined as superoxide anion radical scavenging activity), while 3′-*O*-methylation resulted in a nearly complete loss of antioxidant activity (Harada *et al.*, 1999). Investigation on the bioavailability and effects of biotransformation on the *in vivo* activity of polyphenols should, in addition to identification of the nature and number of the conjugating groups, also assess the position(s) on the polyphenol structure (Manach *et al.*, 2004).

For instance, the conjugation position of quercetin influences whether activity is retained (Day *et al.*, 2000b). Quercetin and two of its glucuronides (3′ - and 4′ -) were found to be potent inhibitors of xanthine oxidase and have superoxide scavenging activity (Table 2). The activity of lipoxygenase, involved in early stages of atherosclerosis, was moderately inhibited by glucuronides of quercetin, with the exception of quercetin-3-glucuronide. Quercetin-3-sulphate and quercetin-3-glucuronide did not effectively retain activity when compared to quercetin.

In another study with EGCG and (−)-epigallocatehin (EGC) (Figure 3), glucuronidation with human, mouse and rat microsomes did not affect loss in activity when evaluated as potential to inhibit the release of arachidonic acid form HT-29 human colon cancer cells (Lu *et al.*, 2003). EGCG-4′-glucuronide was the major EGCG glucuronide obtained with all three different microsomal incubations, while glucuronidation of EGC appeared to be lower than that of EGCG.

Table 2 Summary of studies conducted to investigate the biological activity of phenolic compounds after metabolism to illustrate the effect of biotransformation on bioactivity.

Compound	Metabolite(s)	Assessment of activity	Results	Reference
quercetin	3′-O-methylquercetin	effect on Cu^{2+}-induced oxidation of human LDL	metabolites retain some activity, but half as effective as quercetin	Manach *et al.*, 1998
(+)- catechin	(+)-catechin 5-O-β-glucuronide 3′-O-methyl (+)-catechin	superoxide anion radical scavenging activity (xanthin-xanthinoxidase system)	glucuronidated metabolites displayed similar antioxidant activity as the parent compounds	Harada *et al.*, 1999
(-)- epicatechin	(-)-epicatechin 5-O-β-glucuronide 3′-O-methyl (-)-epicatechin	superoxide anion radical scavenging activity (xanthin-xanthinoxidase system)	3′-O-methylation resulted in a near loss of antioxidant activity	Harada *et al.*, 1999
quercetin	3′-methylquercetin quercetin-3-sulfate quercetin-3-glucuronide quercetin-7-glucuronide quercetin-4′-glucuronide quercetin-3′-glucuronide	inhibition of xanthine oxidase and lipoxygenase	certain metabolites retain activity at expected plasma concentrations depending on conjugation position. Quercetin-4′-glucuronide: very potent inhibitor of xanthine oxidase	Day *et al.*, 2000a
(-)-epigallocatechin gallate (EGCG)	(-)-EGCG-4′-O-glucuronide (-)-EGCG-3′-O-glucuronide (-)-EGCG-7-O-glucuronide	inhibition of the release of arachidonic acid from HT-29 human colon cancer cells	a number of the EGCG glucuronides retain radical quenching and other biological activities of the aglycone	Lu *et al.*, 2003

Table 2 (*Cont.*)

Compound	Metabolite(s)	Assessment of activity	Results	Reference
(-)- epigallocatechin (EGC)	(-)-EGC-3′-*O*-glucuronide (-)-EGC-7-*O*-glucuronide	inhibition of the release of arachidonic acid from HT-29 human colon cancer cells	certain EGC glucuronides retained the activities of their parent compounds in radical scavenging and in inhibiting the release of arachidonic acid from HT-29 human colon cancer cells	Lu *et al.*, 2003
quercetin	glucuronides, sulfoglucuronides and sulfates. conjugation occurs on the 5- and 7-OH groups.	determination of plasma antioxidant status	increased antioxidant status was assumed to be evidence of metabolites retaining antioxidant activity	Justino *et al.*, 2004
quercetin	3′-*O*-methylquercetin (isorhamnetin) 4′-*O*- methylquercetin (tamarixetin) Quercetin 3′-*O*-sulphate Quercetin 4′-*O*-sulphate Isorhamnetin 3-*O*-sulphate Quercetin 3-*O*-glucuronide	FRAP [a], ABTS [b] assay	conjugation results in reduced antioxidant activity	Dueñas *et al.*, 2010
quercetin (-)- epigallocatehin-3-*O*-gallate (EGCG)	3′- or 4′-*O*-methyl-quercetin, quercetin-3-*O*-glucuronide, quercetin-3′-*O*-sulfate, 4″-*O*-methyl EGCG, 4′,4″-di-*O*-methyl-EGCG quercetin	anti-inflammatory effects inhibition of effects on tumor necrosis factor α (TNF α)-induced protein expression of cellular adhesion molecules in human aortic endothelial cells	two of the metabolites: 3′- and 4′-*O*-methyl-quercetin inhibited the expression of intercellular adhesion molecule-1	Lotito *et al.*, 2011

[a] FRAP refers to ferric reducing antioxidant power assay, [b] ABTS refers to the $ABTS^{+\bullet}$ scavenging assay.
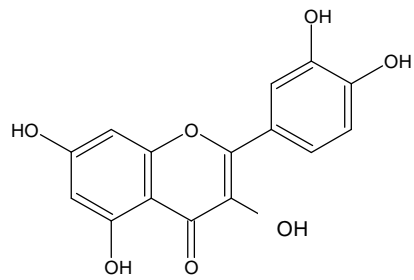
Quercetin
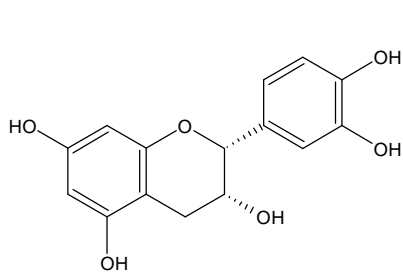
(-) Epicatechin
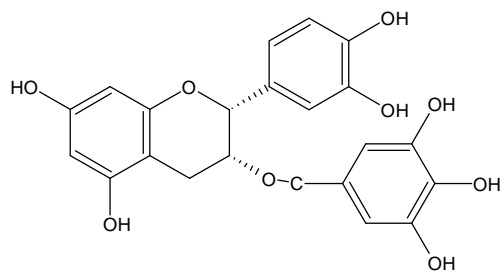
(-) Epicatechin gallate
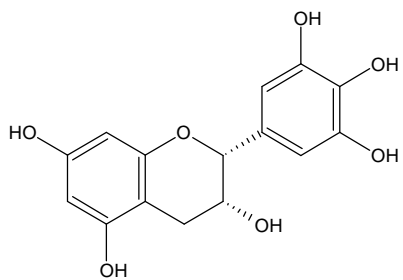
(-) Epigallocatechin
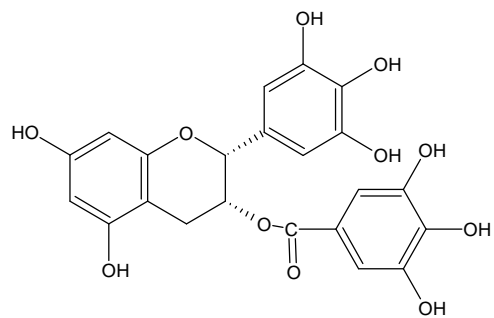
(-) Epigallocatechin gallate

Figure 3 Chemical structures of quercetin (Erlund, 2004) and the major catechins in green tea (*Camellia sinensis*) (Yang *et al.*, 2000).

The glucuronidation of EGCG and EGC in mouse microsomes were more similar to that occurring in human microsomes than to rats. A number of the glucuronides retained the activities of their parent compounds in radical scavenging and inhibiting the release of arachidonic acid from HT-29 human colon cancer cells. Justino *et al.* (2004) investigated the structure-antioxidant activity relationships of quercetin and its metabolites *in vivo* in plasma of rats following intragastrically administeration by stomach intubation (10 mg quercetin/200 g body weight) through a catheter. Quercetin was reported to be primarily metabolized to glucuronides and sulfoglucuronides and to a lesser extend to sulphates. Based on LC-MS/MS analysis, glucuronidation and sulphation is thought to occur on the hydroxyl groups located at position 5 and 7 and not at 3′-OH and 4′-OH groups. It was argued that quercetin metabolites retain antioxidant activity, since the plasma antioxidant status of rats was increased following administration of quercetin.

Dueñas *et al.* (2010) compared the antioxidant activity of several conjugates of quercetin with α-tocopherol in the ferric reducing antioxidant power (FRAP) and ABTS radical cation assays. Substitution of hydroxyl groups of quercetin significantly reduced activity in the antioxidant assays, but the conjugates were more active than α-tocopherol in the ABTS assay, with the exception of isorhamnetin-3-*O*-sulphate. This was attributed to the double substitution of quercitin, i.e. on the B- and C-rings (Table 2).

A recent study on the biological and antioxidant activity of several major metabolites of quercetin and (-) epigallocatehin-3-*O*-gallate (EGCG), that have been identified in humans, confirmed that the activity of metabolites differ from the parent compounds (Lotito *et al.*, 2011). The anti-inflammatory effects were assessed by determining the inhibitory effects on tumor necrosis factor α (TNFα)-induced protein expression of cellular adhesion molecules in human aortic endothelial cells. The metabolites, 3′- or 4′-O-methyl-quercetin, quercetin-3-O-glucuronide, quercetin-3′-O-sulphate, 4″-O-methyl-EGCG and 4′,4″-di-O-methyl-EGCG as well as quercetin were evaluated. The expression of intercellular adhesion molecule-1 was effectively inhibited by 3′-*O*-methyl-quercetin, 4′-*O*-methyl-quercetin, and their parent aglycone compound, quercetin. This study indicated that biotransformation of dietary flavonoids may result in a loss or gain of biological or antioxidant activity which cannot necessarily be predicted from the chemical nature of the flavonoid. Bioactivity and beneficial effects in humans and animals have therefore been attributed to the circulating glucuronides, sulphates and *O*-methylated forms of dietary polyphenols (Spencer *et al.*, 2004).

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POTENTIAL ADVERSE EFFECTS OF DIETARY POLYPHENOLS

In spite of increased interest in using polyphenols as dietary supplements alone or in combination with other drugs as medicine, there are associated risks such as hepatoxicity and possible herb-drug interactions (Prasain *et al*., 2010). Flavonoid concentrates and herbal remedies are aggressively marketed and the nontoxic therapeutic effects are exaggerated, despite the lack of substantiation by regulated clinical trails (Skibola & Smith, 2000)*.* The recommended doses of such products far exceed the level of polyphenols that could be attained with a normal diet, based on the hypothesis that "if a little of something is good, then more is better", resulting in individuals ingesting extremely high levels. An understanding of the role of reactive oxygen species (ROS) in the normal physiology and disease and the interaction of dietary polyphenols is fundamental to the role, protective or detrimental, of polyphenols.

Reactive oxygen species (ROS) and dietary antioxidants

(i) The role of ROS in normal physiology and disease

Reactive oxygen species (ROS) refers to a variety of diverse chemical species, which include superoxide anions, hydroxyl radicals and hydrogen peroxide (Finkel & Holbrook, 2000). The production of ROS in the cell has mostly been viewed as harmful due to association between abnormal production and incorrect regulation with the development of diseases and aging (Finkel & Holbrook, 2000; Holbrook & Ikeyama, 2002). Diseases such as diabetes, Alzheimer's and other neurodegenerative diseases, atherosclerosis contributing to cardiovascular disease and cancer have been attributed to the actions of ROS (Seifried *et al*., 2007). On the other hand, ROS generation is a normal physiological process required for immunocompetence and in coordination and activation of several signal transduction pathways (Seifried *et al*., 2007). Consequently, ROS are referred to as a double-edged sword.

Althought originally implied that the targets of ROS are random, indiscriminate and cumulative, it has been demonstrated that ROS can act as specific signaling molecules under both physiological and pathophysiological conditions (Finkel & Holbrook, 2000). The involvement and importance of oxidants in biological processes is illustrated in Fig. 4. The generation of ROS within limits is therefore required to maintain homoeostasis. Intracellular oxidants have two potential outcomes which are determined by the level present: they may damage various cell components or trigger the activation of specific signaling pathways. Numerous cellular processes are influenced by both these effects and are associated with ageing and development related diseases.

In addition to endogenous generation of ROS, environmental stimuli such as ultraviolet radiation or chemotherapeutic agents may increase oxidative stress disturbing the normal redox

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balance (Finkel & Holbrook, 2000). Survival of the cell will depend on the ability to cope with the stress and to repair or replace damaged molecules. Cells may also respond by undergoing apoptosis, which is described as a process of eliminating the damaged cell as a mechanism of preservation.

(ii) Interaction of dietary antioxidants with ROS

After the link between ROS and certain diseases became apparent, a monumental effort went into antioxidant research (Seifried *et al.*, 2007). Antioxidants are used in normal cellular processes to mediate the immune response, cell proliferation, signal transduction and also quenching excess ROS. Polyphenols are renowned for displaying antioxidant activity (Quideau *et al.*, 2011), because of the ability to scavenge reactive oxygen species (ROS) which includes radicals such as $O_2^{\bullet-}$, $OH^\bullet$, $LOO^\bullet$ and $LO^\bullet$ and non-radical oxygen species such as $HOCL$, H_2O_2, O_2 and O_3 (Halliwell *et al.*, 1995).

Polyphenols also act as anti-oxidants by chelating metal ions such as iron (II)/copper (I) and iron (III)/copper (II) involved in Haber-Weiss and Fenton type reactions that lead to formation of $OH^\bullet$ radicals from H_2O_2 (Collins, 1999; Quideau *et al.*, 2011).

$$Fe^{2+} \quad + \quad H_2O_2 \quad \rightarrow \quad Fe^{3+} + \quad OH^- + \quad OH^\bullet \qquad \text{(Fenton reaction)}$$

$$Fe^{3+} \quad + \quad O_2^{\bullet-} \quad \rightarrow \quad Fe^{2+} + \quad O_2$$

$$H_2O_2 \quad + \quad O_2^{\bullet-} \quad \rightarrow \quad OH^\bullet + \quad OH^- + \quad O_2 \quad \text{(Haber-Weiss reaction)}$$
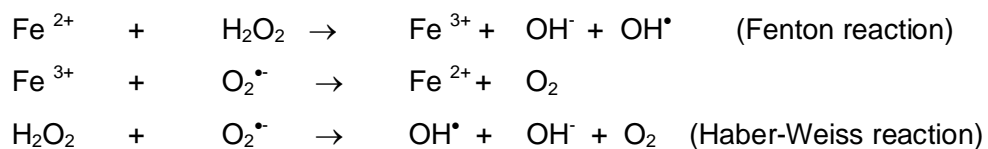
The degree of oxidative stress is determined by the balance between ROS production and antioxidant defenses (Finkel & Holbrook, 2000). Dietary supplementation has been seen as an effective measure and practical approach to increase antioxidant defense and consequently reduce the level of oxidative stress. This is supported by numerous studies conducted *in vitro* (Finkel & Holbrook, 2000), but a link between dietary antioxidant supplementation and the protection against diseases such as cancer has not been established (Hayden *et al.*, 2007; Lin *et al.*, 2009). Limited success has been reported for antioxidant therapy in animal models (McCall & Frei, 1999; Yu, 1999). A greater understanding of the pharmacological properties of many of the agents will be required for successful application of antioxidants to reduce oxidative stress, including knowledge on rates of absorption, tissue distribution, metabolism and the microenvironment where they act (Finkel & Holbrook, 2000). Simply adding a pharmacological agent to achieve increased antioxidant defense will not be effective, since most free-radical scavengers act in oxidation-reduction reactions that are reversible and several can act as antioxidants as well as pro-oxidants, depending on the conditions. Determining the the optimal
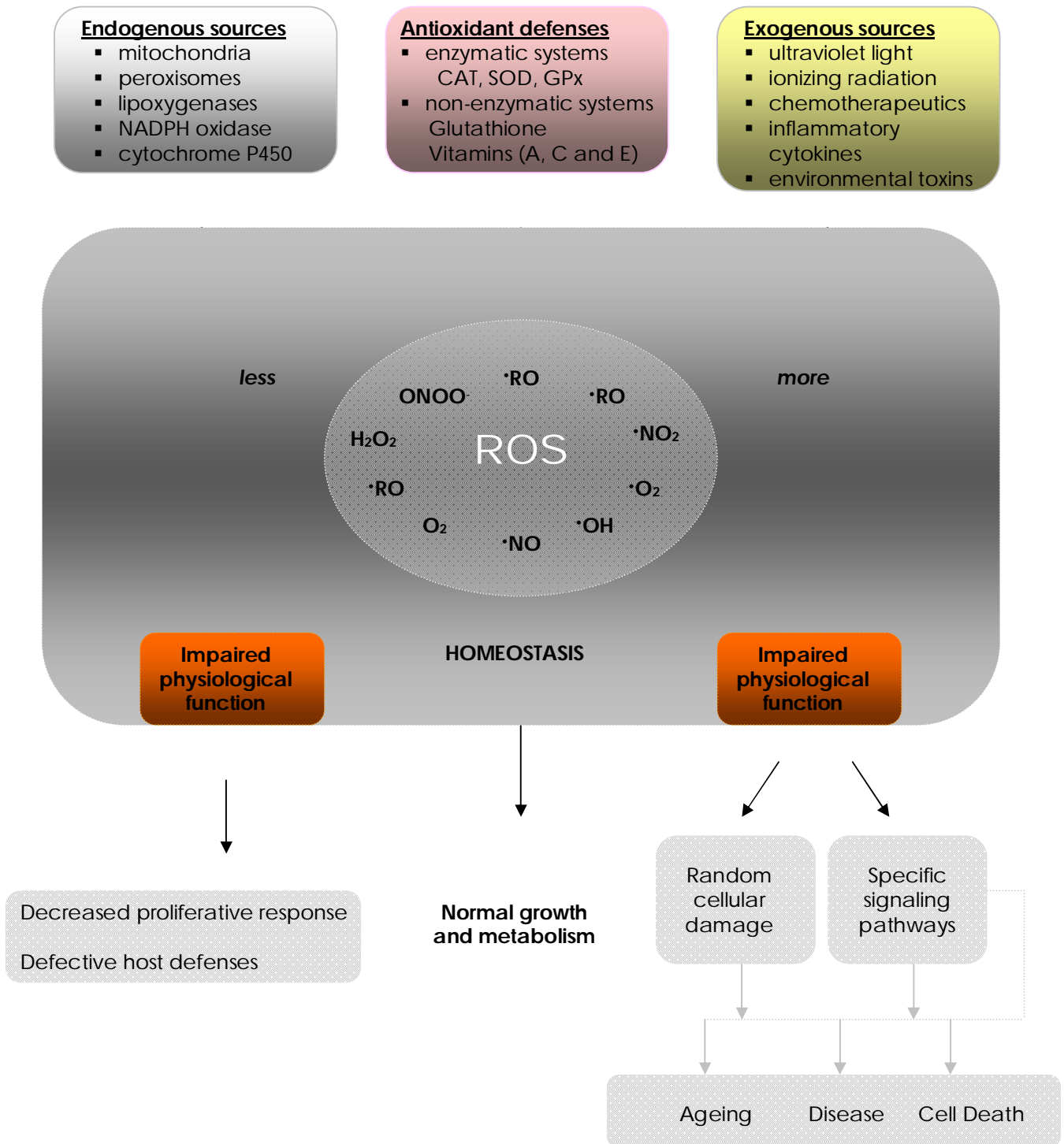
Figure 4 Sources and cellular responses to reactive oxygen species (ROS) (Finkel & Holbrook, 2000). concentration is used to prevent disturbing the delicate redox balance required for the maintenance of normal cell functions.

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dose of supplements is, in addition, complicated by the role of ROS as mediators of normal signaling processes, as discussed above and shown in Fig 4. It is important that the optimal

Dietary polyphenols and toxicity

The use of dietary polyphenol supplements and polyphenol rich food products increased due to the numerous reports on positive effects of polyphenols on disease (Scalbert *et al.*, 2005b). These products do not necessarily contain specified health claims, but different polyphenols have been implicated to reduce oxidative stress and aging, while isoflavones have been recommended to limit hot flushes and to improve bone health in post-menopausal women. Consumption of such products and foods leads to increased intake of polyphenolic compounds beyond the levels of exposure associated with a normal diet and may pose a number of adverse effects and risks. Toxicity has been reported in animals for some herbal products. A green tea (*C. sinensis*) derived preparation, Teavigo, containing more than 90% EGCG, showed toxicity when administered at high doses (2000 mg/kg) in rodents (Isbrucker *et al.*, 2006). Administration of a single dose of 1500 mg/kg EGCG in mice caused hepatotoxicity (Lambert *et al.*, 2010). However, a critical review of the data on the safety of quercetin reported a lack of evidence of *in vivo* toxicity, and also a lack of genotoxic/cardinogenic properties (Harwood *et al.*, 2007). When considering risks and safety of polyphenols, the following hazards are potentially related to polyphenol intake: carcinogenicity/genotoxicity, thyroid toxicity, oestrogenic activity of isoflavones, antinutritional effects, such inhibition of iron absorption and interactions with pharmaceuticals/drugs (Mennen *et al.*, 2005).

(i) Pro-oxidant effects of dietary polyphenols

a) Pro-oxidant acitivity in vitro

Plant-derived antioxidant polyphenols have both pro-oxidative and antioxidative properties that will be determined by their metal-reducing potential, chelating behaviour, pH and solubility characteristics (Babich *et al.*, 2011). Evaluating the pro-oxidant activity of quercetin, p-courmaric acid and their derivatives in NADPH/peroxidase/H_2O_2 and DNA cleavage systems, indicated that the balance between antioxidant and pro-oxidant effects was not apparent (Yang *et al.*, 2012). When considering metal-reducing potential, phenolic compounds with a catechol moiety (aromatic rings with two hydroxyl groups in the ortho position) have pro-oxidant effects under certain conditions by reducing iron (III) or copper (II) ions, thus generating hydroxyl radicals through the Fenton reaction (Scalbert *et al.*, 2005b; Quideau *et al.*, 2011). Furthermore, consumption of high concentrations could potentially lead to pro-oxidant effects (Scalbert *et al.*, 2005b). When considering the role of the structure of polyphenols in pro-

oxidant behaviour, critical factors are the number of hydroxyl groups, hydrophilicity, steric hindrance and the concentration (Rietjens *et al.*, 2002).

When considering protection against lipid peroxidation in cell cultures, flavonoids with a phenol-type substitution pattern on the B-ring may act as pro-oxidants at concentration, where other flavonoids are still active as antioxidants (Galati *et al.*, 1999). The pro-oxidant activity is postulated to result from oxidation of reduced glutathione (GSH) and potentially proceeds as outlined in figure 5 (a). Dietary polyphenols with phenol rings are metabolized by peroxidase to form pro-oxidant phenoxyl radicals in hepatocytes (Galati *et al.*, 2002). The flavonoid semquinone radical is then generated by enzymatic and/or chemical (auto)oxidation (Galati *et al.*, 1999) and is scavenged by GSH regenerating the flavonoid while generating the thiyl radical of glutathione. This anion radical then rapidly reduces molecular oxygen to superoxide anion radicals. Under specific conditions, the phenoxyl radicals are able to oxidize both GSH and NADH and lead to extensive oxygen uptake and reactive oxygen species formation (Galati *et al.*, 2002). Incubation of hepatocytes with dietary polyphenols containing phenol rings partially oxidizes hepatocyte GSH to GSSG.

Flavonoids with a catechol type substituent pattern in their B-ring, especially a 3′,4′-catechol type moiety, are related to the formation of quinone type oxidation products (Rietjens *et al.*, 2002). Scavenging by GSH for these compounds seems to rather occur via conjugate formation, instead of chemical reduction and the formation of GSH flavonoid adducts have been demonstrated (Galati *et al.*, 2001; Galati *et al.*, 2006). For gallic acid and gallic acid tea derivatives, GSH conjugates formation in primary hepatocytes was attributed to their low redox potentials (Galati *et al.*, 2006). Studies in hepatocytes have shown that the catechol B-ring-containing flavonoids with low redox potentials, such as luteolin and quercetin, depleted hepatocyte GSH without causing GSSG formation (Galati *et al.*, 2002). Polyphenols containing a phenol ring were found to generally be more pro-oxidant than polyphenols containing a catechol ring (Galati *et al.*, 2002).

b) Pro-oxidant activity in vivo

Inspite of numerous *in vitro* studies reporting on pro-oxidant and mutagenic potential of polyphenols, quinone reductase, catechol-*O*-methyltransferase and other conjugating enzymes are expected to limit the formation of quinones in endogenous tissues (Scalbert *et al.*, 2005b). Very few studies have been conducted to investigate the pro-oxidant activity of phenolic compounds *in vivo* in laboratory animals or humans (Babich *et al.*, 2011). The low dose of polyphenols resulting from habitual diets together with food matrix effects are posed as reasons for lack of reports on toxicity of polyphenols in epidemiologic studies (Mennen *et al.*, 2005).
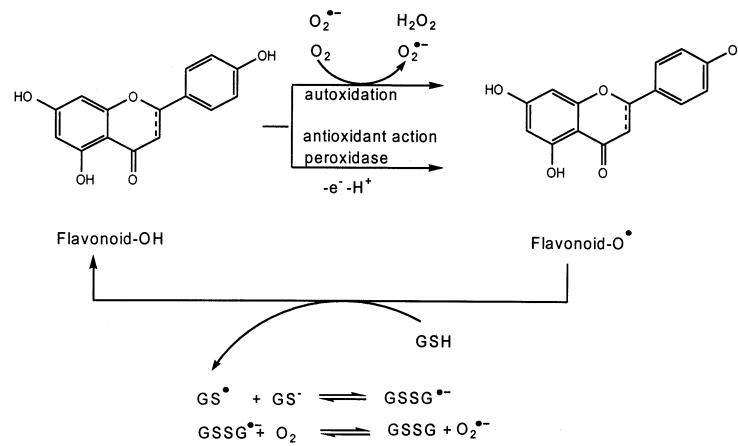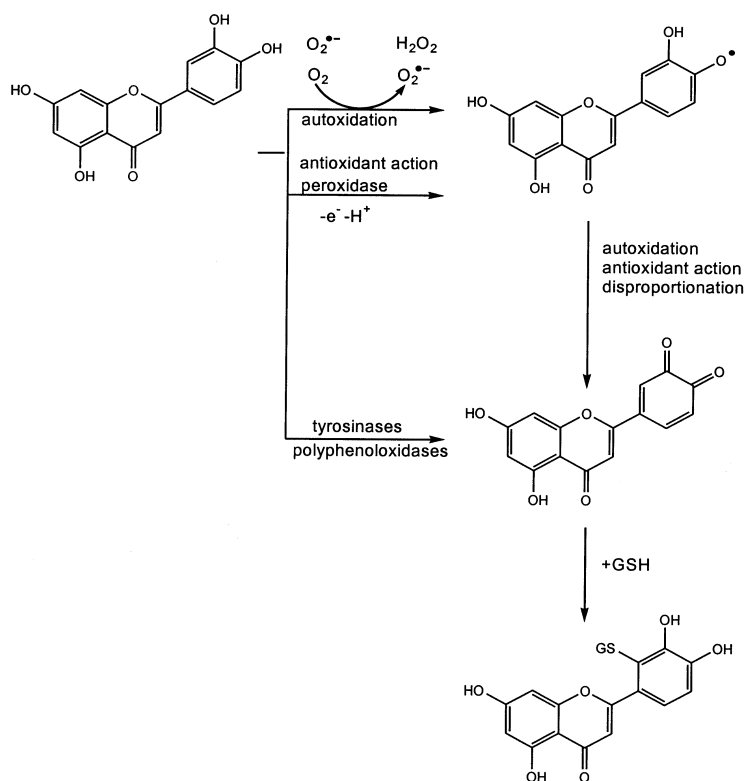
Figure 5 (A) Pro-oxidant chemistry of phenol-type flavonoids and (B) catechol-type flavonoids (Rietjens *et al.*, 2002).

However, carcinogenic and endocrine system-disrupting effects of certain polyphenols reported in animal studies question the ethics of human trials with high doses of these polyphenols.

(iii) Pro-mutagenic/pro-carcinogenic activity of dietary polyphenols

Despite *in vitro* genotoxicity, carcinogenicity studies of flavonoids in mammals are predominantly negative (Middleton & Kandaswami, 1993; Das *et al.*, 1994). Studies in cell cultures have indicate that some polyphenols, including quercetin, could be mutagenic (Scalbert *et al.,* 2005b)*,* yet numerous studies with quercetin showed anticarcinogenic effects in rodents (Scalbert *et al.*, 2005b). However, reports of pro-carcinogenic effects of quercetin in rat models of nitrosomethylurea-induced pancreatic cancer (Barotto *et al.*, 1998) and azoxymethane-induced colon cancer (Pereira *et al.*, 1996), together with consistent findings of positive mutagenic effects *in vitro* have resulted in a intense debate around carcinogenicity of quercetin (Harwood *et al.*, 2007).

Potential interactions of dietary polyphenols with drugs/xenobiotics

(i) Interference with metabolism

Metabolism and elimination of phytochemicals follow the same biochemical pathways and employs the same metabolic enzymes, transporters and efflux pumps as the majority of drugs (Holst & Williamson, 2008). On this basis, phytochemicals may compete with drugs for enzymes, co-factors and transporters and therefore have a significant potential to influence drug uptake, metabolism and clearance from the body (Holst & Williamson, 2008). Although drug-nutrient interactions during absorption and metabolism are very likely to occur and may have enormous health implications, these have rarely been considered (Holst & Williamson, 2008). Changes in metabolism and absorption caused by nutraceuticals might markedly alter the pharmacokinetics of medications given concurrently resulting in potential unwanted effects (Verschoyle *et al.*, 2007). The concentrations of a drug could be either increased or decreased leading to toxicity or decreased efficacy, respectively, or even treatment failure.

(ii) Modulating effect of polyphenols on enzymes

Potential adverse effects of polyphenols may also result from the modulating effects on enzymes. In this regard, the ability of polyphenols to inhibit the activity of a number of enzymes has also been established (Fraga *et al.*, 2010). According to Middleton *et al.* (2000), important enzymes as targets of polyphenols are enzymes with purines, such as adenosine triphosphate

(ATP) as substrates and enzymes with NADPH as a cofactor. Enzymes with purines as substrate include kinases, ATPases, cyclic nucleotide phosphodiesterase, adenylate cyclase, reverse transcriptase, xanthine oxidase, RNA and DNA polymerases, ribonuclease, human DNA ligase, while enzymes with NADPH as a cofactor would comprise aldose reductase, malate dehydrogenase, lactic dehydrogenase, nitric oxide synthase, glutathione reductase, 11-β-hydroxysteriod dehydrogenase. The inhibiting effect of polyphenols on ATP-dependent enzymes is attributed to the competitive binding to the enzyme ATP-binding site (Middleton *et al.*, 2000) of which structural requirements include two hydroxyl substitutions in the 5,7 position in the flavonoid A-ring and 2, 3 unsaturation together with a 4-keto group in the C-ring (Lotito & Frei, 2006).

There is an increasing focus on the importance of considering herb-drug interactions (Chavez *et al.*, 2006). The effect of polyphenols on phase I enzymes, cytochrome P450 (CYP), are of critical importance in this regard as these enzymes are involved in the majority of drug metabolism. The effects of polyphenols on phase I enzymes as reviewed in a systematic evaluation of 60 polyphenols and related compounds on human CYP 3A4 and CYP2C9 activity, revealed that some dietary polyphenols may have the potential to inhibit the metabolism of clinical drugs (Kimura *et al.*, 2010). These included three coumarins and 12 flavonoids displaying inhibitory activities against CYP3A4 or CYP2C9.

Regarding the effects of polyphenols on Phase II enzymes several flavonoids induce UGTs (Zhang *et al.*, 2007). The flavonoid chrysin has been shown to induce UGT1A1 in human hepatic and intestinal cell lines, Hep G2 and Caco-2 (Walle & Walle, 2002). Another study demonstrated that quercetin and kaempferol induced the expression of UGT1A1 and CYP1A2 in HepG2 cells, but not in human primary hepatocytes (Li *et al.*, 2009).

GLOBAL MARKETING TRENDS AND REGULATION OF HEALTH AND SAFETY ASPECTS OF PRODUCTS CONTAINING DIETARY POLYPHENOLS

Nutraceuticals, functional foods and supplements containing polyphenols

Bioactive compounds are currently available in a variety of pharmaceutical forms including pills, capsules, solutions, gels, powders or granules or incorporated in food extracts or phychemical or polyphenol-enriched extracts (Palthur *et al.*, 2010). These products are directly or indirectly implicating beneficial physiological functions, but cannot be classified as food, nutrients or pharmaceuticals and are often refered to as nutraceuticals. Nutraceuticals are defined as "a substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease" (Palthur *et al.*, 2010). Inspite of the

term being recognized internationally, there is no general consensus on its definition and meaning and it creates an open environment for new products that promise novel solutions to health-related issues.   Phytochemicals available on the nutraceutical market include polyphenols such as anthocyanins, proanthocyanidins, flavonols, stilbenes, hydroxycinnamates, coumarins, ellagic acid (EA), isoflavones, lignans and many more. This product type/food group is expected to contribute greatly to future therapeutic developments, but many challenges lie within public policy and regulatory aspects. Although functional foods are regulated in the EU and nutraceuticals are not, the distinction between the two is not always clear (Palthur *et al.*, 2010).   Functional foods are defined as foods that exert a specific health-beneficial effect beyond their nutritional properties, of which the effect must be scientifically substantiated.

The doses of freely available nutritional supplements rich in polyphenols, whether registered, regulated or not, are most alarming. Recommendations for some products are within that attainable in a normal diet, eg. a dose of 50 mg of isoflavones per day could be obtained from soy products in Japan and 100–300 mg grape seed extracts rich in proanthocyanidins per day may be equivalent to that obtained from wine consumption in European countries (Mennen *et al.*, 2005). Others are recommended at intakes far exceeding dietary levels. Examples of capsules available on the internet include 300 mg quercetin, 1 g citrus flavonoids, or 20 mg resveratrol recommended to use 1–6 capsules per day, resulting in a dose of approximately 100 times that obtainable from a Western diet. Furthermore methods of extraction used to produce the supplements may influence the nature of the compounds and consequently also their safety.

Global regulation of safety and health claims for dietary polyphenols in food products and supplements

Dietary supplements and functional foods are not classified as drugs and therefore do not require approval to be marketed in most countries. Subsequently, potential toxicities and drug interactions of these products are mostly unknown. Though not regulated, potential safety issues regarding daily consumption of large doses of flavonoids and isoflavones have been stated (Galati & O'Brien, 2004).

In the European Union (EU), the European Directive 2004/24/EC, released by the European Parliament and by the Council of Europe in 2004 (European Commission, 2004), provided the basis for the use of herbal medicines in Europe. The Directive established that herbal medicines to be released on the market need authorization by the national regulatory authorities of each European country and that these products must have a recognized level of safety and

efficacy. The safety of herbal medicinal products is evaluated on the basis of existing scientific literature, i.e. data from clinical studies, case reports, pre-clinical studies. When data on safety is not sufficient, it is required to be communicated to consumers. According to the criteria of safety and efficacy, herbal medicinal products are classed as: i) 'well established use herbal medicinal products' (medicinal herbs with a recognized level of safety and efficacy); and (ii) 'traditional use herbal medicinal products'. A transitional period of 7 years to register traditional medicinal products that were already on the market was given, which expired on 30 April 2011. All herbal medicines therefore currently require full licensing throughout the EU. There is, however, often controversy over foods being classified as a functional food, drug or a foodstuff, which complicates regulation thereof. This has resulted in dietary supplements being included in regulations of health claims on foodstuffs by some regulatory authorities. Regulation and requirements for health claims in the United States of America (USA), Japan and Codex Alimentarius are relatively different (Verhagen *et al.*, 2010). The categories of health claims in USA, i.e. nutrient content, structure/function claims and health claims, are similar to that used in the EU, but the regulatory status and procedures for use and approval are essentially different. Japanese and USA regulatory provisions for health claims are regarded as quite liberal when compared to the high level of scientific substantiation required in the EU.

The first harmonised legislation governing the use of nutrition and health claims regarding food in the EU came into effect in 2007 (European Commission, 2006). Claims on food labels in the EU were previously controlled on member state level, of which many did not have any legislation (Gilsenan, 2011). The current regulations include the listing of all voluntary beneficial claims relating to food and beverages targeting consumers, and also food supplements and dietetic foods. This has major implications for supplements produced from herbal extracts and functional foods containing increased polyphenols which supposedly increase antioxidant activity and associated health properties. Regulation of claims is not restricted to the packaging, but also includes advertising material, promotional campaigns, websites, catering establishments, menus and also branded images and trademarks that may be interpreted as a nutritional or health claim (Gilsenan, 2011).

Currently there are no regulations regarding the marketing, testing and sales of herbal products in South Africa. The Medicines Control Council (MCC) has attempted to include complementary medicines in the medicine legislation, but this has been rather problematic. Nevertheless, complementary medicine account for a significant share of the medicine market in South Africa. Herbal remedies and dietary supplements are not included in the strict regulation that governs pharmaceutical drugs (Starling, 2011) and are therefore not required to be tested or certified as safe. Regarding health claims, the current labeling regulations in South Africa

(R.146) stipulate that the words "health" or "healthy" or other words or symbols implying that a food, or a substance contained therein, has health-giving properties is prohibited. Futhermore, the word "cure" or any other medicinal claim, which includes prophylactic and therapeutic claims are also prohibited. With regards to a claim for antioxidant activity, the Foodstuffs, Cosmetics and Disinfectants ACT (Act No. 54 of 1972) Regulations relating to the labeling and advertising of foodstuffs (R.146/2010), specify that the claim shall be subjected to pre-market approval and registration by the South African Health Products Regulatory Authority (SAHPRA) for efficacious daily and single serving levels. However, initiation and set-up of SAHPRA is still in progress (personal communications with the South African Department of Health) and currently no health claims are allowed on food packaging in South Africa.

SOUTH AFRICAN HERBAL TEAS: ROOIBOS AND HONEYBUSH AS DIETARY SOURCES OF PHENOLIC AND POLYPHENOLIC COMPOUNDS

The South African herbal teas, rooibos and honeybush, were relatively unknown internationally less than a decade ago, but have become exceedingly popular worldwide (Heinrich & Prieto, 2008). The many traditionally and scientifically established health properties of rooibos and honeybush have been comprehensively discussed in recent reviews (Joubert *et al.*, 2008a, Joubert *et al.*, 2009, Joubert & De Beer, 2011). The following section, therefore, incorporates a brief summary on the background, current research, marketing and the major chemical constituents of rooibos and honeybush. A discussion of the metabolism and bioavailability of the major phenolic compounds present in rooibos and honeybush are included and possible adverse effects of rooibos and honeybush polyphenols are considered.

Rooibos (*Aspalathus linearis*)

(i) Background, current research and marketing trends

Rooibos tea is produced from *Aspalathus linearis* (Burm.f.) Dahlg., belonging to the genus Fabaceae, Tribe Crotalarieae, and is endemic to the Cape Floristic Region (Joubert & De Beer, 2011). This herbal tea is currently consumed in more than 37 countries and a report by the Swiss Business Hub South Africa (Anon, 2007) predicted that rooibos may become the second most commonly consumed beverage tea ingredient in the world after ordinary tea (*Camellia sinensis*).

Potential pharmaceutical product applications of rooibos include use as an anti-diabetic agent (Mose Larsen *et al.*, 2008; Kawano *et al.*, 2009; Joubert *et al.*, 2010), treatment of neurological and psychiatric disorders of the central nervous system (Bruno & Dimpfel, 2009)
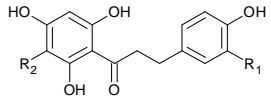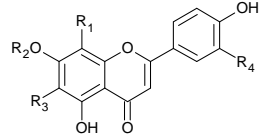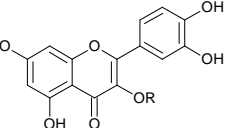
and in topical skin products (Marnewick *et al*., 2005). Many South Africans cannot afford pharmaceuticals and rely on traditional remedies and herbal beverages such as rooibos for its medicinal properties. Rooibos has been associated with numerous health claims over the years, some of which were often inflated and not based on scientific research. However, since the establishment of the South African Rooibos Council (SARC, www.sarooibos.org.za) interpretation and communication of new findings relating to the potential health-benefits of rooibos is handled in a more responsible manner (Joubert *et al*., 2011). The SARC consist of producers, processors, manufacturers and local marketers and exporters and was established in April 2005 to promote the interests of the South African rooibos industry locally and internationally. Additional to its use as herbal tea, rooibos is also applied as intermediate value-added products such as extracts for food, nutraceuticals and cosmetics (Joubert & De Beer, 2011). Current and on-going research projects involving the SARC and conducted in South Africa on health properties of rooibos include investigation of rooibos on satiety, anti-stress potential by inhibition of cortisol, the effect of rooibos on sport performance and ongoing research on anti-cancer properties of rooibos. Other aspects of rooibos currently researched include objective quality and sensory parameters as well as chemical composition.

(ii) Chemical composition of rooibos (Aspalathus linearis)

A major selling point of rooibos as a herbal tea, when compared to tea (*Camellia sinensis*), is that it is naturally caffeine-free (Blommaert & Steenkamp, 1978). Rooibos has also been marketed as a low tannin tea, but it is estimated that an aqueous extract contains approximately 50 % tannin-like substances (Joubert *et al*., 2008a).

The chemical composition and structures of the major monomeric phenolic compounds present in rooibos are summarised in Table 3. Rooibos has an unique flavonoid composition due to the presence of the dihydrochalcone *C*-glucoside, aspalathin (Koeppen & Roux, 1965) and a cyclic dihydrochalcone, aspalalinin (Shimamura *et al*., 2006). Aspalathin is the principle monomeric flavonoid in unprocessed rooibos tea (Koepen & Roux, 1965). Nothofagin, another rare dihydrochalcone *C*-glycoside present in rooibos, is structurally similar to aspalathin, except for the hydroxylation pattern of the B-ring (Table 3). Other phenolic compounds present in rooibos regarded as major include flavones, and flavonols (Koeppen *et al*., 1962; Koeppen & Roux, 1965; Marais *et al*., 2000; Rabe et al., 1994; Shimamura *et al*., 2006). These include the flavones isoorientin, orientin (Koeppen *et al*., 1962; Koeppen & Roux, 1965), vitexin, isovitexin, chrysoeriol (Rabe *et al*., 1994) and luteolin (Snyckers & Salemi, 1974) and the flavonols rutin, isoquercitrin (Koeppen *et al*., 1962) and quercetin (Snyckers & Salemi, 1974). Phenolic acids

Table 3 The major phenolic compounds and contents in unfermented and fermented rooibos (*Aspalathus linearis*) plant material (Joubert & De Beer, 2011).

Polyphenol subgroup	Compound	Substitution	Unfermented Polyphenol concentration [a] g/ 100 g extract	Fermented Polyphenol concentration [a] g/ 100 g extract
Dihydrochalcone				
	aspalathin	R_1=OH, R_2=*C*-β-D-glucopyranosyl	2.56 [b] ± 0.70	0.42 ± 0.02
	nothofagin	R_1=H, R_2=*C*-β-D-glucosylpyranosyl	0.25 ± 0.23	0.04 ± 0.022
Flavone	isoorientin	R_1, R_2=H, R_4=OH, R_3=*C*-β-D-glucopyranosyl	0.45 ± 0.16	0.33 ± 0.05
	orientin	R_1=C-β-D-glucopyranosyl, R_2, R_3=H, R_4=OH	0.26 ± 0.09	0.20 ± 0.03
	vitexin	R_1=*C*-β-D-glucopyranosyl, R_2, R_3, R_4=H	0.04 ± 0.02	0.04 ± 0.01
	isovitexin	R_1, R_2, R_4=H, R_3=*C*-β-D-glucopyranosyl	0.05 ± 0.03	0.04 ± 0.01
	luteolin	R_1, R_2, R_3=H, R_4=OH	0.01 ± 0.01	0.01 ± 0.01
	luteolin-7-*O*-glucoside	R_1, R_3=H, R_2=β-D-glucopyranosyl, R_4=OH	0.02 ± 0.01	0.02 ± 0.01
	chrysoeriol	R_1, R_2, R_3=H, R_4=OCH$_3$	0.003 ± 0.001	0.01 ± 0.002
Flavonol	rutin	R=rutinosyl	0.25 ± 0.14	0.17 ± 0.02
	quercetin	R=H	0.001 ± 0.001	0.01 ± 0.001
	hyperoside	R=galactopyranosyl	0.02 ± 0.01	0.02 ± 0.02

[a] The same plant material was used to produce unfermented and fermented rooibos, [b] Values are the means ± SD given as g/100 g dry plant material of three samples (n=3) as determined with HPLC analyses.

that have been isolated from rooibos tea include *p*-hydroxybenzoic, protocatehuic, vanillic, caffeic, *p*-coumaric and feurulic acids (Rabe *et al.*, 1994). A phenylpyruvic acid glycoside was identified by Marais *et al.* (1996).   Other compounds identified include: lignans, flavone diglycosides, (+)-catechin, the flavonol quercetin-3-*O*-robinobioside and the coumarins, esculetin and esculin (Beltrán-Debón *et al.*, 2011; Breiter *et al,* 2011; Shimamura *et al.*, 2006); patuletin 7-glucoside, esculin, safflomin A, carlinoside, isocarlinoside, neocarlinoside, 2″-*O*-β-arabinopyranosylorientin and vicenin-2, eriodictyol 5,3′di-*O*-glucoside, quercetin-3-*O*-arabinoglucoside and scoparin was reported for the first time by Iswaldi *et al* (2011). The presence of a new luteolin-6-*C*-pentoside-8-*C*-hexoside and a novel C-8-hexosyl derivative of aspalathin in rooibos were reported recently (Beelders *et al.*, 2012).

    Traditional preparation of rooibos includes fermentation to produce the oxidized form (Joubert & De Beer, 2011).   Recent investigation of the oxidative products of aspalathin, showed in addition to flavanones and flavones, the formation of dimers and dibenzofuran during fermentation (Heinrich *et al.*, 2012).   Processed tea is often referred to as fermented, while unfermented tea refers to processed tea (crushed and dried) where processing did not involve chemical oxidation (fermentation).   The fermentation process results in significant quantitative changes with regards to the phenolic composition as shown in Table 3 and is discussed in detail by Joubert *et al.* (2008a), Joubert & De Beer (2011) and Beelders *et al.* (2012).   Limited information was available on the mineral content of rooibos (Joubert & De Beer, 2011) until recently when the presence and levels of 11 minerals including Na, Mg, K, Ca, P, S, Fe, Mn, Zn, Cu and Al was reported (Olivier *et al.*, 2012).

(iii) Metabolism and bioavailability of aspalathin and other major phenolic compounds in rooibos

Several of the flavonoids in rooibos are glycosylated and therefore it would be expected that transformation to their corresponding aglycones by human intestinal bacteria is required for absorption (Joubert *et al.*, 2008a). A study conducted on the absorption of aspalathin as the pure compound and also from an aspalthin-enriched rooibos extract used human abdominal skin in Franz diffusion cells and Caco-2 cell monolayers (Huang *et al.*, 2008) (Table 4). A low level of permeation was evident through skin and distribution in skin layers. The transport of aspalathin was fast and concentration-dependent when considering Caco-2 cells. A significant finding was that transport of aspalathin from the extract preparation, containing aspalathin at a similar concentration than the preparation with the pure compound, showed significantly higher transport, potentially attributed to the presence of other phytoconstituents. It was implied that higher blood levels of aspalathin would be achieved through oral intake of rooibos (aqueous

extract) rather than with a supplement formulation containing pure aspalathin (Huang *et al.*, 2008). Biotranformation was however not considered.

The *in vitro* methylation of aspalathin was evaluated by Courts & Williamson (2009), with human liver cytosolic and human intestinal cytosolic fractions. Two isomeric products evident after incubation of both fractions with different concentrations of aspalathin were identified as 3-*O*-methylaspalathin and 4-*O*-methylaspalathin. The position of hydroxyl methylation was not confirmed, but assumed based on previous research on catechol and quercetin methylation. The relative *O*-methylation rate of the intestinal extract was slightly higher than the liver extract for both metabolites. The potential for hepatic biotransformation, specifically sulphation and glucuronidation, of aspalathin and glucuronidation of nothofagin was demonstrated *in vitro* with rat liver fractions (van der Merwe *et al.*, 2010). Two aspalathin and two nothofagin monoglucuronidated metabolites were present and one sulphated aspalathin metabolite. No sulphation was evident for nothofagin. Glucuronidation resulted in a loss of antioxidant activity of aspalathin in HPLC-ABTS$^{+\bullet}$ and HPLC-DPPH$^{\bullet}$ assays, which may have important implications with regards to *in vivo* bioactivity of aspalathin. It was proposed that the two likely sites of conjugation for aspalathin are 4-OH or 3-OH on the A-ring, while for nothofagin, the 4-OH (A-ring) and 6′-OH (B-ring) seemed to be involved.

In vivo investigation of the metabolism and absorption of aspalathin was first conducted in pigs over an 11 day period (Kreuz *et al.*, 2008) (Table 5). No aspalathin or metabolites were detected in the plasma with LC-MS analyses, with the exception of traces of aspalathin with an increased dose. Aspalathin and five metabolites were detected in the urine and included *O*-methylated and/or glucuronidated aspalathin, a glucuronidated aglycone of aspalathin and an *O*-methylated and glucuronidated eriodictyol metabolite.

Following a bioavailability study of aspalathin in humans (n=6) after consumption of a single oral dose of 300 mL of an aqueous extract of rooibos containing 91.2 mg of aspalathin, two methylated metabolites were present in the urine, 3-*O*-methylaspalathin and 3-*O*-methylaspalathin glucuronide (Courts & Williamson, 2009). However, 4-*O*-methylaspalathin obtained with *in vitro* methylation could not be detected in the urine of human volunteers. The presence of unhydrolysed methylated metabolites of aspalathin in human urine served as indication that deglycosylation is not necessarily a prerequisite for *C*-glycosyl flavonoid absorption in humans. After ingestion of 500 mL of either fermented or unfermented rooibos by 10 volunteers no metabolites or none of the parent compounds could be detected in the plasma (Stalmach *et al.*, 2009). Eight metabolites were detected in the urine including *O*-linked methylated, sulphated and glucuronidated metabolites of aspalathin and an eriodictyol-*O*-sulphate.

Table 4 *In vitro* studies on the metabolism and absorption of aspalathin.

Compound/Extract	Model	Analytical Method	Outcome	Reference
aspalathin	measure transport across human abdominal skin in vertical Franz diffusion cells Caco-2 cell monolayers	HPLC	low level of permeation through skin and distribution in skin layers	Huang *et al.* 2008
unfermented aspalathin enriched rooibos extract	measure transport across human abdominal skin in vertical Franz diffusion cells Caco-2 cell monolayers	HPLC	fast, concentration-dependent transport of aspalathin; higher transport from extract than pure aspalathin	Huang *et al.* 2008
aspalathin	human liver cytosolic fraction with SAM [a] (co-factor)	LC-MS/MS	3-*O*-methylaspalathin, 4-*O*-methylaspalathin	Courts & Williamson, 2009
	human intestine cytosolic fraction with SAM (co-factor)	LC-MS/MS	3-*O*-methylaspalathin, 4-*O*-methylaspalathin	Courts & Williamson, 2009
aspalathin	liver biotransformation of aspalathin and nothofagin and the effect glucuronidation on antioxidant activity of aspalathin rat liver cytosolic fraction with PAPS [b] (co-factor)	HPLC/ LC-MS and LC-MS/MS	one sulfated asplathin metabolite; no sulfated nothofagin	Van der Merwe *et al.*, 2010
	rat liver microsomal fraction for glucuronidation with UDPGA (co-factor)	HPLC/ LC-MS and LC-MS/MS Online radical scavenging assays	two glucuronidated metabolites for both aspalathin and nothofagin	Van der Merwe *et al.*, 2010

[a] S-adenosyl methionine (SAM), [b] 3′-phosphoadenosine-5′-phosphosulfate (PAPS).

Table 5 *In vivo* studies on the metabolism and bioavailability of aspalathin.

Substance Consumed	Administration/ Dose	Subjects (no.)	Method of Analysis	Aspalathin and metabolites	Reference
aspalathin-enriched unfermented rooibos (*Aspalathus linearis*)	oral dose 157-167 mg aspalthin/kg bw [a] per day for 11 days	pigs (n=3)	LC-MS	no metabolites detected in plasma aspalathin and 5 metabolites detected in the urine: *O*-methylated and/or glucuronidated aspalathin, glucuronidated aglycone of aspalathin *O*-methylated and glucuronidated eriodictyol metabolites	Kreuz *et al.*, 2008
unfermented *Aspalathus linearis* (aqueous extract)	single oral dose 91.2 mg	humans (n=6)	LC-MS/MS	3-*O*-methylaspalathin and 3-*O*-methylaspalathin glucuronide present in the urine	Courts & Williamson, 2009
unfermented and fermented rooibos *Aspalathus linearis* (aqueous extract)	single oral dose	humans (n=10)	HPLC-MS	no metabolites detected in the plasma nine metabolites detected in the urine: *O*-linked methyl, sulfate and glucuronide metabolites of aspalathin and an eriodictyol-*O*-sulfate	Stalmach *et al.*, 2009
2 rooibos drinks: (i) unfermented *Aspalathus linearis* (aqueous extract) (ii) active fraction from unfermented *Aspalathus linearis* isolated by HSCCC (aqueous extract)	single oral dose (water, unfermented dose or active fraction) 287 mg	humans (n=12)	HPLC-MS/MS	trace amounts of unchanged flavonoids in plasma aspalathin and nothofagin detected intact in urine seven metabolites detected in the urine: sulfated, glucuronidate and methylated aspalathin and methylated glucuronidated aspalathin. methylated aspalathin was the main metabolite.	Beiter *et al.*, 2011

[a] bw=body weight.

The main compounds excreted differed for the unfermented and fermented tea and were *O*-methyl-aspalathin-*O*-glucuronide and eriodictyol-*O*-sulphate, respectively. Poor bioavailability of the dihydrochalcone aspalathin and the flavanone *C*-glucosides from unfermented and fermented rooibos tea were indicated by excretion of only trace amounts in urine up to 24 h after consumption. It was assumed that most rooibos flavonoids pass from the small to the large intestine where they are subjected to the action of colonic microflora followed by cleavage of the sugar moiety and ring fission producing low molecular weight phenolic acids. These catabolites would then be absorbed into the portal vein (Stalmach *et al.*, 2009).

More recently a human study with twelve volunteers was conducted with rooibos prepared from the unfermented tea and a drink prepared from an isolated active fraction from rooibos (Breiter *et al.*, 2011). Seven metabolites of aspalathin and nothofagin were identified in the urine samples. Intact aspalathin and nothofagin were also detected in urine. The metabolites included sulphated, glucuronidated and methylated aspalathin and also a methylated glucuronidated aspalathin. Methylated aspalathin was the main metabolite excreted. Other compounds present were the glucuronidated aglycone of aspalathin (2,3,4,4',6-pentahydroxy-dihydrochalcone) and glucuronidated nothofagin and its glucuronidated aglycone (glucuronidated 2',4,4',6'- tetrahydroxy-dihydrochalcone).

Honeybush (*Cyclopia spp.*)

(i) Background, current research and marketing trends

Honeybush tea is made from *Cyclopia* spp., which belongs to the endemic fynbos biome genus *Cyclopia* Vent. (family Fabaceae; tribe Podalyrieae) and occurs on the coastal plains and mountainous regions of the Western and Eastern Cape provinces of South Africa (Joubert *et al.*, 2011). It is a traditional South African herbal tea and is becoming increasingly popular as a result of a growing amount of scientific evidence implicating its potential health benefits, specifically with regards to its anticancer, phytoestrogenic properties (Joubert *et al.*, 2008a) and recently the potential to treat diabetes (Muller *et al.*, 2011). It was a very small, also referred to as "cottage" industry until the mid-1990's, but during the last three years honeybush has been sold in 25 countries, which includes the Netherlands and Germany (Joubert *et al.*, 2011). A recent comprehensive review of South African herbal teas (Joubert *et al.*, 2008a) included honeybush and focused on the phenolic composition and biological properties. Research activities during the past 20 years including propagation, production, genetic improvement, processing, composition and the potential for value-adding are discussed in detail in the review by Joubert *et al.* (2011).
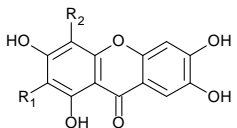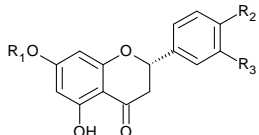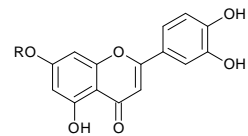
Current developments include patent protection with regards to the anti-diabetic potential for extracts produced from honeybush (*Cyclopia*) (Mose Larsen *et al.*, 2008). An aqueous extract of *C. intermedia* was recently shown to be effective in reducing plasma glucose concentration in a streptozotocin (STZ)-induced diabetic rat model (Muller *et al.*, 2011). With regards to value-adding potential of *Cyclopia* the demand of the tea still exceeds the supply (Joubert *et al.*, 2011). A small quantity of extracts is however produced for use in food products, cosmetics and toiletries.

(ii) Chemical composition of honeybush (*Cylopia* spp.)

Due to increased interest in honeybush (*Cyclopia* spp.) as a herbal tea and its potential as a nutraceutical, characterisation of the phenolic composition has become important (De Beer & Joubert, 2010). The major phenolic compounds present and their content in three commercially important *Cyclopia* spp., *C. genistoides*, *C. subternata* and *C. genistoides,* are summarised in Table 6. Similar to rooibos, honeybush is caffeine free (Greenish, 1881) with a low tannin content (Terblanche, 1982).   Processing of honeybush, as for rooibos, is important for development of its characteristic sweet aroma and flavour and the dark reddish brown colour (Joubert *et al.*, 2011). The process is also referred to as fermentation and results in a significant reduction of the total polyphenol content (Joubert *et al.*, 2008b). Unfermented or unprocessed (green) honeybush is produced by preventing oxidation and browning of the plant material (Joubert *et al.*, 2011).

De Nysschen *et al.* (1996) screened methanol extracts from the leaves of 22 unprocessed *Cyclopia spp.* for the presence of phenolic compounds and reported three major constituents, a xanthone *C*-glycoside, mangiferin, and the two flavanones hesperetin and isosakuranetin. The polyphenol composition of only two of the *Cyclopia* spp., *C. intermedia* and *C. subternata*, has been studied in depth (Ferreira *et al.*, 1998; Kamara *et al.*, 2003; Kamara *et al.*, 2004). De Beer & Joubert (2010) recently developed a HPLC method for the phenolic analysis of *C. subternata*, which was applied to the three other species, *C. intermedia*, *C. genistoides* and *C. sessiliflora*. This study analysed the unfermented as well as the fermented plant material.   The major compounds present in all four species are the xanthones, mangiferin and isomangiferin and the flavanone, hesperidin (De Beer & Joubert, 2010).   Several other compounds have been identified and include flavanones, flavones, flavonols, coumestans and other compounds (De Nysschen *et al.*, 1996; Ferreira *et al.*, 1998; Kamara *et al.*, 2003, Kamara *et al.*, 2004; Kokotkiewicz *et al.*, 2012). Flavanones identified in *Cyclopia* spp. include eriocitrin, narirutin, naringenin, hesperetin, eriodictyol-5-*O*-β-D-glucopyranoside, eriodictyol, naringenin-5-*O*-β-D-glucopyranoside, eriodictyol-7-*O*-β-D-glucopyranoside and an unidentified flavanone-glycoside.

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Table 6 The major phenolic compounds and contents in green (unfermented) honeybush (*Cyclopia* spp.) plant material (De Beer & Joubert, 2010).

Polyphenol subgroup and structure	Compound/Substitution	*C. genistoides* Polyphenol concentration	*C. subternata* Polyphenol concentration	*C. intermedia* Polyphenol concentration
Xanthone	mangiferin ($R_1 = C$-β-D-glucopyranosyl, $R_2 = H$)	$9.55^a \pm 3.0$	2.73 ± 1.65	4.35 ± 1.93
	isomangiferin ($R_1 = H$, $R_2 = C$-β-D-glucopyranosyl)	2.72 ± 0.67	0.86 ± 0.28	1.4 ± 0.49
Flavanone	hesperidin ($R_1 =$ rutinosyl, $R_2 = OH$, $R_3 = OCH_3$)	0.71 ± 0.09	0.62 ± 0.17	0.62 ± 0.16
	eriocitrin ($R_1 =$ rutinosyl, $R_2 = R_3 = OH$)	traces	0.32 ± 0.07	0.13 ± 0.05
	Eriodictyol-glucoside (unidentified)	nd	0.35 ± 0.07	0.07 ± 0.04
Flavone	scolymoside ($R =$ rutinosyl)	traces	0.68 ± 0.62	0.04 ± 0.01

[a] Values represent the mean ± SD given as g/100 g dry plant material of six samples (n=6) as determined with HPLC analysis.

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Flavones shown to be present were luteolin, 5-deoxyluteolin, scolymoside and diosmetin, isoflavones were formononetin, a formononetin-diglucoside, aformosin, calycosin, wistin, orobol, pseudobaptigenin, fujikinetin and isosakuranetin and flavonols included several kaempferol glucosides. Coumestans comprise medicagol, flemmichapparin and sophoracoumestan and other compounds present are EGCG, *p*-coumaric acid, tyrosol and several tyrosol derivatives.

The method developed by De Beer & Joubert (2010) enabled quantification of several well-known as well as unidentified phenolic compounds and is suitable for quantification of the major phenolic compounds present in the water extracts of unfermented and fermented *C. subternata, C. intermedia, C. genistoides* and *C. sessiliflora*. Compounds previously identified and reported following analysis with this method included mangiferin, isomangiferin, eriocitrin, hesperidin and scolymoside, while narirutin and luteolin were detected in most samples, but could not be quantified due to low concentrations and/or co-elution with other compounds. Unknown compounds were identified according to their retention time and UV-Vis spectra based on the evaluations with UV-Vis, LC-ESI-MS and LC-ESI-MS/MS data. These included a flavone glycoside, eriodictyol-glucoside and an unidentifed compound; all quantified as hesperidin equivalents, exept an unidentified hydroxycinnamic acid derivative quantified as mangiferin equivalents. Isolation of the unidentified compounds and investigation of bioactivities were recommended.

Variations found in phenolic composition within the same *Cyclopia* species has been mostly attributed to differences in solubility of phenolic compounds, due to the solvents used for analysis, but it has also been postulated that growth and the leaf to stem ratio of the plant material used in production influences the phenolic composition of the final product (Joubert *et al.*, 2003). Variations in the polyphenolic content of two types of *C. genistoides* due to date of harvesting have been demonstrated. Large variation in samples of the same species, attributed to the nature of the plant material, was also reported by De Beer & Joubert (2010). Qualitative and quantitative differences are evident between *Cyclopia* species when considering the phenolic composition. Fermentation is used as a high-temperature process that involves non-enzymatic oxidation to develop the traditional sensory attributes. It is, however, also responsible for a decrease in the content of most phenolic compounds (De Beer & Joubert, 2010), and also antioxidant activity of all species, with the exception of the ability of *C. genistoides* to inhibit lipid peroxidation *in vitro* (Joubert *et al.*, 2008b). The unfermented plant material of different *Cylopia* spp. has a higher mangiferin content than their fermented counterpart after processing.

It is apparent that the phellenolic composition of *Cyclopia* spp. is influenced by wide-ranging factors, which necessitate proper characterization of the extracts when investigated for

biological properties. Regarding supplementary chemical anaysis, the volatile fraction of unfermented and fermented *C. genistoides* has been studied by headspace gas chromatography with mass spectrometry (GC-MS) and olfactory (GC-O) detections (Le Roux *et al.*, 2008; Le Roux *et al.*, 2012) reporting major quantitative differences in fermented and unfermented preparations.

(iii) Metabolism and bioavailability of mangiferin and other major phenolic compounds in honeybush

The outcome of a number of studies on the absorption and metabolism of mangiferin is summarised in Table 7. It appears that deglycosylation is not a requirement for absorption of mangiferin as demonstrated by the presence of the parent compound in plasma, urine, bile and tissue (Li & Bi, 2003; Lai *et al.*, 2003; Wang *et al.*, 2006; Wang *et al.*, 2007; Li *et al.*, 2008; Ma *et al.*, 2008; Liu *et al.*, 2011). The oral bioavailability has, however, been shown to be relatively poor in the pig and rats (Bock *et al.*, 2008; Han *et al.*, 2010).

The metabolites, 1,7-dihydroxyxanthone (euxanthone) and its glucuronidated derivative, were detected in rabbit urine after administration of an oral dose of mangiferin (Krishnaswamy *et al.*, 1971). An HPLC method was developed for determination of mangiferin in plasma and urine of rats after intravenous administration of 10 mg/kg (Geodakyan *et al.*, 1992a). This was followed by a pharmacokinetic study of mangiferin, the main component of the drug Alpizarin in rats with intravenous injection of the drug in a single dose of 0.3, 1, 3, 10 and 30 mg/kg and also after oral administration in a single doses of 50–500 mg/kg (Geodakyan *et al.*, 1992b). HPLC analysis of plasma did not detect mangiferin. Detection of mangiferin in rat plasma followed by determination of pharmacokinetic parameters after administration of Zhimu, a traditional Chinese medicine (TCM) containing mangiferin, indicated that mangiferin is absorbed slowly (Li & Bi, 2003). Intravenous administration of three doses of mangiferin (10, 30 and 100 mg/kg) resulted in the presence of mangiferin in rat blood as determined using dialysis (Lai *et al.*, 2003). With regards to pharmacokinetics, a linear relation was evident for the lower dose, 10 and 30 mg/kg, while higher doses resulted in a nonlinear pharmacokinetics. Metabolites identified in the bile dialysate with LC-MS/MS included methylated glucuronidated, sulphated and methylated mangiferin. Phase II metabolites of mangiferin that have been detected include *O*-methylated-*O*-monoglucuronidated and *O*-methylated mangiferin in rat urine (Ma *et al.*, 2008) and bile (Lai *et al.,* 2003) and glucuronidated mangiferin (Lai *et al.,* 2003; Ma *et al.*, 2008). Sulphated mangiferin was found in rat bile (Lai *et al.*, 2003), but sulphation could not be confirmed in pigs (Bock et al., 2008).

Table 7 A summary of studies conducted on bioavailability and *in vivo* detection of mangiferin.

Substance Consumed	Administration/ Dose	Days	Subjects	Method of Analysis	Mangiferin and Metabolites	Reference
mangiferin			rabbit	HPLC	two metabolites in urine 1,7-dihydroxyxanthone (euxanthone) and its glucuronide	Krishnaswamy *et al.*, 1971
mangiferin	oral 50-500 mg/kg	single dose	rat	HPLC	no mangiferin detected in plasma	Geodakyan *et al.*, 1992
Zhimu decoction TCM [a] (1g/mL)	oral 20 mL/kg	single dose	rat	HPLC	mangiferin detected in rat plasma	Li & Bi., 2003
		single dose		pharmacokinetic application HPLC		Li & Bi., 2003
mangiferin	intravenous administration 10, 30 and 100 mg/kg	single dose	rat	dialysate collected every 10 min and injected directly into microbore HPLC system. Pharmacokinetic Application HPLC	mangiferin detected in rat blood 10–30 mg/kg - linear relation, 30–100 mg/kg - nonlinear pharmacokinetic relation	Lai *et al.*, 2003
	intravenous administration 100 mg/ kg			Phase I and II metabolites bile dialysate LC- MSMS	m/z 611 (methylated glucuronidated mangiferin), m/z 501 (sulphated mangiferin), m/z 435 (methylated mangiferin)	Lai *et al.*, 2003

[a] Traditional Chinese Medicine (TCM).

Table 7 (*Cont.*)

Substance Consumed	Administration/ Dose	Days	Subjects	Method of Analysis	Mangiferin and Metabolites	Reference
mangiferin	oral 120 mg/kg	single dose	rat	pharmacokinetic application HPLC	mangiferin detected in plasma and urine	Wang *et al.*, 2006
mangiferin	oral 120 mg/kg	single dose	rat	HPLC	four metabolites detected in rat urine: 1,3,7-trihydroxyxanthone, 1,3,6,7-tetrahydroxyxanthone, 1,3,6-trihydroxy-7-methoxyxanthone 1,7-dihydroxyxanthone	Wang *et al.*, 2007
Rhizoma Anemarrhenae extract	15 g/kg	single dose	rat	pharmacokinetic application HPLC	mangiferin in plasma mangiferin was extensively distributed in most of the in tissue samples (brain, heart, liver, spleen, lung, kidney, stomach, intestine, smooth muscle, skeletal muscle and testis) of rats	Li *et al.*, 2008
unfermented *C. genistoides*	oral 74 mg mangiferin/kg	per day for 11 days	pig	metabolites LC- MS LC-MSMS	norathyriol in plasma methylated mangiferin, norathyriol and glucuronidated norathyriol in urine	Bock *et al.*, 2008
unfermented *C. genistoides*	oral 74 mg mangiferin/kg	per day for 11 days	pig	metabolites LC- MS LC-MSMS	phenolic acids detected in feces. 3,4-dihydroxybenzoic acid, 3,4 dihydroxyphenylacetic acid, 2,4,6-trihydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid	Bock *et al.*, 2010
mangiferin	intravenous administration 10, 25 and 50 mg mangiferin /kg		rat	HPLC	5.69 ± 1.48 µg/ml mangiferin in the retina 0.5 h after administration (50 mg/kg) plasma concentration was dose-dependent.	Hou *et al.*, 2010
mangiferin	oral 0.1, 0.3 and 0.9 g	single dose	human (male)	HPLC-MS	38.64 ± 6.75 ng/mL mangiferin in plasma 1 h after administration of 0.9 g elimination half-life ($t_{1/2}$) 7.85 ± 1.72 absorption increase with dose nonlinear pharmacokinetics	Hou *et al.*, 2012

A study with *Rhizoma Anemarrhenae* (Z*himu*), one of the herbs used in TCM, reported that mangiferin was distributed extensively in different tissue samples (brain, heart, liver, spleen, lung, kidney, stomach, intestine, smooth muscle, skeletal muscle and testis) after oral administration in rats. The highest level was present in the stomach with a concentration of 193 µg/g at 0.5 h (Li *et al.*, 2008). Peak concentrations of mangiferin in the smooth and skeletal muscle, small intestine, heart and lung was evident after 4h and then declined.

Oral administration of a green *Cyclopia genistoides* extract failed to show mangiferin or phase II metabolites in the plasma of pigs, while methylated mangiferin was detected in the urine (Kreuz *et al.*, 2010). The transformation of mangiferin into norathyriol by human fecal flora has been demonstrated *in vitro* (Hattori *et al.*, 1988, Sanugal *et al.*, 2005a; Sanugal *et al.*, 2005b) indicating an important role of bacteria metabolizing mangiferin in the disposition of norathyriol and its subsequent metabolites *in vivo*. Phenolic acids detected in faeces of pigs after oral consumption of unfermented *C. genistoides* included 3,4-dihydroxybenzoic acid, 3,4 dihydroxyphenylacetic acid, 2,4,6-trihydroxybenzoic acid and 3,4,5-trihydroxybenzoic acid (Bock *et al.*, 2010). The first pharmacokinetic study of mangiferin in humans reported a plasma concentration of 38.64 ± 6.75 ng/mL plasma after oral administration of 0.9 g mangiferin and an apparent elimination half-life ($t_{1/2}$) of 7.85 ± 1.72 h (Hou *et al.* 2012).

Potential adverse effects of the South African herbal teas, rooibos and honeybush

The historical use of rooibos and honeybush as herbal teas in South Africa has led to a general assumption of its safety (Joubert *et al.*, 2008a). The safety of herbal medicines can, however, not always be guaranteed by long-standing use. For instance, modern phototherapy often incorporates the use of plants in a new manner resulting in unknown risks (Calapai, 2008). This is specifically applicable to development of value added products from rooibos or honeybush.

To date, no reports of toxicity for the normal use as a herbal tea has been documented for rooibos, with the exception of a case report by Sinisalo *et al.* (2010), indicating possible hepatotoxicity due to rooibos ingestion in a female with low-grade B-cell malignancy. However, the adverse effects were attributed to possible contamination of the tea with hepatotoxic compounds, genetic predisposition and/or adverse drug interactions with the tea. *In vitro* pro-oxidant activity has been reported for rooibos (Joubert *et al.*, 2005). This would imply that a hydroxyl radical could be generated in the presence of iron, which could result in significant adverse effects *in vivo* under disease conditions when iron is mobilized. It can, however, be predicted that the absorption and biotransformation will have significant effects on the activity of the parent compound and therefore the pro-oxidant activity displayed *in vitro* is not necessarily an indication of pro-oxdiant activity *in vivo.* A mutagenic effect was evident with rooibos when

considering sex coupled recessive lethal mutations in *Drosophila* (Neethling *et al.*, 1988). The concentration used was, however, 220–230 times that of the concentration of rooibos consumed in a normal cup of tea (Joubert *et al.*, 2008a).

Limited information is available on the *in vivo* effects of *Cyclopia* spp. Most studies on the health associated properties of honeybush have been conducted *in vitro.* Regarding, mangiferin, the major phenolic present in honeybush, no lethal effect was observed in rats when administrated up to the very high level of 1000 mg/kg body weight (Prabhu *et al.*, 2006). A study on the protection of mangiferin against isoproterenol (ISPH)-induced myocardial infarction (MI) and contribution toward the tissues defense system against cardiac damage (Prabhu *et al.*, 2006) included four different treatment groups, i.e. control, ISPH treated, mangiferin treated and pre-treated with mangiferin followed by ISPH treatment. In the mangiferin treated group receiving 100 mg/kg bodyweight i.p. for 28 days, glutathione and antioxidant enzymes in the heart tissue of rats were not significantly altered when compared to the control group. However these parameters were significantly improved in mangiferin pre-treated rats compared to the ISPH induced group (Prabhu *et al.*, 2006).

An important factor in the promotion of rooibos and honeybush teas in future would be knowledge on the potential herb-drug interaction and consequent adverse effects. Following treatment of rats with rooibos for three days, the expression of CYP2C11, known to metabolise a variety of drugs, was inhibited (Jang *et al.*, 2004). Consequences of inhibition of this CYP would potentially include an increased half life and could lead to toxicity. In another study administering rooibos in the drinking water of rats, the activity and expression of intestinal CYP3A were increased (Matsuda *et al.*, 2007). This resulted in the increased hydroxylation of the drug midazolam, which is a short acting benzodiazepine derivative. CYP3A catalyzes the metabolism of numerous therapeutic drugs and it is therefore apparent that rooibos might interfere with xenobiotic metabolism presumably by interfering with the formation of the substrate enzyme complex. It was recently shown that polyphenol-enriched rooibos and honeybush (*C. subternata* and *C. genistoides*) herbal extracts differentially alter the expression of genes associated with xenobiotic metabolism in rat liver and kidneys after administering in the feed for 28 days (Abrahams *et al.*, 2009; Abrahams, 2012). This study was the first investigation of the *in vivo* effects of polyphenolic-enriched extracts of the South African herbal teas on the expression of genes encoding xenobiotic metabolising enzymes in rat liver and kidneys. The herbal teas significantly altered the gene expression of xenobiotic metabolising enzymes in the liver and kidneys of male Fischer rats, suggesting that rooibos and honeybush herbal teas can inhibit and/or enhance the metabolism of both foreign and endogenous compounds. Greater effects were observed for *C. subternata* as compared to *C. genistoides*

inspite of significantly higher xanthone content (mangiferin and isomangiferin). Further investigation into the effects on protein expression of the altered genes and enzyme binding was proposed to provide insight into the downstream effects.

Other potential adverse effects of rooibos and honeybush and their products would be their effect on iron absorption. The effect of tea on the absorption of iron has been a controversial issue for many years. Marnewick *et al.* (2003) did not report any effect on the serum iron levels following consumption of unfermented and fermented rooibos and honeybush (*C. intermedia*) in rats for 10 weeks. The effect of rooibos on iron absorption was investigated in 10 human volunteers receiving 200 ml fermented rooibos, black tea (fermented *Camellia sinensis*) or water with 20 mL milk and 20 g sugar added to each sample (Hesseling *et al.*, 1979). The serum parameters were monitored after 14 days and included hemoglobin, ferritin, transferring, serum iron and iron binding capacity. No effect was indicated for rooibos, while black tea reduced iron uptake. A study in school children consuming two 200 mL servings of fermented rooibos and black tea daily for a more extended period of 16 weeks, also did not report any adverse effects on the iron levels (Breet *et al.*, 2005). In addition, a recent study on the effect of rooibos on biochemical and oxidative stress parameters in adults at risk for cardiovascular disease did not report any effect on the serum iron levels after six weeks of consuming six cups of the fermented rooibos herbal tea per day (Marnewick *et al.*, 2011).

CONCLUSIONS

Only rigorous scientific testing adhering to the principles of evidence-based medicine will assist in the use of herbal medicine to become more than a fashionable trend (Stickel & Schuppan, 2007). It is essential to confirm the effects observed with aglycones through studies using physiologic concentrations of the metabolites that have been identified in the body (Manach *et al.*, 2004). The major problems in research on the health effects of polyphenols include limited knowledge on biotransformation and absorption, biological activity of metabolites, absorption, cellular levels and excretion of these compounds. Further complications arise from challenges with regards to application of the available knowledge in order to unravel the mechanisms involved in health effects observed for dietary polyphenols and their modulatory effects on enzymes. Significant progress has nevertheless been made with respect to reseach on the health properties and contents of the South African herbal teas, rooibos and honeybush, however, many areas, such as safety and effective dose levels require further clarification.

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CHAPTER 3

IN VITRO HEPATIC BIOTRANSFORMATION OF ASPALATHIN AND NOTHOFAGIN, DIHYDROCHALCONES OF ROOIBOS (*ASPALATHUS LINEARIS*), AND ASSESSMENT OF METABOLITE ANTIOXIDANT ACTIVITY

Part of this work was presented* at:

- *9th Annual AstraZeneca Research Day, Groote Schuur, 14 February 2008, Cape Town, South Africa.*

- *South African Society of Biochemistry and Molecular Biology (SASBMB) meeting as part of Bio-08 conference, 23-24 January 2008, Grahamstown, South Africa.*

- *24th International Conference on Polyphenols, 8-11 July 2008, Salamanca, Spain.*

* Details listed in Addendum A.

Van der Merwe, J.D., Joubert, E., Manley, M., De Beer, D., Malherbe, C.J. & Gelderblom, W.C.A. (2010). *Journal of Agricultural and Food Chemistry*, **58**, 2214–2220.

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ABSTRACT

Aspalathin (2′,3,4,4′,6′-pentahydroxy-3′-*C*-β-D-glucopyranosyldihydrochalcone) is the major flavonoid present in the South African herbal tea, rooibos. *In vitro* metabolism of aspalathin and a structural analogue nothofagin, lacking the A-ring catechol group, was investigated by monitoring the formation of glucuronyl and sulphate conjugates using Aroclor 1254-induced and un-induced rat liver microsomal and cytosolic subcellular fractions. Following glucuronidation of both aspalathin and nothofagin, HPLC-DAD, LC-MS and LC-MS/MS analyses indicated the presence of two metabolites, one major and one minor. Only one aspalathin metabolite was obtained after sulphation, while no metabolites were observed for nothofagin. Two likely sites of conjugation for aspalathin are 4-OH or 3-OH on the A-ring. For nothofagin, the 4-OH (A-ring) and 6′-OH (B-ring) seem to be involved. The glucuronyl conjugates of aspalathin lack any radical scavenging properties in on-line post-column DPPH radical and ABTS radical cation assays.   Deconjugation assays utilizing glucuronidase and sulfatase resulted in the disappearance of the metabolites, with the concomitant formation of the unconjugated form in the case of the glucuronidated product.   The balance between conjugated and unconjugated forms of aspalathin could have important implications regarding its role in affecting oxidative status in intra- and extra-cellular environments *in vivo.*

INTRODUCTION

The biological properties of polyphenols have been the topic of many research investigations. Assessing biological activity *in vitro* does not give a true reflection of their activity *in vivo*, since polyphenols are known to be metabolized, especially by phase I and phase II enzymes in the liver, leading to high levels of conjugates in the plasma and urine (Walle, 2004). Studies in animals and humans indicated that these conjugation reactions also occur in the kidneys (McGurk *et al*., 1998). This could explain why certain metabolites are not detected in blood, but high levels are present in urine. Metabolic alterations possibly reduce or abolish the biological properties associated with the parent compound *in vitro* (Day *et al*., 2000).   However,

conjugation/deconjugation reactions in the liver related to sulphate and glucuronide metabolites play an important role in the physiological regulation of xenobiotics (O'Leary *et al.*, 2001).

The health promoting properties of rooibos (*Aspalathus linearis*) have been reported in different *in vitro* and *in vivo* assays, stimulating studies on the bioactivity of its polyphenolic constituents, as they relate to their bioavailability and metabolism (Joubert *et al.*, 2009). Aspalathin and nothofagin (Fig. 1), two *C*-linked dihydrochalcone glucosides, are the major flavonoid constituents in green rooibos (Joubert & Shulz, 2006) with nothofagin demonstrating slightly less potent antioxidant activity in an aqueous environment when using *in vitro* radical scavenging assays (Krafczyk *et al.*, 2009; Snijman *et al.*, 2009). Furthermore, nothofagin exhibits a far less protecting effect against Fe(II)-induced lipid peroxidation in a lipid environment when using a hydrophobic biomembrane assay system (Snijman *et al.*, 2009). Conformational differences and the absence of the catechol moiety in the nothofagin molecular structure were postulated to explain differences in their antioxidant effects in hydrophobic/hydrophilic environments.
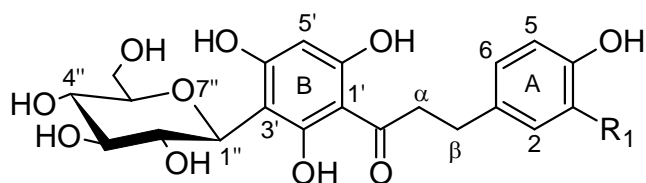
Figure 1 Structures of the *C-β*-D-glucopyranosyl dihydrochalcone aspalathin (R_1 = OH) and nothofagin (R_1 = H).

Recent studies on *in vivo* metabolism of aspalathin showed that its deglycosylation is not a prerequisite for its absorption. *O*-linked methyl, sulphate, glucuronide and *O*-methyl-*O*-glucuronide aspalathin metabolites were found to occur in the urine of human subjects after ingesting rooibos tea (Courts & Williamson, 2009; Stalmach *et al.*, 2009). No nothofagin, nor any of its metabolites, could be detected in urine in spite of a very low detection limit (Stalmach *et al.*, 2009). Ingestion of an aspalathin-enriched green rooibos extract over a two week period showed no increase in the plasma antioxidant status of humans (Sauter, 2004), probably due to either poor bioavailability and/or loss of potency as a result of aspalathin metabolism. A feeding

study with pigs conducted with a similar green rooibos extract showed that aspalathin could not be detected in the plasma, except in trace quantities, when a high dose (ca 480 mg/kg body weight) was ingested (Kreuz *et al.*, 2008). In contrast to Stalmach *et al.* (2009), Laue *et al.* (2009) found unmetabolized aspalathin in the plasma samples of humans.

The aim of the present study was to investigate *in vitro* metabolism of aspalathin by subcellular rat liver fractions obtained from induced (Aroclor 1254) and un-induced (control) male Fischer rats. The metabolism of nothofagin was also investigated to provide information on the possible site of conjugation since it lacks the catechol moiety. The effect of the *in vitro* biotransformation on the antioxidant activity of aspalathin was investigated with two on-line HPLC radical scavenging assays. Both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ were used as they display different scavenging mechanisms.

MATERIALS AND METHODS

Chemicals and water purification

Tris [hydroxymethyl] aminomethane (TRIZMA base) ($\geq$99.9%), bovine serum albumin (BSA), Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid, 97%), citric acid (anhydrous), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), 3′-phosphoadenosine-5′-phosphosulphate (PAPS), β-glucuronidase type IX-A from *E. coli* (1 134 600 units/g solids), sulfatase from *Helix pomatia* (15 000 units/g solids), β-nicotinamide adenine dinucleotide phosphate (NADPH) and Sepharose 2B were obtained from Sigma Chemicals Co. (St. Louis, USA). Sodium dithionite ($\leq$85%) (sodium hydrosulfite; $Na_2S_2O_4$ + H_2O; assay idiometric) and Triton-X-100 were supplied by BDH Chemicals Ltd. (Poole, United Kingdom). Carbon monoxide ($\geq$99.3%) was supplied by AFROX, BOC Special Products (Cape Town, South Africa). Dimethyl sulfoxide (DMSO for UV-spectroscopy, $\geq$99.8%) and 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied by Fluka (Steinheim, Germany). BCA protein assay reagent A containing sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 N sodium hydroxide and BCA protein assay reagent B were purchased from Separations (Cape Town, South Africa). Aroclor 1254 was obtained from Monsanto (St. Louis, USA). Methylene chloride (99%) was supplied by Merck (Darmstadt, Germany). Aspalathin and nothofagin (purity of both ≥95% as determined by HPLC and LC-MS) were isolated from green rooibos and supplied by the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) Unit of the Medical Research Council (MRC) (Bellville, South Africa). De-ionized water was prepared using a Modulab Water Purification System (Separations, Cape Town, South Africa). For HPLC eluant

and sample preparation, de-ionized water was further purified by means of a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, MA). The solvents required for HPLC analysis were HPLC grade acetonitrile (Merck, Darmstadt, Germany) and formic acid (98%) supplied by BDH Chemicals Ltd. (Poole, England). Solvents for LC-MS and LC-MS/MS analyses were HPLC grade acetonitrile from Romil (Cambridge, United Kingdom) and formic acid from Sigma Chemicals Co. (St. Louis, USA).

Preparation of rat liver microsomes and cytosolic fractions

Induced and un-induced livers were obtained from male Fischer rats. The induction of drug metabolizing enzymes was conducted according to the method described by Czygan *et al.* (1973). The induction procedure involved dilution of Aroclor 1254 in sunflower oil to a concentration of 200 mg/mL and administration of a single intraperitoneal injection of 500 mg/kg to each Fischer rat (ca 200 g) 5 days before sacrifice. Freshly excised livers were weighed and washed several times in chilled KCl before being homogenised in 0.15 M KCl (3:1 w/v) for 1 min, using a Thomas homogeniser (Maron & Ames, 1983). The homogenate was filtered through double-layered cheesecloth and homogenised using 10 strokes in a glass tissue grinder with a tight plunger. The homogenate was centrifuged at 9 000 x g for 10 min and aliquots of the supernatant (S9 fraction) were stored in glass vials at -80 ºC until use.

Microsomal and cytosolic fractions were prepared by applying the S9 fraction to a Sepharose 2B column (2.8 x 30 cm, 1.7 mL/min flow rate) and eluting with a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl. The microsomal and cytosolic fractions were collected based on visual separation and stored at -80 °C. The fractions were thawed at room temperature on the day of the assay and diluted to the required protein concentration using ice-cold Tris-HCl buffer. The protein concentration of the microsomes and cytosol was determined according to the method described by Kaushal and Barnes (1986), using BSA as standard. The use of experimental animals was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (MRC) (Belleville, SA).

Phase II conjugation

The phase II conjugation of aspalathin and nothofagin was investigated using microsomal and cytosolic fractions as the respective sources of glucuronosyltransferase and sulfotransferase. Co-factors comprised UDPGA and PAPS. The optimum protein concentration and incubation period for biotransformation were determined with aspalathin in preliminary experiments. Microsomal protein concentrations of 0.5, 1 and 2 mg/mL and cytosolic protein concentrations of 1, 2 and 4 mg/mL were used, while incubation times investigated were 30, 60, 90, 120 and 180

min. Different co-factor and aspalathin (50–200 µM) concentrations were also employed to optimize glucuronidation. Solvents for reconstitution of samples were evaluated for optimal recovery prior to HPLC analyses and included 50% acetonitrile-water, DMSO, methanol and de-ionized water.

To estimate extraction recoveries of aspalathin, a spiked sample with a known concentration of aspalathin added prior to incubation and clean-up was included in every experiment. Controls were incubated in the absence of the co-factor, while sample blanks, which did not contain the polyphenol or co-factor, were used to screen for interfering substances in the matrix during HPLC and LC-MS analyses.

The stabilities of aspalathin, nothofagin and their metabolites in the reconstituted samples were investigated at room temperature by repeated HPLC analysis every 4 h over a period of 24 h. The stabilities of aspalathin and its metabolites during storage at −20 °C were monitored by analyzing two samples at day 0 and again after 21 days.

Glucuronidation

A typical reaction mixture (490 µL) for glucuronidation, consisting of 100 µM nothofagin or 200 µM aspalathin, 2 mg/mL microsomal protein, 2 mM NADPH, 0.02% Triton-X-100 and 50 mM Tris-HCl buffer (pH 7.4) was equilibrated for 3 min at 37 °C. The reaction was initiated by adding 10 µL of a 100 mM UDPGA stock solution to obtain 2 mM in the final reaction volume (500 µL). Incubations were carried out at 37 °C for 120 min in a shaking waterbath. Each reaction was terminated by addition of an equal volume of ice-cold methanol to precipitate proteins, followed by centrifugation at 10 000 x g for 10 min at 4 °C. The supernatant was extracted with 500 µL methylene chloride to remove lipids and Triton-X-100, followed by centrifugation at 10 000 x g for another 10 min at 4 °C. The supernatant was evaporated to dryness under a stream of nitrogen gas and stored at −20 °C. The dried samples were reconstituted in purified water (200 µL) and vortexed prior to HPLC analysis.

Sulphation

The final reaction mixture (500 µL) for sulphation contained either 100 µM nothofagin or 200 µM aspalathin, 25 mM $MgCl_2$, 50 µM PAPS and cytosol (4 mg/mL in 50 mM Tris-HCl buffer, pH 7.4). The reaction was initiated by adding 10 µL of a 2.5 mM PAPS stock solution to obtain 50 µM PAPS in the final reaction volume. Samples were incubated for 60 min. Termination and subsequent sample preparation were conducted as described above.

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Enzymatic hydrolysis

Glucuronidation and sulphation of aspalathin were performed as described above. Following the respective incubation periods, the conjugation reaction was terminated by centrifugation at 16 000 x *g* at 4 °C for 30 min. Aliquots (200 µL) of the respective supernatants were incubated for 120 min at 37 °C with 20 µL β-glucuronidase (5000 U/mL) or 20 µL sulfatase (125 U/mL). This reaction was terminated by adding an equal volume of ice-cold methanol and further prepared for HPLC analysis as described above. The control sample contained a buffer instead of β-glucuronidase or sulfatase.

HPLC analysis of metabolites

HPLC separations were conducted using an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, autosampler, in-line degasser, column thermostat, diode-array detector and Chemstation software for LC 3D systems (Rev. B.02.01). The column used was a 150 x 4.6 mm i.d., 3 µm, Phenomenex Luna Phenyl-Hexyl, with a RP/C_{18} 5 µm Jour Guard column (Separations, Johannesburg, South Africa). Column temperature was maintained at 30 °C and the following solvent gradient with acetonitrile (eluent A) and 1% formic acid (eluent B) at a flow rate of 1 mL/min was applied: 12% A (0−6 min); 12−18% A (6−7 min); 18-25% (7−14 min); 25-40% A (14−19 min); 40-90% A (19−24 min); 90% A (24−29 min); 90−12% A (29−35 min); 12% A (35−40 min). Samples were filtered through Millex-HV hydrophilic PVDF syringe filter units (0.45 µm, 4 mm, Millipore) directly into HPLC sample vials for duplicate injection (10 µL). Tentative identification was made on the basis of the UV/Vis spectra and retention times of the aspalathin and nothofagin standards. Quantification of compounds at 288 nm for calculation of recoveries was based on peak area, obtained with valley-to-valley integration. The concentration range of the respective calibration series used for quantification was based on the expected concentration of the compounds in the spiked samples.

Liquid chromatography-mass spectrometry (LC-MS) analyses

LC-MS analysis of glucuronidated and sulphated samples was performed using a Waters API Quattro Micro triple quadropole mass spectrometer connected to a Waters Alliance 2690 quaternary HPLC pump with a 966 diode-array detector (Waters, Milford, MA.). Sample preparation and separation conditions were the same as described for HPLC analysis. Electrospray ionization in the negative mode was carried out under the following conditions:

desolvation temperature, 370 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.5 kV and cone voltage, 18 V.

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis

For further confirmation of aspalathin conjugation LC-MS/MS analysis was performed using a Waters API QTOF Ultima apparatus with a Waters UPLC system. Sample preparation and separation conditions were the same as described for HPLC analysis. Electrospray ionization in the negative mode was carried out under the following conditions: desolvation temperature, 350 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.7 kV; cone voltage, 35 V and collision energy 20.

Qualitative assessment of radical scavenging ability of aspalathin metabolites

The sample mixture containing aspalathin and its glucuronidated metabolites was subjected to on-line HPLC-DPPH$^\bullet$ and HPLC-ABTS$^{+\bullet}$ analyses. The on-line radical scavenging methods were set up as described by Dapkevicius *et al.* (2001) and Pellegrini *et al.* (2003), respectively, with some modifications to improve radical intensity and prevent precipitation. The on-line system consisted of an LKB Bromma 2150 HPLC pump (Bromma, Sweden), in-line Phenomenex Degasser Model DG-4400 and LKB Bromma 2151 UV/Vis variable wavelength detector and is presented in a diagram in Addendum B. Mixing of the HPLC effluent and the radical solution, at a flow rate of 0.5 mL/min, was achieved with a high pressure static mixing tee (Upchurch, Anatech, Cape Town, South Africa). The reaction coil was made of 5.24 m PEEK tubing (0.25 mm i.d.) to give a reaction time of 0.5 min. Data were captured using DataApex Clarity v2.4.1.91 software (DataApex, Prague, Czech Republic). Scavenging of the DPPH$^\bullet$ and ABTS$^{+\bullet}$ was detected as negative peaks at 515 and 430 nm, respectively. An injection volume of 30 μL was used.

The DPPH$^\bullet$ stock solution (58 mg/L) was freshly prepared in acetonitrile on the day of analysis and kept in a flask protected from light. The working solution, containing 250 mL of a 0.068 mM citric acid phosphate buffer (6.8 mL of 0.01 M citric acid added to 93.2 mL of 0.02 M Na_2HPO_4 and made up to 1000 mL with de-ionized water) and 750 mL DPPH$^\bullet$ in acetonitrile was filtered (0.45 μm PVDF-filter, Milipore) before use.

The ABTS$^{+\bullet}$ stock solution (7 mM) was prepared as described by Pelligrini *et al.* (2003). A working solution was prepared on the day of the analysis and contained 250 mL citric acid phosphate buffer (6.8 mL 0.01 M citric acid and 93.2 mL 0.02 M Na_2HPO_4 made up to 1000 mL with de-ionized water), 25 mL ABTS$^{+\bullet}$ stock solution and 750 mL acetonitrile. The temperature of the ABTS$^{+\bullet}$ solution was maintained at 4 °C using a thermostatically-controlled cooling bath.

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The antioxidant activity of the sulphated aspalathin and glucuronidated nothofagin was not investigated since only a low level of biotransformation was achieved. It would not have been likely to observe activity of the metabolites at such a low concentration in the on-line system.

RESULTS

Optimization of phase II conjugation

The solvent used for reconstitution of the samples had a significant effect on the compound separation and repeatability of the HPLC chromatograms. De-ionised water proved to be the optimum solvent for reconstitution (data not shown). Incubation for 120 min was sufficient to obtain a relatively high level of glucuronidated metabolites, while 60 min was adequate for sulphation. Aspalathin was stable in the reconstituted samples at room temperature over 24 h and when stored at -20 ºC for 21 days, with its extraction recoveries varying from 90−100%. In contrast, nothofagin and its metabolites were rapidly degraded over time. Samples were therefore reconstituted less than 10 min before injection. No matrix peaks were observed in sample blanks in either microsomal or cytosolic fractions with HPLC and LC-MS analyses.

Glucuronidation

No metabolism was observed when incubating with the microsomal fraction (induced and un-induced) in the absence of the co-factor UDPGA (control samples) (Fig. 2A and 2C). Glucuronidation of aspalathin with induced microsomes resulted in two conjugates, represented in the HPLC chromatogram (Fig. 2B) by one minor peak (AG1) and one major peak (AG2). The retention times of AG1 and AG2 (10.65 and 11.54 min, respectively) corresponded to molecular species more polar than aspalathin (t_r = 11.95). Incubation with un-induced microsomes resulted in only one peak with the same retention time as AG2. In this case a substantially smaller metabolite peak was obtained (data not shown). The LC-MS analysis confirmed the presence of a pseudomolecular ion ([M-H]⁻) with *m/z* 627 (Table 1) for both peaks, indicating two regio-isomers of monoglucuronidated aspalathin. Treatment with β-glucuronidase resulted in the disappearance of both peaks (AG1 and AG2), with concomitant formation of aspalathin observed as an increase in the aspalathin peak (data not shown). LC-MS/MS analysis resulted in the characteristic *C*-linked glycoside losses of 90 and 120 amu, giving the respective fragment ions *m/z* 361 and 331 for aspalathin, and *m/z* 537 and 507 for both aspalathin glucuronides, AG1 and AG2. Although the same fragment ions were produced for these metabolites, their relative abundance differed (Table 2). The neutral loss of 176 amu to give a fragment ion with *m/z* 451 is due to the cleavage of the glucuronide moiety.

Glucuronidation of nothofagin with induced microsomes also resulted in two metabolites, one major (NG1) and one minor peak (NG2) (Fig. 2D). LC-MS analysis of the two metabolites gave [M-H]$^-$ ions at m/z 611 (Table 1), indicating that NG1 and NG2 correspond to two regio-isomers of monoglucuronidated nothofagin. Incubation with un-induced microsomes only resulted in the presence of the major metabolite (NG1) (Table 1) and the extent of biotransformation was less than with induced microsomes (data not shown).

Sulphation

No metabolism was observed when incubating the compounds with the cytosolic fraction (induced and un-induced) in the absence of the co-factor PAPS (control samples) (Fig. 2E). Sulphation of aspalathin with the Aroclor-induced cytosolic rat liver fraction resulted in one metabolite (AS) (Fig. 2F). A metabolite eluting at the same retention time was present when incubating aspalathin with un-induced cytosol. The retention time of AS (16.15 min) corresponds to a molecular species less polar than aspalathin (t_r = 11.71). LC-MS analysis of the sulphated samples of both induced and un-induced cytosolic fractions gave a pseudomolecular ion [M-H]$^-$ with m/z 531 (Table 1). Treatment with sulfatase resulted in disappearance of AS, but also decreased the concentration of aspalathin. The sulphated metabolite (m/z 531) produced a fragment ion with m/z 451 (aspalathin) upon loss of 80 amu (sulphate moiety). The other two fragment ions present were m/z 361 and 331, characteristic of aspalathin. No sulphation of nothofagin was observed under the experimental conditions. HPLC analysis did not show any additional peaks to those present in the controls after incubation.

Qualitative assessment of radical scavenging ability of metabolites

The combined HPLC and DPPH$^•$ quenching chromatograms of aspalathin and its glucuronidated metabolites (AG1 and 2) are shown in Fig. 3A. Aspalathin scavenged DPPH$^•$, as manifested by the decrease in absorbance, while no activity was obtained for the conjugates, even though considering that AG2 was present in a slightly higher concentration than aspalathin. This difference in peak height between AG2 and aspalathin was even more prominent in the sample used for testing the ABTS$^{+•}$ scavenging ability of the metabolites (Fig. 3B). In spite of this, and the greater sensitivity of the ABTS$^{+•}$ scavenging assay, as implicated by the positive/negative peak ratio's for aspalathin in the respective assays, no activity could be demonstrated for the metabolites.
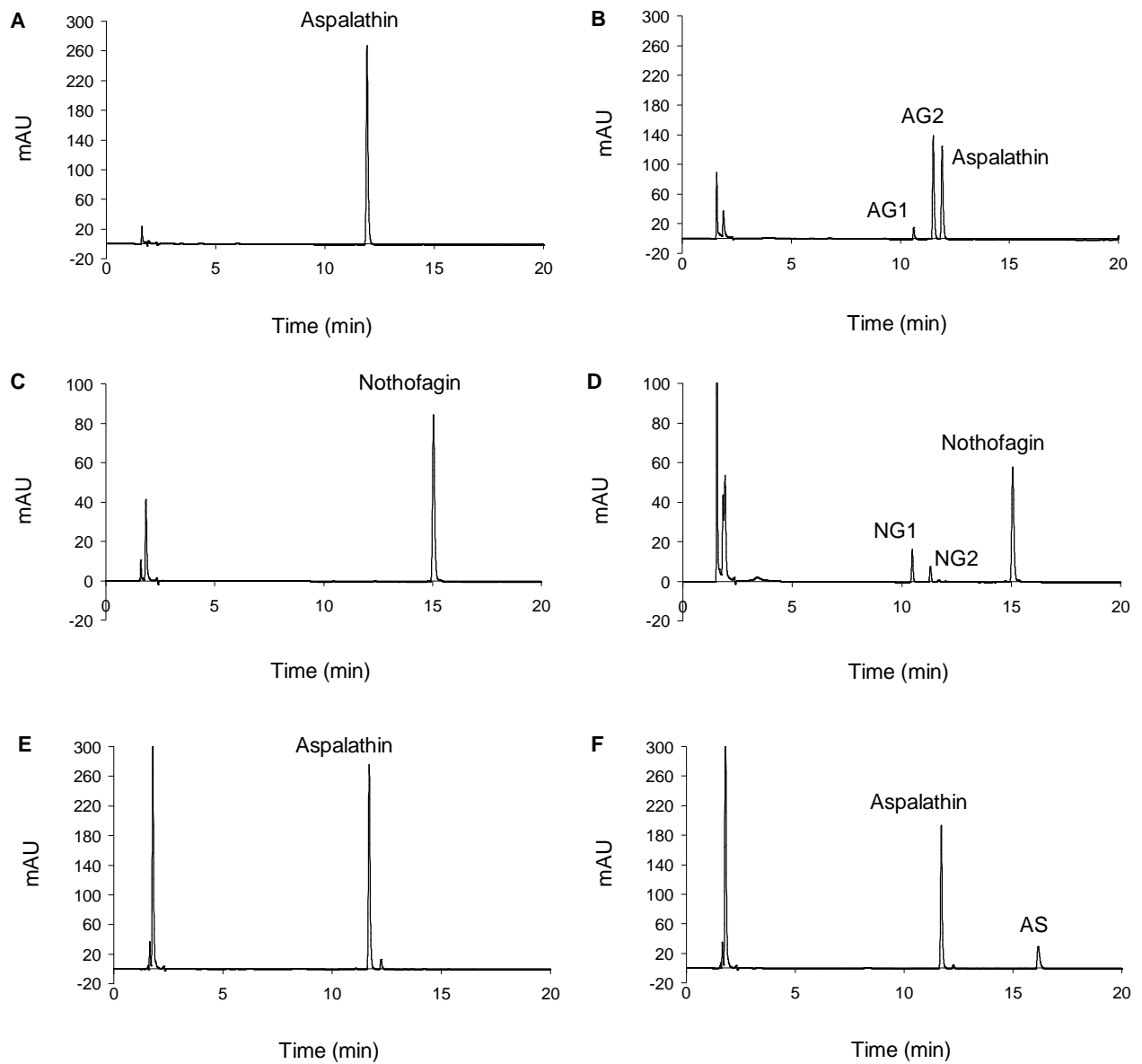
Figure 2 Representative HPLC chromatograms showing a control (absence of UDPGA) sample spiked with aspalathin (A), a sample containing aspalathin glucuronides (AG1 and AG2) (B), a control sample spiked with nothofagin (absence of UDPGA) (C), a sample containing nothofagin glucuronides (NG1 and NG2) (D), a control (absence of PAPS) sample spiked with aspalathin (E), and a sample containing sulphated aspalathin (F).

Table 1 LC-MS of aspalathin, nothofagin and glucuronidated and sulphated metabolites.

Liver fraction	Compound	Mr	LC-MS ions (*m/z*)
	Aspalathin	452	451
Induced microsomes	Aspalathin monoglucuronide (AG1)	628	627
Induced microsomes	Aspalathin monoglucuronide (AG2)	628	627
Un-induced microsomes	Aspalathin monoglucuronide (AG2)	628	627
Induced cytosol	Aspalathin monosulphate (AS)	531	531
Un-induced cytosol	Aspalathin monosulphate (AS)	531	531
	Nothofagin	436	435
Induced microsomes	Nothofagin monoglucuronide (NG1)	612	611
Induced microsomes	Nothofagin monoglucuronide (NG2)	612	611
Un-induced microsomes	Nothofagin monoglucuronide (NG1)	612	611
Induced cytosol	Nothofagin sulphate	nd [a]	nd [a]

[a] nd not detected.

Table 2 LC-MS/MS of aspalathin and glucuronidated and sulphated metabolites.

Compound	Precursor ion (*m/z*)	LC-MS/MS product ions (*m/z*) (% relative abundance)
Aspalathin	451	361 (13); 331 (100); 209 (52); 167 (35)
Aspalathin monoglucuronide (AG1)	627	627 (7); 537 (100); 507 (84); 361 (48); 331 (43)
Aspalathin monoglucuronide (AG2)	627	627 (13); 537 (74); 507 (100); 361 (26); 331 (33)
Aspalathin monosulphate	531	531 (13.57); 451 (41.65); 361 (100); 331 (62.42)
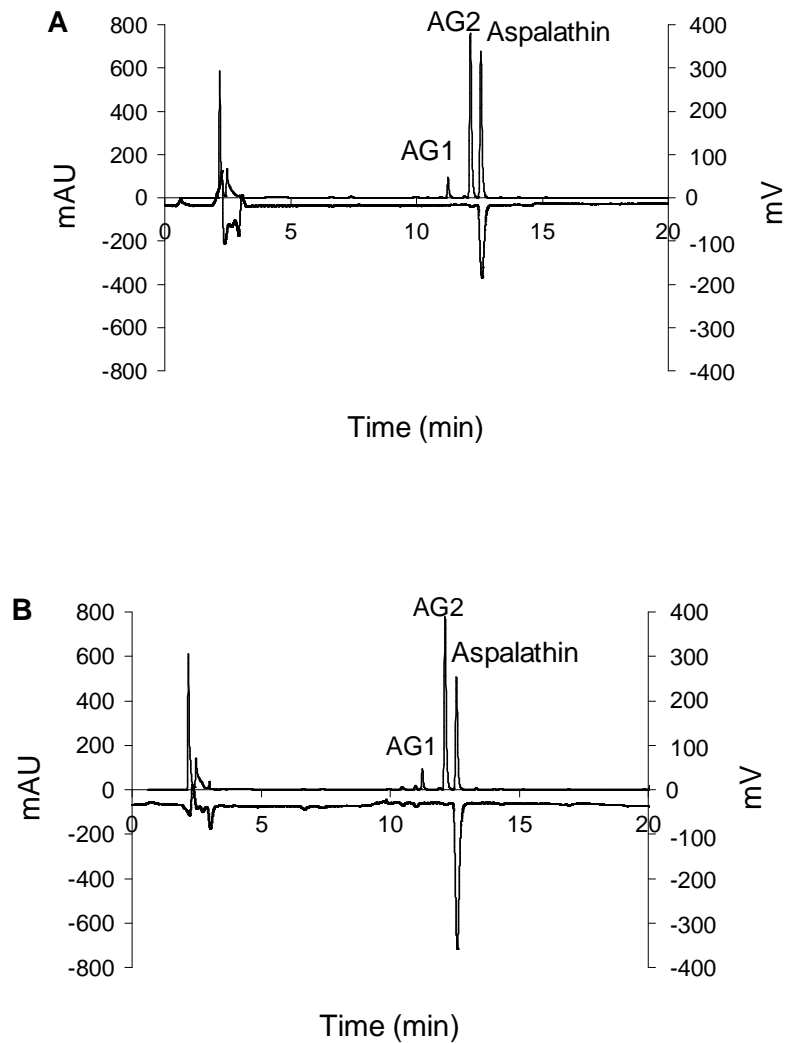
Figure 3 Combined UV and radical quenching chromatograms of aspalathin and glucuronidated metabolites. DPPH$^\bullet$ (A); ABTS$^{+\bullet}$ (B).

DISCUSSION

Conjugation of catechols is one of the major metabolic reactions that occur in biological systems and allows for their rapid excretion into the bile and urine (Antonio *et al.*, 2002). Conjugation reactions are mainly catalyzed by UDP-glycuronosyltransferases (UGT), sulfotransferases (SULT), and the catechol *O*-methyl transferases (COMT) (Manach *et al.*, 2004). Competition between the different conjugation reactions for the same substrate depends on various factors. These include the dosage of the polyphenol and the hydrophobic/hydrophilic nature of the subcellular environment (Koster *et al.*, 1981; Antonio *et al.*, 2002). An increased dose will result in a shift from sulphation toward glucuronidation, since sulphation is generally a higher-affinity, lower-capacity pathway than glucuronidation (Koster *et al.*, 1981). Methylation by COMT also results in a shift of conjugation of polyphenols from sulphation to glucuronidation (O'Leary *et al.*, 2003).

Aspalathin, due to its prominence and radical scavenging activities, has been implicated in many of the antioxidant properties of rooibos (Joubert *et al.*, 2009). Nothofagin is also a good ABTS$^{\bullet+}$ scavenger (Snijman *et al.*, 2009), and is of particular interest for the present study due to the absence of the catechol group, from which insight might be gained on the relative position of conjugation of dihydrochalcone *C*-glycosides. Aroclor 1254 was selected as an enzyme inducer, since it has been shown to enhance the glucuronidation rate of catechols up to 15-fold in rat liver microsomes (Elovaara *et al.*, 2004). One major and one minor glucuronidated product of both aspalathin and nothofagin were observed after incubation with Aroclor 1254-induced microsomes. The presence of the aspalathin glucuronides was confirmed by LC-MS as indicated by a neutral loss of *m/z* 176, representing the glucuronide moiety. The fragmentation pattern of aspalatin and aspalathin-*O*-glucuronide, which is characteristic of *C*-linked glycosides (Kazuno *et al.*, 2005), was in accordance with that previously reported (Stalmach *et al.*, 2009; Kreuz *et al.*, 2008).

Only one minor product was noticed for aspalathin and nothofagin when incubated with un-induced microsomes. PCB inducers such as Aroclor 1254 have been found to induce several UGT activities and, in particular, high-affinity enzyme isoforms (Elovaara *et al.*, 2004), which explains the higher level of biotransformation and additional metabolite observed when using induced microsomes. This study indicates that the major glucuronidated product of aspalathin and nothofagin results from conjugation at the 4-OH, since nothofagin lacks the 3-OH on the A-ring. Glucuronidation studies of quercetin with human liver microsomes indicated four products of which the catechol (4′-OH) also represents the major conjugate, followed by the 3′-OH (Day *et al.*, 2000). The minor glucuronidated product of aspalathin may result from conjugation of the

3-OH on the catechol group or one of the OH groups on the B-ring, presumably the 6′-OH, due to the steric hindrance of the C-glycoside involving the 2′- and 4′-OH groups. This became evident as it is known that conformational distribution of these dihydrochalcone exists due to hydrogen bonding between the C2′ and C4′-OH groups and the C2′-OH and the ethereal oxygen of the glucopyranosyl moiety (Snijman *et al.*, 2009). The minor glucuronidated metabolite of nothofagin will therefore most likely result from conjugation on the 6′-OH.

Sulphation yielded one minor conjugated product for aspalathin with both induced and un-induced rat liver cytosolic fractions. The MS fragmentation pattern obtained after sulphation was analogous to that reported for an aspalathin-*O*-sulphate (Stalmach *et al.*, 2009). Sulphation seems to be one of the major pathways in the conjugation of aspalathin, since aspalathin-*O*-sulphate, in addition to *O*-methyl glucuronide and *O*-methylsulphate conjugates has been identified in human urine (Stalmach *et al.*, 2009). Nothofagin was not sulphated, which implies that the catechol is required for sulphation, or that the sulphated product is unstable under *in vitro* and *in vivo* conditions.

The relative contribution of the conjugation reactions in the metabolism of aspalathin will depend on the distribution of aspalathin between the aqueous and lipid phases within the cell. At physiological pH of 7.4 most polyphenols are associated with the polar head groups of the membrane phospholipids. This makes the OH groups more accessible to glucuronidation (Verstraeten *et al.*, 2003). However, more polar metabolites will largely favor the aqueous compartment phase, making them more accessible to cytosolic conjugation such as methylation and sulphation. As the major urinary metabolite of aspalathin in humans is *O*-methyl glucuronide (Stalmach *et al.*, 2009) it would appear that methylation in the cytosol and the subsequent glucuronidation in the endoplasmic reticulum are the major conjugating pathways involved. This agrees with the notion that methylation diverts the conjugation reaction away from sulphation (O'Leary *et al.*, 2003).

Although conjugation of polyphenols has been recognized for many years, most biological studies have been carried out with the unmetabolized, naturally occurring, plant polyphenols. Glucuronidated products are one of the predominant forms of flavonoids in blood circulation and their biological activities have been of interest (Zhang *et al.*, 2007). However, very little is known about the biological properties of conjugated polyphenolic derivatives due to the lack of commercial standards. Conjugation renders them more hydrophilic than the parent compounds, which may affect their site of action and their interaction with antioxidants in the cell if they retain their antioxidant properties. In the present study the on-line HPLC antioxidant assays, utilizing DPPH$^\bullet$ and ABTS$^{\bullet+}$, showed that glucuronidation of aspalathin eradicated its antioxidant

properties under the assay conditions. The assay conditions, however, do not allow detection of slow radical scavenger due to the reaction time (Dapkevicius *et al.*, 2001).

Although aspalathin metabolites apparently lost their radical scavenging properties the conjugated products are likely, as in the case of quercetin conjugates, to exhibit some other biological properties (Day *et al.*, 2000; Zhang *et al.*, 2007). Quercetin conjugates retain differential inhibitory function of xanthine oxidase and lipooxygenase with the 4'-*O*-glucuronidated product exhibiting an inhibitory effect relative to the parent compound with respect to inhibition of the xanthine oxidase (Day *et al.*, 2000). Glucuronides of quercetin and epicatechin also possess biological activity, including antioxidant activity and inhibition of the growth of human lung cancer cells (Zhang *et al.*, 2007). The antioxidant activity of the nothofagin metabolite was not tested in the present study, but based on its structure and relative antioxidant activity in the ABTS$^{\bullet+}$ assay (Snijman *et al.*, 2009), it may be assumed that these metabolites would also display no activity. The antioxidant activity of the sulphated aspalathin was not investigated, since only a low level of biotransformation was achieved and it would not have been likely to observe activity of the metabolite at such a low concentration in the on-line system.

Unconjugated polyphenols and some of their conjugates may accumulate in the body to produce pharmacological activities (Zhang *et al.*, 2007). Therefore, despite the poor bioavailability of aspalathin and nothofagin (Joubert *et al.*, 2009; Stalmach *et al.*, 2009) they are likely to accumulate in tissue and body fluids and still exert effects due to being available in both conjugated and unconjugated forms. In this regard, unmetabolized aspalathin has been detected in human plasma (Laue *et al.*, 2009). In addition to the liver, glucuronidation could also occur in the kidneys and contribute to the presence of the conjugated products of aspalathin found in the urine of humans after consumption of rooibos (Courts & Williamson, 2009; Stalmach *et al.*, 2009). Some isoforms such as UGT1A8/9 that are highly expressed in the human kidney (McGurk *et al.*, 1998) exhibit a broad spectrum of substrate specificity, and could be involved in the metabolism of aspalathin. Low concentrations of nothofagin in rooibos and/or the poor stability of the parent compound and metabolites could be reasons why they were not detected in urine. In this regard phase II metabolism and subsequent bile excretion coupled to microbial metabolism is likely to lead the enterohepatic recycling of conjugated and deconjugated forms. As the deconjugating enzyme β-glucuronidase is present in the blood, liver and kidneys, it will result in sustained hepatic and/or blood circulation of polyphenols and/or their metabolites (O'Leary *et al.*, 2001). Lysosomal β-glucuronidase released from neutrophils can deconjugate flavonoid glucuronides to the aglycone when injury such as inflammation occur

(O'Leary *et al.*, 2001). The turnover rate of the conjugated and unconjugated forms of aspalathin will be important to evaluate its biological properties *in vivo*.

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CHAPTER 4

MANGIFERIN GLUCURONIDATION: IMPORTANT HEPATIC MODULATION OF ANTIOXIDANT ACTIVITY

Part of this work was presented* at:

- *19th Biennial SAAFoST Congress & Exhibition, 2-5 September 2007, Durban, South Africa.*

- *Indigenous Plant Use Forum (IPUF) 2009, 6-9 July 2009, Stellenbosch, South Africa.*

- *MRC Research Day, 14-15 September, Tygerberg, South Africa.*

* Details listed in Addendum A.

MANGIFERIN GLUCURONIDATION: IMPORTANT HEPATIC MODULATION OF ANTIOXIDANT ACTIVITY

ABSTRACT

Mangiferin displays an extensive spectrum of pharmacological properties, including antioxidant activity. Its phase II metabolism in the presence of Aroclor 1254-induced and un-induced microsomal and cytosolic fractions from rat liver and the antioxidant potency of the glucuronidated conjugates were investigated. Mangiferin was not a substrate for the cytosolic sulfotransferases. Glucuronidation led to the formation of two monoglucuronidated metabolites of mangiferin and a monoglucuronidated metabolite of homomangiferin (a minor constituent of the mangiferin standard). Deconjugation utilising glucuronidase resulted in the disappearance of the metabolites, with the concomitant formation of the two parent compounds. Considering steric hinderance caused by the C-2 glucosyl moiety and the relative acidity of the xanthone OH groups, the 6-OH of mangiferin and, to a lesser degree the 7-OH, are likely to be the primary glucuronidation targets. The ferric iron reducing ability of the glucuronidated reaction mixture was reduced, while the free radical scavenging abilities of mangiferin, utilising on-line post-column HPLC-DAD-DPPH$^{\bullet}$ and HPLC-DAD-ABTS$^{\bullet+}$ assays, were eliminated, providing further evidence that the catechol arrangement at C-6 and C-7 was the preferred site of conjugation. This paper provides the first evidence that the glucuronidated metabolites of mangiferin resulted in a loss in free radical scavenging and ferric iron reducing ability.

INTRODUCTION

Mangiferin (1,3,6,7-tetrahydroxy-xanthone-C2-β-D-glucoside) (Fig. 1) is increasingly receiving attention as a major biologically active compound in plant extracts used in traditional medicines (Li, 2002; Pinto *et al.*, 2005). A wide spectrum of pharmacological properties has been attributed to mangiferin with the antioxidant properties forming the basis for some of the biological effects, including anti-inflammatory and immunomodulatory effects (Pinto *et al.*, 2005). An aqueous extract of mango bark with mangiferin as major constitutent has recently been commercialised as the antioxidant nutritional supplement, Vimang$^{®}$ (Núñez-Sellés *et al.*, 2002).

Occurrence of mangiferin in the normal diet is limited, with exposure largely due to consumption of mango, and recently also the herbal tea, honeybush, prepared from *Cyclopia* species (family Fabaceae; tribe Podalyrieae) (Joubert *et al.*, 2009). Use of this South African

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herbal tea has become very popular during the past 10 years and apart from the major markets (Germany, the Netherlands, United Kingdom and United States of America) also includes traditional tea-drinking countries such as India, Japan and China (Joubert *et al.*, 2011). The dietary burden of mangiferin contributed by honeybush will depend on the *Cyclopia* species and the state of processing of the plant material. Traditionally, honeybush was consumed in 'fermented' (oxidised) form, which contains much lower levels of mangiferin than the plant material as a result of its degradation during 'fermentation'. The subsequent decrease in *in vitro* antioxidant and antimutagenic activity of the herbal tea (Van der Merwe *et al.*, 2006; Joubert *et al.*, 2008a) resulted in the development of green honeybush tea for the preparation of mangiferin-enriched antioxidant extracts for the food and nutraceutical markets.
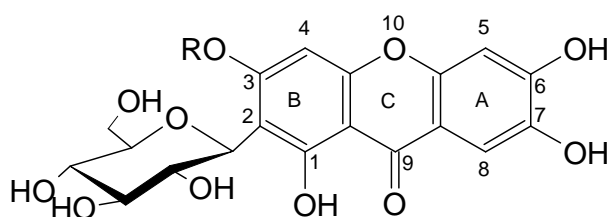
Figure 1 Structures of mangiferin (R = H) and homomangiferin (R = CH$_3$).

In vitro antioxidant activity observed for plant extracts does not necessarily lead to an *in vivo* biological effect due to poor bioavailability, rapid metabolism and excretion and the role of efflux transporters (Liu & Hu, 2007). Polyphenols are treated by the body as xenobiotics, undergoing detoxification by phase II conjugating enzymes. Although information on the changes in biological activity of the metabolites is limited, some studies showed that at least some of the antioxidant activity of the parent compound is retained. Investigations of dietary polyphenols have focussed on the ubiquitous flavonol, quercetin, tea flavan-3-ols, the citrus flavanones, naringenin and hesperetin, and the grape stilbene, resveratrol (Manach *et al.*, 1998; Day *et al.*, 2000a; Lu *et al.*, 2003; Justino *et al.*, 2004; Pollard *et al.*, 2006; Hoshino *et al.*, 2010; Dueñas *et al.*, 2011).

Studies using the pig and rat confirmed poor oral bioavailability for mangiferin (Bock *et al.*, 2008; Han *et al.*, 2010). Oral administration of a green *Cyclopia genistoides* extract failed to show mangiferin or phase II metabolites in the plasma of pigs, while methylated mangiferin was detected in the urine. Studies on rats reported the presence of the parent compound in plasma, urine, bile and tissue of rats (Lai *et al.*, 2003; Li & Bi, 2003; Wang *et al.*, 2006; Wang *et al.*, 2007; Li *et al.*, 2008; Ma *et al.*, 2008). Phase II metabolites, specifically *O*-methylated-*O*-monoglucuronidated and *O*-methylated mangiferin, have been detected in rat urine (Ma *et al.*, 2008) and bile (Lai *et al.*, 2003). Sulphated mangiferin was found in rat bile (Lai *et al.*, 2003), but sulphation could not be confirmed in pigs (Bock *et al.*, 2008). Sulfotransferases are either absent in pigs or some of the isoforms are present at low levels when compared to UDP-glucuronyltransferases (Witkamp & Monshouwer, 1998). The interaction between sulfotransferase and glucuronidation pathways is of interest and it appears that the former is active at low concentrations, while the latter comes into play at higher concentrations of plant polyphenols (Williamson *et al.*, 2000).

Mangiferin is the major antioxidant of *Cyclopia* species and has been recently implicated in the chemopreventative properties of *Cyclopia intermedia* (Marnewick *et al.*, 2005; Marnewick *et al.*, 2009; Sissing *et al.*, 2011), necessitating insight into the biological properties of its conjugated metabolites. The aim of the present study was to investigate phase II conjugation, i.e. glucuronidation and sulphation, of mangiferin by subcellular rat liver fractions obtained from Aroclor 1254-induced and un-induced male Fischer rats. Aroclor 1254 enzyme induction (Lilienblum *et al.*, 1982) was aimed at achieving a higher concentration of conjugated metabolites that would facilitate the screening of their potential antioxidant activity. The effect of the *in vitro* biotransformation on the antioxidant activity of the reaction mixture was investigated with the ferric reducing antioxidant power (FRAP) assay. In lieu of isolating the metabolites for testing of activity, two on-line HPLC-DAD (d*iode array detector)* radical scavenging assays, using the synthetic radicals, 2,2′-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS$^{\bullet+}$), were applied to qualitatively assess the *in vitro* antioxidant activity of the individual metabolites relative to the parent compound.

MATERIALS AND METHODS

Chemicals

General analytical grade laboratory reagents were purchased from Sigma-Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany). Mangiferin from *Mangifera indica* leaves ($\geq$ 98%), bovine serum albumin (BSA), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), uridine 5′-diphos−phoglucuronic acid trisodium salt (UDPGA), 3′-phosphoadenosine-5′-phosphosulphate (PAPS), β-glucuronidase type IX-A from *E. coli* (1 134 600 Units/g solids), β-nicotinamide adenine dinucleotide phosphate (NADPH), Sepharose 2B, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) (Fluka), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) (Fluka) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich) were obtained from Sigma-Aldrich. The BCA^{TM} protein assay kit was obtained from Pierce (Pierce Perbio Science France, Brebières, France), while Aroclor 1254 was from Monsanto (St. Louis, USA). General purpose laboratory grade de-ionised water was prepared using a Modulab Water Purification System (Continental Water Systems Corp., San Antonio, USA). The solvents required for HPLC analysis were HPLC grade acetonitrile (Merck), formic acid (BDH Chemicals Ltd) and de-ionised water, further purified by means of a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, USA). Solvents for liquid chromatography−mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were HPLC grade acetonitrile (Romil, Cambridge, United Kingdom) and formic acid (Sigma).

Preparation of rat liver microsomes and cytosolic fractions

The induction of drug metabolising enzymes in male Fisher 344 rats with Aroclor 1254 was conducted according to the method described by Czygan *et al.* (1973). Preparation of the S9 fraction of induced and un-induced rat livers and the microsomal and cytosolic fractions were carried out as described by Van der Merwe *et al.* (2010). The protein concentration of the microsomal and cytosolic fractions was determined with the BCA protein assay kit according to the method described by Kaushal & Barnes (1986), using BSA as standard. The use of experimental animals was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (MRC) (Belleville, SA).

Phase II conjugation

Preliminary studies

Microsomal and cytosolic fractions were used as the respective sources of glucuronosyltransferase and sulfotransferase. Optimum conditions for glucuronidation of mangiferin, including protein and co-factor concentrations and incubation period, were determined. A spiked sample containing a known concentration of mangiferin (added before incubation and cleanup) was included to estimate extraction recoveries. Control samples, incubated in the absence of the co-factor and sample blanks (without co-factor and mangiferin), were used to screen for interfering substances in the matrix during HPLC and LC-MS analysis. Two methods for sample clean-up before analysis were investigated, i.e. solid phase extraction and protein precipitation using methanol. Samples were dried after clean-up and different reconstitution solvents, i.e. 50% acetonitrile-water, dimethyl sulfoxide (DMSO), methanol and de-ionised water were evaluated in terms of HPLC separation of peaks and repeatability. Differences in solubility of mangiferin and its metabolites between reconstitution solvents resulted in major variation of the HPLC results and poor separation in some cases. De-ionised water proved to be the optimum solvent for reconstitution, resulting in consistent and repeatable HPLC results. An incubation time of 60 min resulted in a high level of glucuronidated metabolites. No interfering matrix peaks were observed in the sample blanks of either the microsomal or cytosolic fractions with HPLC and LC-MS analyses.

Protein precipitation with methanol and liquid-liquid extraction with methylene chloride for sample clean-up before HPLC and LC-MS was achieved by addition of an equal volume of ice-cold methanol, which also terminated the conjugation reaction, followed by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was extracted with 500 µL of methylene chloride to remove lipids and Triton-X-100, followed by centrifugation at 10 000 × g for 10 min at 4 °C. The supernatant was evaporated to dryness under a stream of nitrogen and stored at −20 °C. The dried samples were reconstituted in 200 µL purified water and vortexed prior to HPLC analysis.

Solid phase extraction (SPE) was investigated as a further clean-up method, after protein precipitation and liquid-liquid extraction. Samples were reconstituted to 1 mL with water, mixed thoroughly and 900 µL applied on a SPE column (PS-RP, 3 mL/500 mg, Chromabond; Macherey-Nagel, Düren, Germany), pre-conditioned with 2 mL methanol and 5 mL potassium phosphate buffer (50 mM, pH 3). After applying the sample, it was washed with 5 mL each of buffer and de-ionised water, eluted with 8 mL methanol and analysed directly with HPLC. The

remaining 100 µL was subjected to HPLC analysis without SPE cleanup. An extremely low amount of mangiferin was detected after elution with methanol (less than 50%). Subsequently, only the protein precipitation method using ice-cold methanol followed by methylene chloride for sample clean-up was used before HPLC and LC-MS analyses. Extraction recoveries of mangiferin from the microsomal fraction varied between 75 and 83%. All peaks in the reaction mixture after glucuronidation of the mangiferin standard were stable at room temperature over a 24 hour period and all analyses were carried out within this period.

Glucuronidation

The reaction mixture for glucuronidation contained 200 µM mangiferin, 2 mg/mL microsomal protein, 2 mM NADPH, 0.02% Triton-X-100 and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 500 µL. Samples were equilibrated for 3 min at 37 °C and the reaction was initiated by adding 2 mM UDPGA in 50 mM Tris-HCl buffer. Incubations were carried out at 37 °C for 60 min by continuous shaking. The reaction was terminated by the addition of an equal volume (500 µL) of ice-cold methanol, followed by methylene chloride extraction and sample preparation as described. The stability of mangiferin and the glucuronides were monitored over 24 hrs at room temperature.

Sulphation

The sulphation of mangiferin (100 µM) was conducted in the presence of 25 mM $MgCl_2$, 50 µM PAPS and cytosol (4 mg/mL in 50 mM Tris-HCl buffer, pH 7.4) in a final volume of   500 µL. The reaction was initiated by addition of 10 µL of a 2.5 mM PAPS stock solution prepared in 50 mM Tris-HCl buffer. The samples were incubated for 60 min in a shaking water bath at 37 °C. The reaction was terminated by the addition of ice cold methanol, followed by methylene chloride extraction and sample preparation as described.

Enzymatic hydrolysis of glucuronidated metabolites

Glucuronidation was performed as described. Following the incubation period, the conjugation reaction was terminated by centrifugation at 16 000 × g at 4 ºC for 30 min. Aliquots (200 µL) of the supernatant were incubated for 120 min at 37 ºC with 20 µL of β-glucuronidase (5000 Units/mL). The reaction was terminated by adding an equal volume of ice-cold methanol, followed by methylene chloride extraction and sample preparation as described for glucuronidation. The control sample contained an equal amount of buffer instead of β-glucuronidase.

HPLC analysis of glucuronidated metabolites

HPLC separations were conducted using an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, autosampler, column thermostat, diode-array detector and Chemstation software for LC 3D systems. Separation was achieved at 30 °C on a Phenomenex Synergy Fusion-RP column (150 × 4.6 mm i.d.; 4 μm particle size, 80 Å pore size), protected with a RP/C_{18} 5 μm particle size Jour Guard column (Vici AG International, Schenkon, Switzerland). The following solvent gradient with acetonitrile (eluant A) and 0.1% formic acid (eluant B) at a flow rate of 1 mL/min was applied: 10% A (0–5 min); 10–15% A (5–12 min); 15–90% A (12–16 min); 90–40% A (16–17 min); 40–10% A (17–21 min). Samples were filtered through Millex-HV hydrophilic PVDF syringe filters (0.45 μm pore size, 4 mm diameter, Millipore) directly into a HPLC sample vial for duplicate injection (10 μL). Tentative identification was achieved on the basis of the UV/Vis spectrum and retention time of mangiferin. Quantification of compounds at 320 nm for calculation of recoveries was based on peak area. The glucuronidated conjugates and the impurity in the mangiferin standard were quantified in terms of mangiferin, assuming the same molar extinction coefficients. The concentration range of mangiferin used for the calibration standards was based on the concentration used in the spiked samples.

Liquid chromatography-mass spectrometry (LC-MS) and -mass spectrometry/mass spectrometry (LC-MS/MS) analysis

LC-MS analysis of glucuronidated samples was performed using a Waters API Quattro Micro triple quadropole mass spectrometer, connected to a Waters Alliance 2690 quaternary HPLC pump with a 966 photodiode array detector (Waters, Milford, USA), applying the same separation conditions as for HPLC analysis. Electrospray ionisation in the negative mode was carried out under the following conditions: desolvation temperature, 350 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.5 kV; and cone voltage, 18 V.

For further confirmation of mangiferin conjugation, LC-MS/MS analysis of mangiferin glucuronides was performed using a Waters API QTOF Ultima apparatus equipped with a Waters UPLC system, consisting of a quarternary pump and autosampler. The same separation conditions as for HPLC were applied. Electrospray ionisation in the negative mode was carried out under the following conditions: desolvation temperature, 350 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.7 kV; cone voltage, 35 V; and collision energy, 30 arbitrary units.

Ferric reducing activity of the glucuronidated reaction mixture

The FRAP activity of the mangiferin glucuronidation reaction mixture was determined following the procedure described by Benzie & Strain (1996), modified for 96-well format, using Greiner polystyrene 96-well microplates and a Bio-Tek Synergy HT microplate reader equipped with Gen5 Secure software (Winooski, USA). The FRAP reagent was prepared by mixing 100 mL acetate buffer (300 mM, pH 3.6), 10 mL 10 mM TPTZ (in 40 mM HCl) and 10 mL 20 mM $FeCl_3.6H_2O$. The assay was optimised using standard mangiferin solutions (10–400 µM). Samples of the glucuronidated mixture were reconstituted in de-ionised water to obtain an estimated concentration of 40 mM of mangiferin in the control samples. A 40 µL sample aliquot was mixed with 160 µL FRAP reagent, incubated at 37 ºC and the absorbance measured after 4 min at 593 nm. For preparation of the calibration curve, a stock solution of 5 mM Trolox was prepared in ethanol and diluted with water to obtain a concentration range of 50–600 µM. The FRAP value of the reaction mixture was expressed as µmole Trolox/mL. All analyses were performed in triplicate for two separate experiments.

Radical scavenging ability of individual metabolites

The on-line HPLC-DAD-DPPH[•] and HPLC-DAD-ABTS[•+] assays, based on the respective methods described by Dapkevicius *et al.* (2001) and Pellegrini *et al.* (2003), with some modifications to improve intensity and decrease precipitation, were set up as described by Van der Merwe *et al.* (2010). The on-line system consisted of an LKB Bromma 2150 HPLC pump (Bromma, Sweden), in-line Phenomenex Degasser Model DG-4400 (Santa Clara, CA, USA) and a LKB Bromma 2151 variable wavelength detector. Mixing of the HPLC effluent (1 mL/min) and the radical solution (0.5 mL/min) was achieved with a high pressure static mixing tee (Upchurch, Oak Harbor, USA). The reaction coil consisted of 15.24 m polyether ether ketone (PEEK) tubing (0.25 mm i.d.) to give a reaction time of ca 45 s. Data were captured using DataApex Clarity version 2.4.1.91 software (DataApex, Prague, Czech Republic). Compounds in the reaction mixture after glucuronidation of the mangiferin standard were separated by HPLC and reacted post-column with the radical reagents. Scavenging of DPPH[•] and ABTS[•+] was detected as negative peaks at 515 and 430 nm, respectively.

DPPH[•] reagent

The freshly prepared DPPH[•] stock solution (750 mL; 58 mg/L in acetonitrile) was added to 250 mL of a 0.068 mM citric acid phosphate buffer to obtain the working solution and filtered (0.45 µm Millipore PVDF-filter) before use. The working solution was protected from light and maintained at 20 ºC using a temperature-controlled cooling bath during the analysis.

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ABTS$^{•+}$ reagent

A 7 mM ABTS$^{•+}$ stock solution was prepared in water before adding 440 µL of 140 mM $K_2S_2O_8$ to convert ABTS to ABTS$^{•+}$during storage in the dark for ca 16 hours. The ABTS$^{•+}$ buffered working solution was prepared on the day of the analysis and contained 250 mL 0.068 mM citric acid phosphate buffer, the ABTS$^{•+}$stock solution (25 mL) and 750 mL acetonitrile. The ABTS$^{•+}$ working solution was maintained at 4 °C using a temperature-controlled cooling bath during the analysis.

Statistical Analysis

Statistical analyses were conducted on the HPLC and FRAP data of the reaction mixtures. The normality of the parameters among the groups as well as the homogeneity of the group variance were investigated using the Kolmogorov Smirnof Test and Levene's Test, respectively. Variables complying with normality and homogeneity were tested using a One-way ANOVA and post-hoc Tukey Test. Where sample sizes were unequal the Tukey-Cramer adjustments were made automatically. The non-parametric Wilcoxon Rank-Sums Test was used along with the Tukey-Type post-hoc test where necessary. Statistical analyses were performed with SAS v9.2 and STATA v11 and statistical significance was considered at 5% ($P<0.05$).

RESULTS

Phase II conjugation

Glucuronidation

No metabolism occurred when incubating the mangiferin standard with the microsomal fractions (Aroclor 1254-induced and un-induced) in the absence of the co-factor UDPGA (control samples) (Fig. 2). A minor peak (t_r = 10.91 min), present as an impurity in the mangiferin standard obtained from *Mangifera indica* (estimated at <2% according to the supplier), and assumed to be homomangiferin (Wu *et al.*, 2010), gave a pseudo-molecular ion with *m/z* 435 and fragment ions with *m/z* 315, 287 and 272 (Tables 1 and 2). Glucuronidation of the mangiferin standard with Aroclor 1254-induced microsomes resulted in three metabolites eluting before mangiferin (t_r = 9.43 min) (Fig. 3A).

Two minor peaks (MG1 and HMG; t_r = 5.93 and 8.06 min, respectively) and one major peak (MG2; t_r = 6.70 min) were observed. A similar chromatogram was obtained when using the un-induced microsomes, although the prevalence of the metabolites was ca 20 times less

(quantified as mangiferin equivalents) than when using Aroclor 1254-induced microsomes (data not shown). The presence of the conjugates after incubation with un-induced conjugates was confirmed with LC-MS analysis (Table 1).

LC-MS analysis of the reaction mixtures demonstrated the presence of a pseudo-molecular ion ([M−H]$^-$) with m/z 597 (421 + 176) for both MG1 and MG2, indicating two regio-isomers of monoglucuronidated mangiferin. The third conjugate (HMG) with m/z 611 (435 + 176) is attributed to the glucuronidation of homomangiferin (Table 1). LC-MS/MS analysis of mangiferin resulted in neutral losses of 90 and 120 amu from the pseudo-molecular ion (m/z 421) giving the fragment ions m/z 331 and 301, respectively (Table 2). Cleavage of the glucuronide moiety of MG1 and MG2 resulted in fragment ions with m/z 421 (neutral loss of 176 amu), which fragmented further to give m/z 331 and 301. The fragment ions for MG1 and MG2 were the same, but their relative abundance differed (Table 2). LC-MS/MS analysis of HMG also resulted in cleavage of the glucuronide moiety (neutral loss of 176 amu), although no other major fragments were observed (Table 3). A proposed fragmentation scheme for mangiferin and its glucuronidated metabolites, adapted from Ma *et al.* (2008), is illustrated in Fig. 4.
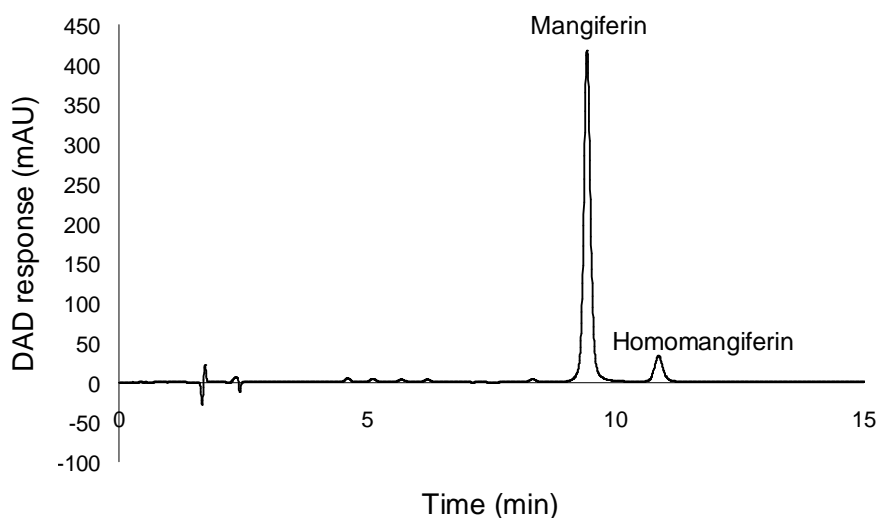
Figure 2 HPLC chromatogram of the control (absence of UDPGA) sample included in the investigation of mangiferin glucuronidation. The sample was spiked with mangiferin standard that contains homomangiferin as a minor impurity.
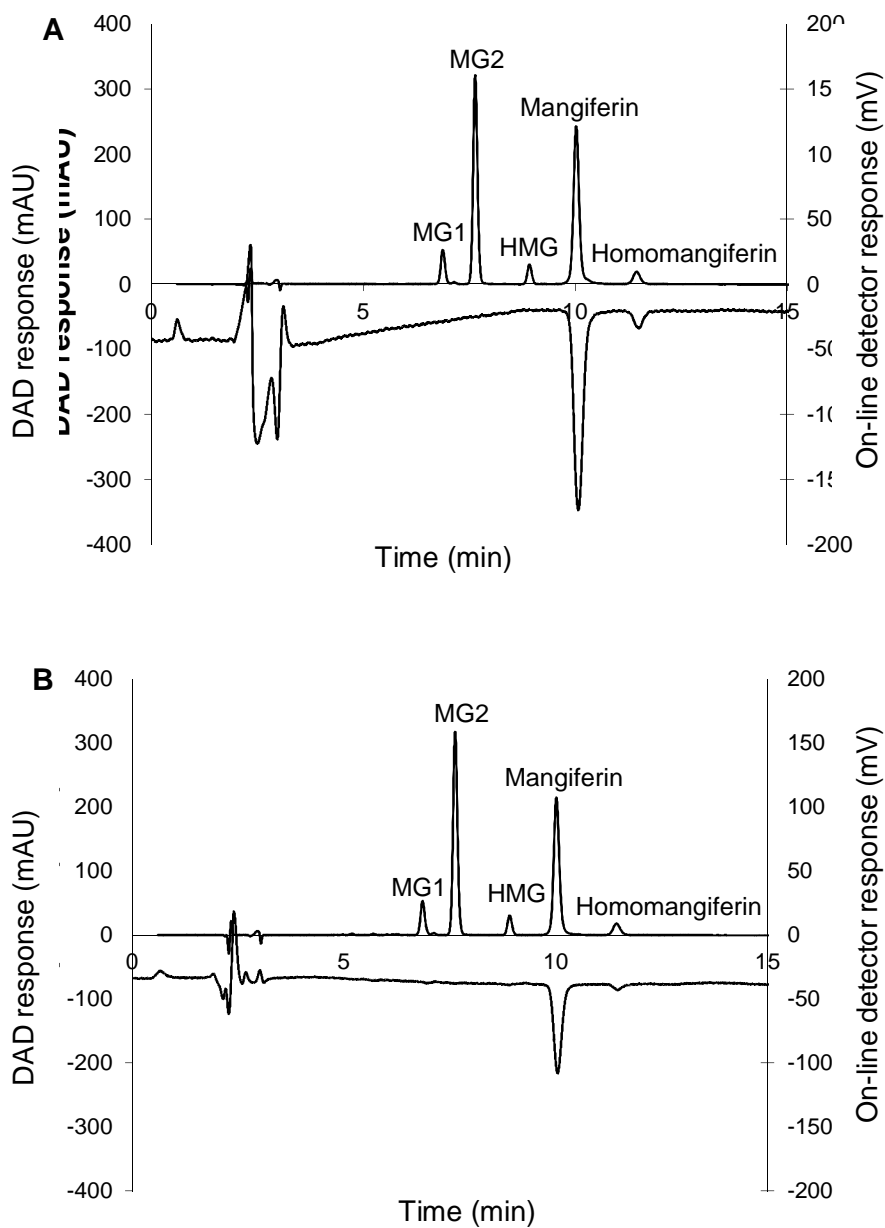
Figure 3 Combined HPLC-DAD and HPLC-on-line radical quenching chromatograms using DPPH$^{\bullet}$ (A) and ABTS$^{\bullet+}$ (B) of a sample containing glucuronidated mangiferin (MG1, MG2) and homomangiferin (HMG). Glucuronidation was mediated by liver microsomes from Aroclor 1254-induced rats and UGT with UDPGA.

Table 1 LC-MS characteristics of mangiferin, homomangiferin and glucuronidated metabolites.

Compound	[M-H]⁻
Mangiferin	421
Homomangiferin	435
Metabolites with induced microsomal fraction	
Mangiferin monoglucuronide (MG1)	597
Mangiferin monoglucuronide (MG2)	597
Homomangiferin monoglucuronide (HMG)	611
Metabolites with un-induced microsomal fraction	
Mangiferin monoglucuronide (MG1)	597
Mangiferin monoglucuronide (MG2)	597
Homomangiferin monoglucuronide (HMG)	611

Table 2 LC-MS/MS characteristics of mangiferin, homomangiferin and glucuronidated metabolites.

Compound	Precursor ion (*m/z*)	Product ions (*m/z* (% Relative abundance))
Mangiferin	421	**331** [a] (48); 313 (6); **301** (100); 272 (15); 271 (22); 259 (31); 258 (7)
Homomangiferin	435	435 (83); 315 (66); 287 (100); 272 (82)
Mangiferin monoglucuronide (MG1)	597	422 (6); **421** (34); 403 (7); 332 (4); **331** (77); 302 (7); **301** (100)
Mangiferin monoglucuronide (MG2)	597	**421** (40); 403 (12); 332 (6); **331** (100); 302 (5); **301** (90)
Homomangiferin monoglucuronide (HMG)	611	435 (100)

[a] Numbers in bold represent fragment ions characteristic of mangiferin.
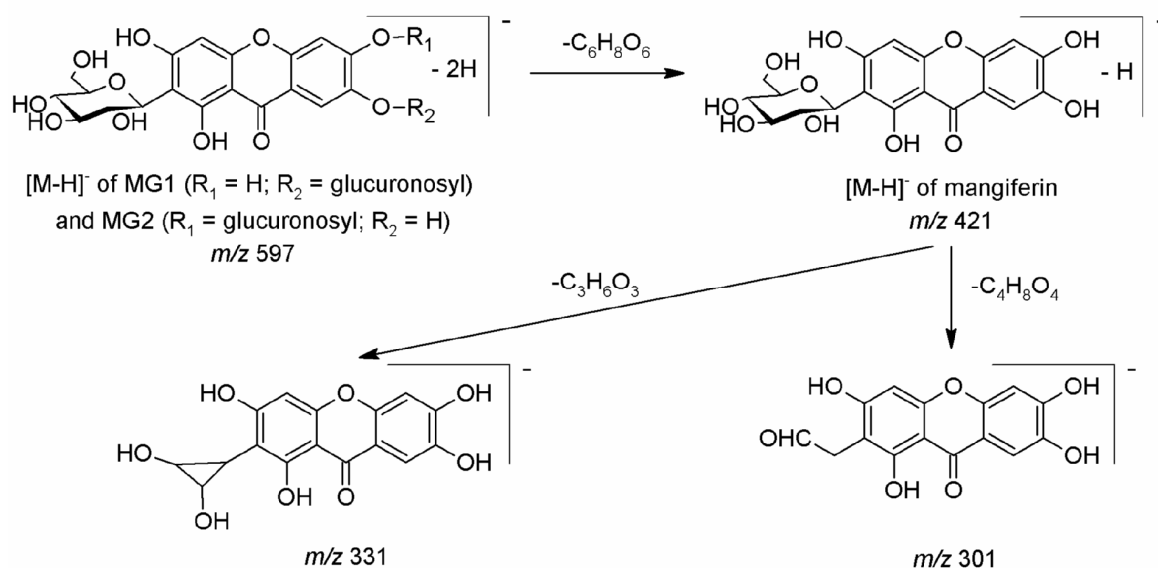
Figure 4 Proposed fragmentation mechanism for mangiferin and glucuronidated metabolites showing the major daughter ions (adapted from Ma *et al*., 2008).

Enzymatic hydrolysis of glucuronidated metabolites

Treatment with β-glucuronidase resulted in the disappearance of all three conjugates with concomitant increase in the peak area of mangiferin and homomangiferin. The chromatogram obtained after deconjugation is similar to that depicted in Fig. 2.

Sulphation

Sulphation of mangiferin with the Aroclor 1254-induced cytosolic rat liver fraction did not result in any detectable metabolites in the absence or presence of the co-factor PAPS. LC-MS analysis confirmed the absence of any sulphated metabolites. The *m/z* values for all possible pseudo-molecular ions ([M-H]⁻) that could result from sulphation of mangiferin were calculated and a search conducted for each after a full scan acquisition in the mass range 300 to 1000 amu.

Ferric reducing activity of the glucuronidated reaction mixture

Samples used to assess the effect of metabolism on the antioxidant activity of the reaction mixture (sample) in the FRAP assay were also subjected to quantification with HPLC analysis. Mangiferin was quantified with an external standard, while homomangiferin and the metabolites HMG, MG1 and MG2, were quantified as mangiferin equivalents. Approximately 27% of mangiferin remained unconjugated, contributing 25% to the total mangiferin equivalents (Table 3). Glucuronidation of mangiferin was accompanied by ca 60% decrease in its ferric reducing ability in the FRAP assay compared to the control, despite the fact that both the reaction mixture and the control contained a similar amount of total mangiferin equivalents (Table 3). MG2 was the most prominent metabolite after glucuronidation and accounted for approximately 60% of the total mangiferin equivalents present in the metabolite reaction mixture.

The homomangiferin concentration, similarly to that of mangiferin, was also extensively reduced (ca 70%) after glucuronidation. The concentration of homomangiferin appears to be overestimated, since mangiferin used was of 98% purity according to the supplier and homomangiferin was quantified as 11.7% mangiferin equivalents. Irrespective of this overestimation, its 'quantification' enables comparison of the relative content of the parent compounds and metabolites in the samples and the change in the activity with glucuronidation.

Radical scavenging ability of individual metabolites

Typical HPLC biograms for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging are depicted in Figs 3A and 3B, respectively. No activity was observed for any of the metabolites, while both mangiferin and homomangiferin scavenged DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, resulting in a decrease in absorbance, observed as negative peaks.

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Table 3 Concentration [a] of compounds and metabolites in the reaction mixtures (samples) and their resulting FRAP [b] activity.

	Mangiferin	Homomangiferin	MG1	MG2	HMG	Total	FRAP
	µg /mL	µg mang eq [c]/mL	µg mang eq [c]/mL	µg mang eq [c]/mL	µg mang eq [c]/mL	µg mang eq [c]/mL	µmoles Trolox/mL
Control	116.67 ± 8.39 a [d]	11.74 ± 0.82 a	-	-	-	120.19 ± 14.35	0.50 ± 0.05 a
Reaction mixture							
I	31.77 ± 3.00 b	3.48 ± 1.51 b	10.42 ± 1.35 a	76.12 ± 4.87 a	8.59 ± 1.61 a	127.98 ± 9.47	0.19 ± 0.04 b
II	33.69 ± 7.94 b	3.74 ± 1.88 b	10.01 ± 1.59 a	73.28 ± 6.11 a	8.44 ± 1.66 a	126.47 ± 15.72	0.21 ± 0.05 b
III	30.11 ± 3.74 b	3.35 ± 1.50 b	9.80 ± 1.50 a	72.98 ± 6.33 a	8.28 ± 1.88 a	122.19 ± 12.02	0.18 ± 0.02 b
Average % of total mang eq in reaction mixture	25.37	2.81	8.02	59.05	6.72		

[a] HPLC analysis was conducted in duplicate for two independent experiments, each containing three samples, [b] FRAP analysis was conducted in triplicate for two independent experiments, each containing three samples, [c] Mang eq refers to mangiferin equivalents quantified with HPLC using mangiferin as standard, [d] Values in a column followed by different letters differed significantly ($P < 0.05$).

DISCUSSION

The polyphenol, mangiferin, occurs widely in various plants (Li, 2002; Pinto *et al.*, 2005) and is one of the major antioxidants of *Cyclopia* species (*Fabaceae*), a South African herbal tea belonging to the Cape fynbos biome (Joubert *et al.*, 2008b). It occurs in relatively high concentrations (up to 4% m/m) in unfermented honeybush (Joubert *et al.*, 2009) and is therefore expected to contribute substantially to the antioxidant properties of this herbal tea (Joubert *et al.*, 2008a).   Apart from its antioxidant properties, mangiferin exhibits anti-apoptotic, immunomodulating, anti-inflammatory, cardioprotective and anti-diabetic potential (Pinto *et al.*, 2005; Prabhu *et al.*, 2009). The exact mechanisms of these biological effects are still under investigation, but its ability to scavenge reactive oxygen species seems to play an integral role. However, the modulating role of metabolism on the antioxidant/antiradical functions of mangiferin has not been clarified as yet. Human intervention studies provide evidence that polyphenol conjugates retain some biological activity (Williamson *et al.*, 2005). Investigations on the biological activity of glucuronidated polyphenols are increasing, as they constitute some of the predominant forms in circulation (Zhang *et al.*, 2007), while the unconjugated forms are virtually absent (Scalbert *et al.*, 2002).

The presence of mangiferin has been reported in rat plasma after administration of Rhizoma Anemarrhenae extract (Li *et al.*, 2008). Norathyriol, the aglycone of mangiferin, was found to be the major metabolite in the plasma of pigs, following ingestion of an unfermented honeybush (*C. genistoides*) extract (Bock *et al.*, 2008). Other metabolites were *O*-glucuronidated norathyriol and *O*-methylated mangiferin (Bock *et al.*, 2008). The *C-C* link between the glucose moiety and aglycone would confer stability to mangiferin against hydrolysis by enzymes in the brush border of the small intestine (Day *et al.*, 2000b). On the other hand, Sanugal *et al.* (2005) described an intestinal bacterium that produces an enzyme that transforms mangiferin to norathyriol. Greater insight into the biotransformation of mangiferin in rats was obtained in a recent study by Liu *et al.* (2011) that detected monoglucuronidated mangiferin in the urine and intestinal content after oral administration. Other phase II metabolites included different variants of methylated and glucuronidated mangiferin and norathyriol.   Sulphated and *O*-glycosylated products of norathyriol were also detected.

Mangiferin was not a substrate for sulphation under the experimental conditions of the present study; conditions that were conducive to the sulphation of aspalathin, a *C*-glucosyl dihydrochalcone (Van der Merwe *et al.*, 2010). In spite of the extensive investigation by Liu *et al.* (2011), no sulphated mangiferin was detected in the plasma and urine of rats treated with this xanthone. The only previous evidence for sulphated mangiferin has been detected by Lai *et*

al. (2003) in bile dialysate of rats after intravenous administration of mangiferin. It would thus appear that mangiferin is not a major substrate of the sulfotransferase. Of interest is that several sulphated-glucuronidated conjugates of norathyriol were detected in the urine and/or plasma of rats treated with mangiferin (Liu *et al.*, 2011). Sulphation is a competitive pathway for glucuronidation (Witkamp & Monshouwer, 1998) and polyphenols may therefore not be sulphated *in vivo* due to glucuronidation being predominant. Catechol-*O*- methyltranferase (COMT) also compete with UDP-glucuronosyltransferase (UGT) in the metabolism of polyphenols (Lambert & Yang, 2003) and therefore could also be an important metabolic pathway of mangiferin *in vivo*. The presence of methylated and glucuronidated metabolites of mangiferin in the urine and the bile of rats (Lai *et al.*, 2003; Ma *et al.*, 2008) supports this. An investigation of the metabolites of green tea catechins demonstrated that COMT seems to be the preferred reaction at low concentrations while the UGT metabolic pathway is favoured at higher doses (Lu *et al.*, 2003).

In the present study, a major (MG2) and two minor (MG1 and HMG) metabolites were identified after glucuronidation of the mangiferin standard using LC-MS and LC-MS/MS analyses. The neutral loss of 176 amu was indicative of the glucuronide moiety for MG1 (*m/z* 597), MG2 (*m/z* 597) and HMG (*m/z* 611). The aglycones of MG1 and MG2 further fragmented to give the typical fragmentation pattern of *C*-linked glycosides (Lai *et al.*, 2003; Bock *et al.*, 2008; Zhang *et al.*, 2011), i.e. loss of 90 and 120 amu, confirming that these metabolites are monoglucuronides of mangiferin. Homomangiferin, a xanthone present in *Mangiferia indica* (Wu *et al.*, 2010), from which mangiferin was isolated, was identified based on a similar fragmentation pattern compared to that reported by Qin *et al.* (2009) and Zhang *et al.* (2011). HMG was identified as a monoglucuronide of homomangiferin based on the mass of the aglycone fragment ion (*m/z* 435).

UDP-glucuronosyltransferase (UGT) consists of many isoforms with broad and overlapping substrate specificities and different tissue distribution (Fisher *et al.*, 2001). The selectivity of each UGT is determined by the position of the OH group on the substrate molecule (Wong *et al.*, 2009). In the present study, Aroclor 1254, containing a mixture of polycyclic hydrocarbons, specifically increased the UGT1A6 isoform of the enzyme in rats (Bock *et al.*, 1998), which particularly catalyses the glucuronidation of small catechols (Harding *et al.*, 1988). As the glucuronidation rate of catechols is enhanced 10 to 15-fold by a standard polychlorinated biphenyl (PCB) inducer, Aroclor 1254 (Elovaara *et al.*, 2004; Antonio *et al.*, 2001), it resulted in the higher levels of glucuronidated mangiferin metabolites observed and facilitated investigations into their antioxidant activity in the present study.

UGT mediated glucuronidation occurs via bimolecular nucleophilic substitution (SN2) with the OH carrying out a nucleophilic attack on the C-1 of the UDP-glucuronic acid (UDPGA) (Yin *et al.*, 1994). The reactivity of substrates such as flavonoids can be influenced by steric hindrance, intra-molecular hydrogen bonding, as well as electron-donating and electron-withdrawing effects of substituents on the rings (Zhang *et al.*, 2007). Lowering of the electron density on the oxygen of an OH-group, due to delocalisation, would reduce its nucleophilicity and overall glucuronidation activity (Zhang *et al.*, 2006). In spite of this, the C4'-OH of flavonoids seems to be one of the most favoured sites for conjugation (Zhang *et al.*, 2007). According to Rose *et al.* (2009), studying indazoles, the site of glucuronidation is likely to be the most acidic proton. The same factors would also be relevant for the reactivity of xanthones such as mangiferin. The most acidic proton of mangiferin is the 6-OH (lowest pK_a value), followed by 3-OH, 7-OH and 1-OH (Gómez-Zaleta *et al.*, 2006). The high acidity of the protons of 6-OH and 3-OH, due to their position being located *para* to the keto function, would lead to a greater proportion of ionised forms present at pH 7.4. The C-2 glucosyl moiety would, however, hinder the approach to the carbon of UDGPA, probably making glucuronidation at 3-OH less favourable than at 7-OH. The low acidity of 1-OH as a result of intra-molecular hydrogen bonding with the adjacent keto group at C-9 (Zhang *et al.*, 2006) and steric hindrance caused by C-2 glucosyl moiety would make it the least likely position to carry out a nucleophillic attack on UDPGA. Taking this into account, MG2 and MG1 correspond most probably with C-6 and C-7 conjugates, respectively.

The on-line antioxidant assays performed in the present study provided some further insight into the position of glucuronidation and confirmed glucuronidation at either 6-OH or 7-OH. Loss of the catechol arrangement through conjugation at C-6 or C-7 would result in a loss of antioxidant activity of xanthones as demonstrated for DPPH$^\bullet$ scavenging activity by Lee *et al.* (2005). In the current study glucuronidation of mangiferin resulted in loss of antioxidant activity in the FRAP assay, as well as in the on-line HPLC antioxidant assays using DPPH$^\bullet$ and ABTS$^{\bullet+}$, indicating that the structural requirements for antioxidant activity were compromised by biotransformation. HMG, the glucuronidated metabolite of homomangiferin, also did not display any radical scavenging. In this case glucuronidation at 3-OH is not possible due to the presence of a methyl group. However, the low quantity of homomangiferin in the mangiferin standard used, may explain why a second metabolite was not observed. Future studies on the phase II metabolism of homomangiferin will provide further insight into the specific hydroxyl groups involved.

Conversely, certain glucuronidated metabolites of quercetin have been shown to retain some biological properties by inhibiting xanthine oxidase and lipoxygenase, enzymes

associated with oxidative stress to related human diseases (Day *et al.*, 2000a). Therefore diverse biological effects of mangiferin metabolites in the body need to be investigated, since the loss of antioxidant activity may not necessarily indicate a loss in other biologically relevant activities. As the catechol moiety seems to be a structural requirement for the iron reducing property of mangiferin, its glucuronidation is likely to prevent recycling of iron under pro-oxidative conditions in the cell. On the other hand, loss of the catechol moiety and thus the ability to complex iron, would affect the secondary antioxidant activity of mangiferin in the cell (Pardo Andreu *et al.*, 2005). Interaction of mangiferin with iron, through complexing involving the catechol moiety, has been shown to be important in the protection against mitochondrial lipid peroxidation and DNA damage by inhibiting iron-mediated oxyradical formation (Pardo Andreu *et al.*, 2005, 2007). The latter also constitutes a potential protective mechanism against the pro-oxidant action of mangiferin. The present study indicated that glucuronidation is likely to modulate these anti- and pro-oxidant properties of mangiferin via the impairment of the catechol moiety.

The presence of β-glucuronidase in different organs and the notion that it is increased during inflammatory disease could play an important role in the turnover of polyphenols in the body and may result in the parent molecule becoming available to exhibit its antioxidant potential (O'Leary *et al.*, 2001; Wong *et al.*, 2009). Alternatively, conjugated polyphenols may pass through the vascular monolayers and be converted to aglycones by hydrolysing enzymes (Shimoi, 2004). Glucuronide metabolites might also be directly accessible to endothelial cells, although it is bound to albumin to a lesser extent than the aglycone (O'Leary *et al.*, 2001). The complex nature of polyphenol metabolism involving the liver and extra-hepatic organs, such as the lungs, kidneys and gastrointestinal mucosa, will create a delicate balance of conjugated and deconjugated metabolites that will likely contribute to the beneficial biological properties of mangiferin.

The current study presented the first evidence of the impairment of the antioxidant activity of mangiferin through conjugation catalysed by the glucuronyltransferases. It provides new perspectives regarding the potential of phase II mangiferin metabolites to modulate the redox status of the cell and hence their role in protecting against oxidative stress.

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CHAPTER 5

EFFECT OF SHORT-TERM AND SUB-CHRONIC DIETARY EXPOSURE TO POLYPHENOL-ENRICHED ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.) EXTRACTS ON RAT LIVER FUNCTION AND ANTIOXIDANT STATUS

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EFFECT OF SHORT-TERM AND SUB-CHRONIC DIETARY EXPOSURE TO POLYPHENOL-ENRICHED ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.) EXTRACTS ON RAT LIVER FUNCTION AND ANTIOXIDANT STATUS

ABSTRACT

Polyphenol-enriched extracts (PEEs) of unfermented rooibos [*Aspalathus linearis* (PER)] and honeybush [*Cyclopia. subternata* (PECsub) and *C. genistoides* (PECgen)] were fed to male Fischer rats in two independent studies for 28 and 90 days. PECgen was only included in the 28 day study. Dietary intake of the PEEs at the administrated dose (2.0 g PER/kg diet and 2.5 g PECsub and PECgen/kg diet) did not significantly ($P \geq 0.05$) affect body weight gain (bwg) or relative liver and kidney weight. PECsub resulted in a significant ($P < 0.05$) increase in the total serum bilirubin (Tbili) after 28 days while the serum ALP levels only significantly ($P < 0.05$) increased after 90 days, suggesting that the biliary system was possibly affected. No other clinical biochemical serum parameters were adversely affected, although AST was significantly ($P < 0.05$) reduced by PECsub and markedly ($P < 0.1$) by PECgen after 28 days, while Tbili was significantly ($P < 0.05$) reduced by PECsub after 90 days. Both PER and PECsub significantly ($P < 0.05$) reduced serum iron, but only after 90 days, indicating that the duration and level of exposure to dietary polyphenols are of importance. Endogenous antioxidant enzyme activity, i.e. catalase (CAT) and superoxide dismutase (SOD), was not significantly ($P \geq 0.05$) altered in the liver after 28 or 90 days. Glutathione reductase (GR) was significantly ($P < 0.05$) increased after 28 days of all three PEE treatments. Reduced glutathione (GSH) was significantly ($P < 0.05$) decreased after 90 days following treatment with the PER extract, resulting in a notable, but not significant ($P \geq 0.05$) decrease in the GSH/GSSG ratio. Similar changes, although not significant, were evident for PECsub. GSH conjugation with the catechol type polyphenols via *o*-quinone formation is likely to be involved implying altered GSH metabolism and consequent affects on cellular oxidative status and defense. Differential effects of the herbal teas were attributed to the diversity in the polyphenolic composition, with the dihydrochalcones, aspalathin and nothofagin, being prominent in rooibos and the xanthones, mangiferin and isomangiferin being major constituents of honeybush. The total polyphenol consumption in the current study was in excess compared to the customarily use of these herbal teas. The observed adverse biological effects as a function of the duration and level of polyphenol exposure may be valuable in future clinical studies investigating the safety and efficacy of value-added products and nutraceuticals derived from PEEs of rooibos and honeybush.

INTRODUCTION

Phytochemicals, such as plant polyphenols are associated with beneficial effects in humans, but considerable scientific evidence exists to suggest that their chronic consumption may lead to adverse biological effects (Lambert *et al.*, 2007). Polyphenols can display pro-oxidative activity and this has been demonstrated for a major green tea (*Camellia sinensis*) flavonoid (−)-epigallocatechin gallate (EGCG) *in vitro* and *in vivo* at high dose levels (Galati *et al.*, 2006; Lambert *et al.*, 2007; Lambert *et al.*, 2010). The induction of oxidative stress by EGCG results from auto-oxidation and quinone formation with the production of H_2O_2, which in spite of potential adverse effects, is also linked to its anticancer properties (Yang & Landau, 2000; Yang *et al.*, 2001). When considering cancer cells, a relative narrow therapeutic window exist between the effective dose required to kill a cancer cell and the dose that results in an adverse toxic effect to the adjacent normal cells (Tascilar *et al.*, 2006). In general, polyphenols can thus play a dual role of which the outcome is determined by the levels consumed in conjunction with the physiological conditions of the cell (Skibola & Smith, 2000).

Reactive oxygen species are produced from several reactions within a cell and is closely regulated by a multiple biological network of protection (Valko *et al.*, 2007). This network includes antioxidants, such as vitamin E together with the antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and co-factors such as glutathione (GSH), ascorbate, β-nicotinamide adenine dinucleotide phosphate (NADP) and glutathione reductase (GR) to regenerate the reducing molecules (Fang *et al.*, 2002). However, excessive exposure to polyphenols may overwhelm these antioxidant defense mechanisms in the cell, resulting in the over production of reactive oxygen intermediates (Skibola & Smith, 2000). This ultimately contributes to cell injury and DNA damage, with the potential induction of irreversible preneoplastic lesions and carcinogenesis. Beneficial antioxidant properties can thus be outweighed by adverse effects and consequently the unregulated use of commercially available polyphenol-enriched foods or dietary supplements are of concern (Skibola & Smith, 2000; Halliwell, 2007). Many uncertainties exist about the relative safe levels for such products.

Due to the historical use of the South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), they are generally regarded as safe for human consumption with an increasing number of reports establishing their beneficial biological effects related to their antioxidant, anticancer and phytoestrogenic properties (Joubert *et al.*, 2008a; Joubert & De Beer 2011; Joubert *et al.* 2011). No reports of toxicity resulting from rooibos and honeybush consumption have been documented, with the exception of a case report by Sinisalo *et al.* (2010). Possible hepatotoxicity was observed following the ingestion of rooibos tea, flavoured

with small amounts of strawberry, chamomile and petals of daisy, in a female with low-grade B-cell malignancy when consuming 1 L/day. However, the adverse effects were attributed to possible contamination of the tea with hepatotoxic compounds, genetic predisposition and/or adverse drug interactions with the tea. A study in rats indicated that exposure to aqueous extracts of unfermented and fermented rooibos and honeybush (*C. intermedia*) as sole source of drinking fluid for 10 weeks did not affect the liver and/or kidney function (Marnewick *et al.*, 2003).

There have been a number of recent advances regarding the health promoting properties of rooibos and honeybush. *Cyclopia subternata* has potential for use in a phytoestrogen product aimed at the nutraceutical market (Mfenyana *et al.*, 2008). *Cyclopia genistoides* showed potential for production of an antioxidant product due to high levels of the bioactive *C*-glucosyl xanthone, mangiferin, the major phenolic compound in *Cyclopia* spp. (Joubert *et al.*, 2009; Joubert *et al.*, 2011a). Currently, rooibos extracts are used as intermediate value-added products by the food, nutraceutical and cosmetic industries (Joubert & De Beer, 2011) and patent protection with regards to the anti-diabetic potential has been obtained for extracts produced from rooibos and honeybush (Mose Larsen *et al.*, 2008a; Mose Larsen *et al.*, 2008b; Muller *et al.*, 2008; Joubert *et al.*, 2011b).   An aqueous extract of *C. intermedia* was recently demonstrated to be effective in reducing plasma glucose concentration in a streptozotocin (STZ)-induced diabetic rat model (Muller *et al.*, 2011) and mangiferin was reported to have potent antipyretic activity (Singh *et al.*, 2011). The major phenolic compound in rooibos, aspalathin, has hypoglycemic effects in animal models (Mose Larsen *et al.*, 2008a; Kawano *et al.*, 2009).

Due to these advances and the resultant potential use of rooibos and honeybush as value-added products, food supplements and/or pharmaceutical products, it is becoming essential to assess possible adverse biological effects. The aim of the present study was to evaluate changes in the serum clinical biochemical parameters and oxidative parameters in rat liver following the short-term and sub-chronic ingestion of dietary polyphenol-enriched extracts (PEEs) of unfermented rooibos (PER) and two unfermented honeybush species, *C. subternata* (PECsub) and *C. genistoides* (PECgen).

MATERIALS AND METHODS

Chemicals and water purification

Deionized water was prepared using a Modulab Water Purification System (Continental Water Systems Corp., San Antonio, USA). For HPLC eluant and sample preparation, deionized water

was further purified by means of a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, MA, USA). The solvents required for HPLC analysis, acetonitrile 'Far UV' for gradient analysis was from BDH Chemicals Ltd. (Poole, England) and glacial acetic acid (>99.8%) from Fluka (Buchs, Switzerland). Dimethyl sulfoxide (DMSO), ferrous sulphate ($Fe(II)SO_4$), ascorbic acid and trichloro-acetic acid (ACS reagent; TCA) (>99%) were supplied by Sigma Chemicals Co. (St. Louis, MO, USA) and methanol and Fe(III)Cl (anhydrous $FeCl_3$) were from Riedel-de Haën (Seelze, Germany). The standards for quantification of rooibos flavonoids, isoorientin, vitexin and luteolin-7-glucoside were from Roth (Karlsruhe, Germany), while orientin, isovitexin, hyperoside, quercetin, luteolin and chrysoeriol were obtained from Extrasynthese (Genay, France). Aspalathin and nothofagin (purity of both ≥95% as determined by HPLC and LC-MS), isolated from green rooibos, were supplied by the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) Unit of the Medical Research Council (MRC, Bellville, South Africa). Standards for quantification of phenolic compounds of the honeybush extracts included mangiferin from Sigma-Aldrich (Steinheim, Germany) and narirutin, eriocitrin, hesperidin, eriodictyol, naringenin and hesperetin from Extrasynthese (Genay, France).

Folin-Ciocalteu's phenol reagent, potassium chloride (KCl) (≥99.9%; ACS reagent) ethylenediaminetetra-acetic acid dipotassium salt (EDTA) and anhydrous sodium carbonate were obtained from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT, 2,[6]-di-tert-betyl-p-cresol) (≥99%), 2-thiobarbituric acid (TBA) (≥98%), 6-hydroxydopamine (6-OHD), diethylenetriaminepentaacetic acid (DETAPAC), L-glutathione oxidized (GSSG, ≥98%), β-nicotinamide adenine dinucleotide phosphate reduced trisodium salt (94%), glutathione reductase (GR) from baker's yeast (*S. cerevisiae*), ammonium sulphate suspension, 100 – 300 units/mg protein), hydrogen peroxide (30% H_2O_2 w/w), sodium acetate-3-hydrate (AR, PAL Chemicals), bovine serum albumin (BSA) and gallic acid were from Sigma Chemicals Co. (St. Louis, MO, USA). TRIS ($N_2C(CH_2OH)_3$ was obtained from Amersham (Ohio, USA). Triton-X-100 was supplied by BDH Chemicals Ltd. (Poole, England). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 5,5′ dithiobis-(2-nitrobenzoic acid) (DTNB), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and ferrous sulphate heptahydrate (99% $FeSO_4$) were obtained from Sigma-Aldrich (Steinheim, Germany). Fluorescein disodium, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium (ferrozine), 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP) (>98%) and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were obtained from Fluka (Buchs, Switzerland). BCA protein assay reagent A (containing sodium carbonate, sodium bicarbonate, BCA detection

reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA protein assay reagent B were purchased from Separations Scientific (Cape Town, South Africa). Analytical grade solvents and chemicals were used except if stated otherwise.

Preparation of polyphenol-enriched rooibos and honeybush extracts (PEEs)

Powdered green rooibos (*A. linearis*) and green honeybush (*C. subternata* and *C. genistoides*) extracts, enriched in aspalathin and mangiferin respectively, through organic solvent extraction (Grüner-Richter *et al.*, 2008), were supplied by the Raps Foundation (Freising-Weihenstephan, Germany). The polyphenol-enriched extracts (PEEs) from rooibos (PER) and *C. subternata* (PECsub) and *C. genistoides* (PECgen) were stored desiccated in the dark.

Antioxidant activity analysis of PEEs

Total polyphenol content

The total polyphenol (TP) content was determined in triplicate according to the method described by Singleton & Rossi (1965), adapted to microplate scale (Arthur *et al.*, 2011). A stock solution of gallic acid (1 mg/mL) was prepared in deionised water to obtain a standard series of 0.1−10 µg/mL in the final reaction volume. Stock solutions of the PEEs were prepared at concentrations of 0.2 mg/mL for PECsub and PECgen and 0.1 mg/mL for PER, by dissolving the extract in 1 mL DMSO and further dilution in deionised water to a final volume of 10 mL. Assays were performed in Greiner 96-well clear flat-bottomed polystyrene plates (LASEC, Cape Town, South Africa) by adding 100 µL Folin-Ciocalteau reagent (1:10 dilution in deionised water) to 20 µL of water (blank), gallic acid standard (0.05–0.5 mg/mL) or 20 µL diluted extract. This was followed by 80 µL of Na_2CO_3 (7.5% w/v) added to each well within less than 8 min. Following incubation at 30 °C, absorbance values at 765 nm were recorded after 2 h on a Bio-Tek SynergyHT microplate reader (Winooski, Vermont, USA) equipped with Gen5 Secure software. Total polyphenol content was determined in triplicate and expressed as g gallic acid equivalents (GAE)/100 g extract.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Free radical scavenging activity was determined using the method of Rangkadilok *et al.* (2007) with slight modification for microplate format (Arthur *et al.*, 2011). Stock solutions of PEEs were prepared as described for total polyphenol analysis. A Trolox stock solution (1 mM) was prepared in 10% ethanol and diluted with deionised water to obtain a concentration range of 5−40 µM in the final reaction volume. For the reaction 270 µL of the DPPH solution (100 µM prepared in methanol and kept in the dark) was added to 30 µL of the blank (distilled water),

Trolox standards or PEEs in Greiner 96-deepwell polypropylene plates (LASEC, Cape Town, South Africa). The plate was sealed with a silicon mat to prevent evaporation of methanol and incubated at room temperature for 2 h, protected from light. Following the incubation, 200 µL of the reaction mixture was pipetted into the corresponding wells of Greiner 96-well flat bottomed polystyrene plates and the absorbance measured at 515 nm using a BioTek SynergyHT microplate reader. The assay was performed in triplicate and results were expressed as µmol Trolox equivalents (TE)/g extract.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) of the PEEs was determined using the method of Benzie & Strain (1996) with slight modifications for microplate format (Arthur *et al.*, 2011). Trolox was used as standard with a concentration range of 5–60 µM in the final reaction volume. The freshly prepared FRAP reagent contained 100 mL of acetate buffer (300 mM, pH 3.6), 10 mL of TPTZ (10 mM) in HCl (40 mM), and 10 mL of $FeCl_3$ (20 mM). Stock solutions of the PEEs were prepared at concentrations of 0.3 mg/mL for honeybush (PECsub and PECgen) and 0.14 mg/mL for PER, by dissolving the extract in DMSO (1 mL) and further dilution in deionised water. The assay was performed by adding 20 µL of distilled water as control, standards and dissolved tea extracts to their allocated wells in Greiner 96-well flat-bottomed polystyrene plates followed by addition of 180 µL of the FRAP reagent to each well. Absorbance, due to the formation of a coloured TPTZ-Fe^{2+} complex, was monitored spectrophotometrically at 593 nm after a reaction time of 4 min at 37 °C, using a BioTek SynergyHT microplate reader (Winooski, Vermont, USA). Determinations were in triplicate and results expressed as µmol TE/g extract.

Oxygen radical absorbance capacity (ORAC)

The oxygen radical antioxidant capacity (ORAC) of the PEEs was evaluated according to the method of Huang *et al*. (2002). Stock solutions (1 mg/mL) of the herbal extracts were prepared in DMSO (1 mL) and further diluted with potassium phosphate buffer (75 mM, pH 7.4). For calibration, a stock solution of Trolox (500 µM) was prepared by dissolving 0.25 g in 50 mL phosphate buffer (75 mM, pH 7.4), and diluting with the phosphate buffer to give calibration standards (5, 10, 15, 20, 25 and 30 µM).

A stock solution of fluorescein (8×10^{-2} mM) was prepared in phosphate buffer (75 mM, pH 7.4) and kept at 4 °C protected from light. A freshly prepared fluorescein working solution (8.16×10^{-5} mM) was prepared daily by dilution of the stock in the phosphate buffer. AAPH (0.414 g) was dissolved in 10 mL of phosphate buffer (153 mM) and was kept on ice. The AAPH solution not used within 8 h was discarded.

For the assay, 25 µL of sample, water or Trolox was added to the allocated wells in a black flat-bottomed Corning® polystyrene 96-well plate (Scientific Group, Cape Town, South Africa) with clear bottom. Pre-incubation and fluorescence readings were measured using a Biotek Synergy HT microplate reader. A thermal barrier was created by filling a single row of the outside wells with 300 µL water. Pre-incubation occurred for 10 min at 37 ⁰C after addition of 150 µL of fluorescein working solution. Following pre-incubation, 25 µL APPH solution was added to each well using the BioTek automatic reagent injection system. Fluorescence measurements ($k_{excitation}$ = 485 nm, $k_{emission}$ = 530 nm) were recorded from the bottom every minute over 35 min.

The quantification of the antioxidant activity was based on the calculation of the area under the curve (AUC) of the sample after subtraction of the AUC for the blank, termed the AUC_{net}. The antioxidant activity by ORAC was calculated as µmol TE/g extract from the calibration curve of AUC_{net} for Trolox against Trolox concentration in the reaction volume. All samples were analysed in triplicate at three dilutions, and the mean value was used to obtain the ORAC value.

HPLC analysis of PEEs

HPLC analysis was conducted using an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, autosampler, in-line degasser, column thermostat, diode-array detector and Chemstation software for LC 3D systems (Rev. B.02.01).

A Phenomenex Gemini C18 110Å column (150 x 4.6 mm i.d.; 5 µm) protected by a Gemini C18 guard cartridge (Phenomenex, Santa Clara, LA, USA) was used for analysis of PER. Separation was achieved with the solvent program described by Joubert (1996), adapted as follows: 0–3 min (20% B), 15-18 min (30% B), 27-29 min (35% B), 35 min (40% B), 54 min (60% B), 62 min (80% B), 68 min (60% B), 81 min (20% B), 90 min (20% B) where A and B were 2% aqueous acetic acid (v/v) and methanol, respectively. A flow rate of 0.4 mL/min was used for elution and 1.2 mL/min for reconditioning. A stock solution of 6 mg/mL of PER was prepared by dissolving in 20% DMSO-water and ultra-sonication. The standard calibration mixtures of the polyphenols and sample solution were filtered using 4 and 33 mm diameter 0.45 mm pore-size Millex-HV syringe filter devices (Millipore), respectively, prior to HPLC analysis.   An injection volume of 5 µL was used and separations were carried out at 38 ˚C.

Analysis of the phenolic compounds of PECgen and PECsub was carried out using a Zorbax Eclipse XDB -18 column (150 x 4.6 mm i.d., 5 µm) from Agilent Technologies at 30 ⁰C and a flow rate of 1 mL/min, using the following solvent gradient program: 0–6 min (12% B), 7 min (18% B), 14 min (25% B), 19 min (40% B), 24 min (50% B), 29 min (12% B), where A and B were 0.1% aqueous formic acid (v/v) and acetonitrile, respectively (De Beer & Joubert, 2010).

The extracts were diluted in purified water prior to analysis to a concentration of approximately 6 mg/mL. The calibration mixtures containing the standards and sample solutions were filtered as described for rooibos, prior to HPLC analysis. Injection volumes of 5 µL and 10 µL were used for PECgen and PECsub, respectively.

Linear regression using the least squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA, USA) was performed on calibration curve data for each compound. For determination of isomangiferin a response factor was determined relative to mangiferin as described by De Beer & Joubert (2010). Phloretin-3′,5′-di-C-β-glucoside (dihydrochalcone), iriflophenone 3-C-β-glucoside (benzophenone), an eriodictyol-glucoside (flavanone) and a hydroxycinnamic acid derivative were identified according to their retention time and UV-Vis spectra based on the tentative identification by De Beer & Joubert (2010) and Kokotkiewicz *et al.* (2012) using UV-Vis, LC-ESI-MS and LC-ESI-MS/MS data. The latter compounds, for which no authentic standards were available, were quantified as hesperidin equivalents.

Short-term (28 day) and sub-chronic (90 day) feeding studies in rats

Animals and diets

Male Fischer 344 rats (weighing between 150–200g; 7–8 weeks old) were obtained from the Primate Unit of the Medical Research Council of South Africa. Animals were individually housed in stainless steel wire-bottomed cages fitted with Perspex houses in a closed environment (24–25 ºC), with a 12 h light-dark cycle and 50% humidity. The rats received a mash prepared from feed cubes (Epol Ltd, Cape Town, South Africa) by milling (Christie & Norris Laboratory Mill, Sheffield, UK). As the antioxidant activity of rooibos exceeds that of honeybush (Joubert *et al.*, 2008a), the rooibos (PER) and honeybush (PECsub and PECgen) extracts were incorporated in the diet at levels of 2.0 g/kg feed and 2.5 g/kg feed respectively. The PEEs were mixed into the mash using a food mixer and stored under nitrogen at −20 ºC. The use of experimental animals was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council of South Africa (Addendum C).

Experimental design and sample collection

The PEE-mash mixtures were fed to the rats for 28 and 90 days in two separate experiments. Rats were randomly divided into the three treatment groups (10 rats/group) for the 90 day study (control, PER and PECsub) and four treatment groups (10 rats/group) for the 28 day study (control, PER, PECsub and PECgen). The same PER and PECsub extracts were used in both the 28 and 90 day feeding studies. The treatment groups received the PEE-mash mixture *ad*

libitum, while the control groups (28 and 90 days) received only the mash. Feed intake was monitored every second day and the body weight (bw) recorded weekly.

Animals in the 90 day study were fasted overnight prior to termination and sacrificed under pentobarbital anesthesia. Blood was obtained from the abdominal aorta and the liver and kidneys harvested, weighed and quickly frozen in liquid nitrogen. The samples were stored at −80 °C until use. The rats of the 28 day study were fasted overnight and terminated by cervical dislocation, blood collected and livers and kidneys harvested, weighed and treated as described above. Upon sacrifice, a separate sample from each liver was rapidly snap-frozen in liquid nitrogen and stored at −80 ºC for investigating changes in the gene expression profiles addressed in Chapter 6. Blood was collected in BD Vacutainer SST II advance (BD, Plymouth, UK) tubes and serum prepared by centrifugation (Sorvall Superspeed RC2-B centrifuge, Separations Scientific, Cape Town, South Africa) at 2 000 $x\ g$ for 10 min at 4 ºC and stored at −80 ºC until analysed.

Clinical biochemical parameters

Clinical biochemical parameters, including total cholesterol (Chol), alkaline phosphatase (ALP), total iron, alanine transaminase (ALT), gamma-glutamyl transferase activity (GGT) aspartate transaminase (AST), total bilirubin (Tbili), unconjugated bilirubin (DBili), creatinine (Creat) and total protein levels were determined using a Technicon RA 1000 automated analyzer at the Nutritional Intervention Research Unit (NIRU) of the Medical Research Council of South Africa (MRC, Bellville, South Africa).

Oxidative parameters in the liver

Five samples were randomly selected from each treatment and control group of the 28 and 90 day feeding studies and liver homogenates prepared for assessment of the activity of antioxidant enzymes, glutathione analysis and the extent of Fe(II)-induced hepatic lipid peroxidation.

Antioxidant enzyme assays
Preparation of liver homogenates

Liver homogenates were prepared on ice by homogenizing ± 200 mg of tissue in 2 mL of sodium phosphate buffer (50 mM, 0.5% (v/v) Triton X-100, pH 7.5) with a Teflon Potter-Elvehjem homogenizer using five strokes. The homogenates were sonicated for two 15 s bursts, followed by centrifugation (Heraeus HS-F16/3 Megafuge 1.OR fitted with a #2704 rotor, Kendro Laboratory Products, Germany) at 15 000 × g for 10 min at 4 °C and stored at −80 ºC

until used. An aliquot was diluted (100x) in 2% SDS (2 mM EDTA and 20 mM $NaHCO_3$) and incubated for 15 min at 37 ºC before the protein concentration was determined in duplicate according to the method of Kaushal & Barnes (1986) using bovine serum albumin (BSA) as standard.

Catalase (CAT) assay

Catalase activity was determined by monitoring the decomposition of hydrogen peroxide at 240 nm according to the method described by Aebi (1984). A reaction buffer was prepared by diluting 30% commercial H_2O_2 stock (~9.4 M) in sodium phosphate buffer (50 mM, pH 7.0) to obtain a final 10.3 mM H_2O_2 solution. Reactions were carried out in 1 mL UV grade PLASTIBRAND micro disposable cuvettes (Sigma Chemical Co., Cape Town, South Africa) and initiated by adding 10 µL of the liver homogenate supernatant (0.1 µg /µL of protein) to 490 µL of the reaction buffer. The absorbance was recorded every 12 s at 240 nm against a blank containing 10 µL supernatant and 490 µL reaction buffer without H_2O_2 using the time drive mode (UVIKON 923 Double Beam UV/VIS Spectrophotometer (Analytical & Diagnostic Products, South Africa) for 2 min.

The activity was calculated from the change in absorbance per min (blank subtracted), utilising linear regression and the molar extinction coefficient (ε) for H_2O_2 (0.0394 mM^{-1} cm^{-1} at 240 nm). Results were expressed as nmol H_2O_2/min/µg protein and represent the mean of duplicate determinations.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was determined according to the method of Ellerby & Bredesen (2000) as adapted for microplate reader format by Marnewick et al. (2009). The auto-oxidation of 6-OHD was measured using a Dynex Opsys MR Microplate Reader (IMRA1396) (Dynex Technologies, Medical Electronic Distributors (Pty) Ltd., Cape Town, South Africa). Liver homogenate samples were diluted to a concentration of 0.1 µg protein/µL in sodium phosphate buffer (50 mM, pH 7.4). A stock solution of 0.1 mM DETAPAC was prepared using the same phosphate buffer. For the reaction four different volumes (5, 10, 15 and 20 µL) of each diluted sample were added to 160 µL DETAPAC stock solutions in a Greiner 96-well clear flat-bottom plates. Phosphate buffer (50 mM, pH 7.4) was added (15, 10 and 15 µL) to the wells to obtain a volume of 180 µL followed by addition of 20 µL of 6-OHD to initiate the reaction (final volume 200 µL). A stock solution of 6-OHD (1.6 mM) was prepared in 0.35% $HClO_4$ (purged with nitrogen for 15 min) on the day of the experiment. The reaction was recorded in duplicate at 490 nm for 5 min at room temperature, using the kinetic mode of the plate reader. The

percentage inhibition of autoxidation was obtained (Revelation Quicklink, version 4.25) and used to construct an inhibition curve with the quantity of protein added to the reaction. The SOD activity was expressed as the amount of protein (ng) required to produce a 50% inhibition of auto-oxidation of 6-OHD and represent the mean of duplicate determinations.

Glutathione reductase (GR) assay

Glutathione reductase activity was measured spectrophotometrically (UVIKON 923 Double Beam UV/VIS spectrophotometer, Analytical & Diagnostic Products, South Africa) as described by Ellerby & Bredesden (2000) by following the oxidation of NADPH. Samples were diluted with buffer (50 mM potassium phosphate, pH 7) to achieve a concentration of approximately 10 mg protein/mL. A stock solution of oxidized glutathione (GSSG, 8 mM) was prepared in distilled water. A NADPH (15 mM) solution was prepared by dissolving in a $NaHCO_3$ solution (1% w/v). For the reaction 20 µL of the diluted sample was added to 960 µL of assay buffer (50 mM Tris/HCL, 1 mM EDTA, pH 8) and incubated at 20 ºC for 10 min. Following incubation, NADPH (20 µL) was added, thoroughly mixed and the linear A_{340} decrease recorded in duplicate for 5 min using 1 mL semi-micro poly-methylmethacrylate (PMMA) disposable cuvettes (Sigma Chemical Co.) with the assay buffer as blank. Enzyme activity was expressed as pmole NADPH used/min/µg protein, using the mmolar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$, for duplicate determinations.

Glutathione analysis

Reduced (GSH) and oxidized forms (GSSG) of glutathione were determined according to the method of Tietze (1969) as adopted for microplate reader format by Abel *et al.* (2004) with some modifications. Homogenates were prepared by grinding ± 250 mg liver tissue in liquid nitrogen using a mortar and pestle (Akerboom & Sies, 1981). Approximately 20 mg was weighed for protein determination according to the Lowry method as described by Markwell *et al.* (1978) using BSA as standard.

Approximately 100 mg of each ground sample was weighed for GSH determination and deproteinized with 1 mL 15% (w/v) TCA containing EDTA (1 mM). For GSSG determination an equal amount of the ground tissue was weighed and 1 mL 6% (v/v) perchloric acid ($HClO_4$), containing freshly prepared 3 mM M2VP (dissolved in 0.1N HCl and EDTA (1 mM), was added. Samples were homogenized on ice with a Polytron PT3100 (LASEC, Cape Town, South Africa) at 14 000 rpm for 25 s and stored at −80 ºC until analyzed.

A stock solution of NADPH (1 mM) was prepared with the assay buffer (500 mM sodium phosphate buffer, 1 mM EDTA pH 7.5) and the glutathione reductase (GR) enzyme stock

solution was prepared by adding 167 µL of the enzyme to 833 µL of the assay buffer to obtain 5 U in 50 µL. DTNB (0.6 mM) was prepared in the assay buffer. After thawing, homogenates were centrifuged (Heraeus HS-F16/3 Megafuge 1.OR) at 15 000 x g for 10 min at 4 ºC and the supernatant diluted with 0.5 M sodium phosphate buffer containing 1 mM EDTA (pH 7.5) at 1:600 for GSH and 1:30 for GSSG. For the assay, 50 µL of samples or standards and 50 µL DTNB and 50 µL GR stock solution were added to the respective wells of Greiner 96-well clear flat-bottom polystyrene plates. The final reaction mixture contained 0.25 U GR, 0.15 mM DTNB and 0.25 mM NADPH in sodium phosphate buffer. Following mixing and incubation for 5 min at room temperature, 50 µL of NADPH was added to initiate the reaction and absorbance was monitored in the kinetic mode (Dynex Opsys MR Microplate Reader (IMRA1396)) at 405 nm for 5 min. Determinations were done in duplicate. The concentration of GSH and GSSG in samples was determined by using a standard curve (0.5–3 µM) for each and the results expressed as nmol GSH or GSSG/mg protein.

Determination of Inhibition of lipid peroxidation in rat liver homogenates

Conjugated dienes (CD) in rat livers are an indicator of early stage lipid peroxidation and were determined according to the method by Hu *et al.* (1989) with slight modifications. Livers were homogenized on ice in 19 volumes potassium phosphate buffer (10 mM, 1.15% KCl, pH 7.4) with a Teflon motorized dounce and stored at −80 ºC until used. An aliquot (800 µL) of the homogenate (containing approximately 2 mg protein) was extracted with chloroform/methanol (CM 1:2, v/v), vortexed for 30 s before adding chloroform (1 mL) and vortexed again for 30 s. This was followed by addition of saline [1 ml 0.9% NaCl, saturated with CM (1:2)], vortexing for 30 s and centrifugation (Heraeus HS-F16/3 Megafuge 1.OR) at 1000 x g for 10 min at 4 ºC. The organic layer was transferred to a new tube and extraction of the aqueous phase with chloroform repeated. The combined organic phases were evaporated to dryness under a stream of nitrogen, the lipids dissolved in 1 mL hexane and the absorbance recorded at 233 nm in quartz cuvettes (1 mL) using a UVIKON 923 Double Beam UV/VIS spectrophotometer. Lipid peroxidation was expressed as nmole CD/mg protein, using a molar extinction coefficient of 27 000 M^{-1} cm^{-1}. Determinations were done in duplicate.

For determination of the thiobarbituric acid reacting substances (TBARS), liver homogenates (1 mg protein) were prepared as described above and an aliquot (containing ± 1 mg protein) was pre-incubated with 0.2 mL 2.5 mM Fe(II)SO$_4$ for 1 hr at 37 ºC in a shaking waterbath. Potassium phosphate buffer (1.15% KCl 10 mM, pH 7.4) was added to untreated control samples to serve as the negative control and the positive control contained 0.2 ml 2.5 mM Fe(II)SO$_4$. Samples were deproteinised using 2 mL 10% trichloroacetic acid containing 1 mM

EDTA and 0.01% BHT. Lipid peroxidation in rat liver homogenates was determined by the formation of TBARS over a 4 h time period, measured as malondialdehyde (MDA), according to the method described by Beuge & Aust (1978) with the following modifications: Samples (2 mL) were mixed with 2 mL ice-cold TCA reagent (10%) containing BHT (12.5 μM) and EDTA (0.372 g/L). After centrifugation (Heraeus HS-F16/3 Megafuge 1.OR) at 2000 x g for 15 min at 4 ºC the supernatant (2 mL) was reacted with an equal volume of 0.67% (w/v) TBA solution, added to inhibit further oxidation. Samples were incubated at 90°C for 20 min, and after cooling to room temperature the absorbance was measured at 532 nm (Esterbauer & Cheeseman, 1990). Lipid peroxidation was calculated as μmole MDA equivalents/mg protein, using a molar extinction coefficient of 1.56 x 105 M^{-1} cm^{-1} for MDA (Beuge & Aust, 1978). Determinations were done in duplicate.

Statistical analyses

Normality among the groups for all parameters was investigated using the Kolmogorov Smirnof Test. The homogeneity of group variances for all parametric parameters were tested using Levene's Test. Group differences for these parametric parameters were then tested using One-way ANOVA's and post-hoc Tukey tests, which is a post-hoc comparison of all pairs after a significant F-test. Where sample sizes were unequal the Tukey-Cramer adjustments were made automatically.

For non-parametric parameters, significant group differences were investigated using the Kruskal-Wallis test, as well as the post-hoc Tukey-type test to ascertain which groups differed. Comparisons with only two parametric groups were tested with T-tests. For those parameters with a time component (repeated measures), an ANOVA was used to test the two main effects, i.e. time and group, as well as an interaction effect. Data for one of the rats receiving PECgen in the 28 day study were omitted due to inconsistent body weight gain (bwg) and clinical biochemical results compared to the other rats. Statistical analyses were performed using SAS v9.2 and statistical significance was set at 5% ($P<0.05$).

RESULTS

Antioxidant activity analysis of PEEs

PER displayed significantly ($P<0.05$) higher antioxidant activity when compared to PECsub and PECgen in the TP, FRAP, DPPH, and ORAC assays (Table 1). Of the honeybush extracts, PECsub exhibited the higher ($P<0.05$) antioxidant activity in all the assays.

Feed and extract intake parameters

The rats receiving PECsub consumed significantly (P<0.05) more feed on average than the control and the PER treated rats during the 28 day feeding period (Table 2). Feed consumption did not differ significantly (P≥0.05) between the PECsub and PECgen treated rats for this time

Table 1 Antioxidant activity analysis of polyphenol-enriched extracts (PEEs) used in the 28 and 90 day feeding studies.

	A. linearis (PER)	*C. subternata* (PECsub)	*C. genistoides* (PECgen)
TP [a]	39.22 ± 1.10 [b] [a] [c]	24.83 ± 0.82 b	21.88 ± 0.59 c
FRAP [d]	2128.03 ± 27.01 a	1169.51 ± 15.85 b	992.65 ± 14.10 c
DPPH [d]	2773.62 ± 118.95 a	1478.44 ± 60.51 b	1257.26 ± 44.97 c
ORAC [d]	12989 ± 105 a	10219 ± 54 b	9679 ± 24 c

[a] TP (Total Polyphenol) expressed as g gallic acid equivalents (GAE)/100 g extract, [b] Values represent the mean ± standard deviation of triplicate analysis, [c] Means in the same row followed by different letters, differ significantly (P<0.05), [d] Total antioxidant activity using the FRAP, DPPH radical scavenging and ORAC assays is given as μmole TE/g extract.

Table 2 Intake parameters of male Fisher rats receiving polyphenol-enriched extracts (PEEs) [a] for a period of 28 and 90 days.

	Control	*A. linearis* (PER)	*C. subternata* (PECsub)	*C. genistoides* (PECgen)
28 days				
Feed intake [b]	9.73 ± 0.42 [c] [a] [d]	9.65 ± 0.39 a	10.35 ± 0.45 b	9.97 ± 0.49 ab
PEE intake [e]		0.19 ± 0.01 a	0.26 ± 0.01 b	0.25 ± 0.01 b
TP intake [f]		75.66 ± 3.04 a	64.27 ± 2.77 b	54.55 ± 2.70 c
90 days				
Feed intake [b]	7.27 ± 0.49 a	7.97 ± 0.57 b	7.74 ± 0.8 ab	
PEE intake [e]		0.16 ± 0.01 a	0.19 ± 0.01 b	
TP intake [f]		62.70 ± 8.87 a	48.05 ± 6.23 b	

[a] Concentration of PEEs (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER/kg and 2.5 g PECsub and PECgen/kg diet, [b] Feed intake was calculated as g feed/100 g bw/day, [c] Values represent the mean ± SD of ten rats (n = 10) per group with the exception of PECgen (n = 9), [d] Means in the same row followed by different letters are significantly different (P<0.05), [e] Intake of PEE was calculated as g extract/100 g bw/day per, [f] Total polyphenol (TP) intake was calculated as mg GAE/100 g bw/day.

period.   In contrast, during the 90 day feeding study, rats receiving the PER diet had a significantly (P<0.05) higher average daily intake than the control group, while that of the PECsub treated rats were not significantly higher (P≥0.05). Due to the higher concentration of PECsub and PECgen mixed into the feed, the average PEE intake was significantly (P<0.05) higher when compared to the PER treated rats for both the 28 day and 90 day studies. However, the average TP intake was significantly (P<0.05) higher in the rats receiving the PER diet in both studies attributable to the higher TP concentration.

The average feed, PEE and TP intake over the 90 day period was notably lower than that for 28 days (Table 2) attributed to a reduced feed intake in older rats. For comparative purposes, the feed intake indices were also calculated for three consecutive months (taken as 30 days) (Table 3). The feed intake and thus intake of PEEs and TP decreased, presumably as a result of the bwg, with time. The average feed intake was significantly (P<0.05) lower in all the treatment groups at 90 days than after 60 days. The intake of PER and PECsub was significantly (P<0.05) higher during the first two months of the 90 day study period.

Table 3 Intake parameters of Male Fisher rats receiving polyphenol-enriched extracts (PEEs) [a] for a period of 90 days, calculated on a monthly basis (1 month taken as 30 days).

	Control	*A. linearis* (PER)	*C. subternata* (PECsub)
Feed intake [a]			
30 days	8.01 ± 0.49 a [b *c]	8.95 ± 0.63 b *	8.63 ± 0.84 ab *
60 days	7.51 ± 0.48 a [#]	8.23 ± 0.56 b *	7.93 ± 0.43 ab *
90 days	6.30 ± 0.6 a [$]	6.70 ± 0.60 a [#]	6.65 ± 0.58 a [$]
PEE intake [c]			
30 days		0.18 ± 0.01 a *	0.22 ± 0.02 b *
60 days		0.17 ± 0.01 a *	0.20 ± 0.01 b *
90 days		0.13 ± 0.01 a [#]	0.17 ± 0.01 b [#]
TP intake [d]			
30 days		70.22 ± 5.39 a *	53.59 ± 5.19 b *
60 days		64.95 ± 4.31 a *	49.25 ± 2.66 b *
90 days		52.92 ± 4.57 a [#]	41.30 ± 3.62 b [#]

[a] Concentration of PEEs (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER/kg and 2.5 g PECsub and PECgen/kg diet, [a] Feed intake was calculated as g feed/day/100 g bw, [b] Values represent the mean ± SD of ten rats per group (n = 10), Means in the same row followed by different letters are significantly different (P<0.05), [c] Means in the same column marked with different symbols (*, #, $) indicate a significant difference (P<0.05) between for the groups, [d] Total polyphenol (TP) intake was calculated as mg GAE/100 g bw/day.

Concentration in PEE and intake of polyphenol compounds
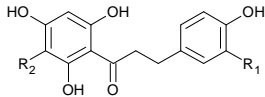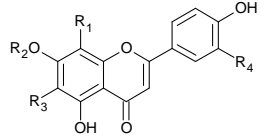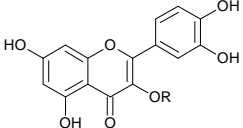
The phenolic composition of PER is characterized by dihydrochalcones, flavones and flavonols (Table 4). Aspalathin (18.73%) was the major flavonoid present followed by nothofagin (1.45%), isoorientin (1.45%) and orientin (0.81%).   A flavonol *O*-diglycoside in fermented and unfermented rooibos, tentatively identified as quercetin-3-*O*-robinobioside, was recently reported to co-elute with rutin (Beelders *et al.*, 2012). Under the separating conditions used in the present study, quercetin-3-*O*-robinobioside co-eluted with rutin and isoquercitrin. The combined content obtained for these three quercetin glycosides was 2.10%. The average aspalathin intake was 35.13 and 29.94 mg/100 g bw/day for the 28 and 90 day studies, respectively, which was more than 10 fold when compared to the other phenolic compounds. Typical chromatograms of the PEEs are presented in Addendum D. The average daily intake of the respective flavonoids was less during the 90 day study due to the significantly ($P<0.05$) lower aforementioned average feed intake.

Qualitative and quantitative differences were evident between the two honeybush extracts. Mangiferin was the major compound in both extracts with PECgen containing approximately 2x the concentration present in PECsub (Table 5). In addition to mangiferin PECgen contained isomangiferin and iriflophenone 3-*C*-glucoside at >1%. Other major compounds (≥1%) present in PECsub were isomangiferin, eriocitrin, scolymoside, iriflophenone 3-*C*-glucoside and phloretin-3′,5′-di-*C*-glucoside. Eriocitrin and scolymoside were not detected in PECgen, while phloretin-3′,5′-di-*C*-glucoside was present at a very low concentration. The hesperidin content of the extracts was between 0.8% and 1%. Several minor compounds, including luteolin and naringenin, were present in very low to trace quantities in both extracts. The average daily intake of total xanthones was ca 30 mg/100 g bw for rats consuming PECgen compared to less than 13 mg/100 g bw for rats consuming PECsub. On the other hand, intake of phloretin-3′,5′-di-*C*-glucoside averaged 6 mg/100 g bw for PECsub during the 28 day study compared to 0.3 mg/100g bw for PECgen. For both species the average intake of iriflophenone 3-*C*-glucoside was substantial (> 4 mg/100 g bw) (Table 5).

Body weight gain, relative organ weights and clinical biochemical parameters
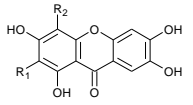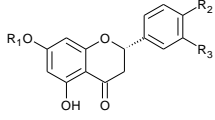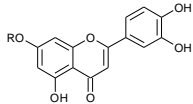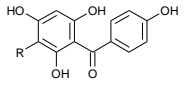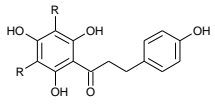
No deaths or obvious clinical signs were observed when conducting the short-term (28 day) and sub-chronic (90 day) studies with rats. The bwg and relative kidney and liver weights were not significantly ($P≥0.05$) affected by the administration of the PEE diets (Fig. 1 and Table 6). The herbal tea extracts did not significantly affect the liver function enzymes during the 28 day

Table 4 HPLC quantification of phenolic compounds present in the polyphenol-enriched rooibos (PER) extract and the average daily polyphenol intake during the 28 and 90 day studies with male Fisher rats.

Polyphenol subgroup	Compound	Substitution	Polyphenol concentration [a] g/ 100 g extract	Daily Intake [b] 28 days mg/100 g bw	Daily Intake [b] 90 days mg/100 g bw
Dihydrochalcone					
	aspalathin	R_1=OH, R_2=C-β-D-glucopyranosyl	18.73	36.13 ± 1.45	29.94 ± 2.03
	nothofagin	R_1=H, R_2=C-β-D-glucosylpyranosyl	1.45	2.80 ± 0.11	2.32 ± 0.16
Flavone	isoorientin	R_1, R_2=H, R_4=OH, R_3=C-β-D-glucopyranosyl	1.45	2.80 ± 0.11	2.32 ± 0.16
	orientin	R_1=C-β-D-glucopyranosyl, R_2, R_3=H, R_4=OH	0.81	1.56 ± 0.06	1.29 ± 0.09
	vitexin	R_1=C-β-D-glucopyranosyl, R_2, R_3, R_4=H	0.30	0.58 ± 0.02	0.48 ± 0.03
	isovitexin	R_1, R_2, R_4=H, R_3=C-β-D-glucopyranosyl	0.39	0.75 ± 0.03	0.62 ± 0.04
	luteolin	R_1, R_2, R_3=H, R_4=OH	0.06	0.12 ± 0.005	0.10 ± 0.01
	luteolin-7-O-glucoside	R_1, R_3=H, R_2=β-D-glucopyranosyl, R_4=OH	0.04	0.08 ± 0.003	0.06 ± 0.004
	chrysoeriol	R_1, R_2, R_3=H, R_4=OCH$_3$	0.02	0.04 ± 0.001	0.03 ± 0.002
Flavonol	quercetin-3-O-robionobioside	R=robinobiosyl			
	isoquercitrin	R=β-D-glucopyranosyl	co-elute 2.10	4.05 ± 0.23	3.36 ± 0.23
	rutin	R=rutinosyl			
	quercetin	R=H	traces	traces	traces
	hyperoside	R=galactopyranosyl	0.29	0.56 ± 0.02	0.46 ± 0.03

[a] Values represent the percentage (g/100 g extract) and are the mean of duplicate analyses, [b] Values represent the average daily polyphenol intake per treatment group (10 rats/group), bw = body weight.

Table 5 HPLC quantification of phenolic compounds present in polyphenol-enriched *C. subternata* (PECsub) and *C. genistoides* (PECgen) and average daily intake during the 28 and 90 day studies in male Fisher rats.

Polyphenol Subgroup	Compound	Substitution	Polyphenol concentration [a] g/100 g extract		Daily Intake [b] mg/100 g bw		
			PECsub	PECgen	PECsub 28 days	PECgen 28 days	PECsub 90 days
Xanthone							
	mangiferin	R$_1$=C-β-D-glucopyranosyl, R$_2$=H	3.79	9.29 [a,b]	9.81 ± 0.42	23.16 ± 1.15	7.34 ± 0.45
	Isomangiferin	R$_1$=H, R$_2$=C-β-D-glucopyranosyl	1.16	2.94	3.01 ± 0.13	7.33 ± 0.36	2.25 ± 0.14
	total xanthones				**12.82**	**30.49**	**9.59**
Flavanone	eriocitrin	R$_1$=rutinosyl, R$_2$=R$_3$=OH	1.10	-	2.85 ± 0.12	-	2.13 ± 0.13
	narirutin	R$_1$=rutinosyl, R$_2$=OH, R$_3$=H	nq [c]	nq [c]	–	-	–
	hesperidin	R$_1$=rutinosyl, R$_2$=OH, R$_3$=OCH$_3$	0.99	0.83	2.56 ± 0.11	2.07 ± 0.10	1.92 ± 0.12
	eriodictyol	R$_1$=H, R$_2$=R$_3$=OH	nq	nq [c]	–	-	–
	naringenin	R$_1$=R$_3$=H, R$_2$=OH,	0.01	0.01	0.03 ± 0.001	0.02 ± 0.001	0.02 ± 0.001
	hesperetin	R$_1$=H, R$_2$=OH, R$_3$=OCH$_3$	nq		nq		nq
	eriodictyol–O-glucoside	Unidentified (t$_r$ = 10.72 min) [d]	0.23	0.03	0.60 ± 0.03	0.07 ± 0.004	0.45 ± 0.03
Flavone	luteolin	R=H	0.06	0.09	0.16 ± 0.01	0.22 ± 0.011	0.12 ± 0.015
	scolymoside	R=rutinosyl	1.29	-	3.34 ± 0.14	-	2.50 ± 0.15
Benzophenone							
	iriflophenone-3-C-β-glucoside	R=C-β-D-glucopyranosyl	1.66	2.53	4.30 ± 0.19	6.31 ± 0.31	3.21 ± 0.20
Dihydrochalcone							
	phloretin-3′,5′-di-C-β-glucoside	R=C-β-D-glucopyranosyl	2.35	0.11	6.08 ± 0.26	0.27 ± 0.01	4.55 ± 0.28
	hydroxycinnamic acid derivative	Unidentified (t$_r$ = 11.01 min) [d]	0.72	-	1.86 ± 0.08	-	1.39 ± 0.09

feeding period, with the exception of PECsub that significantly (P<0.05) decreased AST levels, while increasing Tbili (P<0.05). PECsub resulted in a significant (P<0.05) increase in ALP in the serum during the 90 day feeding period. For the same period both the PER and PECsub extracts resulted in significantly (P<0.05) decreased serum Fe and TBili levels.

Oxidative parameters in the liver

Activity of antioxidant enzymes

The PEEs of rooibos and honeybush did not significantly (P≥0.05) alter the activity of the antioxidant liver enzymes CAT and SOD compared to the respective controls at 28 and 90 days (Table 7). The activity of GR was, however, significantly (P<0.05) elevated following the treatment with PEEs for 28 days.

Glutathione status

The GSH concentration in the livers was not significantly affected by the PEEs during the 28 day feeding period, although the honeybush extracts markedly increased the GSH level and GSH/GSSG ratio (Table 7). However, in the 90 day study the PER containing diet significantly (P<0.05) lowered the GSH concentration in the liver when compared to that of the control rats, while rats receiving the PECsub diet also displayed marginal (P<0.1) reduction. No significant (P≥0.05) effect was noted in the GSSG levels, while the GSH/GSSG ratio was markedly decreased in the rats, specifically those receiving the PECsub diet.

Inhibition of lipid peroxidation in rat livers

Consumption of PEEs for 28 and 90 days showed no significant (P≥0.05) effect on the CD levels in the liver although the PER diet resulted in a slight elevated CD level after 90 days. (Table 7). The MDA levels of the Fe(II)-induced lipid peroxidation in the liver of the rats that received PECsub for 28 and PER extracts for 90 days, were slightly, but not significantly (P≥0.05) elevated.

A

B
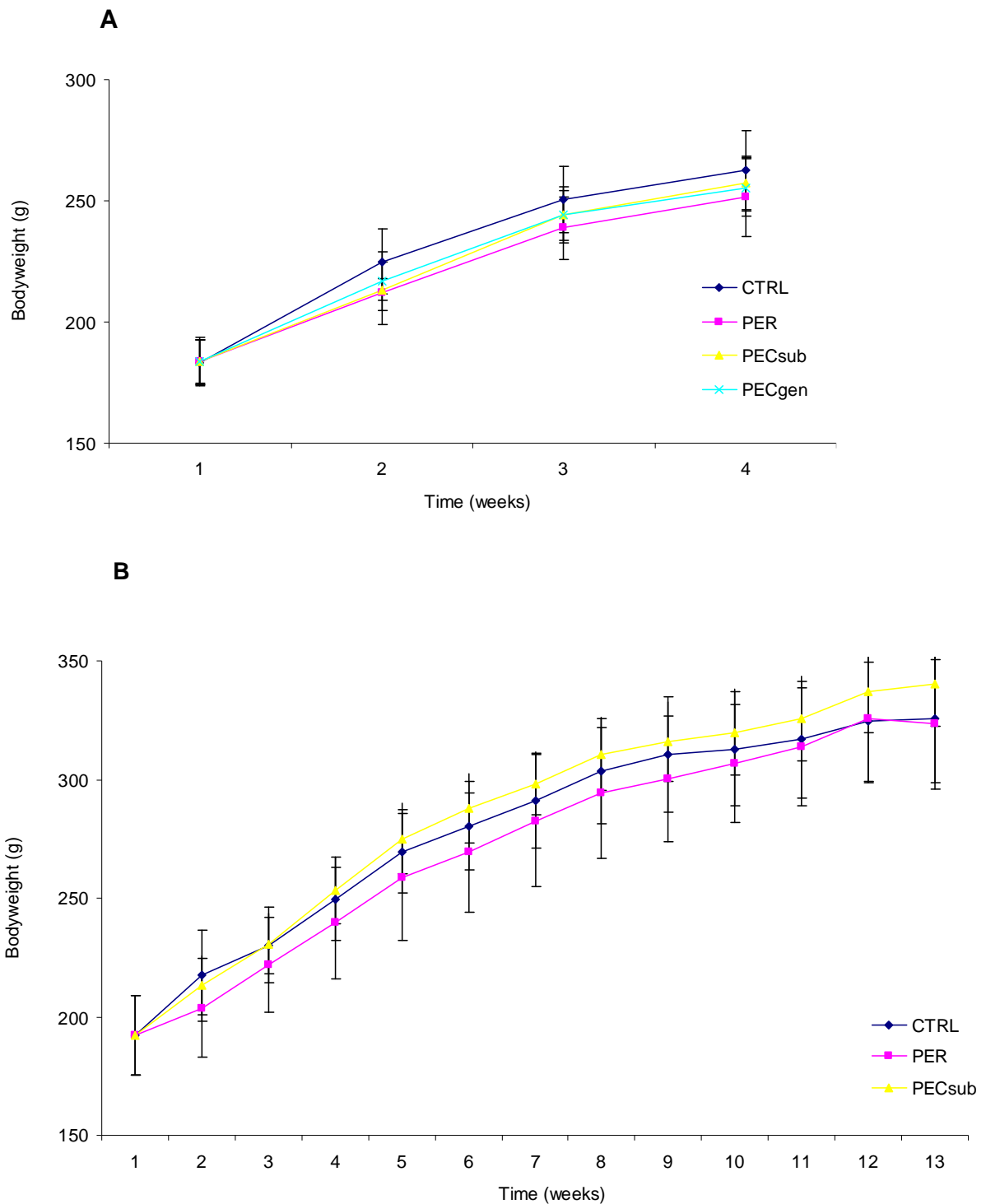
Figure 1 Mean body weight (bw) (average $\pm$ standard deviation) of the control (CTRL) and treatment groups that received polyphenol-enriched extracts (PEEs) (PER, PECgen, PECsub) for 28 days (A) and 90 days (B). Values represent the mean $\pm$ SD of ten rats (n=10) per group, with the exception of *C. genistoides* (n = 9).

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Table 6 Body weight gains (bwg), relative liver and kidney weights and serum clinical biochemical indicators of liver function after dietary consumption of polyphenol-enriched extracts (PEEs) [a] by male Fisher rats for 28 and 90 days during two respective studies.

	Control	A. linearis (PER)	C. subternata (PECsub)	C. genistoides (PECgen)
28 days				
Body weight gain (bwg) (g) [b]	94.50 ± 10.20 [c] a [d]	89.50 ± 12.94 a	92.00 ± 9.70 a	93.50 ± 12.83 a
Relative liver weight (%) [e]	3.57 ± 0.18 a	3.70 ± 0.18 a	3.81 ± 0.25 a	3.57 ± 0.19 a
Relative kidney weight (%) [e]	0.65 ± 0.03 a	0.63 ± 0.03 a	0.67 ± 0.03 a	0.66 ± 0.03 a
Chol (mmol/L)	1.17 ± 0.18 [b] a [c]	1.15 ± 0.09 a	1.19 ± 0.13 a	1.18 ± 0.07 a
ALP (U/L)	126.8 ± 11.36 a	136.90 ± 6.43 a	128.0 ± 7.31 a	135.11 ± 10.92 a
GGT	2.70 ± 2.75 a	1.80 ± 2.70 a	2.90 ± 1.60 a	2.30 ± 1.95 a
ALT (U/L)	44.20 ± 9.90 a	42.40 ± 5.04 a	41.90 ± 4.41 a	43.67 ± 5.87 a
AST (µmol/L)	104.90 ± 21.93 a	105.40 ± 12.96 a	88.30 ± 5.10 b	89.33 ± 6.73 ab
Tbili (µmol/L)	8.26 ± 1.75 a	7.78 ± 1.21 a	10.51 ± 1.38 b	8.60 ± 2.55 ab
Dbili (µmol/L)	0.99 ± 0.41 a	0.84 ± 0.21 a	1.24 ± 0.33 a	1.05 ± 0.57 a
Creat (µmol/L)	46.19 ± 4.54 a	46.09 ± 2.72 a	45.66 ± 2.92 a	45.34 ± 1.22 a
Iron (µmol/L) [f]	11.04 ± 1.06 a	11.34 ± 1.27 a	11.41 ± 1.30 a	11.74 ± 2.12 a
90 days				
Body weight gain (bwg) (g) [b]	126.85 ± 19.21 a	139.86 ± 17.41 a	146.75 ± 25.56 a	
Relative liver weight (%) [e]	2.73 ± 0.27 a	2.68 ± 0.20 a	2.76 ± 0.19 a	
Relative kidney weight (%) [e]	0.80 ± 0.14 a	0.75 ± 0.07 a	0.77 ± 0.05 a	
Chol mmol/l	1.37 ± 0.25 a	1.15 ± 0.13 a	1.17 ± 0.26 a	
ALP U/l	131.38 ± 21.89 a	143.76 ± 19.31 ab	183.73 ± 21.29 b	
GGT	3.00 ± 1.89 a	3.67 ± 2.00 a	3.90 ± 0.57 a	
ALT U/l	76.33 ± 7.57 a	83.00 ± 14.02 a	80.30 ± 31.61 a	
AST µmol/l	107.80 ± 25.73 a	121.00 ± 16.17 a	114.30 ± 33.59 a	
Tbili µmol/l	2.46 ± 0.36 a	2.09 ± 0.22 b	1.86 ± 0.24 b	
Dbili µmol/l	1.24 ± 0.23 a	1.30 ± 0.33 a	1.08 ± 0.21 a	
Creat µmol/l	64.70 ± 8.41 a	60.33 ± 3.77 a	60.80 ± 5.35 a	
Iron µmol/l	15.10 ± 1.82 a	11.37 ± 1.55 b	10.94 ± 1.56 b	

[a] Concentration of PEEs (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER /kg and 2.5 g PECsub and PECgen/kg diet, [b] Bwg was calculated by subtracting the bw on the first day from the bw on the day of termination, [c] Values represent the mean ± SD of ten rats (n=10) per group, with the exception of *C. genistoides* (n = 9), [d] Means in the same row followed by different letters are significantly different (P<0.05), [e] Relative liver and kidney weights were calculated as a percentage of the bw on the day of termination. [f] Iron refers to the total iron determined in the plasma.

Table 7 Effect on lipid peroxidation, oxidative enzyme parameters and reduced glutathione (GSH), oxidized glutathione (GSSG) and the ratio GSH:GSSG in rat liver after dietary consumption of polyphenol-enriched extracts (PEEs) [a] by male Fisher rats for 28 and 90 days during two respective studies.

	Control	*A. linearis* (PER)	*C. subternata* (PECsub)	*C. genistoides* (PECgen)
28 days				
CAT [b]	320.69 ± 36.85 [c] a [d]	296.56 ± 28.56 a	340.47 ± 40.76 a	330.73 ± 27.04 a
SOD [e]	0.64 ± 0.06 a	0.58 ± 0.06 a	0.62 ± 0.05 a	0.58 ± 0.08 a
GR [f]	41.71 ± 2.49 a	48.92 ± 0.85 b	51.64 ± 4.97 b	49.28 ± 3.67 b
GSH [g]	18.34 ± 4.88 a	19.70 ± 7.45 a	25.95 ± 4.56 a	24.12 ± 3.93 a
GSSG [h]	1.40 ± 0.42 a	1.66 ± 0.57 a	1.56 ± 0.26 a	1.59 ± 0.24 a
GSH/GSSG [i]	12.80 ± 4.44 a	11.95 ± 5.77 a	16.00 ± 4.17 a	16.12 ± 3.28 a
CD [j]	10.43 ± 2.08 a	10.20 ± 2.13 a	10.63 ± 2.13 a	10.88 ± 0.86 a
MDA [k]	1.58 ± 0.53 a	1.67 ± 0.66 a	1.92 ± 0.08 a	1.74 ± 0.33 a
90 days				
CAT [b]	323.47 ± 33.29 a	320.85 ± 37.48 a	290.91 ± 19.83 a	
SOD [e]	0.55 ± 0.09 a	0.53 ± 0.06 a	0.60 ± 0.09 a	
GR [f]	45.54 ± 3.47 a	44.96 ± 5.14 a	46.12 ± 6.20 a	
GSH [g]	19.69 ± 3.07 a	12.39 ± 0.59 b	12.87 ± 1.04 ab	
GSSG [h]	1.68 ± 0.33 a	1.26 ± 0.37 a	2.04 ± 0.94 a	
GSH/GSSG [i]	11.35 ± 3.79 a	9.66 ± 3.01 a	6.79 ± 4.11 a	
CD [j]	10.57 ± 1.68 a	12.27 ± 1.44 a	10.68 ± 1.56 a	
MDA [k]	1.50 ± 0.59 a	1.78 ± 0.43 a	1.63 ± 0.18 a	

[a] Concentration of PEE (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER/kg and 2.5 g PECsub and PECgen/kg diet, [b] Catalase (CAT) activity measured as nmole/min/µg protein, [c] Values represent the mean ± SD of duplicate analysis of five random samples selected from each treatment group of ten rats (n = 10), with the exception of C. genistoides (n = 9), [d] Means in the same row followed by different letters are significantly different (P<0.05), [e] Superoxide dismutase (SOD) activity given as the amount of protein (µg) required to produce a 50% inhibition of auto-oxidation of 6-hydoxydopamine, [f] Glutathione reductase (GR) activity measured as pmole NADPH used per min/µg protein, [g] Reduced glutathione (GSH) concentration given as nmole/µg protein, [h] Oxidized glutathione (GSSG) concentration given as nmole/µg protein, [i] GSH/GSSG ratio, [j] Conjugated diene (CD) concentration is given as nmole/mg protein, [k] Malondialdehyde (MDA) concentration given as µmole/mg protein.

DISCUSSION

Investigation on specific health effects and required concentrations of polyphenols should incorporate studies establishing a "safety level" to avoid toxic or adverse effects (Finley *et al.*, 2011). Studies in cell cultures and experimental animals indicated that antioxidants may cause toxicity, particularly when administered or consumed at high dose levels. In relation to tea and tea products, a green tea (unfermented *C. sinensis*) derived preparation, Teavigo, containing more than 90% of the potent antioxidant EGCG, exhibited toxicity when administered at a high single dose (2000 mg/kg) to rats (Isbrucker *et al.*, 2006). Oral administration of a single bolus dose 500 mg/kg to dogs resulted in proximal tubular necrosis in the kidneys and elevated serum bilirubin and AST levels in males, with the female dogs presented with liver necrosis. Administration of a single dose of 1500 mg/kg EGCG also resulted in hepatotoxicity in mice (Lambert *et al.*, 2010). In humans, consumption of high doses of tea-based (*C. sinensis*) dietary supplements showed elevated ALT and bilirubin levels which were resolved following cessation of the supplement consumption (Bonkovsky, 2006). Upon the re-introduction, the serum markers related to liver toxicity re-appeared indicating a causative role of the supplement.

Detailed characterization of the chemical and biological properties of herbal extracts used in animal and human studies is imperative to facilitate the benchmarking of the biological properties and avoidance of potential adverse effects. This has become important as differences in the chemical composition and biological activity exist and was illustrated in variations in the antimutagenic and antioxidant properties of rooibos and honeybush extracts (van der Merwe *et al.*, 2006; Manley *et al.*, 2006; Joubert *et al.*, 2008b). A recent study on rooibos indicated that the polyphenol content and the antioxidant activity varies between individual batches of plant material used, depending on stem content and the particle size of the raw material used (Joubert & De Beer., 2011). Therefore, comparison of the biological properties of the PEEs in the current study to *in vivo* studies conducted with aqueous extracts of rooibos and honeybush, should also compare differences in chemical composition and the *in vitro* antioxidant activities of extracts. These parameters could potentially be applied as quality parameters or used to obtain prediction of outcomes *in vivo*.

Comparative TP content and antioxidant activities

The TP content of PER in the present study (39.22 g GAE/100 g extract) was higher than the averages of 35.08 and 35.12 g GAE/100 g reported for aqueous extracts of unfermented rooibos by Joubert *et al.* (2008b) and De Beer *et al.* (2011), respectively. Enrichment of the current rooibos preparation (PER) resulted in an aspalathin content of 18.73 g/100 g extract,

approximately 2–3 fold the concentration when compared to the aqueous unfermented rooibos extracts, while the levels of nothofagin and the flavone-glucosides, isoorientin, vitexin and isovitexin, were also increased (van der Merwe, 2004; Joubert *et al.*, 2005, Beelders, 2011). The PER extract displayed increased antioxidant activity in the FRAP, DPPH and ORAC assays when compared to aqueous unfermented rooibos extracts (De Beer *et al.*, 2011). Increased activity is mainly attributed to the 2–3 fold enrichment in aspalathin, known to be a potent rooibos antioxidant when compared to the radical scavenging activity of quercetin and the green tea (*C. sinensis*) catechin, EGCG (Snijman *et al.*, 2009).

In contrast the TP content of PECsub and PECgen extracts (24.83 and 21.88 g GAE/100 g, respectively) were lower than the reported levels for aqueous extracts of unfermented *C. subternata* (32.41 g GAE/100 g) and *C. genistoides* (28.74 GAE/100 g) (Joubert *et al.*, 2008b). The PECsub extract contained a higher level of mangiferin (3.79 g/100 g) when compared to aqueous extracts of unfermented *C.subternata* containing 1.19 g/100 g (Joubert *et al.*, 2008b) and 2.73 g/100 g extract (De Beer & Joubert, 2010). The mangiferin content of PECgen (9.29 g/100 g) was similar to 10.04 g/100 g for the aqueous extracts of unfermented *C. genistoides* reported by Joubert *et al.* (2008b) and 9.55 g/100 g by De Beer & Joubert (2010). The isomangiferin (1.16 and 2.94 g/100 g) and hesperidin contents (0.99 and 0.83 g/100g) were higher for both PECsub and PECgen than in these aqueous extracts. In contrast the eriocitrin (1.10 g/100 g) and scolymoside (1.29 g/100 g) contents were slightly increased in PECsub compared to the reported levels in aqueous extracts of *C. subternata* (De Beer & Joubert, 2010).

The PECsub and PECgen extracts used in the current study were less effective antioxidants than unfermented aqueous extracts of these herbal teas, when evaluated in the FRAP antioxidant assay (Joubert *et al.*, 2008b), which is attributed to compositional differences in batches of plant material used for preparation of the aqueous extracts and PEEs. Apart from the mangiferin content in PECsub, the enrichment of the polyphenol content of honeybush (PECsub and PECgen) was not very effective in increasing TP and antioxidant activity. However, when considering these parameters PER was successfully enriched. This emphasises the importance of quality of the raw material used to produce polyphenol-enriched extracts. Furthermore, when benchmarking the chemical and biological properties of herbal extracts, the selection of plant material should be carefully considered.

Modulation of clinical biochemical parameters

No effect on the bwg and relative liver and kidney weights of the rats was observed during the 28 and 90 day treatment periods. However, AST levels was significantly and markedly

decreased by PECsub and PECgen after 28 days, respectively while PECsub significantly (P<0.05) increased serum Tbili levels. The reason for the significant decrease in AST is not known at present. The age of rats should be considered when interpreting the clinical biochemical parameters. The baseline Tbili as higher in the younger rats as is evident form the levels in the 28 and 90 day studies. It is also unclear why Tbili was increased in the serum of rats receiving PECsub for 28 days, while it significantly (P<0.05) decreased after 90. A similar decrease was also evident in the rats receiving the PER extract. In contrast a significant (P<0.05) increase in ALP levels was evident in the rats receiving the PECsub extract after 90 days while a marked increase was noticed for PER. Although increased ALP levels are associated with drug-induced cholestasis (Wright & Vadenberg, 2007), GGT activity is considered to be a more reliable marker for cholestasis than ALP in rats (Ozer *et al.*, 2008). The levels of GGT tended to be markedly, but not significantly, elevated in the serum of rats consuming PER and PECsub for 90 days. The serum ALT levels, Tbili, ALP and bile acids are used supplementary to conventional biomarkers of liver function, in particular with regard to the differential diagnosis of biliary function (Ozer *et al.*, 2008). Total bilirubin is, however, regarded as a better indicator of disease severity than ALT in humans (Dufour *et al.*, 2001). Tbili is a product of hemoglobin degradation used as a marker of hepatobiliary injury, specifically reflecting cholestasis and biliary effects (Ozer *et al.*, 2008). Bilirubin levels may also be increased due to non-hepatic causes such as hemolysis. Although *in vitro* studies indicated that polyphenols affect hemolyses in human erythrocytes due to phenoxyl radical formation (Galati *et al.*, 2002) it is uncertain whether the serum levels of honeybush polyphenols could have reached adequate levels to induce a similar effect *in vivo* in the current study. Dietary consumption of PECsub appears to adversely affect the biliary system after exposure for 28 and 90 days, although differences exist in the manifestation thereof depending on age of the rats and the level of polyphenol exposure and duration.

The different effects in the biochemical serum parameters between the two honeybush extracts (PECsub and PECgen) should be considered in view of their qualitative and quantitative differences in phenolic composition. Firstly, the TP intake of PECsub was significantly higher when compared to PECgen and secondly, PECsub contained phenolic compounds not present in PECgen, i.e. eriocitrin and scolymoside. PECsub also contained relatively high levels of both iriflophenone 3-*C*-glucoside and phloretin-3′,5′-di-*C*-glucoside when compared to PECgen. In contrast the xanthone (mangiferin and isomangiferin) intake was approximately 2.5 fold lower for the rats fed the PECsub extract, suggesting that these polyphenols do not play a determining role in changes in the Tbili and ALP serum levels. The

specific role of the polyphenols and/or the combination thereof in the disruption of biliary function as affected by PECsub is not known at present.

The level of exposure and type and/or specific polyphenol composition of extracts will determine the outcome of the biological effects. The significant higher TP level consumed by the PECsub treated rats during the 28 (64.27 mg GAE/100 g) and 90 day (48.05 mg GAE/100 g) studies is considered to result in the effect on Tbili observed, while no effect were evident at the TP level of 22.90 GAE mg/100 g in the study by Marnewick *et al*. (2003) with aqueous extract of unfermented *C. intermedia* as sole available drinking fluid (10 weeks).

TP exposure and time dependent effects on iron intake

As indicated above the phenolic composition of the PEEs, the level of TP administered, time of exposure and the age of the rats are of importance when considered biological effects. With respect to the duration of exposure, the serum iron levels were significantly decreased by PER and PECsub after the 90 day dietary treatments, while no effect was evident after 28 days. The latter is in agreement with a 10 week study in rats where no changes were observed in serum iron levels when exposed to aqueous fermented and unfermented rooibos and honeybush (*C. intermedia*) in the drinking water (Marnewick *et al*., 2003). Studies in humans also fail to provide evidence of a reduction in serum iron following consumption of fermented rooibos. These include a study with ^{59}Fe isotope in 30 human volunteers consuming a single serving of 200 ml fermented rooibos tea (with 40 ml milk and sugar), black tea or water for 14 days (Hesseling *et al*., 1979) and a study in school children (175) consuming two 200 mL servings of fermented rooibos and black tea (fermented *C. sinensis*) with milk and sugar daily for an extended period of 16 weeks (Breet *et al*., 2005). A recent study on the effect of rooibos on biochemical and oxidative stress parameters in adults at risk for cardiovascular disease also did not record any effect on the serum iron levels after six weeks of consuming six cups of the traditional fermented rooibos herbal tea per day (Marnewick *et al*., 2011). The TP intake of the latter study as well as the 10 week study in rats (Marnewick *et al*., 2003) was approximately 3 fold below that used in the present study over 90 days. It is therefore apparent that sub-chronic exposure over a period of 90 days to high levels of TP for rooibos (62.70 mg GAE/100 g bw/day) and honeybush (48.05 mg GAE/100 g bw/day) adversely affect serum iron presumable by disrupting iron absorption from the gut which could have important implications in human regarding physiological conditions associated with anemia. Inspite of the TP intake for PECsub being much lower than for PER, both significantly reduced iron levels, indicating that a upper limit for TP applies in terms of adversely affecting iron absorption and/or compositional difference.

Tannins from various food sources, including tea (*C. sinensis*) have been implicated in the inhibition of iron absorption (Graham *et al*., 2001). However, the effect of tea consumption on the absorption of iron has been controversial for some time. A recent study with green (unfermented *C. sinensis*) and black teas (fermented *C. sinensis*) in humans reported that the iron status parameters were not affected by their consumption of 1 L/day when using a four week cross over design (Schlesier *et al*., 2012). Blood samples were collected prior to the one week depletion period, at the start of the intervention phase and thereafter weekly during intervention. The only iron status parameter significantly affected was plasma ferritin values significantly reduced after tea consumption, especially in women with low basal ferritin values. Hemoglobin, hematocrit, transferring, total iron and iron binding were not significantly affected in healthy men and women. In rats it has been postulated that habitual tea (*C. sinensis*) consumption protects against the inhibitory effects of tea on iron absorption (Kim *et al*., 2004). The duration of this investigation in rats was however only 24 days, but the findings indicate that rats possess adaptive mechanisms to partially overcome the inhibitory effects of tannins on iron absorption. When considering the observed significantly reduced total serum iron in the 90 day studies, rooibos has been "branded" as low in tannins, but the hot water soluble solids of the fermented tea contains as much as 50% complex tannin-like substances (Joubert *et* al., 2008a). However, the tannin content is still far less when compared to black tea (Blommaert & Steenkamp, 1978). The tannin content of honeybush is much lower and estimated to be approximately 4.34% of the hot water soluble solids in fermented honeybush (Du Toit & Joubert, 1998). Reduced iron-absorption has also been attributed to formation of nontransportable polyphenol-iron complexes (Kim *et al*., 2008), which seems to be the likely contributing factor in reduced iron levels in the current study due to the high levels of TP consumed.

Bioavailability and modulation of cellular redox status in rat liver

The role of phenolic compounds on the oxidative status of the liver and observed effects on the serum biochemical parameters should take cognizance of the bioavailability of these compounds. The presence of both aspalathin and mangiferin, the major polyphenols of rooibos and honeybush, respectively, has been reported in plasma of humans. A concentration of up to 1.97 nmol aspalathin was reported in the plasma, 3 h after consumption of 500 mL (containing 287 mg aspalathin) of rooibos by humans (Breiter *et al*., 2011). Other intact compounds reported in the plasma in this study were isoorientin, orientin, isovitexin, vitexin and rutin, but at much lower levels than aspalathin. A pharmacokinetic study in humans with mangiferin reported a concentration of 38.64 $\pm$ 6.75 ng/mL after oral administration of 0.9 g mangiferin (Hou *et al*., 2011). Aspalathin and mangiferin are deglycosylated and excessively metabolized

by phase II conjugating enzymes following absorption (Bock *et al.*, 2008; Courts & Williamson, 2009; Stalmach *et al.*, 2009; Breiter *et al.*, 2011). Other rooibos phenolic compounds consumed at more than 1 mg/100 g bw/day in the present study included isoorientin, orientin and nothofagin. Orientin has been detected at a level of 45.07–60.74 µg/g in lung, liver and kidney tissue after intravenous administration of the pure compound (20 mg/kg bw) in rats, but no orientin could be detected in the plasma (Li *et al.*, 2008). Poor gastrointestinal absorption of orientin, isoorientin, vitexin and isovitexin was reported following gavage of a bamboo extract in rats (Zhang *et al.*, 2005).

Regarding the honeybush polyphenols, the aglycone of mangiferin, norathyriol, was the main metabolite detected in the plasma of pigs following the consumption of an extract of unfermented *C. genistoides* (Bock *et al.*, 2008). Metabolites reported for eriocitrin in plasma of rats include glucuronidated and/or sulphated conjugates of eriodictyol, homoeriodictyol and hesperetin (Miyake *et al.*, 2000). Small quantities of the free and conjugated form of hydrocaffeic acid were also detected. Urine metabolites included unconjugated and conjugated forms of the flavanones and hydrocaffeic acid. Hesperidin metabolites recovered from human plasma after ingestion of orange juice include glucuronides and sulfoglucuronides of hesperetin (Manach *et al.*, 2003). It would appear that, in general very few unmetabolised polyphenols do occur in the body, although deglycosylated products and metabolites of the phenolic compounds may still contain an intact catechol moiety.

Another measure of indirect evidence of absorption through the gut barrier is an increase in the antioxidant capacity of plasma after consumption of polyphenol-rich foods (Scalbert & Williamson*,* 2000; Tapiero *et al.*, 2002). The antioxidant status in plasma following ingestion of an aspalathin-enriched green rooibos extract over a two week period in humans showed no increase (Sauter, 2004), while Wanjiku (2009) and Villaño *et al*. (2010) reported a significant increase following consumption of rooibos tea. Breiter *et al*. (2011) could also not detect a significant increase in the plasma antioxidant capacity after consumption of different rooibos formulations. An increased total antioxidant capacity in plasma after the consumption of polyphenol-rich foods is often challenged as the effective means of measurement due to the extensive metabolism of dietary polyphenols (Spencer *et al.*, 2008). In relation to the South African herbal teas, both the major phenolic compounds in rooibos and honeybush, aspalathin and mangiferin, loose their antioxidant potency after phase II glucuronidation (Van der Merwe *et al.*, 2010, Van der Merwe *et al.*, 2012). However, since some of the major phenolic compounds in both rooibos and honeybush are bioavailable, albeit to a limited extent, these compounds and their parent compounds and/or metabolites, could affect the oxidative status, activity of antioxidant liver enzymes and subsequently the biochemical parameters.

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In the current study none of the PEEs significantly ($P > 0.05$) effected oxidative stress in the liver although there was a marked increase in lipid peroxidation, as monitored by the CD level in the livers of rats treated with PECsub after 28 days. After 90 days both CD and Fe(II)-induced lipid peroxidation were also markedly increased in the liver of rats receiving PER. An underlying oxidative stress therefore, appears to exist in the liver of the rats following consumption of PER and PECsub. A recent study demonstrated that an aqueous extract of fermented rooibos protects against lipid peroxidation in the liver of hepatotoxic carcinogen, fumonisin B_1 treated rats (Marnewick *et al.*, 2009). No protection was, however, evident for unfermented rooibos, which tended to synergistically increase the hepatotoxic effect of fumonisin B_1. This was attributed to pro-oxidant effects as demonstrated for rooibos (Joubert *et al.*, 2005). Fermented rooibos, in contrast, prevented lipid oxidation and oxidative stress and demonstrated protection against carbon tetrachloride-induced liver damage in rats (Uličná *et al.*, 2003; Uličná *et al.*, 2008). In humans, an aqueous extract of fermented rooibos decreased the level of lipid peroxidation and increased the GSH concentration in red blood cells and plasma of occupationally workers exposed to lead (Nikolova *et al.*, 2007). These studies were conducted with fermented rooibos extracts that are customarily used and protection against oxidative damage was demonstrated under conditions of increased oxidative stress. It is therefore apparent that protective effects of rooibos against lipid peroxidation are evident under conditions that are conducive to oxidative stress. Under normal physiological conditions, as indicated in the present study the marked increase in lipid peroxidation in the liver is rather an indication of an underlying oxidative stress.

The activities of the antioxidant enzymes, CAT and SOD in the liver were not altered under the present experimental conditions. However, the level of GR was significantly increased by all three PEEs after 28 days, while PER and PECsub did not affect GR significantly after consumption for 90 days. The significantly increased activity of GR may result from a disturbance in the glutathione metabolism, not yet manifested in the levels of GSH and GSSG at 28 days. The activity of the phase II enzymes, GSH-S-transferase and UDP-glucuronosyl transferase in the liver was significantly enhanced after 10 weeks of exposure to aqueous extracts of rooibos and *C. intermedia* as sole source of drinking fluid (Marnewick et al., 2003). This could possibly be a result of effective polyphenol metabolism in the liver and subsequent excretion in order to maintain the homeostatic balance regarding oxidative status in the liver. However, during conditions of oxidative stress, polyphenols may still play an important role in the body via the increased release of β-glucuronidase and/or sulfatase by inflammatory cells such as macrophages. This will facilitate de-conjugation reactions, liberating the free unconjugated polyphenol thereby re-establishing its antioxidant characteristics (Kawai, 2011).

The metabolism of the major rooibos and honeybush polyphenols and implications thereof were discussed in detail in Van der Merwe *et al.* (2010) and Van der Merwe *et al.* (2012). Mangiferin was shown to protect against isoproterenol-induced myocardial infarction in rats by contributing toward the tissues defense system (Prabhu *et al.*, 2006) suggested to be related to the antioxidant potential of the unconjugated form *in vivo*. Assessing the effects of rooibos and honeybush polyphenols in humans, specific endpoints should include the monitoring of the ratio of conjugated and unconjugated forms of relevant polyphenolic constituents. In addition, other parameters, apart from monitoring the activities of antioxidant enzymes, would provide a better assessment of the beneficial and/or adverse effects of polyphenol-enriched extracts in *in vivo* models.

Polyphenol and glutathione interactions

Apart from the increased levels of GR after 28 days, additional evidence that the PEEs interfere with the oxidative status in the liver, is obtained from the finding that the GSH level was significantly and markedly decreased after the 90 day exposure regimens by PER and PECsub, respectively. In contrast, the 10 week study with fermented and unfermented rooibos and honeybush (*C. intermedia*) as sole source of drinking fluid significantly reduced oxidized glutathione (GSSG) levels in the liver of rats while GSH was markedly increased (Marnewick *et al.*, 2003). These changes resulted in a significant ($P<0.05$) increase in the GSH/GSSG ratio by the unfermented and fermented rooibos and unfermented honeybush (*C. intermedia*). In humans, consumption of rooibos for six weeks improved several biomarkers of blood lipid status by decreasing lipid peroxidation and improving the redox status in the blood of adults at risk for developing cardio-vascular disease (Marnewick *et al.*, 2011). The plasma antioxidant capacity was not altered, likely related to the phase II metabolism of the polyphenols and the resultant loss in antioxidant activity. However, the level of CD and TBARS in the plasma significantly decreased while GSH and the GSH/GSSG ratio were both significantly increased (Marnewick *et al.*, 2011).

The duration and level of exposure to PEEs appears to be the major determinants when considering glutathione as a biological marker for cellular oxidative stress. GSH is a powerful intracellular antioxidant, present in mammalian cells, and its level is regarded as a good indication of oxidative stress (Green *et al.*, 2006). Depletion of GSH by polyphenols has been reported and may be affected by different mechanisms in the cell (Galati *et al.*, 2002). In cancer cells, GSH depletion by polyphenols has been proposed as a potential strategy to sensitize the cell to oxidative-induced cell death via phenoxyl radical-induced oxidative stress and mitochondrial membrane potential collapse (Galati *et al.*, 2002; Kachadourian & Day, 2006).

Therefore, the generation of H_2O_2 and reactive semi-quinone radicals by dietary polyphenols could be valuable in anticancer therapy. It is however of major concern when utilising supplements containing high doses of polyphenols due to the potential pro-oxidant activity of these compounds (Galati *et al.*, 2002; Heim *et al.*, 2002; Rietjens *et al.*, 2002; Halliwell, 2007; Halliwell, 2008). Pro-oxidant activity of aqueous extracts and their crude polymeric fractions of rooibos have been reported to be linear with the dihydrochalcone (aspalathin and nothofagin) and flavonoid content (Joubert *et al.*, 2005). Pro-oxidant activity was also demonstrated for pure aspalathin *in vitro* in the same study. Fermentation (i.e., oxidation) of rooibos decreased the pro-oxidant activity of aqueous extracts, which was associated with a decrease in the dihydrochalcone content. Pro-oxidant activity of mangiferin at the levels present in unfermented *Cyclopia* spp. has also been suggested (Joubert *et al.*, 2008b).

The chemical structures of flavonoids are an important determining factor when considering their pro-oxidant activity and their interaction with glutathione metabolism (Rietjens *et al.*, 2002). The pro-oxidant activity of phenol-type flavonoids is prone to result in GSH oxidation, while catechol-type flavonoids may lead to GSH-conjugation. Flavonoids with a phenol-type substituent pattern in the B-ring are enzymatically and/or chemically oxidized to generate a flavonoid semiquinone radical (Galati *et al.*, 1999; Rietjens *et al.*, 2002). This radical is scavenged by GSH leading to regeneration of the flavonoid resulting in the formation of the thiyl radical of glutathione (Galati *et al.*, 1999). This radical then reacts with GSH to generate a disulfide radical anion which rapidly reduces molecular oxygen to superoxide anion radicals. Flavonoids with a catechol type substituent pattern in their B-ring, especially a 3′,4′-catechol type moiety, is related to the formation of quinone type oxidation products (Rietjens *et al.*, 2002). These compounds scavenge GSH rather via conjugate formation instead of chemical reduction and the formation of GSH flavonoid adducts have been demonstrated (Galati *et al.*, 2001; Galati *et al.*, 2006). For gallic acid and gallic acid tea (*C. sinensis*) derivatives, GSH conjugates formation in primary hepatocytes was postulated to be attributed to their low redox potentials (Galati *et al.*, 2006), which are much lower than that calculated for the couple $GS^{•}H^{+}/GSH$ ($E_{m7}=$ 930 mV) (Wardman, 1990). Although the exact mechanism of GSH depletion in the liver following the 90 day feeding study is not known at present, GSH oxidation seems not to be involved since the GSSG level was not altered. The formation of GSH conjugates via flavonoid quinone formation is therefore more likely to occur. In this regard, studies in hepatocytes have shown that the catechol B-ring-containing flavonoids with low redox potentials, such as luteolin and quercetin, depleted hepatocyte GSH without causing GSSG formation (Galati *et al.*, 2002). The major B-ring catechol containing flavonoids in PER are aspalathin, isoorientin and orientin followed by the quercetin glycosides rutin, isoquercitrin and quercetin-3-robinobioside. Apart

from the catechol containing xanthones, mangiferin and isomangiferin, other catechol flavonoids of importance in honeybush with regards to GSH interaction in the present study will be eriocitrin, scolymoside and iriflophenone 3-*C*-glucoside.

Conclusions

The use of PEEs and the subsequent increased intake of polyphenols from rooibos and honeybush should be carefully managed, specifically under conditions of iron deficiency. When considering the serum biochemical parameters, PECsub could influence the biliary system in rats when consumed at the high total polyphenol dose of 64.27 mg GAE/100 g/day for more than 28 days. Slightly elevated CD levels and Fe(II)-induced lipid peroxidation (MDA) evident in the liver homogenates following consumption of PER and PECsub are likely associated with depletion of GSH, although other polyphenol/GSH interactions also need to be considered. It is apparent that there should be clear differentiation between effects of phenolic compounds when investigated under a disease state such as cancer or cardiovascular disease as opposed to their effects in healthy subjects. Toxicological risks may arise above a certain threshold limit following ingestion of polyphenol-enriched supplements as a function of the dose (Rietjens *et al.*, 2002). This raises the question about the dose-effect responses for beneficial versus toxic affects of functional food ingredients, which are often unknown.

The current study indicated that dietary PEEs of rooibos and honeybush had limited adverse affects in rats when administrated for 28 and 90 days in two separate studies, although the dose and duration of exposure should be carefully monitored. The observed effects on glutathione is, however, of major concern, since the disruption of GSH metabolism will have a major impact on the cellular redox status as well as xenobiotic metabolism in terms of the normal physiology and defense against xenobiotic metabolism (Dickinson & Forman, 2002). The biochemical serum parameters and oxidative parameters, i.e. antioxidant enzyme activity and glutathione analysis in rat liver are frequently used as indicators of oxidative stress. The lack of sensitivity and specificity of these biomarkers during the safety assessment of high levels of polyphenols (enriched extracts, functional foods or nutraceuticals) is however, questionable and additional approaches may be required to provide a more thorough evaluation.

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CHAPTER 6

CHANGES IN EXPRESSION OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE RELATED GENES IN RAT LIVER FOLLOWING INTAKE OF POLYPHENOL-ENRICHED ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.) EXTRACTS   FOR 28 DAYS

CHANGES IN EXPRESSION OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE RELATED GENES IN RAT LIVER FOLLOWING INTAKE OF POLYPHENOL-ENRICHED ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.) EXTRACTS FOR 28 DAYS

ABSTRACT

The safety of value-added products and nutraceuticals derived from polyphenol-enriched extracts (PEEs) from the South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) has not been sufficiently investigated to date. To provide additional insight into results from conventional biomarkers (serum clinical biochemical parameters and oxidative parameters in the liver), the changes in expression of oxidative stress and antioxidant defense related genes following the dietary consumption of PEEs prepared from these teas was assessed for the first time. PEEs of unfermented rooibos (PER) and honeybush [*Cyclopia. subternata* (PECsub) and *C. genistoides* (PECgen)] were fed to male Fischer rats for 28 days. A quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) array was used for evaluation of the effect on gene expression of 84 oxidative stress and antioxidant defense related genes in the livers of rats. Dietary intake of the PEEs at the administrated dose (2.0 g PER/kg diet and 2.5 g PECsub and PECgen/kg diet) significantly ($P<0.05$) altered the expression of several of these genes, i.e. seven as a result of PER intake and eleven by PECsub and PECgen, respectively, of which seven were mutually affected. Intake of PER resulted in the up-regulated expression of four genes and down-regulated expression of three genes, which included the glutathione peroxidases (*Gpx*), *Gpx2* and *Gpx3*, the aminoadipate semialdehyde synthase (*Aass*), neutrophil cytoxoic factor 2 (*Ncf2*), EH-domain containing 2 (*Edh2*), isocitrate dehydrogenase 1 (*Idh1*) and NAD(P)H dehydrogenase, quinone 1 (*Nqo1*). The expression of genes mutually affected by PECsub and PECgen included antioxidant related genes, prostaglandin-endoperoxide synthase 1 (*Ptgs1*), kinesin family member 9 (*Kif9*), serine (or cysteine) peptidase inhibitor clade B member 1b (*Serpinb1b*) and genes involved in reactive oxygen species (ROS) metabolism, xeroderma pigmentosum complementation group A (*Xpa*), thioredoxin interacting protein (*Txnip*), as well as oxygen transporter related genes fanconi anemia complementation group C (*Fancc*) and vimentin (*Vim*). The largest fold changes were neutrophil cytosolic factor 2 (*Ncf2*) down-regulated 4.78 fold by PER, NADPH oxidase organizer 1 (*Noxo1*) expression increased 19.97 fold by PECsub and a 372 fold increased expression of thyroid peroxidase (*Tpo*) affected by PECgen. Variations and

differential modulation of gene expression was attributed to the differences in phenolic composition between rooibos and honeybush, as well as between the two honeybush species, *C.subternata* and *C.genistoides*. Changes in the expression of the oxidative stress and antioxidant defense related genes could be indicative of an underlying stress affected by the PEEs in the liver.

INTRODUCTION

Polyphenols are known to activate or inhibit primary enzyme systems responsible for metabolism of xenobiotics, such as carcinogens, pharmaceutical drugs and environmental pollutants (Middleton *et al.*, 2000; Moon *et al.*, 2006). This interaction is of critical importance with respect to drug metabolism as subsequent increased or decreased levels of a specific drug could result in toxicity or failure in treatment efficacy. Therefore the effect of polyphenols on the expression of cytochromes P450 (CYP450) and other drug metabolizing enzymes have been the topic of many investigations (Hodek *et al.*, 2002; Moon *et al.*, 2006; Kimura *et al.*, 2010). Furthermore, inhibition of key cellular enzymes, gene transcription and induction of tumor suppressor genes have been proposed as important mechanisms involved in the chemopreventive effects of green tea (unfermented *Camelia sinensis*) and (-)-epigallocatechin gallate (EGCG) in animal models (Lambert *et al.*, 2010). However, limited information is available on the effect of polyphenols and herbal extracts on oxidative stress and antioxidant defense related genes and specifically the potential adverse effects of excessive doses (Leonarduzzi *et al.*, 2010).

The induction of hepatic antioxidant enzymes in rats was demonstrated following ingestion of the phenolic acids, gentisic acid, gallic acid, ferulic acid and *p*-coumaric acid at a dose of 100 mg/kg bw for 14 days (Yeh & Yen, 2006). The increased expression in hepatic superoxide dismutase (*Sod*), glutathione peroxidase (*Gpx*) and catalase (*Cat*) mRNA levels, induced by these phenolic acids, were associated with increased enzyme activity and suggested to be important in the protection against adverse effects related to oxidative stress and mutagenesis. The South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) as sole source of drinking fluid, significantly increased the activity of phase II liver cytosolic enzymes glutathione S-transferase α and UDP-glucuronosyl transferase in a 10-week study with rats (Marnewick *et al.*, 2003). Continuous ingestion of commercial rooibos as sole source of drinking fluid for two weeks resulted in induction of intestinal CYP3A in rats, implying potential herb drug interactions (Matsuda *et al.*, 2007). Effects of rooibos and honeybush and some of their major phenolic constituents on drug metabolizing enzymes have been reviewed

(Joubert *et al.*, 2009). Cardioprotective effects of aqueous rooibos extracts through inhibition of apoptosis were related to its flavonol content (Pantsi *et al.*, 2011). A recent study with polyphenol-enriched extracts of rooibos and honeybush (*C. subternata* and *C. genistoides*) administered in the feed for 28 days, indicated differential alteration of expression of genes associated with xenobiotics metabolism in rat liver and kidneys (Abrahams, 2012). These studies have indicated the influence of rooibos and honeybush extracts on CYP450 and a few phase II xenobiotic metabolising enzymes, but no information is currently available regarding the modulation of the expression of genes associated with the antioxidant status *in vivo*. This is of particular interest as a clinical study showed that consumption of six cups of the traditional fermented rooibos herbal tea over a period of six weeks improved the serum redox status and lipid biomarkers associated with cardiovascular disease (CVD) (Marnewick *et al.*, 2011).

Metabolic processes determining cellular oxidative status are complex and involve numerous genes, proteins and metabolites, making assays based on a few genes and/or physiological endpoints in assessing the beneficial or adverse effects of dietary nutrients, unreliable (Liu-Straton *et al.*, 2004). Conventional liver function assays include amongst others, serum bilirubin, serum albumin and selected hepatic enzymes that are representative of cellular disruption or damage (Amacher, 2010). Although useful and mostly applicable across species, many of the conventional and experimental hepatotoxicity indicators currently used as preclinical biomarkers for liver toxicity are not exclusively liver-specific, or their activity in serum or plasma may be influenced by factors other than liver injury. Due to hepatotoxicity and nephrotoxicity being important and continuing problems in the pharmaceutical industry, there is a need to improve toxicity testing methods (Taboureau *et al.*, 2012). Microarray technologies offer the ability to generate a large amount of gene expression information as an initial step to assess the molecular mechanisms of toxicologic changes, known as toxicogenomics. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) array analysis offers more specific information and is also more sensitive than microarrays (Buluchamy *et al.*, 2010) and hence can be applied as a screening tool to assess the impact of dietary constituents in health and disease. Potential application would therefore be in investigating toxicity as part of a toxicogenomics approach and elucidation of mechanisms involved in protective or adverse properties.

The current study investigated the modulatory effects of PEEs prepared from unfermented rooibos (PER) and honeybush (PECsub and PECgen) on the expression of 84 oxidative stress and antioxidant defense related genes in the liver after dietary administration to male Fisher rats for 28 days by using a commercially available quantitative RT-PCR array.

MATERIALS AND METHODS

Chemicals

Chemicals used for HPLC analysis were described in Chapter 5. The majorities of chemicals for experimental use in this research chapter was obtained as part of experimental kits and are therefore indicated in the text in the methodology section. Solvents and chemicals used were analytical grade except if stated otherwise.

Animals, diet and sample collection

Preparation of the diets containing the polyphenol-enriched herbal extracts (PEE) of rooibos (PER) and honeybush, *C. subternata* (PECsub) and *C. genistoides* (PECgen) are described in Chapter 5.

The feeding study in rats was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research council (MRC) and conducted as described (Chapter 5) over a period of 28 days. Rats were randomly divided into four treatment groups (n=10) consisting of the control, PER, PECsub and PECgen treated rats. As the antioxidant activity of rooibos exceeds that of honeybush (Joubert *et al.*, 2008), the rooibos (PER) and honeybush (PECsub and PECgen) extracts were incorporated in the diet at levels of 2.0 g/kg feed and 2.5 g/kg feed, respectively. Control rats received the untreated dietary mash as described (Chapter 5). Feed intake was monitored every second day and the body weight was recorded weekly. After the 28 days feeding period, rats were terminated by decapitation and the livers were harvested, immediately snap frozen in liquid nitrogen and stored at −80°C until use. The daily intake of the total polyphenols and the individual polyphenols were calculated as described (Chapter 5). The average feed intake was calculated for the three rats in each treatment group and the control used in the RT-PCR analyses. Selection of the rats utilised was based on the RNA quality extracted as described below.

Quantitative RT-PCR array analysis

Extraction of ribonucleic acid (RNA)

Total RNA was isolated from the livers of five randomly selected rats for each of the four treatment groups, consisting of ten rats (n = 10). A QIAGEN RNeasy mini kit (Whitehead Scientific, Cape Town, SA) which combines the selective binding properties of a silica-gel-based membrane with the speed of micro-spin technology to extract RNA in the presence of a specialized high-salt buffer system was used for RNA isolation. Liver was thawed on ice and 20–25 mg of each sample was lysed in 600 µL of a highly denaturing guanidine isothiocyanate

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(GITC)-containing lyses buffer* (RLT) followed by homogenizing in a QIAGEN Tissuelyser II (Separations, Cape Town, SA) for 40 sec. Beta-mercaptoethanol (β-ME) (14.3 M) was added to the buffer RLT prior to use. The lysate was centrifuged (Heraeus HS-F16/3 Megafuge 1.OR fitted with a #2704 rotor, Kendro Laboratory Products, Germany) at 10 000 x g for 3 min and the supernatant was transferred to a clean microcentrifuge tube. Ethanol (75%) (600 µL) was added to the lysate and the mixture (1200 µl) was fractionated on an RNeasy spin column by centrifuging for two 15 sec intervals at 8 000 x g. The RNeasy spin column membrane was washed with RW1 buffer* (700 µl) by centrifugation at 8 000 x g for 15 sec. A final washing step using RPE buffer* (500 µl) was performed by centrifuging again for two 15 sec intervals at 8 000 x g and then for 2 min. For evaluation of RNA quality and integrity, RNase-free water (50 µl) was added directly to the spin column membrane and centrifuged at 8 000 x g for 1 min.

* Buffers are included in the QIAGEN RNeasy mini kit and therefore no information is available regarding the reagents, concentrations or pH.

RNA cleanup and quality assessment

Genomic DNA in the RNA samples was digested using the Ambion® Turbo DNA-free™ kit (Applied Biosystems, Johannesburg, South Africa) according to the manufacturer's recommendations. Briefly, RNA (20 µg), DNase buffer*, DNAse I* (4 units) and nuclease-free water* were mixed and samples incubated for 90 min at 37°C in a final volume of 20 µL. The DNAse enzyme was then deactivated by incubating the mixture with 10 µL DNAse inactivation reagent* for 2 min at room temperature. The RNA-containing supernatant was collected after centrifugation at 8 000 x g.

The RNA quantity and purity were determined with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and RNA integrity was confirmed by determining the RNA 28S:18S ratio with a Bioanalyzer 2100 (Agilent Technologies, Johannesburg, South Africa).

Analysis comprised transferring a gel-dye mixture* (9 µl) to one of the wells on a RNA 6000 Nano LabChip (Agilent Technologies) followed by spreading through plunging 1 ml of air with a syringe into the well for 30 sec. The gel-dye mix (9 µL) was also transferred to two additional wells for quality control purposes. The Nano marker buffer* (5 µL) was added to each of the 12 sample wells and the well containing the RNA ladder. The RNA samples (400 ng/µL) and Ambion RNA 6000 ladder* were denatured at 70 °C for 2 min before pipetting 1 µL into the respective wells. The chip was vortexed for 1 min at 240 rpm followed by analyses on the Bioanalyser using an Agilent 2100 Expert software program. The small (18S) and large (28S)

ribosomal RNA (rRNA) subunits were recorded as bands and peaks on an electrogram and RNA integrity numbers (RINs), displayed as numbers from 1 to 10 were determined from the ratio between the size of the 28S band to the 18S band. Following the RNA quality assessment, samples (isolated RNA) were stored at −80 ºC prior to gene analyses. Of the five randomly selected samples of each treatment group (n=10), three RNA samples were selected for further evaluation with RT^2-Profiler PCR Array analyses based on RNA integrity parameters.

*Reagents were part of the Ambion® Turbo DNA-free™ kit, therefore no specific details are available.

cDNA synthesis

First strand complimentary deoxyribonucleic acid (cDNA) synthesis of the isolated RNA was carried out using the RT^2 First Strand Kit (SABiosciences, Whitehead Scientific, Cape Town, SA) following the manufacturer's protocol. Contamination of genomic DNA was eliminated from total RNA (1 μg) with 2 μL of a five times concentrated genomic DNA elimination buffer* and nuclease-free water to a final volume of 10 μL and incubated at 42 °C for 5 min. The sample was subsequently mixed with oligo-dT primers a five times concentrated reverse transcription buffer* and reverse transcriptase to a final volume of 20 μL and incubated at 42 °C for 15 min. The RT reaction was terminated by heating at 95 °C for 5 min and stored at -20 °C until use.

*Reagents were part of the RT^2 First Strand kit, therefore no specific details are available.

Quantitative RT-PCR array analysis

Samples were diluted in SABiosciences RT^2 qPCR master mix (Whitehead Scientific, Cape Town South Africa) according to the supplier's directions and pipetted into the wells of SABiosciences 96-well RT^2 Profiler rat oxidative stress and antioxidant defense PCR array plates (Whitehead Scientific, Cape Town South Africa) to evaluate the expression of 84 oxidative stress and antioxidant defense genes (See Addendum E, Table 1 for the array layout and Table 2 for a detailed list of all genes evaluated). The RT^2 Profiler Array (SABiosciences) is a commercially available set of optimized quantitative RT-PCR primer assays on a 96-well plate that are available for investigating different biological pathways (URL: http://www.sabiosciences.com /PCRArrayPlate.php). Analysis included a total of four plates, including liver samples from three rats for each treatment group (PER, PECsub and PECgen and Control). The array contains 84 primer pairs of oxidative stress and antioxidant defense pathway-focused genes and five primers of house keeping genes: ribosomal protein large P1

(*Rplp1*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), ribosomal protein L13A (*Rpl13a*), lactate dehydrogenase A (*Ldha*) and β-actin (*Actb*). Seven wells were used to test nontranscribed genomic DNA contamination and PCR performance.

Preparation involved dilution of 20 µL cDNA in nuclease-free water to a final volume of 111 µl. A sub-sample (102 µl) was mixed with a two times concentrated RT^2 qPCR SYBR green I master mix* (550 µl), containing HotStart DNA polymerase*, and nuclease-free water* (448 µl) to a final volume of 1100 µl. For analysis 10 µL of the cDNA mixture was transferred to a PCR profiler array containing 84 genes, coding oxidative stress and antioxidant defense genes, five reference genes and quality control parameters. A summary of the genes included is presented in Table 1. Data was generated in real time from the ABI 7900HT real time PCR machine (Applied Biosystems, Johannesburg, South Africa) with a two-step cycling program (1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and then at 60 °C for 1 min). Data were analyzed using the Excel-based PCR array data analysis template from the SABiosciences website: URL: http://www.superarray.com/pcrarraydataanalysis.php.

*Reagents were part of the RT^2 qPCR master mix, therefore no specific details are available.

Genomic DNA and reverse transcription controls

The cycle threshold (Ct) reflects the cycle number at which the fluorescence generated within a reaction exceeds the background noise and this inversely correlates to the initial amount of target template. The mean Ct for each sample's genomic DNA control (GDC) parameter was determined and was greater than 35 for all the test samples, indicating that the genomic DNA contamination did not affect the gene expression profiling results. The mean Ct value for the control sample was between 30 and 35.   If a sample has a mean Ct between 30 and 35, a gene-by-gene analysis for each gene of interest (GOI) is done using the equation ΔCt = Ct (GDC) – Ct (GOI). If a value of six or greater is obtained with this calculation it indicates that

Table 1 A summary of the genes evaluated by the RT[2] Profiler rat oxidative stress and antioxidant defense PCR array. Genes [a] are grouped according to their function based on the listing by SABiosciences (Frederick, MD, USA).

Grouping of genes	Gene symbol [a]
a) Antioxidant defense related genes	
(i) Glutathione peroxidases (*Gpx*)	*Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7, Gpx8, Gstk1*
(ii) Peroxiredoxins	*Ehd2, Prdx1, Prdx2, Prdx3, Prdx4, Prdx5, Prdx6*
(iii) Other peroxidases	*Aass, Apc, Cat, Ctsb, Duox1, Epx, Kif9, Lpo, Mpo, Ptgs1, Ptgs2, Rag2, Serpinb1b, Slc41a3, Tmod1*
(iv) Other antioxidants	*Gsr, Nxn, Sod1, Sod3, Srxn1, Txnrd1, Txnrd2, Zmynd17*
b) Genes involved in reactive oxygen species (ROS) metabolism	
(i) Superoxide dismutases (*Sod*):	*Sod1, Sod2, Sod3*
(ii) Other genes involved in superoxide metabolism:	*Ccs, Ncf1, Ncf2, Nos2, Nox4, Noxa1, Noxo1, Scd1*
(iii) Oxidative stress responsive genes	*Aass, Als2, Apoe, Cat, Ctsb, Dhcr24, Duox1, Duox2, Epx, Ercc2, Ercc6, Gab1, Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7, Idh1, Mpo, Mpp4, Nqo1, Nudt1, Nudt15, Park7, Ppp1r15b, Prdx1, Prdx2, Prdx6, Prnp, Psmb5, Sod1, Tpo, Txnip, Txnrd2, Ucp3, Xpa.*
c) Oxygen transporter related genes	
	Aqr, Cygb, Dnm2, Fancc, Hba-a2, Hbz (Hba-x), LOC367198 (Atr), Mb, Ngb, Ift172, Slc38a1, Slc38a4, Slc38a5, Vim, Xirp1

[a] A detailed list of gene names, arranged according to function (grouping by SABioSciences) of each enzyme is summarised in Table 2 Addendum E.

the results, fold changes calculated with the mean Ct value, may still be used without further validation. All values calculated for the control samples using this equation were six or greater. The reverse transcription control (RTC) indicates whether impurities in the RNA samples were significant enough to inhibit the reverse transcription reaction. RTC signifies the difference between the average Ct of the RTC and the positive PCR control (PPC) and a value below 5 is evidence of no RT inhibition. All test samples had a value below 5. The value of the control sample was slightly more than 5 at 5.13 indicating some impurities.

Data normalization, analysis and statistics

The mean Ct value was determined for each cDNA sample by calculating the difference in Ct between the target gene and reference gene. The comparative Ct ($\Delta\Delta$Ct) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): $\Delta\Delta$Ct = ΔCt (treated) – ΔCt (control). The fold change for each treated sample relative to the control sample (obtained from rats receiving no tea treatment) was calculated using the formula of $2^{-\Delta\Delta Ct}$. Statistical calculations were conducted based on the ΔCt values using a two-tailed t-test. A P-value equal to and/or less than 0.05 (P$\leq$0.05) was designated as significant.

Statistical analyses of the average feed and polyphenol intake

Normality among the groups for all parameters was investigated using the Komogorov-Smirnof Test. The homogeneity of group variances for all parametric parameters was tested using Levene's Test. Group differences for these parameters were tested using One-way ANOVA's and post-hoc Tukey test, which is a post hoc comparison of all pairs after a significant F-test. Where samples sizes were unequal the Tukey-Cramer adjustments were made automatically. For comparisons where only two parametric groups were present, T-tests were used. The Pooled method was used where variances were equal, and the Satterthwaite method was used for unequal group variances. Statistical analyses were performed with SAS v9.2 and statistical significance was set at 5% (P<0.05).

RESULTS

Daily feed, PEE and total polyphenol intake

The average daily feed and total polyphenol (TP) intake parameters presented in Chapter 5 were recalculated to represent only the data for the three rats for RNA liver samples selected for quantitative RT-PCR array analysis. No significant differences were evident in the feed intake
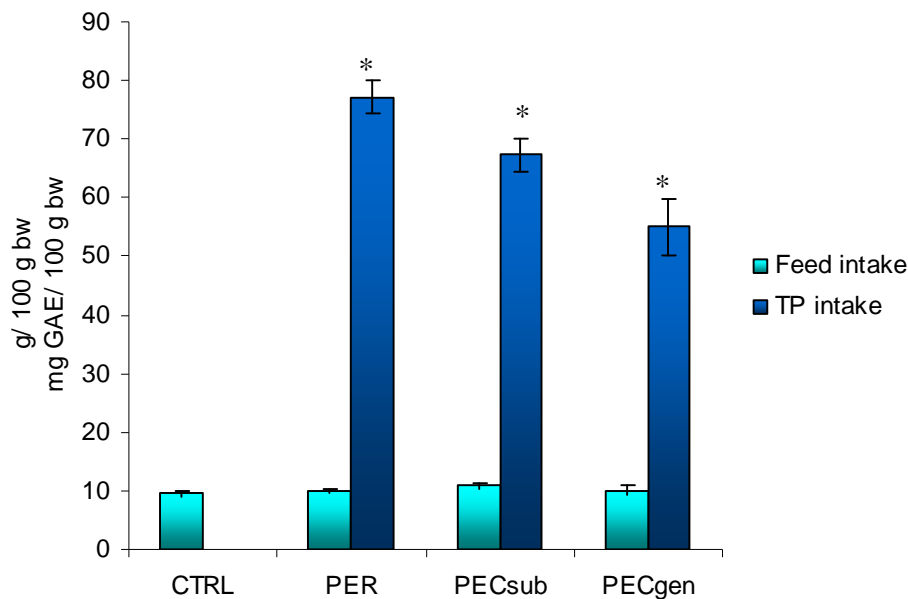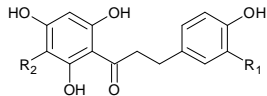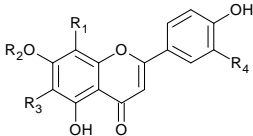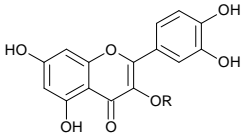
Figure 1 Feed and total polyphenol (TP) intake of male Fisher rats receiving PEEs (2.0 g PER extract/kg and 2.5 g PECsub and PECgen/kg diet). Feed intake was calculated as g feed consumed/100 g bw/day, TP intake was calculated as the mg gallic acid equivalents (GAE)/100 g bw/day. Values represent the mean ± SD of 3 rats per group (n = 3). Statistical significance (P<0.05) is indicated as "*" on the error bars.

of the four different treatment groups (n=3 rats/group), but the PECsub treated group consumed markedly more feed on average than the control and PER treated rats (Figure 1). The average daily TP intake was significantly (P<0.05) higher in the rats receiving PER extracts as compared to the honeybush extracts. The TP intake of the rats receiving PECsub was significantly (P<0.05) higher when compared to the PECgen treated rats.

Intake of polyphenol compounds
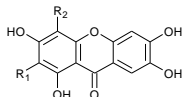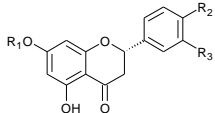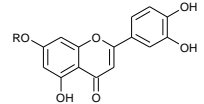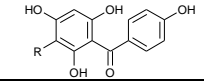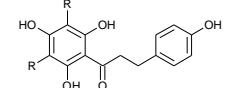
The average daily intake of the individual polyphenols for the rats receiving the PER, PECgen, PECsub is summarised in Tables 2 and 3. The rats receiving the PER extract consumed an average daily intake of aspalathin (36.85 mg/100 g bw) (Table 2). This far exceeded the average daily intake of any of the other phenolic compounds quantified in the extract with nothofagin (2.85 mg/100 g bw) and iso-orientin (2.85 mg/100 g bw), orientin (1.59 mg/100 g bw) and the combined flavonols obtained for the three quesceten glycosideds (4.13 mg/100 g bw)

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Table 2 HPLC quantification of phenolic compounds present in the polyphenol-enriched rooibos (PER) extract and the average daily polyphenol intake during the 28 study with male Fisher rats.

Polyphenol subgroup	Compound	Substitution	Polyphenol concentration [a] g/ 100 g extract	Daily Intake [b] 28 days mg/100 g bw
Dihydrochalcone				
	aspalathin	R_1=OH, R_2= C-β-D-glucopyranosyl	18.73	36.85 ± 1.35
	nothofagin	R_1=H, R_2=C-β-D- glucosylpyranosyl	1.45	2.85 ± 0.10
Flavone	isoorientin	R_1=H, R_2=R_4=OH, R_3= C-β-D-glucopyranosyl	1.45	2.85 ± 0.10
	orientin	R_1=C-β-D-glucopyranosyl, R_2=R_4=OH, R_3= H	0.81	1.59 ± 0.06
	vitexin	R_1=C-β-D-glucopyranosyl, R_2=OH, R_3=R_4=H	0.30	0.59 ± 0.02
	isovitexin	R_1=R_4=H, R_2=OH, R_3= C-β-D-glucopyranosyl	0.39	0.77 ± 0.03
	luteolin	R_1=R_3=H, R_2=R_4=OH	0.06	0.12 ± 0.005
	luteolin-7-gluc	R_1=R_3=H, R_2=O-β-D-glucopyranosyl, R_4=OH	0.04	0.08 ± 0.003
	chrysoeriol	R_1=R_3=H, R_2=OH, R_4=OCH$_3$	0.02	0.04 ± 0.001
Flavonol	quercetin-3-robionobioside	R=O-robinobioside		
	isoquercitrin	R=O-β-D-glucosyl	co-elute 2.10	4.13 ± 0.15
	rutin	R=O-rutinosyl		
	quercetin	R= OH	traces	traces
	hyperoside	R=O-galactopyranosyl	0.29	0.57 ± 0.02

[a] Values represent the percentage (g/100 g extract) and are the mean of duplicate analyses, [b] Values represent the average daily polyphenol intake per treatment group (3 rats/group), bw = body weight.

Table 3 HPLC quantification of phenolic compounds present in polyphenol-enriched *C. subternata* (PECsub) and *C. genistoides* (PECgen) and average daily intake during the 28 study in male Fisher rats.

Polyphenol Subgroup	Compound	Substitution	Polyphenol concentration g/100 g extract		Daily Intake [b] mg/100 g bw	
			PECsub	PECgen	PECsub 28 days	PECgen 28 days
Xanthone						
	mangiferin	$R_1 = C$-β-D-glucopyranosyl, $R_2 =$ H	3.79	9.29 [a,b]	10.28 ± 0.42	24.19 ± 0.95
	isomangiferin	$R_1 =$ H, $R_3 = C$-β-D-glucopyranosyl	1.16	2.94	3.15 ± 0.13	7.65 ± 0.30
Flavanone						
	eriocitrin	R_1=rutinosyl, R_2=R_3=OH	1.10	-	2.98 ± 0.12	-
	hesperidin	R_1= rutinosyl, R_2=OH, R_3=OCH$_3$	0.99	0.83	2.68 ± 0.11	2.16 ± 0.10
	naringenin	R_1=R_3=H, R_2=OH	0.01	0.01	0.03 ± 0.001	0.02 ± 0.001
	eriodictyol–glucoside	Unidentified (t_r = 10.72 min)	0.23	0.03	0.60 ± 0.03	0.07 ± 0.004
Flavone						
	luteolin	R=H	0.06	0.09	0.16 ± 0.01	0.23 ± 0.01
	scolymoside	R=O-rutinosyl	1.29	-	3.50 ± 0.14	
Bensophenone						
	iriflophenone 3-C-β-glucoside	R = glycosyl	1.66	2.53	4.30 ± 0.19	6.59 ± 0.20
Dihydrochalcone						
	phloretin-3′,5′-di-C-β-glucoside	R = glucosyl	2.35	0.11	6.35 ± 0.26	0.26 ± 0.01
Unknown	hydroxycinnamic acid derivative	Unidentified (t_r = 11.01 min)	0.72	-	1.86 ± 0.08	

The average daily intake of both xanthones was significantly (P<0.05) higher in the PECgen (24.19 mg/100 g bw mangiferin and 7.65 mg/100 g bw isomangiferin) when compared to PECsub (10.28 mg/100g bw mangiferin and 3.15 mg/100g bw isomangiferin) treated rats (Table 3).   Other phenolic compounds consumed at relatively high levels daily for honeybush treatments were the flavanone hesperidin (2.68 mg/100 g bw for PECsub and 2.16 mg/100g bw for PECgen) and the bensophenone, iriflophenone-3-*C*-glucoside (4.30 mg/100 g bw for PECsub and 6.59 mg/100g bw for PECgen).   PECsub treated rats consumed to relatively high daily levels of the dihydrochalcone phloretin-3′,5′-di-*C*-glucoside (6.35 mg/100 g bw).   The hydroxycinnamic acid derivative present in PECsub, and absent in PECgen was consumed at 1.86 mg/100 g bw/day.

RNA quality and integrity

The quality of RNA was satisfactory with a RNA integrity number (RIN) greater than 8 and the $A_{260/280}$ greater than 2.   A number of the $A_{230/260}$ values were less than 2, indicating possible salt contamination.   As the RIN number and $A_{260/280}$ was satisfactory and the $A_{230/260}$ values were close to 2, the RNA was deemed as fit for use.

Differential expression of oxidative stress and antioxidant defense related genes

Results obtained with quantitative RT-PCR array analysis of the relative expression of 84 oxidative stress and antioxidant defense genes in rat liver are summarised in Table 4 (Addendum E).   The complete spectrum of differential gene expression and quantitative changes for all the genes tested are shown as 3-D profiles in Figure 2a, b and c.   Treatment with PER for 28 days resulted in significant (P≤0.05) changes in the expression of seven genes, of which one gene was mutually affected by PECsub and one by PECgen.   The treatments with PECsub and PECgen altered the expression of 11 genes, each of which seven were mutual (Table 4).

a) Antioxidant defense related genes

PER treatment significantly affected the expression of two genes encoding for antioxidants, including the up-regulation of glutathione peroxidase 2 (*Gpx2*) with 1.8 fold and down-regulation of *Gpx3* with -1.2 fold change.   PECsub resulted in -1.32 fold change down-regulation of peroxidase EH-domain containing 2 (*Edh2*).   Expression of other peroxidases genes significantly affected by PECsub and PECgen included the down-regulation of kinesin family member 9 (*Kif9*) by -1.87 and -1.96 fold and serine (or cysteine) peptidase inhibitor clade B member 1b (*Serpinb1b*) by -2.00 and -2.29, respectively.   PECsub and PECgen both also

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Table 4 Fold change and P-values of the genes significantly affected by dietary consumption of polyphenol-enriched extracts (PEEs) of rooibos (PER) and honeybush (*C.subeternata* (PECsub) and *C. genistoides* (PECgen)) during the 28 study with male Fischer rats.

Function grouping and name of gene	Symbol	PER		PECsub		PECgen	
		FC [a]	P-value [b]	FC	P-value	FC	P-value
Antioxidant defense related genes							
(i) Glutathione Peroxidases							
Glutathione peroxidase 2	*Gpx2*	+1.80 [c]	0.04				
Glutathione peroxidase 3	*Gpx3*	-1.20 [d]	**0.04**				
(ii) Peroxiredoxins							
EH-domain containing 2	*Ehd2*			-1.38	0.042		
(iii) Other Peroxidases							
Kinesin family member 9	*Kif9*			-1.87	0.009	-1.96	0.005
Prostaglandin-endoperoxide synthase 1	*Ptgs1*			+2.41	0.001	+1.67	0.048
Serine (or cysteine) peptidase inhibitor, clade B, member 1b	*Serpinb1b*			-2.00	0.015	-2.29	0.001

[a] FC indicates the fold change, [b] P≤0.05 designated as significant, [c] values preceded by + indicates up-regulation, values preceded by - indicates down-regulation.

Table 4 (*Cont.*)

Function grouping and name of gene	Symbol	PER		PECsub		PECgen	
		FC	P-value	FC	P-value	FC	P-value
Genes involved in reactive oxygen species (ROS) metabolism							
(i) Superoxide dismutases (SOD)							
Superoxide dismutase 1	Sod1			+1.18	0.002		
Other genes involved in superoxide metabolism							
Neutrophil cytosolic factor 2	Ncf2	-4.78	0.03				
NADPH oxidase organizer 1	Noxo1			+19.97	0.019		
(ii) Oxidative Stress Responsive Genes							
Aminoadipate-semialdehyde synthase	Aass	+1.32	0.01	+1.25	0.005		
Apolipoprotein E	Apoe	-1.27	0.05				
Isocitrate dehydrogenase 1	Idh1	+1.30	0.04				
NAD(P)H dehydrogenase, quinone 1	Nqo1	+1.68	0.02			+1.73	0.013
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	Nudt1					+1.71	0.008
Proteasome (prosome, macropain) subunit, beta type 5	Psmb5					+3.94	0.039
Thyroid peroxidase	Tpo					+372.43	0.049
Thioredoxin interacting protein	Txnip			-1.61	0.045	-1.93	0.038
Xeroderma pigmentosum	Xpa			+1.97	0.005	+2.00	0.005
(iii) Oxygen Transporters							
Fanconi anemia, complementation group C	Fancc			+3.41	0.021	+2.72	0.005
Vimentin	Vim			+1.80	0.026	+1.67	0.031

[a] FC indicates the fold change, [b] P≤0.05 designated as significant, [c] values preceded by + indicates up-regulation, values preceded by - indicates down-regulation.

resulted in the up-regulation of prostaglandin-endoperoxide synthase 1 (*Ptgs 1*) with respective changes of 2.41 and 1.67 fold.

b) Genes involved in reactive oxygen species (ROS) metabolism

(i) SOD and other genes involved in superoxide metabolism

Significantly (P≤0.05) affected genes involved in superoxide metabolism were the down-regulation of neutrophil cytosolic factor 2 (*Ncf2*) with -4.78 fold change by PER, while an almost 20 (19.97) fold up-regulation of NADPH oxidase organizer 1 (*Noxo1*) was evident for PECsub. PECsub also affected up-regulation in the expression of superoxide dismutase 1 (*Sod1*) with a 1.18 fold change.

(ii) Oxidative stress responsive genes

All three tea treatments significantly (P≤0.05) affected oxidative stress responsive genes, although only one was mutual for PER and PECsub, one for PER and PECgen, but four for PECsub and PECgen. PER and PECsub resulted in mutual up-regulation of aminoadipate-semialdehyde synthase (*Aass*) with 1.32 and 1.25 fold changes respectively. PER consumption resulted in the up-regulation of isocitrate dehydrogenase 1 (*Idh1*) and NAD(P)H dehydrogenase, quinone 1 (*Nqo*1) with 1.30 and 1.68 fold, respectively, while apolipoprotein E (*Apoe*) expression was down-regulated, -1.27 fold.

PECgen affected most of these genes and resulted in the up-regulation of *Nqo1* by 1.73, nudix (nucleoside diphosphate linked moiety X)-type motif 15 *(Nudt1)* by 1.71 and proteasome (prosome, macropain) subunit beta type 5 (*Psmb5*) by 3.94. The largest fold increase recorded was the up-regulation of *Tpo* by 372.43 by PECsub. PECsub and PECgen both up-regulated *Xpa* with fold changes of 1.97 and 2.00, respectively and both down-regulated *Txnip* with -1.61 and -1.93, respectively.

(iii) Oxygen transporter related genes

Treatment with PER did not significantly (P>0.05) affect any of the genes for oxygen transporters. The genes fanconi anemia complementation group C (*Fancc*) and vimentin (*Vim*) were up-regulated by both PECsub (3.41 and 1.8 fold, respectively) and PECgen (2.72 and 1.67 fold, respectively).
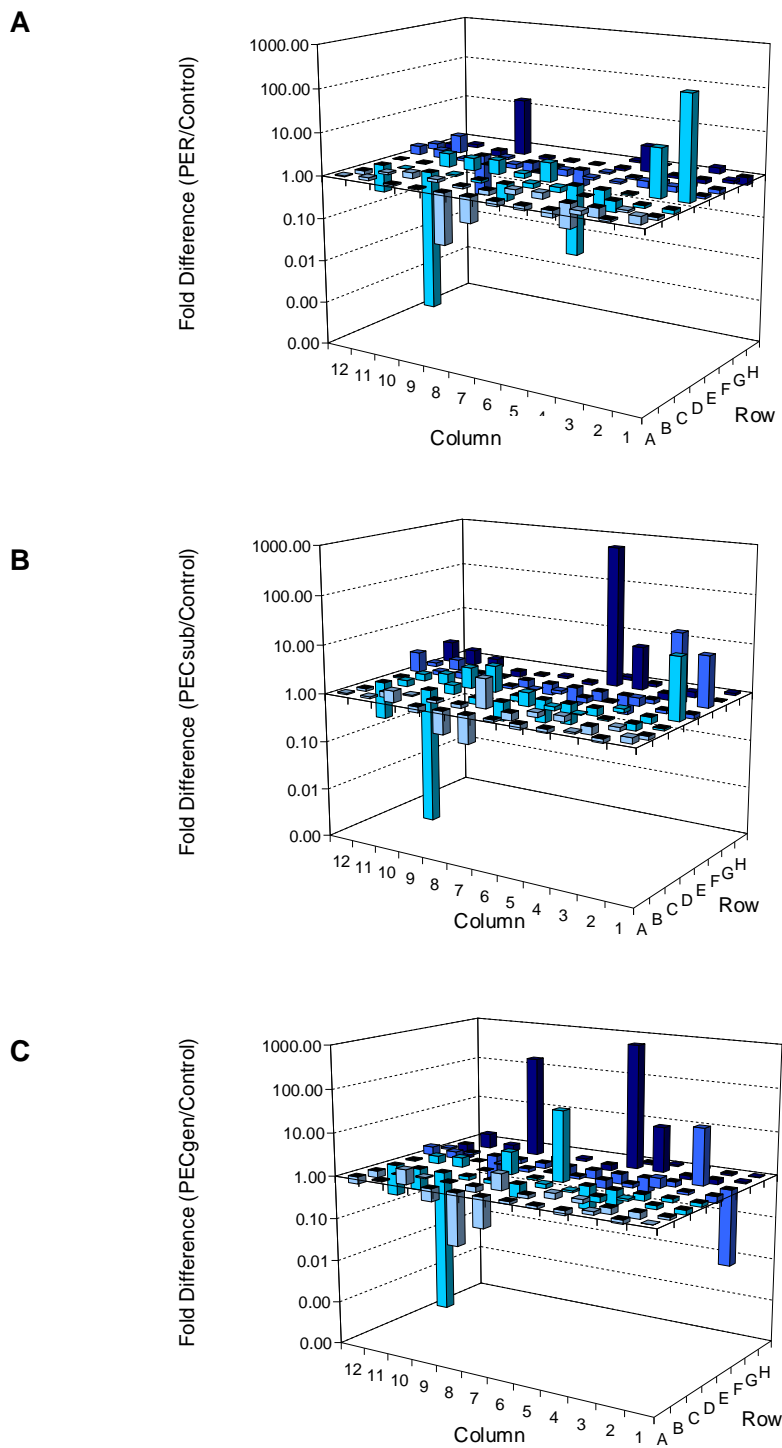
Figure 2 3-D profile graph of the differential expression of 84 oxidative stress and antioxidant defense related genes indicating the fold difference of each gene between the PEE treated and the control rats for the three different dietary treatments with (A) PER, (B) PCsub and (C) PECgen.

DISCUSSION

Quantitative gene expression is regarded as a valuable tool in identifying the differential expression of genes related to signal transduction pathways, several biological processes and disease related pathological states (Buluchamy *et al.*, 2010). A quantitative RT-PCR array approach is recommended instead of whole genome microarrays, since it is regarded as a more reliable and sensitive gene expression profiling technology for analysis of panels of genes. In the current study detection of differential expression of oxidative stress and antioxidant defense related genes in rat liver following exposure to PEEs from rooibos (PER) and honeybush (PECsub and PECgen) was conducted with this approach. The 84 gene transcripts evaluated included the glutathione peroxidases (*Gpx*) and peroxiredoxins (*Tpx*), genes involved in reactive oxygen species (ROS) metabolism and oxygen transporter related genes. It provided a logical starting point in investigating the effect of PEEs from rooibos and honeybush on gene expression in order to assess any possible adverse effects related to oxidative stress in rat liver. The PEEs significantly ($P \leq 0.05$) differentially altered the gene expression of a number of genes indicating the potential of the herbal tea extracts in altering the redox status in the liver.

Comparative TP and polyphenolic and intakes

The TP intake, polyphenol composition and intake of PER, PECsub and PECgen were discussed in detail in Chapter 5, but is shortly summarised again since it as was recalculated to include the data of the 3 rats relevant to the gene analysis. The TP intake for PER treated rats was much higher than for honeybush due to a higher concentration in the enriched extract. The average daily aspalathin intake for the treatment group in this study (n=3 rats per group) for quantitative RT-PCR analysis was 36.85 mg/100 g bw, which is more than 10 fold higher than the daily intake of the other phenolic compounds present in PER. Other compounds consumed at a level of more than 1 mg/100 g bw/day were the dihydrochalcones nothofagin and the flavones, isoorientin and orientin.

The major phenolic compounds present in honeybush (PECsub and PECgen) were the xanthones, mangiferin and isomangiferin, which were consumed at an approximately 2.5 fold lower daily level in the rats receiving PECsub compared to PECgen. Other major differences between these two extracts with regards to their phenolic composition included consumption of high levels of scolymoside and phloretin-3′,5′-di-*C*-glucoside for PECsub treated rats, while not detected and very low levels were present for PECgen. Both PECsub and PECgen treated rats consumed relatively high levels of iriflophenone-3-*C*-glucoside and hesperidin. Differences in phenolic composition between rooibos (PER) and interspecies differences for honeybush

(PECsub and PECgen) are implicated in differential gene expression observed. The role of phenolic composition is deducted from PER only affecting one mutual gene with each honeybush species, i.e. *Nqo1* with PECgen and *Aass* for PECsub. On the contrary, similarities in composition of PECsub and PECgen is likely responsible for mutual modulation of the expression of seven genes. However, the role of other herbal tea constituents, specifically the polymeric anthocyanidins and/or different combinations of the known polyhenolic constituent also needs to be considered.

Modulation of gene expression by PER

Antioxidant defense related genes

Antioxidant defense related genes significant ($P \leq 0.05$) affected by PER included the two glutathione peroxidases, *Gpx2* and *Gpx3*, of which the expression was up- and down-regulated, respectively. The Gpx family is multiple isozymes regarded as a major component in the antioxidant defense of the cell and is responsible for the detoxification of hydrogen peroxide and organic peroxides that use glutathione as an electron donor (Gornicka *et al.*, 2011). Glutathione peroxidase 2 (*Gpx2*) is mainly expressed in the gastrointestinal tract, including the esophagus epithelium, but it is also detectable in human liver (Brigelius-Flohé & Kipp, 2009). The role of *Gpx2* in cell proliferating processes has been implicated and this gene is up-regulated in most preneoplastic cells, depending on the developmental stage of malignant transformation (Brigelius-Flohé & Kipp, 2009). It is also a target for NF-E2-related factor 2 (*Nrf2*), a transcriptor factor that regulates important antioxidant and phase II detoxifying genes (Banning *et al.*, 2005). Implications of effects of PER on *Nrf2* is discussed together with the up-regulation of *Nqo1*. *Gpx2* suppresses cyclooxygenase activity by removal of hydroperoxides required for enzyme activation thereby facilitating an anti-inflammatory function (Brigelius-Flohé & Kipp, 2009). In this regard the up-regulation by PER may indicate an anti-inflammatory response as a result of underlying oxidative stress due to the high levels of polyphenols consumed.

Glutathione peroxidase 3 (*Gpx3*) is expressed in heart, lung, breast and kidney and implicated in providing antioxidant activity extra cellular and a potential oxidant scavenger role in the plasma (Chu *et al.*, 1992). Down-regulation of *Gpx3* by PER could facilitate oxidative stress due to reduced ROS quenching. This gene is down-regulated during neoplastic transformation as compared to healthy tissue where it presumably plays a role as a tumor suppressor (Brigelius-Flohé & Kipp, 2009) and therefore further down-regulation by PER may be seen as unfavorable during various stages of cancer. Increased expression of *Gpx3* was evident in the hearts of diabetic mice, indicating a protective role from hyperglycemia induced oxidative stress

(Iwata *et al.*, 2006), attributed to transcriptional up-regulation as a result of oxidative stress (Brigelius-Flohé & Kipp, 2009). With regards to vascular disease, reduced *Gpx3* expression under disease conditions is presumed to be a protective effect (Westpal *et al.*, 2009). When considering the *Gpx* family and effects of PEE consumption in the current study, no effects were evident for the expression of any of the other glutathione peroxidases, i.e. *Gpx1, 4, 5, 6, 7 or* glutathione reductase (*Gsr*) *and* glutathione S-transferase (*Gstk 1*).

Genes Involved in reactive oxygen species (ROS) metabolism

The expression of genes involved in ROS metabolism affected by PER included *Ncf2*, *Apoe*, *Idh1* and *Nqo1*. The neutrophil cytoxolic factor 2 (*Ncf2/p67phox*) gene was down-regulated (-4.78 fold) by PER. This gene encodes for the cytosolic factor 2, the cytoxolic subunit of the multi-protein complex known as NADPH oxidase in neutrophils (Selemidis *et al.*, 2008; Drummond *et al.*, 2012). The role of this gene in the liver is currently not clear, but if the down-regulation of *Ncf2* does affect NADPH activity in the liver the following applies. The NADPH oxidase enzyme complex produces superoxide anion and other reactive oxygen species (ROS) from molecular oxygen, using NADPH as electron donor and influences a multitude of biological functions including host defense and redox signaling (Chan *et al.*, 2009; Selemidis *et al.*, 2008). Considering risk states, such as hypertention and hypercholesterolemia, for CVD, NADPH oxidase expression and activity are markedly up-regulated in the blood vessel wall and therefore implicated to contribute to artery disease (Selemidis *et al.*, 2008). Therefore, these enzymes are proposed as important therapeutic targets in CVD. The down-regulation of *Ncf2* could therefore possibly be associated with a recent clinical study, demonstrating that rooibos decreased plasma markers of lipid peroxidation in adults at risk for CVD (Marnewick *et al.*, 2011). A study with green tea (unfermented *C. sinensis*) and black tea (fermented *C. sinensis*) in bovine carotid artery endothelial cells implicates a role in protection against CVD and specifically hypertention by down-regulating of the NADPH oxidase subunits p22phox and p67phox (Ying *et al.*, 2003). Of importance is that down-regulation of *Ncf2* expression by PER in rat liver occurred under normal conditions and verification in disease states will be required as part of further investigation on the potential use of rooibos as therapeutic agents in restricting oxidative damage in this regard. None of the other genes encoding for NADPH oxidases proteins, NADPH oxidase 4 (*Nox4*), NADPH oxidase activator 1 (*Noxa1*) or NADPH oxidase organizer 1 (*Noxo*1) were affected PER, and therefore the final effect on NADPH oxidase activity is not clear. However, modulation of oxidative stress related genes, including genes encoding for NADPH oxidase are considered as important in the development of many diseases such as cancer due to ROS levels effecting the growth indices and survival of different cancer

cells (Chan *et al.*, 2009). Therefore the inhibition of development of skin, liver and oesophageal preneoplastic lesions by rooibos in rats is applicable and has been related to the disruption of the growth regulatory mechanisms related to apoptosis (Marnewick *et al.*, 2003, Marnewick *et al.*, 2009; Sissing et al., 2011).

Up-regulation of the expression of the peroxidase, *Aass*, encodes an enzyme that plays an antioxidant protective role in cells whilst also involved in lysine degradation. A defective *Aass* gene is implicated in familial hyperlysinemia and accumulation of lysine, which may cause of neurological symptoms (Seminotti *et al.,* 2008). The implications of PER up-regulating this gene is currently unclear.

The down-regulation of the expression of apolipoprotein E (*Apoe*) gene by PER may impact on cholesterol homeostasis as apolipoprotein E plays a key role in metabolism of cholesterol and triglycerides by binding to receptors in liver contributing to the clearance of chylomicrons, very low density lipoprotein (VLDL) and high density lipoprotein (HDL) from plasma (Issa *et al.*, 2012). This may be related to findings in a recent study with rooibos in humans indicating modulation of the serum lipid profile by significantly ($P<0.05$) decreased triacylglycerol and LDL-cholesterol levels and increased HDL-cholesterol (Marnewick *et al.*, 2011).

These finding are also applicable to the up-regulation of the expression of *Idh1* by PER, which could indicate increased expression for the protein isocitrate dehydrogenase 1 (Idh1), an $NADP^+$-dependent isocitrate dehydrogenase (Reitman *et al.*, 2010). In normal cellular metabolism Idh1 is involved in lipid metabolism and plays a crucial role in glucose sensing. Isocitrate dehydrogenase 1 activity is involved in maintaining cellular cholesterol and fatty acid homeostasis through synthesis and degradation and is therefore suggested as a target enzyme for lipid-lowering pharmacological strategies (Shechter *et al.*, 2003). In addition to the normal cellular role, the activity of Idh1 is required as a major source for cytosolic NADPH (Shechter *et al.*, 2003) and an association with cellular response to insults is evident from increased activity with a variety of oxidative insults (Reitman *et al.*, 2010). Increased activity of Idh1 and NADPH has been associated with less oxidative stress and kidney injury in ischemia/reperfusion conditions in mice presumably due to an increased GSH/total glutathione ratio (Kim et al., 2011). Mutations of *Idh1* in cancer supports the theory that altered metabolism contributes to tumorigenesis (Oermann *et al.*, 2012) and its up-regulation in breast, prostate, neuroglia and brain cancer tissue has been implicated in cancer promotion (Furuta *et al.*, 2010). Therefore up-regulation of *Idh1* expression by PER may have important implications for oxidative stress in the liver, and potentially be involved in some of the advancing effects of unfermented rooibos in fumonisin B_1-induced hepatocarcinogenesis in rats (Marnewick *et al.*, 2009). Dysregulation of *Idh1* is a common phenomen in cancer cells as it functions at a crossroad of cellular metabolism

in lipid synthesis and cellular defense against oxidative stress and cancer cells may gain from the glucose sensing role of Idh1 (Reitman *et al.,* 2010). When considering other disease states such as diabetes, expression and enzymatic activity of cytosolic *Idh1* is up-regulated in the renal cortex of diabetic rats and mice (Lee *et al.*, 2010). Decreased activity of Idh1 is an early marker for hypertrophy before the onset of ventricular dysfunction in transgenic hypertrophic cardiomyopathic mice (Lucas *et al.*, 2003) and spontaneously hypertensive rats (Benderdour *et al.*, 2004) and therefore could be applicable to artherosclerosis and CVD. The outcome of the up-regulation of *Idh1* expression by PER will therefore depend on the specific conditions in terms of oxidative stress and disease.

The *Nqo1* gene encoding for a flavin adenine dinucleotide (FAD)-binding the NAD(P)H: quinone oxidoreductase was up-regulated by PER. It is a cytoplasmic 2-electron reductase known to catalyse the metabolic detoxification of quinones protecting cells from redox cycling, oxidative stress and neoplasia (Long & Jaiswal, 2000).   This FAD-binding protein forms homodimers and reduces quinones to hydroquinones, thereby preventing the unwanted one electron reduction of quinones which results in the production of reactive oxygen species via the redox cycling of semiquinones (Vasilliou *et al.*, 2006). The expression is up-regulated as part of an oxidative stress response and found to be overexpressed in certain types of malignant tissues in the colon, breast, lung and liver (Strassburg *et al.*, 2002). Therefore the increased expression in the liver following PER consumption is of concern and warrants further investigation. NAD(P)H:quinone oxidoreductase 1 is highly regulated by *Nrf2* (Lin *et al.*, 2007) and plays an integral role in cellular responses to oxidative stress and its expression is up-regulated in an Nrf2-dependent fashion in mice following challenges with electrophilic chemicals (Nioi *et al.*, 2003). The expression of *Nrf2* was up-regulated and activated by isoorientin in HepG2 cells leading to increased levels of antioxidant enzyme proteins, particularly Nqo1 (Lim *et al.*, 2007). The modulating effect of the green tea (unfermented *C. sinensis*) polyphenol (-)-epigallocatechin-3-gallate (EGCG) on *Nrf2* has been implicated in its chemoprevention and cytoprotective activities (Na & Surh, 2008). The reversal (increase) of hyperhomocysteinemia associated decrease of Nqo1 activity and expression by red wine polyphenol supplementation is regarded as a potential protective mechanism in CVD (Noll *et al.*, 2011). The so-called "protective" effect resulting from up-regulated expression of *Nqo1* by PER under the current study conditions is, however, questionable. It may rather be an indication of oxidative stress in the liver.

Modulation of gene expression by PEEs from honeybush

Exposure of rats to PEEs from honeybush (PECsub and PECgen) resulted in significant (P≤0.05) changes in the expression of eleven genes respectively of which seven were mutually affected.

Antioxidant defense related genes

PECsub selectively affected the expression of the gene EH domain containing 2 (*Edh2*) by down-regulating the expression with -1.38 fold. This gene encodes for a protein (Edh2), a member of four highly homologous proteins (EHD1-4) that form part of a distinct subfamily of the Eps15 homology (EH) domain-containing protein family (Confalonieri & Di Fiore, 2002). These EH containing and binding proteins form a network of protein:protein interactions that coordinates cellular functions connected to endocytosis, actin remodeling and intracellular signal transduction. *Edh2* is expressed predominantly in thymus, heart, muscle and lung and weakly in the liver (Blume *et al.*, 2007). The enzyme (Edh2) interacts with the insulin-responsive glucose transporter (GLUT4) in rat adipocytes, which is suggested to play a key role in GLUT4 recruitment to the plasma membrane (Park *et al.*, 2004). The up-regulation of *Ehd2* expression is therefore of interest in the anti-diabetic effects of honeybush, recently demonstrated for *C. intermedia* (Muller *et al.*, 2011). PECsub resulted in the up-regulation of *Aass* expression and as for PER the implications is currently unclear. Of the antioxidant defense genes, expression of *Ptgs1* was up-regulated, while that of *RGb156* encoding for *serpinb1b* was down-regulated by both PEEs prepared from honeybush. The up-regulation of *Ptgs1*, or cycloogyenase 1 (*Cox-1*), is applicable to an inflammatory process via the immediate prostanoid response. Up-regulation by PECsub and PECgen, therefore, may indicate an inflammatory response, although excessive or inadequate activation may both have deleterious effects. Conversely, polyphenols are known to exhibit anti-inflammatory properties through the inhibition of *Cox-1* and it has been considered as a molecular target that directly affects inflammation (Yoon & Back, 2005).

The expression of *Serpinb1b* was down-regulated more than 2 fold by both the honeybush PEEs and may result in reduced serpinb1b activity which, together with *Aass* and *Nqo1*, are antioxidant enzymes that protects cells from oxidative stress. *Serpinb1b* inhibits the accumulation of ROS, possibly indirectly by inhibiting inflammation (Polytarchou *et al.*, 2008) hence the down-regulation may result in an increase in ROS production and subsequent oxidative stress.

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Kif 9 is part of the kinesin family member 9 regulating podosomes and of podosomal matrix degradation. Both honeybush PEEs down-regulated *Kif 9*, which as a regulators of podosomes, are actin based matrix contacts in a variety of cell types such as monocytic cells, and apart from matrix degradation, are involved in cell migration and invasion. Impairment of the function of this protein significantly impairs matrix degradation (Corfine *et al.*, 2011). The implications of down-regulation of *Kif9* expression by PECsub and PECgen in the liver is currently not clear.

Genes involved in reactive oxygen species (ROS) metabolism

The oxidative stress responsive genes affected by both PEEs from honeybush included the expression of *Txnip* and *Xpa*, which were down- and up-regulated, respectively. *Txnip* encodes for thioredoxin interacting protein, a glucose-regulated pro-apoptotic factor in beta-cells, which binds and inhibits thioredoxin thereby modulating cellular redox status by promoting oxidative stress (Chen *et al.*, 2008). Increased oxidative stress is known to play a role in vascular disease and diabetes mellitus due to reduced thioredoxin activity as a result of interaction with *Txnip* during hyperglycemia (Schulze *et al.*, 2004). Therefore, the further down-regulation of *Txnip* expression by PECsub and PECgen is likely to play a key role in the antidiabetic effects of honeybush when considering the affects on oxidative stress. Mangiferin, which is the major phenolic compound in mangiferin, which is the major phenolic compound in *Cyclopia* spp. and were consumed at high levels in the current study decreases insulin resistance in mice (Ichiki *et al.*, 1998; Miura *et al.*, 2001) and is implicated in the antidiabetic properties of unfermented *C. intermedia* (Muller *et al.*, 2011). It should, however, be noted that modulation of the expression of the protein is affected by diverse nutrient factors such as glucose, glutamine and fatty acids (Schulze *et al.*, 2004). *Txnip* also exhibits anti-proliferative effects, inhibits tumor cell growth and is down-regulated in many cancer cell lines and tissues (Han *et al.*, 2003; Pang *et al.*, 2009). *Txnip* deficient mice have been shown to be predisposed in developing liver cancer (Sheth et al., 2006).

Further to the anti-tumor properties of honeybush is the up-regulation of *Xpa* expression, encoded for xeroderma pigmentosum complementation group A protein that facilitates the assembly of a pre-incision complex during the processing of DNA damage by the nucleotide excision repair pathway via the interaction with replication protein A (Patrick & Turchi, 2002). The role of this mechanism in the chemopreventive properties of honeybush extracts in liver, skin and oesophagus carcinoma in rats (Marnewick *et al.*, 2003, Marnewick *et al.*, 2009; Sissing *et al.*, 2011) would therefore be of interest.

Modulation of the oxygen transporter related genes is of interest with respect to cacinogenesis. The *Fancc* gene encodes the protein for fanconi anemia group C which delays

the onset of apoptosis and promotes homologous recombination repair of damaged DNA (Niedzwiedz *et al.*, 2004). This protein is frequently up-regulated in cancer cells and functions by preventing protein oxidation and apoptosis by inhibiting the formation of inactivating disulfide bonds within GSTP1 during apoptosis (Cumming *et al.*, 2001). Further up-regulation of the expression by PECsub and PECgen may therefore be of concern.

The *Vim* gene encodes vimentin, a member of the intermediate filament family and is responsible for maintaining cell shape, integrity of the cytoplasm and stabilizing cytosdeletal interactions. It also functions as an organizer of a number of critical proteins involved in attachment, migration and cell signaling. Degradation of vimentin resulted in the morphological manifestation of apoptosis suggesting pro-apoptotic effects induced upon cleavage which could serve as a novel anti-cancer therapeutic target (Lahat *et al.*, 2010). Up-regulation of expression of these enzymes by honeybush could be in response to an underlying oxidative response in the liver due to high levels of polyphenols consumed and as postulated for *Fancc* could selectively promote the growth of cancer cells.

Genes selectively affected by PECsub included *Sod1, Noxo 1* and *Edh2.* The protein encoded by *Sod1* gene binds copper and zinc ions and is one of two isozymes responsible for destroying free superoxide radicals in the body (Zelko *et al.*, 2002; Milani *et al.*, 2011). The encoded isozyme is a soluble cytoplasmic protein, acting as a homodimer to convert naturally-occuring but harmful superoxide radicals to molecular oxygen and hydrogen peroxide. The other two genes encoding for the superoxide dismutase proteins, mitochondrial superoxide dismutase 2 (*Sod2*) and extracellular superoxide dismutase 3 (*Sod3*) were, however, not affected. The increased expression of *Sod1* may be postulated to be an early indication of oxidative stress resulting from the high levels of polyphenols consumed leading to the up-regulation of this antioxidant enzyme.

A large fold change following PEE treatment was evident for PECsub resulting in up-regulation of *Noxo1* (19.97) expression, which encodes for the protein NADPH oxidase organizer (*Noxo1*) (Bedard & Krause, 2007). The function of *Noxo1* is important in the organization of active *Nox1*, *Nox2* and *Nox3* complexes. The Nox family NADPH oxidases are proteins that transfer electrons across biological membranes resulting in the generation of reactive oxygen species. In general, the electron acceptor is oxygen resulting in the formation of superoxide. The other two genes related to NADPH oxidases, *Nox4* and *Noxa1* were, however, not affected by PECsub and the implication of the large fold up-regulation of *Noxo1* in the current study is therefore not clear.

When compared to PECsub, treatment with PECgen selective up-regulated the expression of *Nqo1*, *Tpo, Nudt1* and *Psmb5 genes*. Both PER and PECgen up-regulates *Nqo1* expression,

the outcome of which was discussed above. Specifically applicable to honeybush, mangiferin has been implicated in the hepatoprotective activity due to induction of antioxidant defense via the Nrf2 pathway (Das *et al.*, 2012), which as discussed would involve *Nqo1*. Up-regulation of *Nqo1* in the current study may thus result from the high levels of mangiferin consumed. This also further indicates the potential affects of PER and PECgen on the *Nrf2* pathway although possibly triggered by different compounds, due to the significant differences in phenolic composition.

The largest change following the treatment with PEEs was evident for PECgen resulting in up-regulation of the expression of thyroid peroxidase (*Tpo*) of 372 fold. The *Tpo* gene encodes for the thyroperoxidase (Tpo) enzyme which is mainly expressed in the thyroid and liberates iodine for addition onto tyrosine residues on thyroglobulin for the production of thyroxine (T_4) or triiodothyronine (T_3), thyroid hormones (Ruf & Carayon, 2006). The ability of Tpo to generate reactive oxygen species via Fenton type reactions is one of the mechanisms implicated in thyroid pathogenesis. Alterations in the thyroid, such as hyperthyroidism is known to effect lipid peroxidation in the liver and heart (Venditti *et al.*, 1997), however the reason for the up-regulation in the liver by PECgen under the present experimental conditions is not clear and warrants further investigation. Several flavonoids have been reported to inhibit thyroid peroxidase and interfere with thyroid hormone biosynthesis and ultimately thyroid function (Martin & Appel, 2010).

The protein encoded by *Nudt1* gene, nucleoside diphosphate linked moiety X-type motif 1 (Nudt1), is an enzyme that hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP, 8-oxo-dATP, 2-hydroxy-dATP and 2-hydroxy-rATP resulting from oxidative stress, to monophosphates. Increased expression of *Nudt1* and 8-oxodGTPase activity have been reported under conditions of oxidative stress in cell cultures (Meyer *et al.*, 2000; Haghdoost *et al.*, 2006). Therefore up-regulation of the expression of this gene by PECgen may indicate oxidative stress in the liver. PECgen treatment also resulted in up-regulation of *Psmb5* expression, encoding the protein proteasome (prosome, macropain) subunit, beta type 5. This proteasome form part of the proteolytic machinery for the degradation of normal and damaged proteins (Chondrogianni *et al.*, 2005) and plays a particular role during oxidative stress and aging as it is responsible for the degradation and removal of oxidative-induced damaged proteins, specifically in the nucleus (Breusing & Grune, 2008). The latter could provide additional protection against the accumulation of these proteins during situations of oxidative stress and may be postulated to protect against aging. However, up-regulation of this gene by honeybush could in the present study is indicative of an increased oxidative stress in the liver due to exposure to PECgen.

Conclusions

The PCR array used in the current study indicated that expression of several genes related to the oxidative stress and antioxidant defense are significantly (P≤0.05) affected in rat liver by PEEs prepared from rooibos and honeybush consumed over a period of 28 days. Investigating the effect of rooibos and honeybush on *Nrf2* is an important consideration for future studies, since genes of antioxidant enzymes regulated by *Nrf2* that were affected by rooibos and honeybush, included *Gpx2, Gpx3, Sod1* and *Nqo1*. Approaches focusing on *Nrf2* have been suggested to provide protection against diabetes (Negi *et al.*, 2011), which in the light of the anti-diabetic properties of rooibos and honeybush and their phenolic compounds (Mose Larsen *et al.*, 2008a; Mose Larsen *et al.*, 2008b; Muller *et al.*, 2008; Kawano *et al.*, 2009; Muller *et al.*, 2011; Singh *et al.*, 2011) together with the findings in the current study, may be highly applicable.

Investigations on the translational and post translational modification of the relevant proteins may assist in developing screening tools to assess the impact of the PEE extracts of rooibos and honeybush and their polyphenols in health and disease. Further to conventional biomarkers for oxidative stress, the application of quantitative PCR-arrays may provide additional and more specific information and assist in elucidation of beneficial and adverse effects of consuming high concentrations of rooibos and honeybush polyphenols. The differential modulation of expression of the oxidative stress and antioxidant defense related genes in rat liver by the PEEs of rooibos and honeybush in the current study indicate an underlying oxidative stress. This could have very important implications for the development of certain diseases.

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CHAPTER 7

GENERAL DISCUSSION & CONCLUSIONS

"should we consume supplements of the flavonoid enriched foods now coming onto the market…?" Halliwell (2007)

GENERAL DISCUSSION & CONCLUSIONS

Polyphenol-enriched nutritional supplements are available without prescription for self-medication and mostly lack any scientific substantiation and clear clinical evidence to support their beneficial health effects (Liu & Hu, 2007).   Due to their widely advertised anecdotal "superpowers", polyphenols are very popular in scientific research and in the public media. Key questions regarding complementary and alternative medicine (CAM) therapies concern their safety, effective dose and efficacy in disease prevention (Rapaka & Coates, 2006).

Mechanisms of rooibos and honeybush polyphenols in disease prevention has not been clarified, due to uncertainties and limited investigations on their bioavailability, metabolism and alterations in activity of different enzyme systems and understanding their role in maintaining the cellular redox status. Furthermore, potential herb-drug interactions and altering effects of rooibos and honeybush on signal transduction pathways with respect to different cell growth regulatory pathways are required. This poses many challenges in extrapolating the effects of the polyphenolic constituents, their conjugated metabolites and the sensitivity and specificity of the measured biological endpoints used to evaluate possible beneficial and/or adverse biological effects *in vivo*. Some of these aspects were addressed and critically evaluated in the current study.

Rooibos (*Aspalathus linearis* family; Fabaceae, tribe Podalyrieae) and honeybush (*Cyclopia* spp.; family Fabaceae; tribe Podalyrieae) are indigenous herbal teas unique to South Africa (Joubert *et al*., 2008a). Apart from their caffeine free market leverage, increased research on their health properties have led to international recognition in the herbal tea market (Joubert & De Beer, 2011; Joubert *et al*., 2011a). It has been predicted that rooibos will become the second most consumed beverage tea in the world after green and black teas produced from *Camellia sinensis* and it is already consumed in more than 37 countries. Honeybush is less well-known, but is also sold in 25 countries globally with main markets in the Netherland and Germany. Both herbal teas have been implicated for use as value-added products and use as nutraceuticals (Joubert *et al*., 2008a). Clinical studies with the South African herbal teas are limited to that by Sauter (2004) and Marnewick *et al*. (2011) for rooibos, while no studies for honeybush have been conducted. *In vivo* studies for honeybush are few and recent investigations include effects of *C. intermedia* in a diabetic rat model (Muller *et al*., 2011), chemopreventive carcinogenesis models utilising rats (Marnewick *et al*., 2003; Marnewick *et al*., 2009; Sissing *et al*., 2011) and bioavailability studies with *C. genistoides* in pigs (Bock *et al*., 2008; Bock *et al*., 2010).

Effects of metabolism on antioxidant activity of major rooibos and honeybush polyphenols and implications for cellular oxidative status in the liver

The effect of liver metabolism, specifically glucuronidation, on the antioxidant activity of the major rooibos polyphenols, aspalathin and nothofagin and honeybush polyphenol, mangiferin, was conducted utilising an on-line high performance liquid chromatography (HPLC) testing system. The use of on-line biochemical detection assays coupled to HPLC has been applied to whole plant extracts and is regarded as an effective screening technique that overcomes challenges associated with pre-isolation (Malherbe *et al.*, 2012). This is achieved by measuring the effects of compounds post-column directly after separation. In the current study, this system was applied to overcome losses of aspalathin and mangiferin glucuronides that would have been associated with pre-isolation due to instability of metabolites. The online methodology provides a very effective and realistic model for future *in vitro* evaluation of polyphenol metabolites regarding antioxidant activity. The loss in antioxidant activity reported following glucuronidation of both aspalathin and mangiferin was expected when considering structure related antioxidant activity of aspalathin (Snijman *et al.*, 2009) and a loss of the catechol arrangement shown to eliminate the antioxidant activity of xanthones (Lee *et al.*, 2005). Apart from the loss in antioxidant activity, the modulating effect of conjugates on other cellular processes such as cell signaling and modulation of metabolic and antioxidant enzyme systems should be considered. Phase II conjugates of certain flavonoids display higher activity than the parent compound and therefore it cannot be generally assumed that conjugation results in decreased biological activity (Beekman *et al.*, 2012).

Furthermore deconjugation by β-glucuronidase may assist in the parent molecule becoming available when increased antioxidant activity is required under conditions such as inflammation (Wong *et al.*, 2009). These conjugation-deconjugation reactions enables delivery of the parent compound as effector.   This is evident from the absence of quercetin in plasma, but demonstrable systemic effects in spite of weak *in vitro* bioactivity of plasma conjugates (Perez-Vizcaino *et al.*, 2012).

Chemical composition, dose levels, duration of exposure and the oxidative stress parameters and iron

For comparison of available data from *in vivo* studies with rooibos and honeybush, major shortcomings are the lack of compositional information and dose comparisons. Sufficient chemical characterization of plants is vital for the potential use as herbal remedies, traditional medicines or foods and nutritional supplements and in studies on the biological effects such as

the prevention of DNA damage, antioxidant, anti-inflammatory effects and improvement of immune function (Verhagen *et al.*, 2003; Knasmueller *et al.*, 2012).

Comparing polyphenol contents and antioxidant activities of the PEEs to previous reports for aqueous extracts of rooibos (Joubert *et al.*, 2008b) and honeybush (Joubert *et al.*, 2008b; De Beer & Joubert, 2010, De Beer *et al.*, 2011) indicated that the rooibos extract (PER) was efficiently enriched, whereas enrichment was less effective for the honeybush extracts (PECsub and PECgen). Therefore PER may be regarded as a potential "functional food", whereas the honeybush extracts (PECsub and PECgen) is more closely related to unfermented honeybush extracts, currently available on the market. Comparisons of the dose administrated and calculated human equivalent dose (HED) in terms of total polyphenol (TP), aspalathin (rooibos) and mangiferin (honeybush) intakes in the current study is presented together with estimates for *in vivo* studies conducted with rooibos (Marnewick *et al.*, 2003; Marnewick *et al.*, 2011) and honeybush (Marnewick *et al.*, 2003) in Table 1. The HED was calculated according to the body surface area (BSA) normalization method (Reagan-Shaw *et al.*, 2008).

A decrease in the TP content of rooibos and honeybush and specifically aspalathin and mangiferin content with the non-enzymatic processing step, fermentation, has been confirmed and reviewed (Joubert *et al.*, 2008a; Joubert *et al.*, 2009) and explains large differences in the TP, aspalathin and mangiferin contents between fermented and unfermented extracts (Table 1). The TP consumption resulting from an aqueous extract of unfermented rooibos for 10 weeks as sole source of drinking water in rats (Marnewick *et al.*, 2003), was much higher than the HED calculated for a recent six week clinical study on the protection of rooibos (fermented) against risk indicators of cardiovascular disease (CVD) (Marnewick *et al.*, 2011). Volunteers in the six week study received a total dose of TP equivalent to 382.2 mg GAE/day and based on an average bodyweight (bw) of 60 kg an exposure of 6.37 mg GAE/kg/day could be calculated. This apparent dose is more than eight times below the current administrated HED for PER.

The comparative TP exposure in rats when considering PECsub and PECgen were much lower than for PER for both the 28 and 90 day studies and are implicated in differences observed in biological effects between rooibos and honeybush. To date no clinical studies have been conducted with any of the *Cyclopia* spp. and animal studies reporting on clinical biochemical parameters are limited to that of Marnewick *et al.* (2003) with aqueous extracts of fermented and unfermented *C. intermedia* in rats with a HED dose for TP more than 2 fold below that used in the current study (Table 1). Interspecies differences for honeybush resulted in the HEDs of the major polyphenolic compound, mangiferin, being two fold higher for PECgen than for PECsub in the 28 day study. It was not possible to calculate an estimated intake of mangiferin for the study by Marnewick *et al.* (2003) in rats, since the mangiferin contents of

species vary significantly (Joubert *et* al., 2008b) and a large scale study defining the intakes from a "cup-of-tea" has not been conducted for the respective *Cyclopia* species. Doses of TP and the specific polyphenols in the current study were far higher than doses applicable and relevant for human consumption of the herbal teas from rooibos and honeybush.

The TP doses and that of the major phenolic compounds in rooibos and honeybush, aspalathin and mangiferin, respectively, are implicated in differences observed on the biological effects in the liver including the significant (P<0.05) alteration of activity of glutathione reductase (GR) and the levels of reduced glutathione (GSH), total serum bilirubin (Tbili), and serum iron levels. Considering the potential adverse effects observed for PECsub regarding serum Tbili and alkaline phosphatase (ALP) it was apparent that the age of rats, the polyphenol type and dose as well as the duration of exposure will determine how it is manifested. Irrespective of the specific effects, the current study indicated that PECsub affects the biliary system and therefore warrants further investigation. The marked increase in serum GGT following 28 and 90 days of PECsub consumption is a further indication of a disruptive effect on the biliary system. In this regard, hepatoxicity of green tea (unfermented *Camellia sinensis*) dietary supplements in humans for a dose range of 700–2100 mg/day included elevated transaminases and bilirubin levels amongst other (Mazzanti *et al.*, 2009). Bilirubin is regarded as a better marker than transaminases when evaluating toxicity in humans (Dufour *et al.*, 2001).

Considering the different doses, differential polyphenolic composition and consequent effects of rooibos and honeybush on serum iron, decreased levels has not been reported for rooibos and honeybush previously. The significantly (P<0.05) reduced serum iron levels after 90 days of PER and PECsub consumption is therefore of concern. No significant effects were evident after 28 days in the current study and previous reports with different time-frames on rooibos and honeybush in rats (Marnewick *et al.*, 2003) or rooibos in humans (Hesseling *et al.*, 1979; Breet *et al.*, 2005; Marnewick *et al.*, 2011). Regarding the effects of rooibos and honeybush on iron absorption, emphasis would thus be on the high levels of rooibos and honeybush polyphenols consumed for an extended period such as 90 days. The much higher xanthone (mangiferin and isomangiferin) content of *C. genistoides* compared to PECsub and other honeybush species (Joubert *et al.*, 2008b) and its effect on serum iron levels is an important consideration for future investigation.

Table 1 Summary of the average administrated dose, calculated human equivalent dose (*HED*), effects on serum clinical biochemical parameters and oxidative stress parameters in studies with rooibos and honeybush in rats and a human clinical study with rooibos (*Aspalathus linearis*).

Study Subject	Administered	Group/ Compound	Dose mg/kg bw/day (*HED*) [a]	Observed effects [b]	Days
Effects of fermented and unfermented rooibos and honeybush *C. intermedia* in rat liver (Marnewick *et al.* 2003)					
Rats	unf [c] rooibos	TP [d]	161.20 (*26.14*)	↑ GSH/GSSG; ↓ GSSG	70
	unf honeybush	TP	229.00 (*37.14*)	↑ GSH/GSSG; ↓ GSSG	
	ferm rooibos	TP	64.00 (*10.38*)	↑ GSH/GSSG; ↓ GSSG	
	ferm honeybush	TP	72.90 (*11.82*)	*↑ GSH/GSSG; ↓ GSSG*	
Effects of polyphenol-enriched extracts (PEEs) from unfermented rooibos (PER) and unfermented honeybush [*C. subternata* (PECsub) and *C. genistoides (PECgen)*] in rat liver (current study)					
Rats	PER	TP	756.60 (122.71)	↓ GR;	28
			627.00 (*101.68*)	↓Tbili; ↓ Iron, ↓GSH	90
	PECsub	TP	642.70 (*104.22*)	↑ Tbili, ↓ AST; ↓ GR	28
			480.50 (*77.92*)	↓ Tbili, ↑ ALP; ↓ Iron, ↓ GSH	90
	PECgen	TP	545.50 (*88.46*)	↓ GR	28
	PER	aspalathin	361.30 (*58.59*)		28
			299.40 (*48.55*)		90
	PECsub	mangiferin	98.10 (*15.91*)		28
			73.40 (*11.90*)		90
	PECgen	mangiferin	231.60 (*37.56*)		28
Effects of fermented rooibos cardiovascular disease (CVD) parameters in human plasma (Marnewick *et al.*, 2011)					
Human	ferm rooibos	TP	6.37	↑ TP in plasma ↓ CD ↓TBARS	42
		aspalathin [e]	± 7.0	↑ GSH/GSSG ;↑ GSH; ↓LDL-C; ↑HDL-C	

[a] Human equivalent dose (HED) refers to the human equivalent dose as calculated according to the body surface area (BSA) normalization method (Reagen-Shaw *et al.*, 2007) and is presented as mg/GAE/kg body weight (bw)/day in parenthesis in *italics*, [b] ↑ indicates increased; ↓ indicates decreased significantly (P<0.05); oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase (GR), total bilirubin (Tbili), aspartate transaminase (AST), alkaline phosphatase (ALP), total iron (Iron), low density lipoprotein cholesterol;(LDL-C), high density lipoprotein cholesterol (HDL) [c] fermented aqueous extract (ferm), unfermented aqueous extract (unferm) [d] The total polyphenol (TP) dose is given as the average galic acid equivalents (GAE) mg GAE/100 g bw/day, [e] The average aspalathin intake was calculated according to the estimation of Beelders (2011) analyzing a large number of rooibos infusions (n=114) showed that a "cup-of–rooibos tea" contains 5.84 mg aspalathin/L.

Differences in the administered dose and polyphenolic composition impacted on the glutathione metabolism.   Aqueous extracts of unfermented and fermented rooibos and unfermented honeybush (*C. intermedia*) significantly (P<0.05) increased the GSH/GSSG ratio and significantly (P<0.05) decreased the level of GSSG in rat liver (Marnewick *et al.*, 2003) and fermented rooibos in human plasma (Marnewick *et al.*, 2011), respectively (Table 1). PER had a very different effect in the current study and the activity of glutathione reductase (GR) was significantly (P<0.05) increased after 28 days, while GSH levels were significantly (P<0.05) reduced after 90 days. PECgen and PECsub also resulted in significantly (P<0.05) increased GR after 28 days and a marked decrease in GSH. It is proposed that the high levels of TP led to an underlying oxidative stress and affects the glutathione metabolism.   Although not significantly, the level of Fe(II)-induced lipid peroxidation was markedly increased in the liver after 28 and 90 days by PECsub and PER, respectively. The latter could be related to the reduced GSH level in the liver implying a specific interaction with rooibos and honeybush polyphenols. GSSG levels were not increased and therefore GSH conjugation with the catechol type polyphenols via *o*-quinone formation is likely for both rooibos and honeybush.   GSH conjugation has been demonstrated for cathechol type B-ring-containing flavonoids in hepatocytes (Galati *et al.*, 2002).   In this regard, catechol containing polyphenols present and consumed in high levels for rooibos comprise aspalathin, isoorientin and orientin and for honeybush are the xanthones mangiferin, isomangiferin, eriocitrin, scolymoside and iriflophenone 3-*C*-glucoside.   This observation is of concern as it indicates that rooibos and honeybush with increased polyphenol content as functional foods could lead to oxidative stress when consumed for extended periods and therefore warrants further investigation.

Modulatory effects of rooibos and honeybush PEEs on oxidative stress and antioxidant defense related genes: association with oxidative stress parameters and serum clinical biochemical parameters

Methods such as genomics and proteomics offer "novel" predictive markers to detect liver pathology before alterations in clinical and histopathology parameters occur, although serum biochemical parameters (clinical pathology) still provides valuable information in assessing the extent, severity and type (membrane injury versus cholestasis and hepatic function) of liver damage (Ramaiah, 2007).   Microarray technology has been applied in the pharmaceutical industry and drug development for early prediction and classification of hepatoxins (McMillian *et al.*, 2004) and RT-PCR has become the preferred method for confirming results obtained from techniques involving gene expression (Gaj *et al.*, 2008). Application of RT-qPCR arrays in investigating toxicity of herbal extracts and mechanisms in disease prevention is, nevertheless,

relatively novel. This approach was applied to rooibos and honeybush for the first time in the current study.

The effects of PEEs on the serum clinical biochemical parameters, oxidative stress parameters together with the effects on expression of oxidative stress and antioxidant defense related gene are summarised in Table 2. When considering PEE treatment for 28 days, the only serum biochemical parameters significantly ($P<0.05$) affected was an increase in Tbili and decrease in aspartate transaminase (AST) by PECsub. The only oxidative stress parameters significantly ($P<0.05$) affected was increased GR by all three PEEs. Conversely the significant ($P\leq0.05$) effects on gene expression in rat liver following 28 days of PEE treatment included a whole range of different genes with a variety of functions.

The majority of carcinogens is metabolically activated by cytochrome P450 (CYP450), whereas detoxification is catalyzed by a series of phase II enzymes facilitating conjugation of reactive carcinogenic species with endogenous moieties such as GSH, glucuronic acid, and sulfate for excretion (Na & Surh, 2008). The antioxidant defense system at cellular level incorporates superoxide dismutase (SOD), catalase (CAT), GSH, glutathione peroxidase (GPx), polyphenols, and phase II detoxifying enzymes including glutathione S-transferase, UDP glucuronyltransferase, NADPH quinone oxidoreductase (Nqo1) and microsomal epoxide hydrolase (Negi *et al.*, 2011). Induction of phase II detoxifying enzymes is regarded as one of the most important components of cellular defense mechanisms and assists in eliminating electrophilic and prooxidative toxicants before DNA damage occurs. In this regard the reduction of GSH levels in the liver by PER and PECsub could lower the defense mechanisms in the protection against oxidative stress and drug metabolism. The redox-sensitive transcription factor, nuclear factor erythroid 2 p45 (NR-E2)-related factor (Nrf2) plays a key role in regulating induction of phase II detoxifying and antioxidant enzymes in response to stressful conditions (Wang & Jaiswal, 2006). The activation of Nrf2 by polyphenols has been demonstrated for green tea (unfermented *C. sinensis*) polyphenols (Na & Surh, 2008) and one of the rooibos polyphenols, isoorientin (Lim *et al.*, 2007). Up-regulation of *Nrf2* expression by polyphenols could thus be favourable under conditions of oxidative stress, but in healthy subjects it may pose an indication of oxidative stress, specifically applicable to the current study as a result of high dose levels of TP.

Although no significant effects were evident for SOD and CAT activities in the current study, GSH was significantly ($P<0.05$) lowered by PER after 90 days and relevant genes affected after 28 days include *Gpx2*, *Gpx3* and *Nqo1*. The *Nrf2* system may serve as a potential target for the changing cellular antioxidant capacity by modulating gene expression, also referred to as indirect antioxidant activity (Jung & Kwak, 2010). Antioxidant genes regulated by Nrf2 include

amongst others, *Gpx1, Gpx2, Sod1* and *Nqo1* (Jung & Kwak, 2010), which are applicable in the current study. PER affected expression of *Gpx2, Gpx3* and *Nqo1*, while PECsub and PECgen affected *Sod1* and *Nqo1*, respectively. Therefore investigating the effect of rooibos and honeybush and/or its polyphenols on Nrf2 is an important recommendation for future investigation. Alterations of the cellular redox status due to elevated levels of ROS and electrophilic species and/or reduced antioxidant capacity (eg. lowered levels of GSH) are important factors in the signals triggering the transcriptional response of *Nrf2* (Nguyen *et al.*, 2009). The significant ($P < 0.05$) decrease in GSH by PER after 90 days and increased activity of GR after 28 days together with the up-regulation of *Nqo1* by PER and PECgen (28 days) in the current study points towards an effect of PER and PECgen polyphenols on the *Nrf2* pathway as a result of oxidative stress caused by the high levels of TP consumed.

The up-regulation of *Nqo1* after 28 days could further explain the increased quinone formation by PER manifested as significantly ($P < 0.05$) reduced GSH after 90 days, with no increase in GSSG. Although activity of glutathione reductase (GR) was significantly increased after 28 days by PER and both honeybush extracts no modulatory effects were observed on the expression of glutathione reductase (*Gsr*) in these livers. This discrepancy requires investigation together with the effects of rooibos and honeybush polyphenols on glutathione metabolism.

Other contradicting results regarding gene analysis and oxidative stress parameters were evident for the effect of rooibos on SOD activity and modulation of *Sod* expression. PER significantly ($P \leq 0.05$) up-regulated the expression of *Sod1*, while no significant effect was evident in activity of SOD in the liver. Regarding gene regulation, only the gene for soluble SOD (*Sod1*) was up-regulated, while expression of mitochondrial (*Sod2*) and extracellular (*Sod3*) was not affected. Although the up-regulation of *Sod1* did not result in a measurable increased enzyme (SOD) activity, it may be an early indication of oxidative stress as a result of the high levels of polyphenols consumed in the current study. The need to further investigate alterations in the expression of the different genes on a protein level is therefore evident.

Table 2 A summary of the significant (P<0.05) effects of 28 and 90 day studies with PEEs [a] (PER, PECsub and PECgen) in male Fisher rats on serum clinical biochemical and liver oxidative stress parameters and significant (P≤0.05) effects on expression of oxidative stress and antioxidant defense related genes in the liver after 28 days.

Treatment	Dose TP (HED)	*In vivo* effects [b]	Genes [c] affected	Applicable to *in vivo* findings
PER	756.60 (122.71)	↓ GR (28)	↑ *Gpx2*	
		↓ Tbili (90)	↓ *Gpx3*	
		↓ Iron (90)	↑ ***Aass*** [d]	
		↓ GSH (90)	↓ *Ncf2*	
			↑ *Idh1*	
			↑ ***Nqo1***	
			↓ *Apoe*	
PECsub	642.70 (*104.22*)	↓ AST (28)	↓ ***Edh2***	
		↑ Tbili (28)	↑ ***Aass***	
		↑ ALP (90); ↓ Tbili (90)	↑ *Sod1*	
		↓ Iron (90)	↑ ***Noxo***	
PECgen	545.50 (*88.46*)	↑ GR	↑ ***Nqo1***	
			↑ *Nudt1*	
			↑ *Psmb5*	
			↑ *Tpo*	
Mutual genes affected by PECsub & PECgen			↑ *Kif9*	
			↓ *Ptgs*	
			↓ *Serpinb1b*	
			↑ *Xpa*	
			↓ *Txnip*	
			↑ *Fancc*	
			↑ *Vim*	

[a] Concentration of PEEs (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER /kg and 2.5 g PECsub and PECgen/kg diet. [b] ↑ indicates increased; ↓ indicates decreased significantly (P<0.05); [c] ↑ indicates up-regulation; ↓ down-regulation of significantly affected (P≤0.05) gene expression. Descriptions and gene names of the symbols presented are included in the detailed listing of the genes related to oxidative stress included in the rat oxidative stress and antioxidant defense RT[2] Profiler PCR array in Table 2 Addendum E, [d] genes mutually affected by PEEs indicated in bold.

Further indications of oxidative stress on a gene level is provided by the PEEs significantly (P≤0.05) modulating the expression of thioredoxin interacting protein (*Txnip*)*,* neutrophil cytosolic factor 2 (*Ncf2*)*,* xeroderma pigmentosum complementation group A (*Xpa*) and isocitrate dehydrogenase 1 (*Idh1*)*.* Down-regulation of the expression of *Txnip* by honeybush PEEs (PECsub and PECgen) in the current study correlates with the findings of mangiferin decreasing insulin resistance in mice (Ichiki *et al.*, 1998; Miura *et al.*, 2001) and anti-diabetic properties of *C. intermedia* (Muller *et al.*, 2011). Considering the chemopreventive properties of honeybush extracts (*C. intermedia*) in liver, skin and oesophagus carcinoma in rats (Marnewick *et al.*, 2003; Marnewick *et al.*, 2009; Sissing *et al.*, 2011), upregulation of the expression of *Xpa* by PECsub and PECgen is of importance. Evaluation of anti-tumor effects considering the differences in phenolic composition of *Cyclopia* spp. and effect on gene expression, specifically *Txnip* and *Xpa*, is recommended for elucidating anti-tumor properties of *C. subternata* and *C. genistoides*.

Although PER did not significantly affect serum cholesterol in the current study, genes that may impact on cholesterol affected by PER include *Ncf2*, *Apoe* and *Idh1*. It can be argued that no effect was observed in the current study on serum parameters due to observations being conducted in healthy subjects. The effects observed on a gene level may be applicable to rooibos modulating the serum lipid profile by significantly (P<0.05) decreased triacylglycerol and LDL-cholesterol levels and increased HDL-cholesterol in the human study with subjects at risk for CVD (Marnewick *et al.*, 2011). Up-regulation of the expression of *Idh1* on the other hand, could promote cancer growth as a result of glucose sensing and the associated reduction in oxidative stress. However, potential lowered oxidative stress postulated with increased expression of *Idh1* by PER together with its glucose sensing properties could be involved in anti-diabetic properties of rooibos.

Effects of rooibos and honeybush on antioxidant defense and oxidative stress related genes in rat liver and relevance to protective or adverse effects under disease conditions
The potential of rooibos and honeybush to modulate pharmaceutical drug metabolizing enzymes is evident (Joubert *et al.*, 2008a, Joubert *et al.*, 2009; Abrahams, 2012). On the contrary, limited information is currently available on their impact on the oxidative stress and antioxidant defense related genes *in vivo*. Applying the effects of rooibos and honeybush on oxidative stress and antioxidant defense related genes to gene regulation in cancer, CVD and diabetes illustrates the potential influence on modulating disease progression (Table 3). The postulated effects are speculative and refer to the stages and disease to illustrate the implications of gene modulation by PER, PECsub and PECgen and should be considered in

context of affecting an increase or decrease in oxidative stress. Examples are included for the antioxidant defense related genes, glutathione peroxidases (*Gpx*), *Gpx2* and *Gpx3* and the oxidative stress responsive genes, *Idh1, Txnip* and *Nqo1* (Table 3).

Up-regulation of the expression of *Gpx2* may lead to protection against oxidative stress that will promote the growth of preneoplastic and cancer cells. In this regard the down-regulation of *Gpx3* expression will prevent the development of preneoplastic cells prone to be sensitized to oxidative stress (Schulte-Herman *et al.*, 1997). Up-regulation of the expression of *Idh1* by PER could assist in the survival of cancer cells, but may at the same time have a protective effect in CVD. When considering the down-regulation of *Txnip* expression by both honeybush extracts (PECsub and PECgen) increased oxidative stress is postulated, leading to protection against cancer by possible inhibiting the growth of preneoplastic cells via apoptosis (Shulte-Herman *et al.*, 1997). This may, on the contrary, potentially promote the development of atherosclerosis and diabetes. When considering the anti-proliferating effects of this gene (Han *et al.*, 2003), down-regulation may also lead to cancer promotion and not protection as postulated with regards to the effect of oxidative stress.

Modulation of oxidative stress and antioxidant defense related genes should therefore be interpreted with care as it is clear that the disease and stage of development will determine whether it could have a protective and/or promoting (enhancing disease) effect. Specifically related to cancer, protection against oxidative stress during cancer initiation protects against the development of early lesions, while this protection may on the other hand inhibit apoptosis and stimulate the growth of cancer cells (Salganik *et al.*, 2001). In contrast an increase in oxidative stress is likely to have the opposite effect as it is known that cancer cells are sensitized and undergo oxidative stress-induced apoptosis (Schulte-Hermann *et al.*, 1997). In view of the postulated role of rooibos and honeybush in protection against or advancing diseases such as cancer, CVD and diabetes, verification of effects on gene expression under disease conditions will be required. However, the effects of PEEs of rooibos and honeybush in the current study indicates that caution should be exercised in developing pharmaceutical products with specialised effects focused on one disease as it may promote the development of another.

Table 3 A summary of the modulating effects of rooibos and honeybush PEEs [a] on selected oxidative stress and antioxidant defense related genes in rat liver after 28 days and possible implications in carcinogenesis, diabetes and CVD/arteriosclerosis.

Gene [b]	Modulation [c]	PEE	Disease condition	Expression or activity in diseased condition	Reference	Postulated effect of PEEs [Protect/Inhibit (-) or Promote (+)]
Antioxidant defense related genes						
Gpx2	↑	PER	carcinogenesis (preneoplastic)	↑ up-regulated	Brigelius-Flohé & Kipp, 2009	+
Gpx3	↓	PER	carcinogenesis (neoplastic)	↓ down-regulated	Brigelius-Flohé & Kipp, 2009	-
Genes involved in reactive oxygen species (ROS) metabolism						
Oxidative stress responsive genes						
Idh1	↑	PER	carcinogenesis	↑ up-regulated	Furuta et al., 2010	+
			diabetes	↑ up-regulated	Lee et al., 2010	-
			atherosclerosis/CVD			
			cardiomyopathic mice	↓ down-regulated	Lucas et al., 2003	-
			spontaneously hypertensive rats	↓ down-regulated	Benderdour et al., 2004	-
Txnip	↓	PECsub, PECgen	cancer cell lines and tissue	↓ down-regulated	Han et al., 2003; Pang et al., 2009	+/-
			diabetes			
			regulates oxidative stress	↓ down-regulated	Schulze et al., 2004	+
			atherosclerosis			
			regulates oxidative stress	↓ down-regulated	Schulze et al., 2004	+
Nqo1	↑	PER, PECgen	cancer - certain types of malignant tissue and tumours	↑ up-regulated	Strassburg et al., 2002; Vasiliou et al., 2006	+
			CVD hyperhomocysteinemia	↓ down-regulated	Noll et al., 2011	-

[a] Concentration of PEEs (PER, PECsub and PECgen) in the feed mixture was 2.0 g PER /kg and 2.5 g PECsub and PECgen/kg diet, [c] ↑ indicates up-regulation; ↓ down-regulation of significantly affected (P≤0.05) genes in the current study, Descriptions and gene names of the symbols presented are included in the detailed listing of the genes related to oxidative stress included in the rat oxidative stress and antioxidant defense RT[2] Profiler PCR array in Table 2 Addendum E, [d] cardiovascular disease (CVD).

Conclusions

Potential adverse effects of polyphenols and herbal extracts have mostly been down-played, but it is crucial when researching efficacy and health promoting potential of herbal products for application in nutraceuticals and/or functional foods. Hepatic metabolism, specifically glucuronidation of the major rooibos and honeybush polyphenols affects antioxidant activity and could have implications for the oxidative status in the liver. The *in* vivo studies provide essential information about safety aspects of increased intake of polyphenols from rooibos and honeybush and new perspectives on the dose levels and compositional differences. The importance of chemical characterisation of herbal extracts in the evaluation of toxicity and disease prevention studies is confirmed again.   Effects of rooibos and honeybush polyphenols on oxidative stress and antioxidant defense related genes are considered for the first time and a screening with quantitative RT-PCR array provides a new method of investigating and understanding the effects of rooibos and honeybush administered as functional foods in the form of PEEs. Associations and contradictions were observed and some genes may offer potential therapeutic opportunities for rooibos and honeybush. Development of functional foods from rooibos and honeybush should critically consider potential adverse effects on iron absorption and probable indications of oxidative stress observed at the current doses. The potential of rooibos and honeybush to advance adverse effects under disease conditions was postulated as a result of modulatory effects on gene expression and warrants clarification before applications of rooibos and honeybush at high dose levels.

There is an obvious need for regulatory authorities and academic institutions to develop consensus on the requirements for toxicity and efficacy testing of herbal products, be it as herbal tea, functional food or other therapeutic applications. Irrespective, the current study clearly indicate that considering the modulatory effects on the expression of oxidative stress and antioxidant defense related genes together with conventional biomarkers provides additional insight on safety and potential health attributes of rooibos and honeybush.

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ADDENDUM A

PRESENTATION INDICATED IN CHAPTER 3, 4 AND 5

* Presentations are listed in the order indicated in the research chapters 3, 4 and 5

CHAPTER 3:

Van der Merwe J.D., Gelderblom W.C.A., Joubert E. & Manley, M. Bioavailability of the major phenolic compounds in the unique South African herbal tea, rooibos. AstraZeneca 9th Annual Research Day, Groote Schuur Cape Town, South Africa, 14 February 2008 (Poster presentation).

Van der Merwe J.D., Joubert E., Gelderblom W.C.A. & Manley, M. Liver metabolism of the major phenolic compound in rooibos (*Aspalathus linearis*): glucuronidation. South African Society of Biochemistry and Molecular Biology (SASBMB) meeting as part of Bio-08 conference, Grahamstown, South Africa, 23–24 January 2008 (Poster presentation).

Van der Merwe J.D., Joubert E.,Gelderblom W.C.A., De Beer, D. & Manley, M. Liver metabolism of 2',3,4,4',6'-pentahydroxy-3-C-β-D-glucopyranosyldihydrochalcone: glucuronidation and sulphation. International Conference on Polyphenols ICP2008, Salamanca, Spain, 8–11 July 2008 (Poster presentation).

CHAPTER 4:

Van der Merwe J.D., Gelderblom W.C.A, Joubert E. & Manley, M. Biotransformation of mangiferin, a major phenolic compound in honeybush tea: implications for its bioactivity? 19th biennial SAAFoST Congress & Exhibition Durban, South Africa 2–5 September 2007 (Poster presentation).

Van der Merwe, J.D., Joubert, E., Manley, M., De Beer, D., Malherbe, C.J. & Gelderblom, W.C.A. Liver metabolism of the major phenolic compound present in honeybush. Indigenous Plant Use Forum (IPUF), Stellenbosch, South Africa, 6–9 July 2009 (Oral presentation).

Van der Merwe, J.D., Joubert, E., Manley, M., De Beer, D., Malherbe, C.J. & Gelderblom, W.C.A. Liver metabolism of the major phenolic compound present in honeybush: implications for bioactivity. MRC Research Day, Tygerberg, 14–15 September 2009 (Oral presentation).

CHAPTER 5:

Van der Merwe, J.D., Gelderblom, W.C.A., Joubert, E & Manley, M. Safety evaluation of an aspalathin-enriched rooibos (*Aspalathus linearis*) extract as a cancer chemopreventive agent. 15th World Congress of Food science and Technology, IUFOST 2010, Cape Town, South Africa, 22–26 August 2010 (Oral presentation).

OTHER RELEVANT PRESENTATION:

Van der Merwe J.D., Gelderblom W.C.A., Joubert E. & Manley, M. Bioavailability of the major phenolic compounds in rooibos. CANSA Audit of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit of the Medical Research council (MRC), Cape Town, South African, 11 April 2008 (Oral presentation).

Van der Merwe J.D., Gelderblom W.C.A., Joubert E. & Manley, M. What happens to rooibos in the body? 1st Rooibos Science Café, MTN Science Centre, Century City, Cape Town, South Africa, 9 November 2007 (Oral presentation).

Van der Merwe J.D., Gelderblom, W.C.A, Joubert, E. & Manley, M. Flavonoid metabolism: Implication for the health properties of South African herbal teas *Aspalathus linearis* (rooibos) and *Cyclopia* (honeybush). External Review of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit of the Medical Research council (MRC), Cape Town, South Africa, 14 August 2007 (Oral presentation).

Van der Merwe, J.D., Gelderblom, W.C.A., Joubert, E. & Manley, M. Bioavailability of the major phenolic compound in rooibos tea. Rooibos Council Annual Meeting, Clanwilliam, South Africa, 25 April 2007 (Oral presentation).

ADDENDUM B

HPLC-DPPH ON-LINE DETECTION
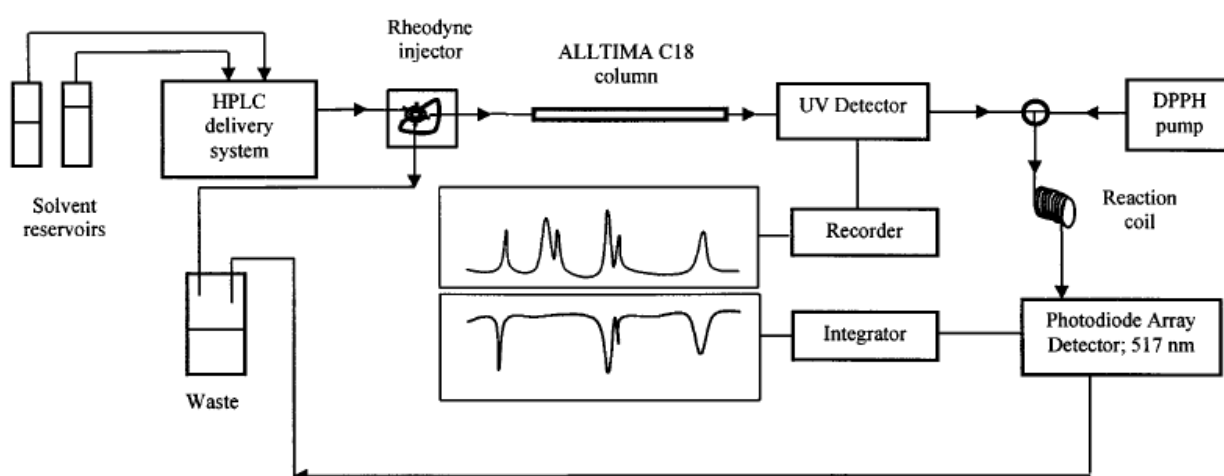
Figure 1 Instrumental set-up for the HPLC-DPPH on-line detection of radical scavenging compounds (Koleva *et al.*, 2000).

Reference

Koleva, I.I., Niederlander, A.G. & van Beek, T.A. (2000). An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. *Analytical Chemistry*, **72**, 2323−2328.

ADDENDUM C

NO. 153   P. 1

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Ethics Committee for Research on Animals (ECRA)

PO Box 19001, Tygerberg, 7505, Cape Town, South Africa
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Http://www.sahealthinfo.org/Modules/ethics/ethics.htm

The
**Medical
Research
Council**

17 April 2007

Ms Debora van der Merwe
Promec Unit
MEDICAL RESEARCH COUNCIL

Dear Ms van der Merwe,

YOUR REVISED APPLICATION TO THE ECRA : REF. 05/07 "Bioavailability of the major polyphenolic compounds in rooibos and honeybush".

The ECRA Committee received your revised application and it has been approved.

You may start with your experiment now.

May I remind you that you are required to submit a brief progress report on the progress of this study at six monthly intervals so the ECRA can be kept informed of the progress you are making and of any problems you may encounter.

Your progress report dates   :   November 2007   and   May 2008.
We will remind you by letter.

Kind regards.

PROF D DU TOIT
Chairperson : ECRA Committee

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ADDENDUM D
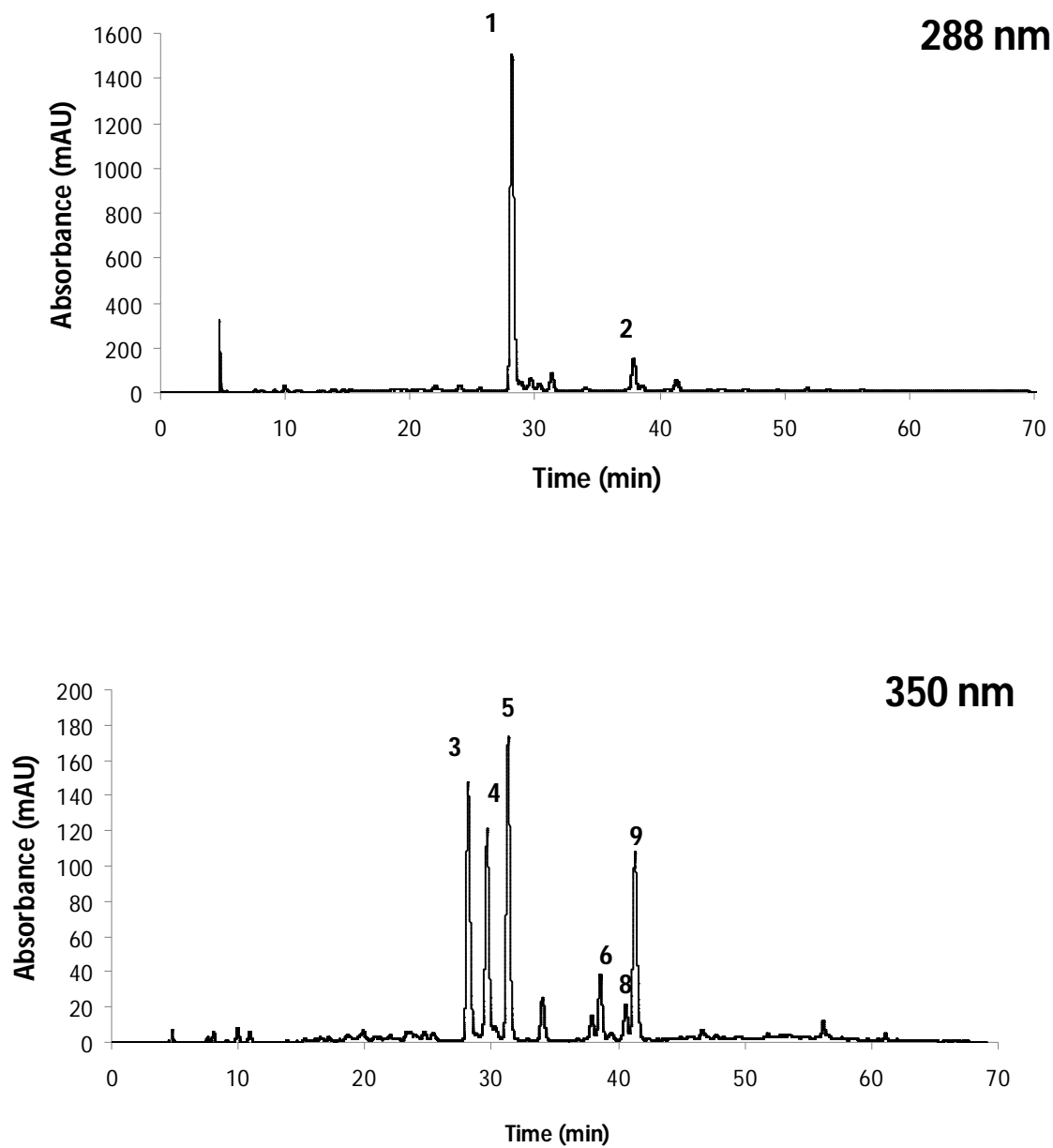
Figure 1 Typical HPLC chromatogram of the unfermented polyphenol-enriched rooibos (PER) extract used in the 28 and 90 day feeding studies with rats. Polyphenolic compounds quantified and indicated on the chromatograms were: 1=aspalathin, 2=nothofagin, 3=orientin, 4=isoorientin, 5=vitexin, 6=isovitexin, 7=luteolin-7-gluc, 8=hyperoside, 9=co-elution of quercetin-3-robionobioside/isoquercitrin/rutin, 10=luteolin, 11=chrysoeriol.
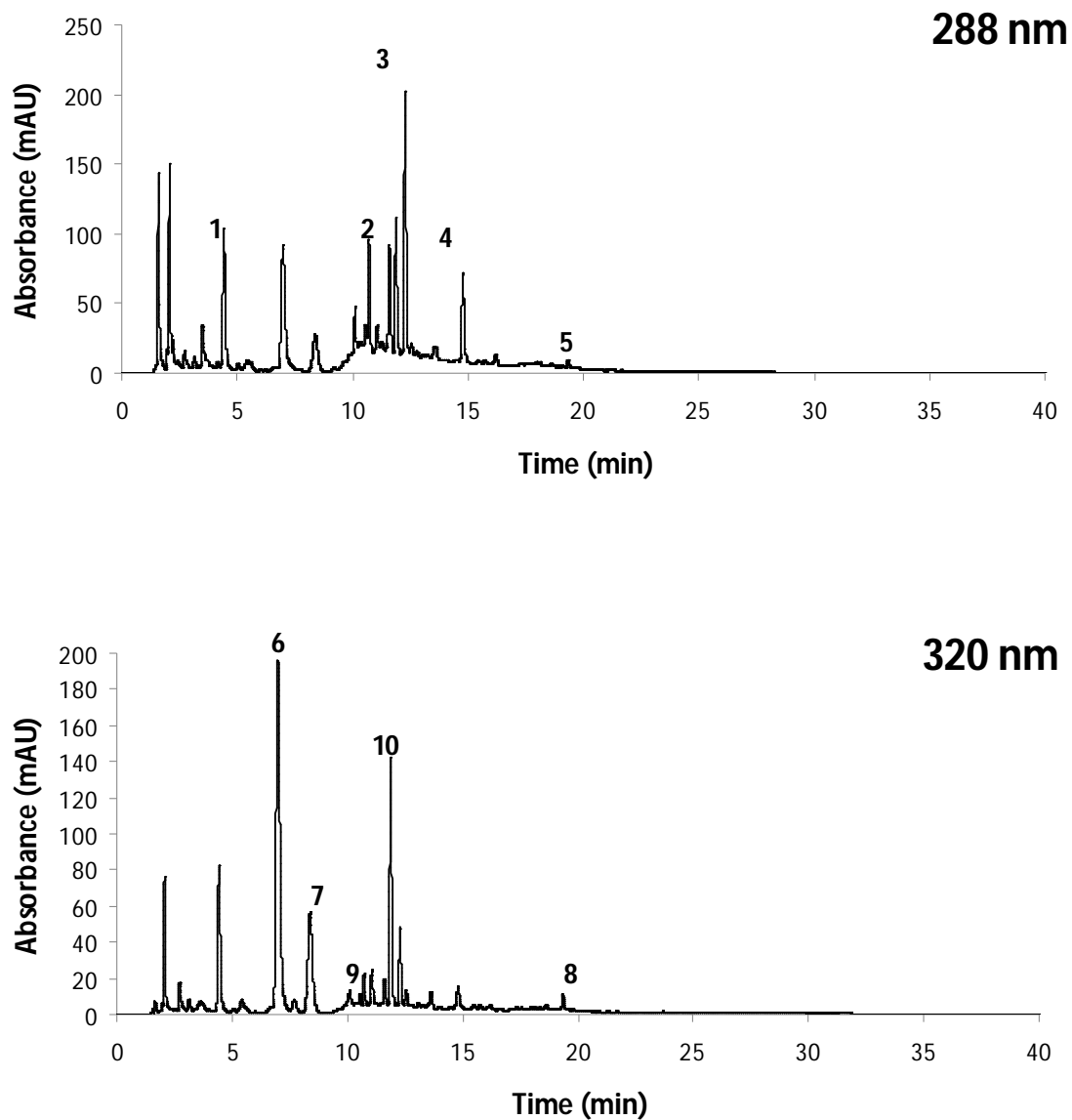
Figure 2 Typical HPLC chromatogram of the unfermented polyphenol-enriched *C. subternata* (PECsub) extract used in the 28 and 90 day feeding studies with rats. Polyphenolic compounds quantified and indicated on the chromatograms were: 1=iriflophenone-3-*C*-glucoside, 2=unknown eriodictyol-glucoside, 3=phloretin-3',5'-di-*C*-glucoside, 4=hesperidin, 5=naringenin, 6=mangiferin, 7=isomangifeirn, 8=luteolin, 9=unknown hydroxycinnamic acid derivative, 10=scolymoside.
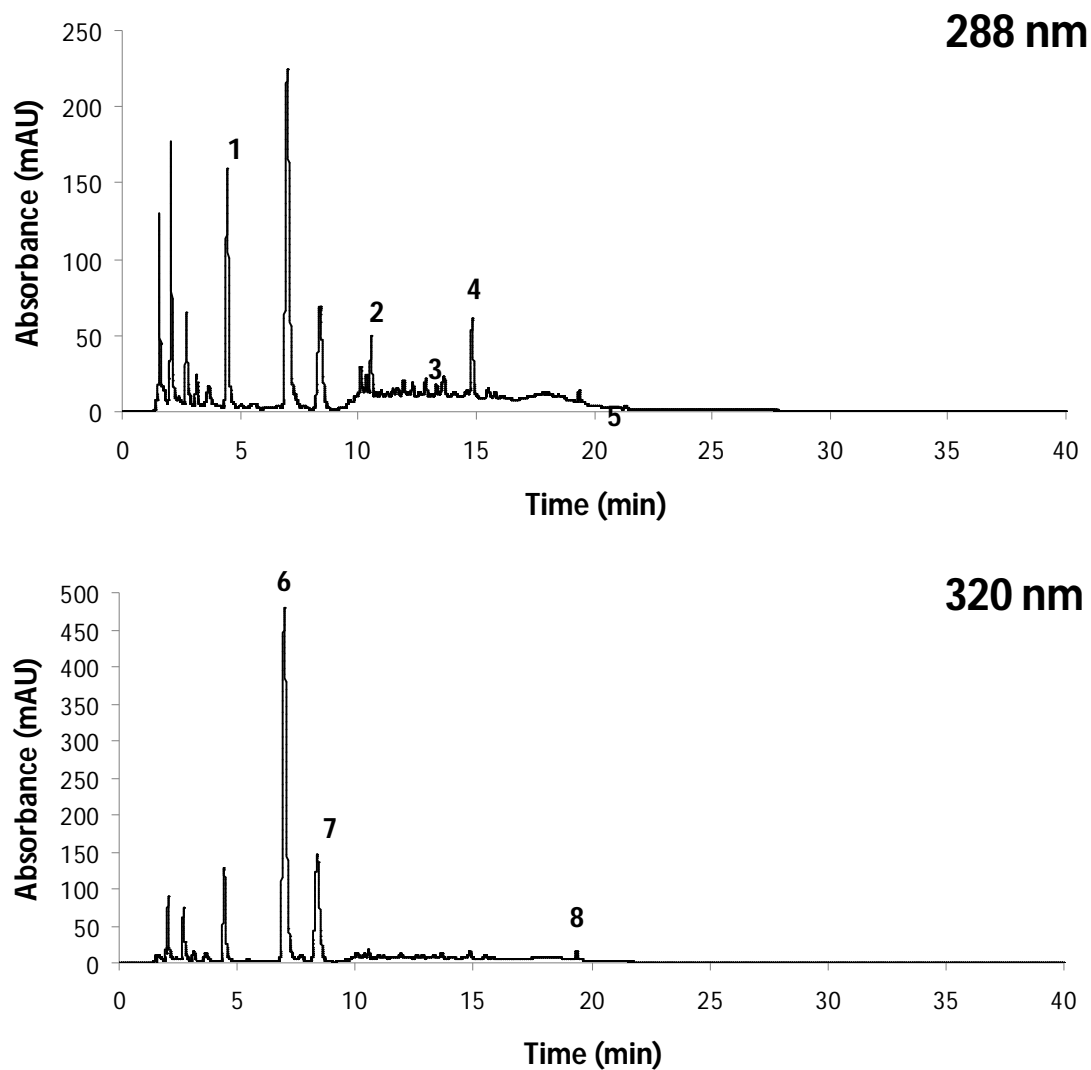
Figure 3 Typical HPLC chromatogram of the unfermented polyphenol-enriched *C. genistoides* (PECgen) extract used in the 28 day feeding studies with rats. Polyphenolic compounds quantified and indicated on the chromatograms were: 1=iriflophenone-3-*C*-glucoside, 2=unknown eriodictyol-glucoside, 3=phloretin-3',5'-di-*C*-glucoside, 4=hesperidin, 5=naringenin, 6=mangiferin, 7=isomangiferin, 8=luteolin.

ADDENDUM E
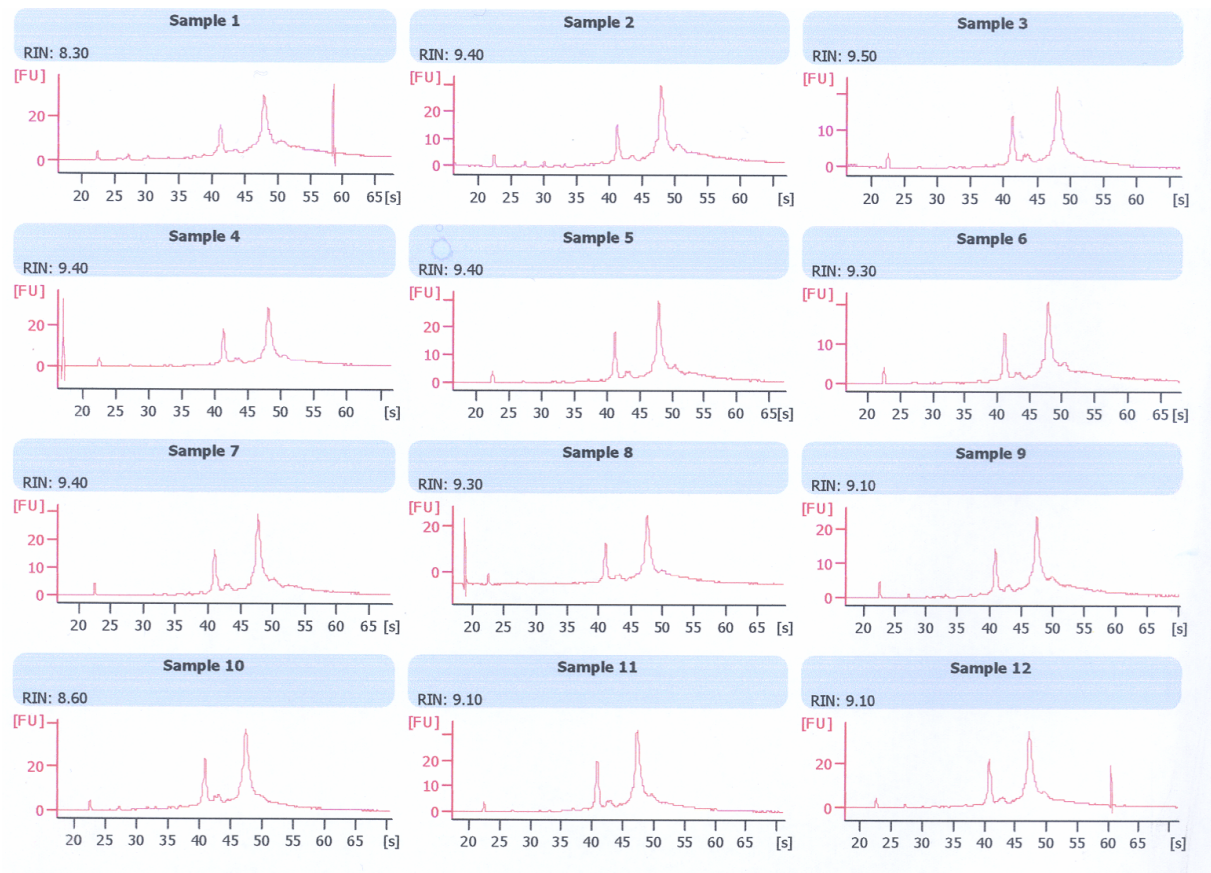
Figure 1. RNA integrity numbers (RINs) of RNA extracted from rat liver tissue as assessed with an Agilent 2100 Bioanalyzer.   Sample 1 – 3 (Control), Sample 4 – 6, (PER), Sample 7 – 9 (PECgen) and Sample 10 – 12 (PECsub).
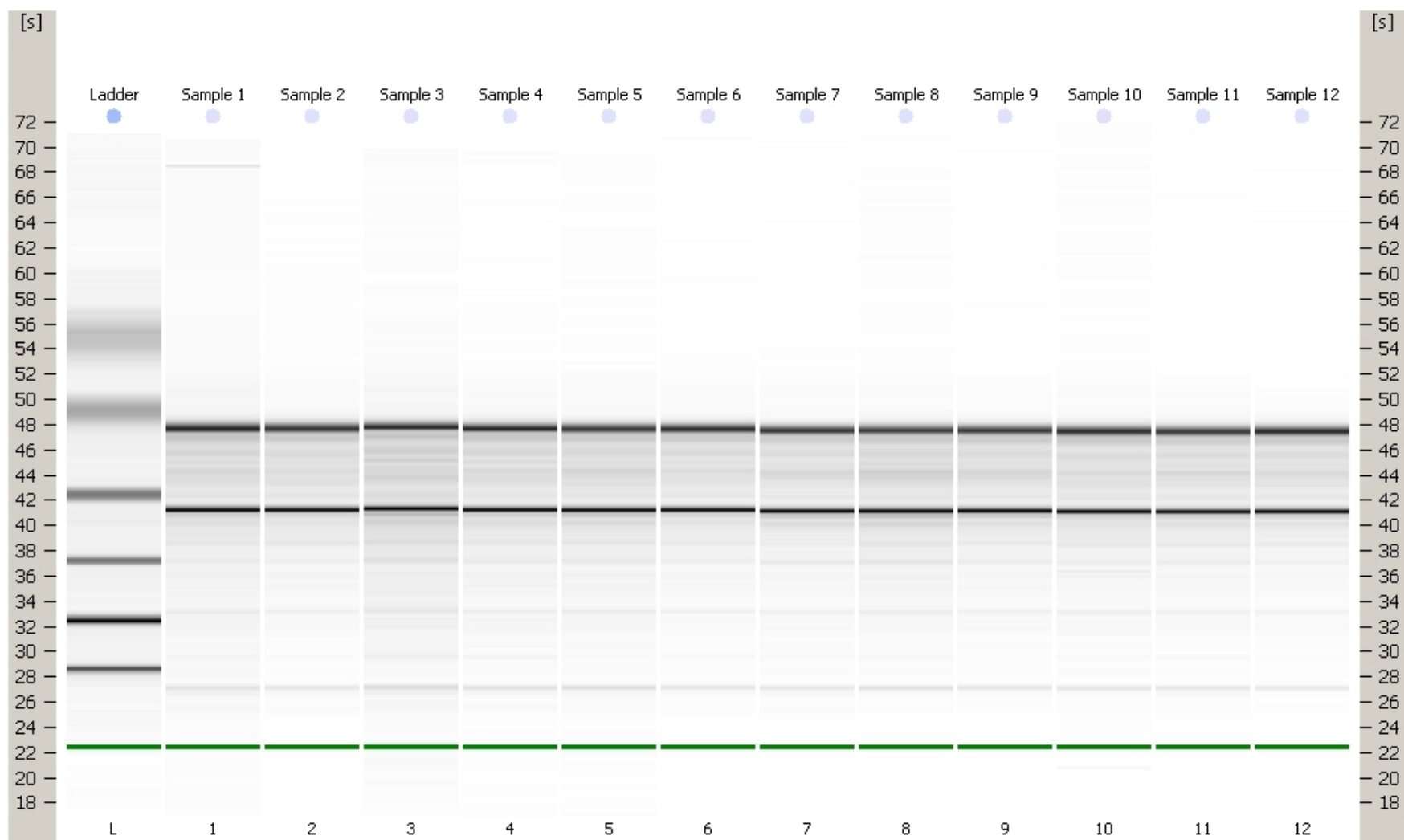
Figure 2 Quality parameters for isolated RNA as determined with a Bioanalyser RNA nanolabchip. Sample 1–3 (CTRL), Sample 4–6, (PER), Sample 7–9 (PECgen) and Sample 10–12 (PECsub).

Table 1 The array layout of the RT² Profiler™ PCR array used to investigate 84 genes related to oxidative stress antioxidant defense after consumption of PEE for 28 days by Male Fisher rats. Table presented as obtained from the www.sabiosciences.com on 1/08/2012.

Aass A01	Als2 A02	Apc A03	Apoe A04	Aqr A05	Cat A06	Ccs A07	Xirp1 A08	Ctsb A09	Duox2 A10	Cygb A11	Dhcr24 A12
Dnm2 B01	Duox1 B02	Ehd2 B03	Epx B04	Ercc2 B05	Ercc6 B06	Fancc B07	Fmo2 B08	Gab1 B09	Gpx1 B10	Gpx2 B11	Gpx3 B12
Gpx4 C01	Gpx5 C02	Gpx6 C03	Gpx7 C04	Gsr C05	Gstk1 C06	Hba-a2 C07	Hbz C08	Idh1 C09	LOC367198 C10	Lpo C11	Mb C12
Mpo D01	Mpp4 D02	Ncf1 D03	Ncf2 D04	Ngb D05	Nos2 D06	Nox4 D07	Noxa1 D08	Noxo1 D09	Nqo1 D10	Nudt1 D11	Nudt15 D12
Nxn E01	Park7 E02	Ppp1r15b E03	Prdx1 E04	Prdx2 E05	Prdx3 E06	Prdx4 E07	Prdx5 E08	Prdx6 E09	Prnp E10	Psmb5 E11	Ptgs1 E12
Ptgs2 F01	Rag2 F02	Gpx8 F03	Serpinb1b F04	Kif9 F05	Scd1 F06	Ift172 F07	Slc38a1 F08	Slc38a4 F09	Slc38a5 F10	Slc41a3 F11	Sod1 F12
Sod2 G01	Sod3 G02	Srxn1 G03	Tmod1 G04	Tpo G05	Txnip G06	Txnrd1 G07	Txnrd2 G08	Ucp3 G09	Vim G10	Xpa G11	Zmynd17 G12
Rplp1 H01	Hprt1 H02	Rpl13a H03	Ldha H04	Actb H05	RGDC H06	RTC H07	RTC H08	RTC H09	PPC H10	PPC H11	PPC H12

Table 2 Detailed listing of the genes related to oxidative stress included in the rat oxidative stress and antioxidant defense RT² Profiler™ PCR array as presented in the array layout (Table 1). Presented as obtained from the www.sabiosciences.com on 1/06/2012.

Position	Symbol	Description	Gene Name
A01	Aass	Aminoadipate-semialdehyde synthase	-
A02	Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	-
A03	Apc	Adenomatous polyposis coli	RATAPC
A04	Apoe	Apolipoprotein E	APOEA
A05	Aqr	Aquarius homolog (mouse)	-
A06	Cat	Catalase	CS1, Cas1, Cs-1, RATCAT01, RATCATL
A07	Ccs	Copper chaperone for superoxide dismutase	-
A08	Xirp1	Xin actin-binding repeat containing 1	Cmya1
A09	Ctsb	Cathepsin B	-
A10	Duox2	Dual oxidase 2	Thox2
A11	Cygb	Cytoglobin	MGC95105, Staap, Stap
A12	Dhcr24	24-dehydrocholesterol reductase	-
B01	Dnm2	Dynamin 2	DYIIAAB
B02	Duox1	Dual oxidase 1	-
B03	Ehd2	EH-domain containing 2	MGEPS
B04	Epx	Eosinophil peroxidase	-
B05	Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	-
B06	Ercc6	Excision repair cross-complementing rodent repair deficiency, complementation group 6	-
B07	Fancc	Fanconi anemia, complementation group C	Facc
B08	Fmo2	Flavin containing monooxygenase 2	-
B09	Gab1	GRB2-associated binding protein 1	-
B10	Gpx1	Glutathione peroxidase 1	GSHPx, GSHPx-1
B11	Gpx2	Glutathione peroxidase 2	GPX-GI, GSHPx-2, GSHPx-GI
B12	Gpx3	Glutathione peroxidase 3	GSHPx-3, GSHPx-P, Gpxp
C01	Gpx4	Glutathione peroxidase 4	Phgpx, gpx-4, snGpx
C02	Gpx5	Glutathione peroxidase 5	-
C03	Gpx6	Glutathione peroxidase 6	OBPII, Ry2d1
C04	Gpx7	Glutathione peroxidase 7	-
C05	Gsr	Glutathione reductase	-
C06	Gstk1	Glutathione S-transferase kappa 1	GST13-13, GSTkappa
C07	Hba-a2	Hemoglobin alpha, adult chain 2	HBAM, Hba-a1, Hba1
C08	Hbz	Hemoglobin, zeta	RGD1307486
C09	Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	-
C10	LOC367198	Similar to Serine/threonine-protein kinase ATR (Ataxia telangiectasia and Rad3-related protein)	-
C11	Lpo	Lactoperoxidase	-
C12	Mb	Myoglobin	-
D01	Mpo	Myeloperoxidase	-
D02	Mpp4	Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)	Dlg6
D03	Ncf1	Neutrophil cytosolic factor 1	Ncf-1, p47phox
D04	Ncf2	Neutrophil cytosolic factor 2	-
D05	Ngb	Neuroglobin	-
D06	Nos2	Nitric oxide synthase 2, inducible	Nos2a, iNos
D07	Nox4	NADPH oxidase 4	-
D08	Noxa1	NADPH oxidase activator 1	-
D09	Noxo1	NADPH oxidase organizer 1	-
D10	Nqo1	NAD(P)H dehydrogenase, quinone 1	Dia4, MGC93075
D11	Nudt1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	Mth1

Position	Symbol	Description	Gene Name
D12	Nudt15	Nudix (nucleoside diphosphate linked moiety X)-type motif 15	-
E01	Nxn	Nucleoredoxin	
E02	Park7	Parkinson disease (autosomal recessive, early onset) 7	CAP1, DJ-1, Dj1, SP22
E03	Ppp1r15b	Protein phosphatase 1, regulatory (inhibitor) subunit 15b	-
E04	Prdx1	Peroxiredoxin 1	Hbp23, MGC108617
E05	Prdx2	Peroxiredoxin 2	Tdpx1
E06	Prdx3	Peroxiredoxin 3	Prx3
E07	Prdx4	Peroxiredoxin 4	MGC72744
E08	Prdx5	Peroxiredoxin 5	Aoeb166
E09	Prdx6	Peroxiredoxin 6	-
E10	Prnp	Prion protein	PrP, Prn
E11	Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	
E12	Ptgs1	Prostaglandin-endoperoxide synthase 1	Cox-3, Cox1, Cox3
F01	Ptgs2	Prostaglandin-endoperoxide synthase 2	COX-2, Cox2
F02	Rag2	Recombination activating gene 2	-
F03	Gpx8	Glutathione peroxidase 8	RGD1307506
F04	Serpinb1b	Serine (or cysteine) peptidase inhibitor, clade B, member 1b	RGD1560658
F05	Kif9	Kinesin family member 9	RGD1565187
F06	Scd1	Stearoyl-Coenzyme A desaturase 1	-
F07	Ift172	Intraflagellar transport 172 homolog (Chlamydomonas)	Slb
F08	Slc38a1	Solute carrier family 38, member 1	Ata1, GlnT, Sat1
F09	Slc38a4	Solute carrier family 38, member 4	Ata3
F10	Slc38a5	Solute carrier family 38, member 5	SN2
F11	Slc41a3	Solute carrier family 41, member 3	-
F12	Sod1	Superoxide dismutase 1, soluble	CuZnSOD
G01	Sod2	Superoxide dismutase 2, mitochondrial	
G02	Sod3	Superoxide dismutase 3, extracellular	ECSODPT
G03	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	Ab2-390, Npn3
G04	Tmod1	Tropomodulin 1	E-Tmod, Tmod
G05	Tpo	Thyroid peroxidase	-
G06	Txnip	Thioredoxin interacting protein	MGC94673, Vdup1
G07	Txnrd1	Thioredoxin reductase 1	MGC93353, Tr
G08	Txnrd2	Thioredoxin reductase 2	MGC93435, Tr3, Trxr2, Trxrd2
G09	Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	-
G10	Vim	Vimentin	-
G11	Xpa	Xeroderma pigmentosum, complementation group A	-
G12	Zmynd17	Zinc finger, MYND-type containing 17	-
H01	Rplp1	Ribosomal protein, large, P1	MGC72935
H02	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase, Hprt, MGC112554
H03	Rpl13a	Ribosomal protein L13A	-
H04	Ldha	Lactate dehydrogenase A	Ldh1
H05	Actb	Actin, beta	Actx
H06	RGDC	Rat Genomic DNA Contamination	RGDC
H07	RTC	Reverse Transcription Control	RTC
H08	RTC	Reverse Transcription Control	RTC
H09	RTC	Reverse Transcription Control	RTC
H10	PPC	Positive PCR Control	PPC
H11	PPC	Positive PCR Control	PPC
H12	PPC	Positive PCR Control	PPC

Table 3 Integrity parameters of the RNA isolated from liver tissue after exposure to PEEs for 28 days as determined with a NanoDrop™ 1000 spectrophotometer.

Sample	ng/ul	260/280[a]	260/230[b]
Control a	399.50	2.06	2.07
Control b	743.96	2.10	1.93
Control c	378.48	2.07	2.08
PER a	445.50	2.05	2.03
PER b	366.82	2.07	2.02
PER c	345.24	2.08	1.91
PECsub a	388.78	2.06	1.60
PECsub b	448.69	2.05	1.97
PECsub c	436.74	2.05	1.87
PECgen a	434.74	2.05	2.06
PECgen b	479.11	2.07	1.91
PECgen c	405.97	2.06	2.06

[a] Indicates protein and/or DNA contamination (Where ≥2 indicates pure RNA); [b] Indicates salt contamination (Where ≥2 indicates pure RNA).

Table 4 Complete list of fold change and P-values of oxidative stress and antioxidant defense related genes evaluated with quantitative RT-PCR array in rat liver following intake of polyphenol-enriched rooibos (PER) and honeybush (PECsub and PECgen) extracts for 28 days.

Position	Unigene	GeneBank	Symbol	Description	Gene Name	PER		PECsub		PECgen	
						FC [a]	P [b]	FC	P	FC	P
A01	Rn.198671	XM_231524	Aass	Aminoadipate-semialdehyde synthase	N/A	1.320	0.014	1.254	0.005	1.080	0.435
A02	Rn.6408	XM_343574	Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	N/A	-1.012	0.843	-1.066	0.591	-1.056	0.643
A03	Rn.88057	NM_012499	Apc	Adenomatous polyposis coli	RATAPC	1.012	0.941	-1.046	0.785	1.060	0.681
A04	Rn.32351	NM_138828	Apoe	Apolipoprotein E	APOEA	-1.274	0.048	-1.040	0.881	-1.159	0.569
A05	Rn.37299	XM_345418	Aqr	Aquarius homolog (mouse)	N/A	-1.134	0.100	-1.217	0.113	-1.102	0.190
A06	Rn.3001	NM_012520	Cat	Catalase	CS1/Cas1	-1.215	0.334	-1.036	0.786	-1.122	0.618
A07	Rn.12311	NM_053425	Ccs	Copper chaperone for superoxide dismutase	Ccs	-1.196	0.531	-1.240	0.510	-1.409	0.454
A08	Rn.27588	XM_236702	Xirp1	Xin actin-binding repeat containing 1	Cmya1	-3.706	0.220	-2.448	0.316	-3.423	0.228
A09	Rn.100909	NM_022597	Ctsb	Cathepsin B	Ctsb	1.004	0.997	-1.081	0.605	-1.282	0.235
A10	Rn.55542	NM_024141	Duox2	Dual oxidase 2	Thox2	-1.398	0.125	1.659	0.110	-1.299	0.596
A11	Rn.105938	NM_130744	Cygb	Cytoglobin	Staap/Stap	1.275	0.116	1.044	0.772	1.111	0.565
A12	Rn.9470	XM_216452	Dhcr24	24-dehydrocholesterol reductase	N/A	1.027	0.778	1.087	0.527	-1.075	0.748
B01	Rn.11231	NM_013199	Dnm2	Dynamin 2	DYIIAAB	-1.528	0.063	-1.385	0.090	-1.317	0.112
B02	Rn.162682	NM_153739	Duox1	Dual oxidase 1	Duox1	-2.537	0.680	-1.571	0.250	-1.683	0.126
B03	Rn.16016	NM_001024897	Ehd2	EH-domain containing 2	MGEPS	-1.441	0.123	-1.385	0.042	-1.177	0.525
B04	Rn.17695	XM_220834	Epx	Eosinophil peroxidase	Epx	1.178	0.874	1.200	0.922	1.254	0.987
B05	Rn.74906	XM_218424	Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	LOC308415	-1.081	0.676	-1.012	0.901	-1.014	0.931
B06	Rn.19370	XM_224627	Ercc6	Excision repair cross-complementing rodent repair deficiency, complementation group 6	LOC306274	-1.212	0.340	-1.701	0.134	-1.539	0.153
B07	Rn.10798	NM_012557	Fancc	Fanconi anemia, complementation group C	Facc	-1.204	0.376	3.410	0.021	2.719	0.005
B08	Rn.3928	NM_144737	Fmo2	Flavin containing monooxygenase 2	Fmo2	-1.030	0.705	1.559	0.579	1.245	0.981
B09	Rn.1725	XM_341667	Gab1	GRB2-associated binding protein 1	Gab1	-1.027	0.923	-1.088	0.629	-1.004	0.995
B10	Rn.11323	NM_030826	Gpx1	Glutathione eroxidise 1	GSHPx/GSHPx-1	2.623	0.161	2.213	0.306	2.780	0.246
B11	Rn.3503	NM_183403	Gpx2	Glutathione eroxidise 2	GPX-GI/GSHPx-2	1.801	0.037	1.284	0.332	1.424	0.135
B12	Rn.108074	NM_022525	Gpx3	Glutathione eroxidise 3	GSHPx-3/GSHPx-P	-1.202	0.037	-1.080	0.715	-1.268	0.414
C01	Rn.3647	NM_017165	Gpx4	Glutathione eroxidise 4	Phgpx/gpx-4	-1.118	0.161	-1.105	0.479	-1.093	0.319

[a] FC indicates the fold change, the comparative Ct (ΔΔCt) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): ΔΔCt = ΔCt (treated) – ΔCt (control). The fold-change for each treated sample relative to the control sample was calculated using the formula of $2^{-\Delta\Delta Ct}$. [b] P indicates the P-value, Statistical calculations was done based on the ΔCt values using a two-tailed t-test. A P-value equal to and/or less than 0.05 was designated as significant.

Table 4 (*Cont.*)

Position	Unigene	GeneBank	Symbol	Description	Gene Name	PER FC	P	PECsub FC	P	PECgen FC	P
C02	Rn.218434	XM_001059839	Gpx5	Glutathione peroxidase 5	Gpx5	-1.167	0.604	-2.268	0.310	-1.445	0.511
C03	Rn.9852	NM_147165	Gpx6	Glutathione peroxidase 6	OBPII/Ry2d1	-1.429	0.353	1.232	0.938	1.594	0.466
C04	Rn.4130	XM_216473	Gpx7	Glutathione peroxidase 7	N/A	-1.014	0.992	1.041	0.557	-1.092	0.412
C05	Rn.19721	NM_053906	Gsr	Glutathione reductase	Gsr	1.193	0.152	1.163	0.066	1.055	0.503
C06	Rn.109452	NM_181371	Gstk1	Glutathione S-transferase kappa 1	GST13-13/GSTkappa	1.101	0.656	1.151	0.507	1.033	0.940
C07	Rn.107334	NM_013096	Hba-a2	Hemoglobin alpha, adult chain 2	HBAM/Hba-a1	-1.880	0.073	-1.774	0.090	-1.350	0.246
C08	Rn.2703	XM_213268	Hbz	Hemoglobin, zeta	RGD1307486	-1.565	0.471	-2.268	0.310	-1.445	0.511
C09	Rn.3561	NM_031510	Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	1.299	0.037	1.400	0.114	1.071	0.624
C10	Rn.112309	XM_346005	LOC367198	Similar to Serine/threonine-protein kinase ATR (Ataxia telangiectasia and Rad3-related protein)	LOC367198	-17.684	0.372	-17.094	0.372	-16.332	0.372
C11	Rn.60583	XM_220831	Lpo	Lactoperoxidase	N/A	1.990	0.621	2.214	0.451	1.483	0.957
C12	Rn.40511	NM_021588	Mb	Myoglobin	Mb	-2.447	0.173	-3.174	0.112	-1.720	0.655
D01	Rn.47782	XM_220830	Mpo	Myeloperoxidase	Mpo	4.087	0.380	1.273	0.526	-1.417	0.533
D02	Rn.163075	NM_021265	Mpp4	Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)	Dlg6	1.570	0.525	-2.268	0.310	1.626	0.453
D03	Rn.38575	NM_053734	Ncf1	Neutrophil cytosolic factor 1	Ncf-1/p47phox	1.003	0.993	1.072	0.688	-1.225	0.612
D04	Rn.162331	XM_344156	Ncf2	Neutrophil cytosolic factor 2	N/A	-4.781	0.034	-1.311	0.682	-1.472	0.592
D05	Rn.64645	NM_033359	Ngb	Neuroglobin	Ngb	-2.197	0.430	1.239	0.965	-1.308	0.556
D06	Rn.10400	NM_012611	Nos2	Nitric oxide synthase 2, inducible	Nos2a/iNos	4.388	0.356	1.748	0.402	2.794	0.386
D07	Rn.14744	NM_053524	Nox4	NADPH oxidase 4	Nox4	-1.220	0.483	-1.435	0.225	-1.220	0.342

[a] FC indicates the fold change, the comparative Ct (ΔΔCt) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): ΔΔCt = ΔCt (treated) – ΔCt (control). The fold-change for each treated sample relative to the control sample was calculated using the formula of $2^{-\Delta\Delta Ct}$. [b] P indicates the P-value, Statistical calculations was done based on the ΔCt values using a two-tailed t-test. A P-value equal to and/or less than 0.05 was designated as significant.

Table 4 (*Cont.*)

Position	Unigene	GeneBank	Symbol	Description	Gene Name	PER FC	P	PECsub FC	P	PECgen FC	P
D08	Rn.162651	XM_231042	Noxa1	NADPH oxidase activator 1	noxa1	1.237	0.603	1.963	0.183	2.225	0.084
D09	Rn.137764	XM_220221	Noxo1	NADPH oxidase organizer 1	N/A	3.413	0.339	19.967	0.019	16.466	0.072
D10	Rn.11234	NM_017000	Nqo1	NAD(P)H dehydrogenase, quinone 1	Dia4	1.681	0.017	1.447	0.188	1.730	0.013
D11	Rn.10669	NM_057120	Nudt1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	Mth1	1.157	0.395	1.429	0.151	1.706	0.008
D12	Rn.144747	XM_214236	Nudt15	Nudix (nucleoside diphosphate linked moiety X)-type motif 15	N/A	-1.271	0.280	-1.089	0.607	-1.133	0.536
E01	Rn.105982	XM_340857	Nxn	Nucleoredoxin	N/A	-1.272	0.093	-1.363	0.141	-1.199	0.099
E02	Rn.30105	NM_057143	Park7	Parkinson disease (autosomal recessive, early onset) 7	CAP1/DJ-1	1.114	0.486	-1.083	0.622	1.014	0.863
E03	Rn.78049	XM_222633	Ppp1r15b	Protein phosphatase 1, regulatory (inhibitor) subunit 15b	N/A	1.246	0.191	1.223	0.322	1.441	0.063
E04	Rn.2845	NM_057114	Prdx1	Peroxiredoxin 1	Hbp23	1.138	0.349	1.026	0.900	-1.035	0.777
E05	Rn.2511	NM_017169	Prdx2	Peroxiredoxin 2	Tdpx1	1.000	0.975	-1.009	0.910	1.014	0.932
E06	Rn.2011	NM_022540	Prdx3	Peroxiredoxin 3	Prx3	1.222	0.092	1.069	0.594	1.002	0.916
E07	Rn.17958	NM_053512	Prdx4	Peroxiredoxin 4	MGC72744	-1.208	0.286	-1.152	0.357	-1.143	0.410
E08	Rn.2944	NM_053610	Prdx5	Peroxiredoxin 5	Aoeb166	2.152	0.308	1.118	0.652	1.368	0.168
E09	Rn.42	NM_053576	Prdx6	Peroxiredoxin 6	Prdx6	1.220	0.228	1.050	0.695	1.209	0.137
E10	Rn.3936	NM_012631	Pmp	Prion protein	PrP/Prn	-1.224	0.410	-1.509	0.314	-1.250	0.359
E11	Rn.2	XM_341314	Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	Psmb5	3.307	0.114	3.279	0.098	3.944	0.039
E12	Rn.44404	NM_017043	Ptgs1	Prostaglandin-endoperoxide synthase 1	Cox-3/Cox1	1.222	0.294	2.408	0.001	1.669	0.048
F01	Rn.44369	NM_017232	Ptgs2	Prostaglandin-endoperoxide synthase 2	COX-2/Cox2	-3.425	0.288	-1.083	0.348	-12.092	0.352
F02	N/A	XM_001079235	Rag2	Recombination activating gene 2	Rag2	-21.013	0.165	-2.544	0.308	-1.643	0.298
F03	Rn.106264	XM_215486	Gpx8	Glutathione peroxidase 8	RGD1307506	-1.259	0.212	-1.078	0.680	-1.131	0.573
F04	Rn.137930	XM_225268	RGD1560658	Similar to serine (or cysteine) proteinase inhibitor, clade B, member 1b	Serpinb1b	-1.900	0.056	-1.995	0.015	-2.294	0.001
F05	Rn.140344	XM_001077207	Kif9	Kinesin family member 9	RGD1565187	-1.157	0.241	-1.875	0.009	-1.958	0.005
F06	Rn.1023	NM_139192	Scd1	Stearoyl-Coenzyme A desaturase 1	Scd1	-1.547	0.327	-1.697	0.155	-1.550	0.134

[a] FC indicates the fold change, the comparative Ct ($\Delta\Delta$Ct) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): $\Delta\Delta$Ct = ΔCt (treated) – ΔCt (control). The fold-change for each treated sample relative to the control sample was calculated using the formula of $2^{-\Delta\Delta Ct}$. [b] P indicates the P-value, Statistical calculations was done based on the ΔCt values using a two-tailed t-test. A P-value equal to and/or less than 0.05 was designated as significant.

Table 4 *(Cont.)*

Position	Unigene	GeneBank	Symbol	Description	Gene Name	PER FC	P	PECsub FC	P	PECgen FC	P
F07	Rn.171849	NM_053792	Ift172	Intraflagellar transport 172 homolog (Chlamydomonas)	Slb	-1.419	0.136	-1.693	0.059	-1.627	0.073
F08	Rn.162022	NM_138832	Slc38a1	Solute carrier family 38, member 1	Ata1/GlnT	-2.754	0.058	-2.549	0.064	-1.708	0.083
F09	Rn.17990	NM_130748	Slc38a4	Solute carrier family 38, member 4	Ata3	-1.080	0.672	-1.179	0.445	-1.094	0.864
F10	Rn.81033	NM_138854	Slc38a5	Solute carrier family 38, member 5	SN2	-2.479	0.197	1.248	0.599	-1.407	0.432
F11	Rn.190829	NM_001037492	Slc41a3	Solute carrier family 41, member 3	Slc41a3	2.115	0.102	1.060	0.969	-1.686	0.612
F12	Rn.6059	NM_017050	Sod1	Superoxide dismutase 1, soluble	CuZnSOD	-1.394	0.784	1.183	0.002	1.212	0.122
G01	Rn.10488	NM_017051	Sod2	Superoxide dismutase 2, mitochondrial	Sod2	1.102	0.445	1.097	0.494	1.209	0.093
G02	Rn.10358	NM_012880	Sod3	Superoxide dismutase 3, extracellular	ECSODPT	-1.275	0.162	1.028	0.906	-1.338	0.295
G03	Rn.2835	XM_215887	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	Ab2-390/Npn3	-1.565	0.471	-1.641	0.425	-1.445	0.511
G04	Rn.1646	NM_013044	Tmod1	Tropomodulin 1	E-Tmod/Tmod	-1.604	0.490	4.000	0.255	4.310	0.209
G05	Rn.91199	NM_019353	Tpo	Thyroid peroxidase	Tpo	-1.565	0.471	47.697	0.145	372.430	0.049
G06	Rn.2758	NM_001008767	Txnip	Thioredoxin interacting protein	Vdup1	-1.434	0.067	-1.606	0.045	-1.930	0.038
G07	Rn.67581	NM_031614	Txnrd1	Thioredoxin reductase 1	MGC93353	-1.200	0.441	-1.216	0.501	-1.059	0.695
G08	Rn.6300	NM_022584	Txnrd2	Thioredoxin reductase 2	Tr3/Trxr2	-1.159	0.099	-1.060	0.606	1.007	0.886
G09	Rn.9902	NM_013167	Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	6.537	0.172	2.340	0.402	18.383	0.212
G10	Rn.2710	NM_031140	Vim	Vimentin	Vim	-1.446	0.386	1.802	0.026	1.672	0.031
G11	Rn.12469	XM_216403	Xpa	Xeroderma pigmentosum, complementation group A	LOC298074	-1.010	0.939	1.967	0.005	1.998	0.005
G12	Rn.25565	XM_214130	Zmynd17	Zinc finger, MYND-type containing 17	N/A	-4.496	0.075	1.400	0.530	-1.375	0.759
H01	Rn.973	NM_001007604	Rplp1	Ribosomal protein, large, P1	MGC72935	-1.250	0.233	-1.072	0.456	1.067	0.622
H02	Rn.47	NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase/Hprt	1.090	0.507	1.070	0.478	-1.070	0.429
H03	Rn.92211	NM_173340	Rpl13a	Ribosomal protein L13A	Rpl13a	1.223	0.441	1.277	0.320	1.317	0.303
H04	Rn.107896	NM_017025	Ldha	Lactate dehydrogenase A	Ldh1	-1.111	0.522	-1.181	0.384	-1.100	0.567
H05	Rn.94978	NM_031144	Actb	Actin, beta	Actx	1.042	0.594	-1.079	0.421	-1.195	0.029
H06	N/A	U26919	RGDC	Rat Genomic DNA Contamination	RGDC						
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC						
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC						
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC						
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC						
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC						
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC						

[a] FC indicates the fold change, the comparative Ct (ΔΔCt) method was used to calculate the relative amount of transcripts in the treated and untreated samples (control): ΔΔCt = ΔCt (treated) – ΔCt (control). The fold-change for each treated sample relative to the control sample was calculated using the formula of $2^{-\Delta\Delta Ct}$. [b] P indicates the P-value, Statistical calculations was done based on the ΔCt values using a two-tailed t-test. A P-value equal to and/or less than 0.05 was designated as significant.