

HEPATIC, RENAL AND ENDOGENOUS ANTIOXIDANT STATUS MODULATORY EFFECTS OF CHRONIC CO-SUPPLEMENTATION OF ROOIBOS (*ASPALATHUS LINEARIS*) AND RED PALM OIL (*ELAEIS GUINEENSIS*) IN MALE WISTAR RATS

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Abstract

Background: This study investigated the hepatic, renal and endogenous antioxidant status modulatory effects of chronic co-supplementation of rooibos and red palm oil (RPO) in male Wistar rats.

Materials and Methods: Rats were randomized into four groups (n=10/group) and fed daily either standard rat chow (SRC) and water (**Group I**), SRC and the aqueous rooibos extract (2% w/v; **Group II**), SRC and RPO (200 µL/day) with water (**Group III**), or SRC and RPO (200 µL/day) with rooibos (2% w/v; **Group IV**) for 22 weeks.

Results: Chronic feeding of rooibos, RPO or their combination did not induce any adverse hepatic or renal effects, as shown by serum levels of alkaline phosphatase, alanine- and aspartate aminotransferase, lactate dehydrogenase, albumin, creatinine, blood urea nitrogen and uric acid. Histopathology analyses showed that liver from the three supplementation groups displayed normal hepatic histoarchitecture. Chronic feeding of RPO did not influence the redox status in the blood and liver significantly. Supplementation of rooibos alone for 22 weeks significantly (P<0.05) increased the reduced glutathione (GSH) level, GSH/GSSG ratio and catalase (CAT) activity in the liver. Co-supplementation of rooibos and RPO significantly (P<0.05) increased plasma total polyphenol content, trolox equivalent antioxidant capacity (TEAC), as well as blood GSH and GSH/GSSG ratio. Plasma conjugated dienes (CD) and hepatic malondialdehyde (MDA) levels were reduced significantly by the combination treatment, which also increased significantly the activity of CAT, glutathione reductase (GR), as well as the GSH/GSSG ratio in the liver.

Conclusion: Our results suggest that chronic feeding of an aqueous fermented rooibos extract alone or in combination with RPO modulate the endogenous antioxidant system in rats. This modulation could be indicative of a threshold effect and/or an additive effect indicating a positive interaction between the bioactive components in the rooibos and RPO; however future studies are needed to elucidate the nature and specific mechanisms involved. Also, the health status of the individual/experimental animal should be considered before antioxidant supplementation, as it is suggested to affect the outcome.

Key words: endogenous antioxidant system; glutathione redox status; lipid peroxidation; rooibos; red palm oil

Introduction

Oxidative stress, the imbalance between the production of free radicals and the natural antioxidant ability of cells has been established as either a cause or consequence of several disease states (Dalle-Donne et al., 2006; Khansari et al., 2009; Lappas et al., 2011). Although, reactive oxygen and nitrogen species (RONS) are generally considered as highly reactive and cytotoxic molecules, recent evidence is suggesting that besides their noxious effects, they participate in important physiological processes such as defence against pathogens, signal transduction and apoptosis (Valko et al., 2007). Reactive oxygen and nitrogen species, including superoxide radical anion ($O_2^{\cdot-}$) and hydroxyl (OH^{\cdot}) radicals produced during oxidative stress, are highly reactive and they can attack important macromolecules within their vicinity, resulting in damage to important cell structures, such as lipids and membranes, proteins and nucleic acids (Dalle-Donne et al., 2006; Valko et al., 2007). Lipid peroxidation as a major consequence of oxidative stress-induced damage may disrupt cellular functions and membrane integrity, leading to pathophysiological alterations and cell death (Dalle-Donne et al., 2006; Shieh et al., 2010). Impairment of the antioxidant defence system of the cell, including inactivation/inhibition of antioxidant enzymes, as well as an alteration of the glutathione (GSH) redox status, that is crucial for cellular thiol-antioxidant defence system and cellular metabolic machinery, also form part of the consequences of oxidative stress (Malireddy et al., 2012).

To prevent redox imbalance and oxidative damage that is consequent to oxidative stress, living cells have developed a biological defence system, consisting of an array of enzymatic and non-enzymatic antioxidants, to prevent damage due to oxidative stress. However, overproduction of RONS, with subsequent oxidative stress may overwhelm the endogenous antioxidant defence system, as is the case during the onset or progression of a disease state. Therefore, dietary antioxidant therapy are seriously sought after and

regarded as a promising strategy to strengthen the cellular antioxidant defence system and prevent oxidative stress-mediated cellular injury. Disease prevention using whole plant extracts is gaining scientific attention worldwide, partly because the plants are sources of bioactive phytochemicals, with antioxidant, disease prevention and therapeutic properties (Malireddy et al., 2012), and also because epidemiological studies are providing convincing evidence that diets rich in fruits, vegetables, teas and spices are associated with lower risk of chronic diseases (Heber, 2013).

Rooibos is a unique South African herbal tea produced from the leaves and stems of *Aspalathus linearis* Dahlg (Leguminosae). It is naturally caffeine-free and has low tannin content when compared to *Camellia sinensis* teas, but high in unique antioxidant polyphenols (Joubert et al., 2008). Rooibos has a flavonoid profile that is distinctly different from those found in *Camellia sinensis*, including the β -dihydroxy-dihydrochalcone glucoside aspalathin, and its cyclic counterpart aspalalinin, both of which are unique to rooibos (Rabe et al., 1994; Shimamura et al., 2006), the dihydrochalcone nothofagin, as well as flavonols including orietin, iso-orientin, vitexin, isovitexin, luteolin, quercetin and chrysoeriol among others (Rabe et al., 1994; Bramati et al., 2002; Joubert et al., 2008). Studies has shown that rooibos exert hepatoprotective effect in acute and chronic liver damage in rats (Ulicna et al., 2003; 2008), and modulate oxidative stress by preventing lipid peroxidation and improving glutathione redox status in rat tissues and in humans (Nikolova et al., 2007; Marnewick et al., 2011; Pansi et al., 2011; Awoniyi et al., 2012).

Red palm oil (RPO) is the edible oil obtained from crude palm oil extracted from the oil palm plant, (*Elaeis guineensis*), after a modified refining process, involving degumming and bleaching, followed by de-acidification and deodorisation by molecular distillation (Nagendran et al., 200). It is an antioxidant-rich oil, with almost equal proportions of saturated (mostly palmitic acid) and unsaturated (mostly oleic and linoleic acid) fatty acids (Sambanthamurthi et al., 2000). Red palm oil is made up of a cocktail of fat soluble antioxidants, including vitamin E (70% as tocotrienols and 30% as tocopherols), carotenoids (mainly α - and β -carotene), as well as lycopene, squalene and coenzyme Q10 (Al-Saqer et al., 2004). The beneficial effects of RPO has been demonstrated in various studies. Red palm oil has been shown to be hypocholesterolemic (Wilson et al., 2005), protect hearts subjected to ischaemia/reperfusion injury (Esterhuysen et al., 2006) and inhibit lipid peroxidation and modulate oxidative stress in different models of oxidant-mediated injury (Narang et al., 2005; Eriyamremu et al., 2008; Budin et al., 2011).

The observed health effects shown by many medicinal plants have been attributed to the additive or synergistic interaction of the various phytochemicals they contain, rather than to one single phytochemical. Also, evidence exists to show that polyphenols and antioxidant vitamins may result in the synergistic modulation of lipid peroxidation, oxidative stress, and co-oxidation of dietary antioxidants (Gorelik et al., 2005; De Kok et al., 2008). Therefore, we hypothesized that chronic feeding of rooibos and RPO together, may result in the synergy of their beneficial effects. Furthermore, most experimental and intervention studies on rooibos and RPO involved either acute or sub-chronic feeding in rodent models of disease or in animals with extensive pathology. This study investigated the hepatic, renal and responses of the endogenous antioxidant system in apparently healthy Wistar rats to chronic supplementation of rooibos and RPO.

Materials and Methods

Red Palm Oil and Aqueous Rooibos Extract Preparations

Fermented rooibos extract (RTE, 2% w/v) was prepared by the addition of freshly boiled tap water to rooibos leaves and stems at a concentration of 2g/100 mL (Marnewick et al 2003). The mixture was allowed to stand at room temperature for 30 minutes with constant stirring, filtered and dispensed into water bottles. The aqueous rooibos extract was fed to rats *ad libitum* and fresh rooibos was prepared every second day. The RPO used in this study (Carotino™ baking fat, Carotino SDN BHD, Johar-Bahru, Malaysia) was fed to the rats orally (200 μ L, equivalent to 7g/kg diet) on a daily basis in the morning, before the animals had access to the standard rat chow.

Animal Treatment and Experimental Design

Forty pathogen-free, male Wistar rats weighing 220 ± 24 g were used in this study. The animals were obtained and housed at the Primate Unit of Stellenbosch University (Tygerberg Campus, South Africa). The rats were housed individually in stainless steel wired-bottom cages fitted with polypropylene houses under controlled environment, maintained at a temperature of between 22-24°C, with a 12 h light dark cycle and 50-54% humidity. The rats were fed standard rat chow (SRC) *ad libitum* and had free access to tap water or the aqueous rooibos extract. The animals received humane care in accordance with the Principle of Laboratory Animal Care of the National Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institute of Health Publication no. 80-23, revised 1978). The study protocol was approved by CPUT's Faculty of Health and Wellness Sciences Research Ethics Committee (Ethics Certificate no: CPUT/HAS-REC 2010/A003). After acclimatization in the experimental animal holding facility for 1 week, the rats were randomized into four groups of 10 animals each, and treated for 22 weeks as follows:

Group I (control group): Fed SRC with access to water as the sole source of drinking fluid for the duration of the study.

Group II (rooibos group): Fed SRC with access to rooibos (2% w/v) as the sole source of drinking fluid for the duration of the study.

Group III (red palm oil group): Fed SRC and red palm oil [200 μ L (equivalent to 7g/kg diet) per day], with access to water as only source of drinking fluid for the duration of the study.

Group IV (rooibos + red palm oil group): Fed SRC and red palm oil (200 μ L per day), with access to rooibos (2% w/v) as the only source of drinking fluid for the duration of the study.

The general conditions of the rats were monitored daily throughout the study and body weights recorded weekly and at sacrifice (end of 22 weeks). Fluid intake was monitored at intervals of 2 days throughout the study. At the end of the experimental period, after an overnight fast, animals in all the groups were sacrificed under sodium pentobarbital anaesthesia (0.15 mL/100g body weight, i.p.). Approximately 8 ml of blood was collected via the abdominal aorta and aliquoted into tubes with (EDTA) and without anticoagulant to obtain plasma and serum, respectively. Plasma/serum was separated immediately by centrifugation at 5 000 g for 5 min

at 4 °C. The liver was removed, washed twice with ice-cold PBS (10 mM phosphate buffered saline pH 7.2) to remove residual blood, blotted to dry, and weighed. A slice of the liver sample was taken and fixed in 10% buffered formaldehyde solution for histological examination. The remaining liver tissue was immediately snap-frozen in liquid nitrogen and stored at -80 °C for biochemical analysis.

Histopathological Analysis

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

Soluble Solids, Total Polyphenols, Flavonol and Flavanol Content of the Aqueous Rooibos Extract

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

In Vitro Total Antioxidant Capacity of Rooibos Extract and Red Palm Oil Oxygen Radical Absorbance Capacity Assay

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

Trolox Equivalent Antioxidant Capacity Assay

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

Ferric Ion Reducing Antioxidant Power Assay

The ferric ion reducing antioxidant power (FRAP) of the rooibos extract and RPO was determined using the method described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in 100 mM HCl) and FeCl₃·6H₂O (20 mM) in a ratio of 10:1:1, v/v/v). Briefly, 10 µL of sample or ascorbic acid (AA) standard was added to 300 µL FRAP reagent in a 96-well clear plate. The plate was incubated at room temperature for 30 min, and absorbance read at 593 nm in a Multiskan Spektrum plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). Results were expressed as µmol ascorbic acid equivalent/litre (AAE/L) or µmol AAE/gram.

High Performance Liquid Chromatography Analysis of the Aqueous Rooibos Extract

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

High Performance Liquid Chromatography Analysis of RPO Vitamin E content of RPO

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

Carotenoids Content of RPO

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

Preparation of Liver Homogenates

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

Antioxidant Capacity of Plasma and Liver Samples

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

Liver and Kidney Function Markers

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

Oxidative Stress and Antioxidant Status Biomarkers Plasma and Hepatic Lipid Peroxidation

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

Hepatic and Erythrocyte Antioxidant Enzymes Activity

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

Hepatic and Whole Blood Glutathione Redox Status

The total glutathione (GSH and GSSG) was measured using a Bioxytech GSH/GSSG-412TM commercial kit (OxisResearchTM, Portland, USA), based on a method described by Tietze (1969). Aliquots of whole blood with 3 mM freshly prepared M2VP (for GSSG determination) or without M2VP (for GSH determination) were precipitated with 5% (w/v) metaphosphoric acid (MPA), while liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 10 000g for 10 min, 50 µL of supernatant (from whole blood or liver homogenate) was added to 50 µL of glutathione reductase (1U) and 50µL of 0.3mM DTNB. The reaction was initiated by addition of 1 mM NADPH to a final volume of 200 µL. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards. GSH concentration was calculated as the difference between total glutathione and 2GSSG.

Statistical Analysis

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

Results

Phytochemical Content and Antioxidant Capacity of Plant Materials

The phytochemical content and *in vitro* antioxidant capacity of the aqueous rooibos extract and RPO are shown in Table 1. The total polyphenolic content of the rooibos extract is 1.06 ± 0.00 mg GAE/mL, 63% of which were made up of flavonols and flavanols. HPLC analysis of the rooibos extract revealed the presence of peaks consistent with patterns showed by standards including aspalathin, iso-orientin, orientin, rutin/hyperoside and others (chromatogram not shown). Quantitatively, the HPLC analysis showed aspalathin (29.98 ± 0.08 µg/mL), iso-orientin (25.98 ± 0.52 µg/mL), orientin (18.61 ± 0.13µg/mL) and hyperoside/rutin (14.55 ± 2.26µg/mL) were the main flavonoids in the aqueous rooibos extract (Table 1). The total polyphenolic content of RPO used is 0.17 ± 0.01 mg GAE/100g. The tocopherol content of the oil is 98µg/g, while the tocotrienol content is 386µg/g. The total carotene content (α and β) is 53 µg/g. The *in vitro* antioxidant capacity of the RPO, measured as ORAC, FRAP and TEAC values are 175 ± 15.19 µmol TE/100g, 12.30 ± 0.28 µmol AAE/100g and 0.16 ± 0.03 µmol TE/100g, respectively (table 1).

Table 1: Phytochemical constituents and *in vitro* antioxidant capacity of aqueous rooibos extract and red palm oil

Aqueous rooibos extract		Red palm oil	
Constituents	Concentration	Constituents	Concentration
Soluble solids (mg/mL)	2.85 ± 0.39	α-tocopherol (µg/g)	71.28 ± 2.30
Total polyphenol (mg GAE/mL)	1.06 ± 0.02	β/γ-tocopherol(µg/g)	6.20 ± 0.94
Flavonol (mg QE/ mL)	0.48 ± 0.01	δ-tocopherol (µg/g)	20.70 ± 1.66
Flavanol (mg CE/mL)	0.19 ± 0.01	α-tocotrienol (µg/g)	102.36 ± 1 .52
Aspalathin (µg/mL)	29.98 ± 0.08	β/γ-tocotrienol (µg/g)	227.48 ± 2.74
Orientin (µg/mL)	18.61 ± 0.13	δ-tocotrienol (µg/g)	56.46 ± 1.55
Iso-orientin (µg/mL)	25.98 ± 0.52	α-carotene (µg/g)	23.74 ± 1.16
Vitexin (µg/mL)	6.07 ± 1.05	β-carotene (µg/g)	29.34 ± 2.92
Iso-vitexin (µg/mL)	7.18 ± 0.20	Total polyphenol (mg GAE/100g)	0.17 ± 0.01
Hyperoside/rutin (µg/mL)	14.55 ± 2.26	SFA (%)	51
Quercetin (µg/mL)	0.89 ± 0.18	MUFA (%)	38
Luteolin (µg/mL)	0.22 ± 0.06	PUFA (%)	11
Chrysoeriol (µg/mL)	0.25 ± 0.01	ORAC (µmol TE/100g)	175.00 ± 15.19
ORAC (µmol AAE/mL)	1.69 ± 0.03	FRAP (µmol AAE/100g)	12.30 ± 0.28
FRAP (µmol TE/mL)	5.20 ± 0.04	TEAC (µmol TE/100g)	0.16 ± 0.03
TEAC (µmol TE/mL)	4.79 ± 0.33		

Values are mean ± SD. Soluble solid is a mean of 12 determinations while other parameters are mean of 5 determinations. AAE (ascorbic acid equivalent), CE (catechin equivalent), GAE (gallic acid equivalent), QE (quercetin equivalent), TE (trolox equivalent), SFA (saturated fatty acid), MUFA (mono-unsaturated fatty acid), PUFA (poly-unsaturated fatty acid).

Daily Intake Profile of Rooibos and Red Palm Oil

The daily phenolic, vitamin E and carotene intakes of the various rat groups consuming the aqueous rooibos and RPO are shown in table 2. These intakes were based only on the rooibos and RPO intakes, and do not take into account the SRC intake. Daily water intake between the control group and the group fed RPO alone was similar. Rooibos intake, total phenolic, flavonol and flavonol

intakes per day between the two groups that were fed rooibos were not significantly different. Also, the daily vitamin E and carotene intakes between the group fed RPO alone and the group fed combination of rooibos and RPO were also similar.

Table 2: Daily phenolic, vitamin E and carotenoid intakes of the various rat groups consuming the aqueous rooibos extract and RPO

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

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

Body and Liver Weight Change

The effect of chronic feeding of rooibos, RPO or their combination on body weight gain, liver weight and relative liver weight is shown above in table 3. Chronic feeding of rooibos, RPO or their combination for 22 weeks, did not have any deleterious effect on the body weight gains across all the groups, however, consuming these plant products alone or in combination did significantly (P<0.05) decrease the absolute liver weight and the relative liver weight of the rats when compared to the control animals.

Liver and Kidney Function Markers and Histopathology

Table 3 also shows the effect of chronic feeding of rooibos and RPO on markers of liver and kidney functions. The serum levels of hepato-specific enzymes (ALP, ALT, AST and LDH) remained similar to that of the control, after feeding with the rooibos extract, RPO or their combination for 22 weeks. Data from the histopathological examination of liver tissues showed that none of the feeding regimens had a deleterious effect on the liver, with the liver tissues showing normal histoarchitecture (figure 1). Also the levels of ALB, BUN, CREA and uric acid which can be used to assess damage to the kidney, was unaffected by chronic feeding of the rooibos extract, RPO or their combination for 22 weeks.

Table 3: Effect of chronic feeding of aqueous rooibos extract, RPO or their combination on weight changes and markers of liver and kidney functions.

Treatment	Control	Rooibos	RPO	Rooibos + RPO
Body weight (g)	294.00 ± 39.61	285.80 ± 22.06	281.40 ± 32.40	281.90 ± 21.40
Liver weight (g)	13.62 ± 1.32	11.77 ± 1.87*	11.30 ± 1.37*	11.75 ± 1.48*
Relative liver weight (%)	2.81 ± 0.15	2.46 ± 0.32*	2.39 ± 0.16*	2.42 ± 0.17*
ALB (g/L)	33.14 ± 0.88	33.74 ± 0.60	34.72 ± 1.05	33.78 ± 1.10
ALP (U/L)	74.11 ± 11.16	68.05 ± 11.55	67.28 ± 11.73	66.25 ± 10.76
ALT (U/L)	91.07 ± 21.24	82.46 ± 21.73	95.11 ± 27.60	82.64 ± 13.29
AST (U/L)	145.37 ± 19.53	126.83 ± 45.46	143.01 ± 42.89	122.99 ± 21.37
LDH (U/L)	262.84 ± 73.20	192.86 ± 86.00	216.00 ± 71.05	200.68 ± 35.52
CREA (µmol/L)	30.50 ± 2.63	31.06 ± 2.87	30.50 ± 2.89	32.78 ± 1.47
BUN (mmol/L)	5.94 ± 0.50	6.21 ± 0.47	6.26 ± 0.52	6.04 ± 0.53
Uric Acid (µmol/L)	121.00 ± 21.31	94.89 ± 28.38	100.11 ± 34.79	95.11 ± 24.94

Values are mean ± SD (n=8-10). *Significantly different from control at p<0.05. ALB (albumin), ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), BUN (blood urea nitrogen), CREA (creatinine), LDH (lactate dehydrogenase).

Plasma and Hepatic Antioxidant Capacity and Lipid Peroxidation

Effect of chronic feeding of aqueous rooibos extract, RPO or their combination on the antioxidant capacity in the plasma and liver is presented in table 4. In the plasma, total polyphenol and TEAC status were unaffected by chronic feeding of the aqueous rooibos extract or RPO when compared with control. However, combined feeding of the rooibos extract and RPO significantly (P<0.05) increased the total polyphenol content and TEAC status compared to control animals. The ORAC and FRAP status of the rats remained similar (P>0.05) to those of the control group after being fed the aqueous rooibos extract, RPO or their combination for 22 weeks. In the liver, the ORAC, TEAC and FRAP status in the groups chronically fed the aqueous rooibos extract, RPO or their combination was not statistically (P>0.05) different when compared to the control group. Lipid peroxidation was assessed as conjugated dienes (CD) and malondialdehyde (MDA) levels. Plasma CD remained unchanged (P>0.05) compared to control rats when aqueous rooibos extract alone was supplemented. Plasma CD level in the rats consuming RPO either alone or combined with rooibos was significantly (P<0.05) reduced when compared to control rats (table 4). Supplementation of rooibos, RPO and their combination did not have any effect on plasma MDA as the MDA level was similar across all treatment groups. In the liver, the level of CD was not affected by chronic

supplementation of the fermented rooibos extract, RPO and their combination. Hepatic MDA was also not affected by chronic supplementation of the fermented rooibos extract or RPO alone, however, when both extract were given together, hepatic MDA was significantly ($P<0.05$) lowered when compared to the control group (table 4).

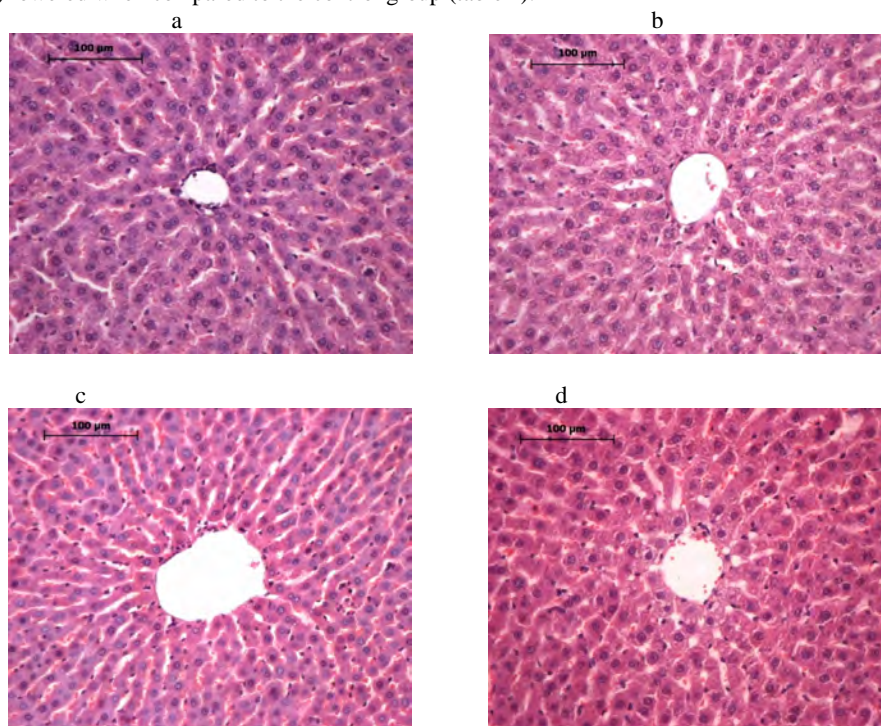


Figure 1: Hematoxylin and eosin stained liver sections showing the effect of chronic feeding of aqueous rooibos and red palm oil on liver histoarchitecture. (a) control group, (b, c and d) rooibos, red palm oil and rooibos + red palm oil group respectively, showing normal histoarchitecture of the liver.

Table 4: Effect of chronic supplementation of rooibos, red palm oil and their combination on plasma and liver antioxidant capacity and lipid peroxidation

Tissue	Parameter	Control	RTE	RPO	RTE + RPO
Plasma	TP ($\mu\text{mol GAE/L}$)	64.44 ± 3.75^b	67.28 ± 5.32^b	63.92 ± 3.40^b	71.77 ± 4.39^a
	TEAC ($\mu\text{mol TE/L}$)	6130.42 ± 360.24^b	6199.80 ± 268.22^b	6252.25 ± 244.40^b	6586.39 ± 343.77^a
	ORAC ($\mu\text{mol TE/L}$)	1412.14 ± 264.98^a	1613.96 ± 303.09^a	1462.84 ± 394.63^a	1625.70 ± 381.97^a
	FRAP ($\mu\text{mol AAE/L}$)	268.67 ± 30.65^a	266.37 ± 15.63^a	260.35 ± 18.49^a	271.74 ± 17.36^a
	CD (nmol/L)	81.41 ± 6.67^a	76.21 ± 7.50^{ab}	71.52 ± 6.39^b	69.90 ± 5.28^b
	MDA ($\mu\text{mol/L}$)	1.56 ± 0.31^a	1.29 ± 0.28^a	1.39 ± 0.42^a	1.51 ± 0.33^a
Liver	TEAC ($\mu\text{mol TE/g}$)	65.38 ± 6.72^a	66.30 ± 3.12^a	63.03 ± 2.11^a	65.08 ± 4.46^a
	ORAC ($\mu\text{mol TE/g}$)	17.23 ± 1.69^a	16.14 ± 1.85^a	16.37 ± 1.89^a	16.91 ± 1.43^a
	FRAP ($\mu\text{mol AAE/g}$)	2.85 ± 0.30^a	2.87 ± 0.35^a	3.03 ± 0.35^a	3.03 ± 0.39^a
	CD (nmol/L)	10.29 ± 0.29^a	10.15 ± 0.28^a	11.00 ± 1.08^a	11.05 ± 1.17^a
	MDA ($\mu\text{mol/L}$)	11.31 ± 2.33^a	10.97 ± 1.47^a	11.35 ± 1.56^a	9.06 ± 1.13^b

Values are mean \pm SD (n=10). Values in the same row with different superscript are significantly different from each other at $p<0.05$. CD (conjugated dienes), FRAP (ferric reducing ability of plasma), MDA (malondialdehyde), ORAC (oxygen radical absorbance capacity), TEAC (trolox equivalent antioxidant capacity), TP (total polyphenol), RTE (fermented rooibos extract), RPO (red palm oil), AAE (ascorbic acid equivalent), GAE (gallic acid equivalent), TE (trolox equivalent).

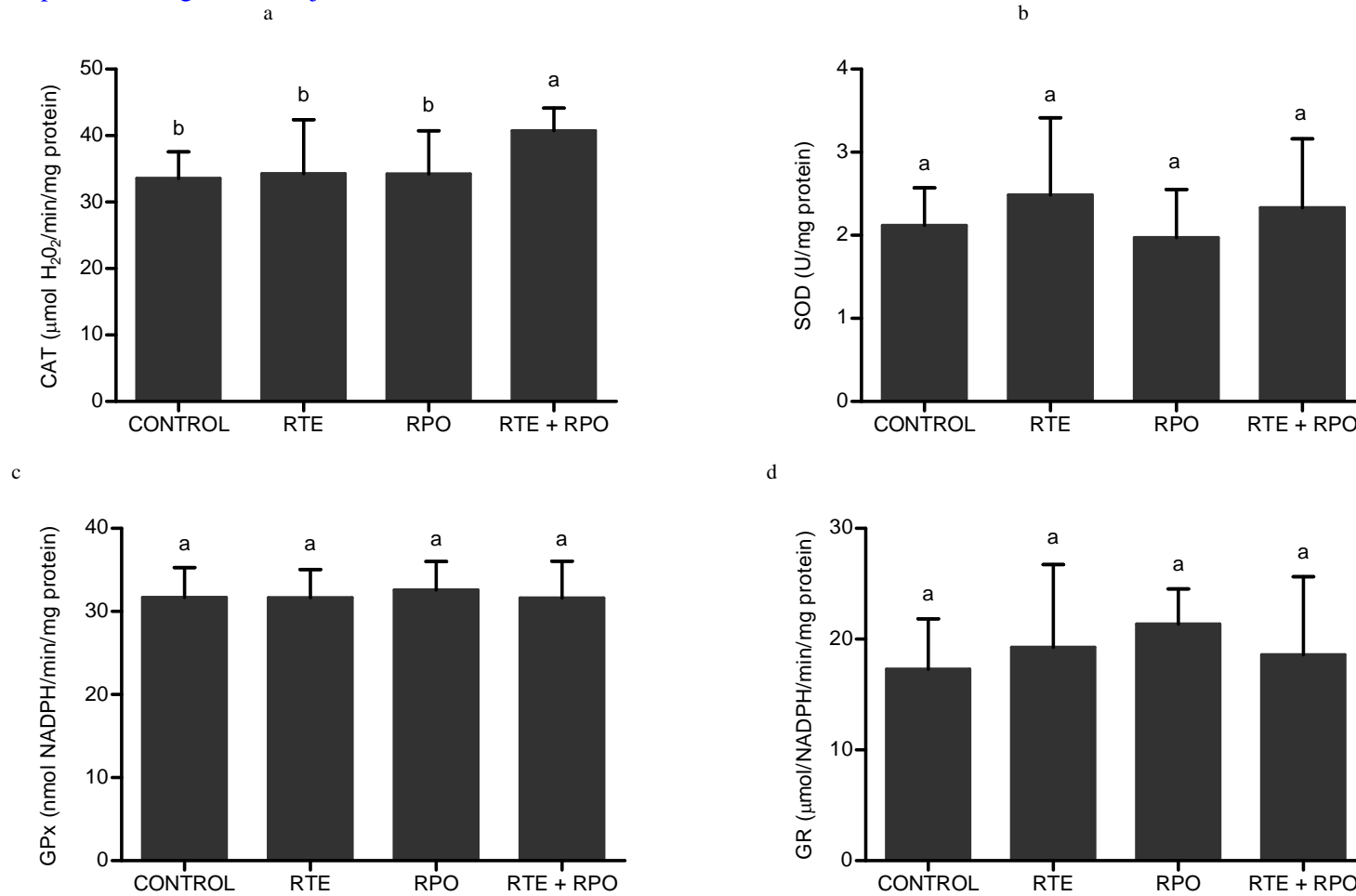


Figure 2: Effect of chronic supplementation of rooibos, red palm oil and their combination on erythrocyte (a) catalase (CAT), (b) superoxide dismutase (SOD), (c) glutathione peroxidase (GPx) and (d) glutathione reductase (GR). Bars represent mean \pm SD (n=9-10). Bars with different letters are significantly different from each other at P<0.05. RTE (fermented rooibos extract), RPO (red palm oil).

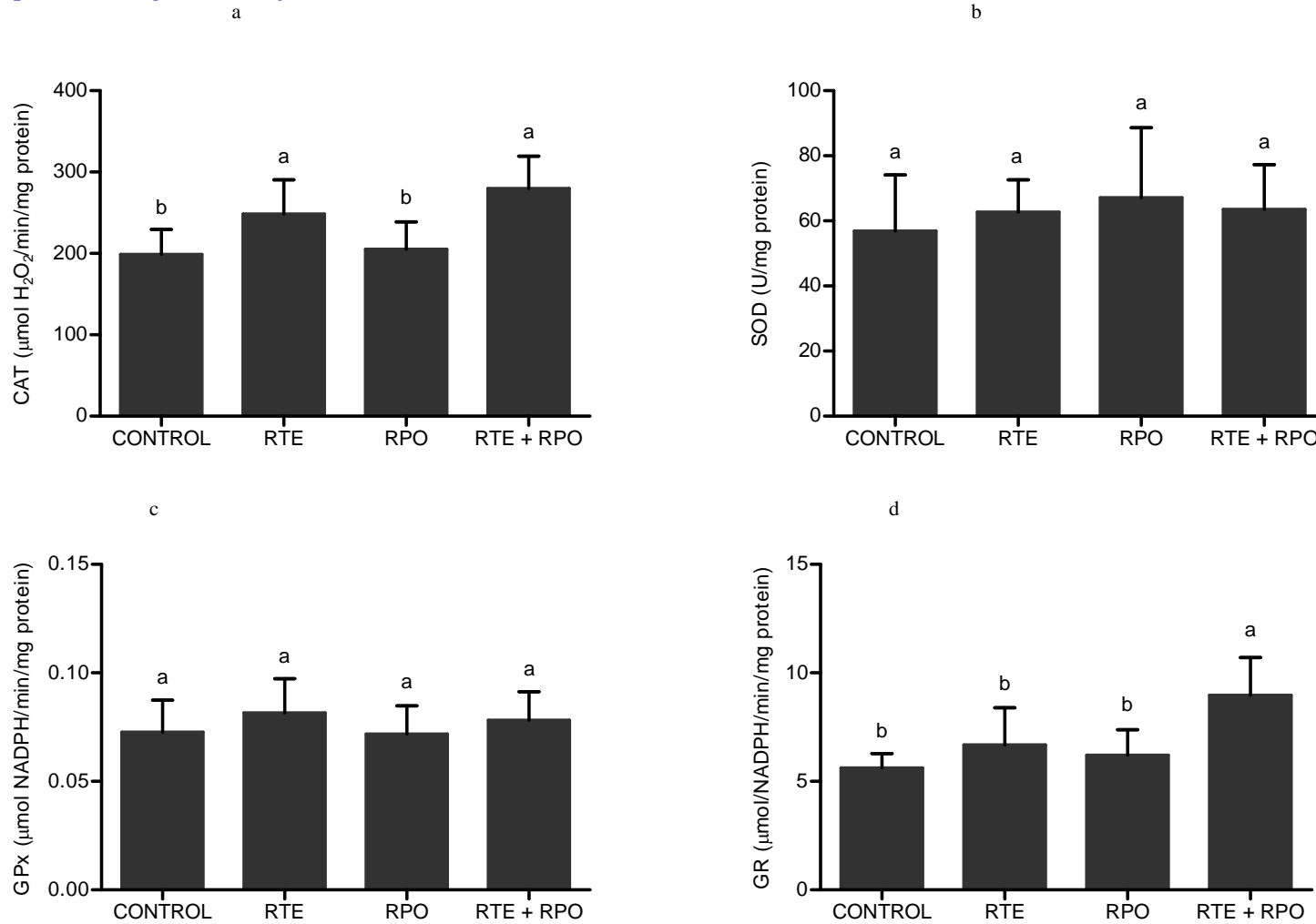


Figure 3: Effect of chronic supplementation of rooibos, red palm oil and their combination on hepatic (a) catalase (CAT), (b) superoxide dismutase (SOD), (c) glutathione peroxidase (GPx) and (d) glutathione reductase (GR). Bars represent mean \pm SD (n=9-10). Bars with different letters are significantly different from each other at P<0.05. RTE (fermented rooibos extract), RPO (red palm oil)

Fig 2 and 3 shows the results of the effect of chronic feeding of rooibos and RPO on erythrocyte and hepatic antioxidant enzymes. In the erythrocyte, the activities of SOD, GPx and GR remained unchanged as a result of chronic feeding of the aqueous rooibos extract, RPO or their combination, when compared to the control group (figures 2b, 2c & 2d). The activity of CAT in rats chronically fed the rooibos extract or RPO alone was similar to those of control rats. However, combined feeding of the rooibos extract and RPO significantly ($P < 0.05$) increased the erythrocyte CAT activity when compared to the control group (Fig 2a). Hepatic SOD and GPx remain unchanged across all treatment groups when compared to control group (figures 3b & 3c). Feeding the fermented rooibos extract alone or combined with RPO significantly ($P < 0.05$) increased the CAT activity when compared with the control group (figure 3b). GR activity was not affected by supplementation of fermented rooibos or RPO alone. However when the two plant extract were supplemented together, the activity of GR was significantly ($P < 0.05$) increased compared to the control group (figure 3d).

Whole Blood and Hepatic Glutathione Status

Table 5 shows the glutathione status across all experimental groups. In the blood, supplementation of the fermented rooibos extract alone, or together with RPO for 22 weeks, significantly ($P < 0.05$) increased the level of reduced glutathione (GSH) when compared to the control. Rats that were fed RPO alone for 22 weeks had GSH values that were comparable to those of the control. Oxidized glutathione (GSSG) was not affected by chronic supplementation of fermented rooibos extract, RPO or their combination as GSSG level remain similar in all the treatment groups compared with the control group. The GSH/GSSG ratio was increased significantly in rats supplemented with fermented rooibos extract either alone or in combination with RPO, when compared with control rats. In the liver, supplementation of fermented rooibos extract, RPO or their combination for 22 weeks did not affect GSH level when compared to control. Chronic supplementation of fermented rooibos extract alone or together with RPO significantly ($P < 0.05$) decreased the liver GSSG levels and significantly increased the GSH/GSSG ratio when compared to the control.

Table 5: Effect of chronic supplementation on rooibos, red palm oil and their combination on whole blood and hepatic glutathione redox status in Wistar rats

Tissue	Parameter	Control	RTE	RPO	RTE + RPO
Whole blood	GSH ($\mu\text{mol/L}$)	728.76 ± 85.47^b	893.68 ± 86.33^a	792.95 ± 115.07^b	915.28 ± 90.98^a
	GSSG ($\mu\text{mol/L}$)	26.96 ± 1.75^a	27.09 ± 2.74^a	27.02 ± 2.80^a	26.57 ± 3.00^a
	GSH/GSSG	27.06 ± 2.85^c	33.11 ± 2.65^{ab}	29.29 ± 4.01^{bc}	34.30 ± 4.90^a
Liver	GSH ($\mu\text{mol/g}$)	9.10 ± 0.76^a	9.13 ± 1.11^a	8.53 ± 0.97^a	9.06 ± 1.30^a
	GSSG ($\mu\text{mol/g}$)	0.35 ± 0.08^a	0.25 ± 0.09^b	0.30 ± 0.05^{ab}	0.24 ± 0.06^b
	GSH/GSSG	27.79 ± 6.30^b	37.06 ± 7.44^a	28.95 ± 5.33^b	34.50 ± 3.34^a

Values are mean \pm SD (n=10). Values in the same row with different superscript are significantly different from each other at $p < 0.05$. GSH (reduced glutathione), GSSG (oxidized glutathione), RTE (fermented rooibos extract), RPO (red palm oil).

Discussion

The sensitive balance between the pro-oxidant and antioxidant systems in the body is of utmost importance when determining the state of health, well-being and survival of organisms. A loss in the functional activity of the antioxidant systems, as a result of overproduction of reactive free radicals or depletion of the antioxidant molecules, might result in the disruption of the pro-oxidant to antioxidant ratio, creating a state of oxidative stress, which has been implicated as either a cause or consequence of many disease states (Dalle-Donne et al., 2006). The fact that oxidative stress has been implicated in the pathophysiology of most disease states suggest that augmenting the cellular antioxidant defence system may be a promising and pragmatic approach to prevent or slow down the progression of such diseases. Epidemiological findings and experimental data from animal and human studies has shown that diets rich in plant-derived foods, containing high levels of natural antioxidants, may contribute to reduced mortality from diseases (Heber, 2004; 2013), thus attention is now centred on dietary phytochemicals not only as an effective intervention in disease onset and progression, but also as an intervention for sustaining and promoting overall health. Fruits, vegetables, herbal teas and spices are rich sources of dietary phytochemicals, which individually or in combination may benefit health. These benefits are attributed to the presence of essential dietary micronutrients, fibres, antioxidant vitamins, trace elements, and polyphenolic compounds, mostly flavonoids (Yahia, 2010).

In this study, the effects of a sustained feeding of two antioxidant-rich natural plant products on the endogenous antioxidant system in male Wistar rats were investigated. Specifically, the activities of antioxidant enzymes and the glutathione redox status were measured in the liver and blood of these rats. Prior to the feeding experiment, the phytochemical composition as well as the *in vitro* antioxidant capacity of the aqueous rooibos extract and red palm oil were quantified in order to determine whether the phenolic and antioxidant content of the plant extracts played an important role in their observed health effects. In accordance with previously published studies, HPLC quantification of the rooibos extract showed that aspalathin is the major flavonoid present in rooibos, along with others such as iso-orientin, orientin, vitexin, iso-vitexin and hyperoside/rutin (Rabe et al., 1994; Bramati et al., 2002; Shimamura et al., 2006). Different isoforms of vitamin E, α - and β -carotene were also quantified in the RPO used in this study.

Literature on whether consumption of a polyphenol-rich diet will lead to an increase in plasma total polyphenol level and plasma total antioxidant capacity (TAC) has been inconclusive, with some studies reporting an increase (Leontowicz et al., 2003; Gorinstein et al., 2006), while others have reported no increase (Alia et al., 2003; Garcia-Solis et al., 2008; Kim et al., 2008). It has been

suggested that since assays for antioxidant capacity lack specificity, a single measurement of TAC is insufficient (Halliwell, 2009). Therefore measuring TAC using a battery of assay methods is recommended. Thus, for the current study, we measured the plasma and hepatic antioxidant capacity using three well known TAC (ORAC, FRAP and TEAC) assays. Our results showed that chronic consumption of fermented rooibos extract or RPO for 22 weeks did not increase the plasma total polyphenol or plasma and liver TAC measured as ORAC, FRAP or TEAC. However, when the rooibos extract and RPO were fed together, the combination resulted in a significant increase in plasma total polyphenol and plasma TAC measured only as TEAC. Some studies in humans have shown that the plasma total polyphenol content parallels the plasma TAC after the intake of polyphenol-rich diets (Henning et al., 2004; Torabian et al., 2009; Wang et al., 2012), suggesting that phenolic compounds are the major contributors to the plasma TAC, a fact that we also suspected may be true in animals. The TEAC assay is based on the inhibition of the absorbance of the ABTS radical cation (ABTS^{•+}) by antioxidants. The ABTS^{•+} radical can be solubilised in both aqueous and organic media, allowing the assay to measure lipophilic antioxidants like carotenes and tocopherols (Karadag et al., 2009) that are found in RPO. Based on this, we can assume that the TEAC assay is more sensitive and more applicable to our study, since we supplemented with plant extracts that are rich in both hydrophilic and lipophilic antioxidants. The fact that the combined supplementation resulted in an increase in plasma total polyphenol levels and TAC measured as TEAC, showed that there may be a positive interaction in the ability of both extracts to increase the plasma polyphenol and TAC and that there may be a threshold that has to be reached before a significant increase is evident.

Results from this study showed that chronic supplementation of the aqueous rooibos extract, RPO or their combination for 22 weeks did not have any deleterious effects on the growth of the rats, as body weight gains remained similar across all treatment groups. However, all the rats in the treatment groups maintained lower absolute- and relative liver weights compared to the control rats, and no clear reason could be offered to explain this. Furthermore, no mortality was recorded throughout the 22 weeks of supplementation and fluid intakes in all the supplementation groups were similar to those of the control group. A widely used measure of safety of medicinal and plant extracts in literature is to determine their effect on serum levels of liver and kidney function markers. Increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) are used as surrogate markers for hepatic injury. In this study, we observed that chronic feeding of aqueous rooibos extract, RPO or their combination for 22 weeks resulted in no significant changes in the serum levels of ALP, ALT, AST and LDH and that it was similar to those of the control animals. The same trend was also observed in the serum levels of kidney function markers (albumin, creatinine, blood urea nitrogen, and uric acid), that were also similar to those of the control group consuming water. Therefore, our result showed that chronic feeding of rooibos; RPO or their combination for 22 weeks did not exhibit adverse hepatic or renal effects. The result of the effect of rooibos, RPO or their combination on the liver was further corroborated by the results of the histopathological examination of the liver tissues, which showed that liver tissues from all the supplementation groups exhibited normal histoarchitecture, similar to those of the control. This observation from our study becomes all the more important, especially with recent cases of possible adverse hepatic effects of rooibos. Sinisalo et al. (2010) reported a case of a 42-year-old patient diagnosed with a low grade B-cell malignancy, Waldenstrom's macroglobulinemia, six years earlier, who temporarily experienced elevated liver enzymes after consuming relatively large amount of rooibos. The study however re-affirms the excellent safety record of rooibos and concluded that contamination of the rooibos herbal tea by some hepatotoxic compound, genetic predisposition and/or diseased state of the patient may be responsible for the effect. A recent study also reported a case of acute hepatitis and liver failure associated with daily ingestion of rooibos and buchu herbal tea in a 52-year-old man with a history of hyperlipidemia and stage III chronic kidney disease secondary to IgA nephropathy (Engels et al., 2013). Previous studies have reported on the safety of rooibos showing that it is hepatoprotective and does not influence liver functions negatively in animals (Ulicna et al., 2003; 2008; Marnewick et al., 2003) and humans (Marnewick et al., 2011), a fact that was confirmed by the current study. Again these results draw attention to the health-status of the individual being very important when considering supplementation with dietary antioxidants.

Increased ROS generation during oxidative stress may result in lipid peroxidation, which causes considerable alteration in the functions and structural organization of the cell membrane. Since lipid peroxidation is a well-known mechanism of toxicity exhibited by many xenobiotics, it is expected that antioxidant supplementation will augment the cell's endogenous antioxidant defence system, and therefore inhibit lipid peroxidation. Results from this study showed that in the plasma, chronic feeding of RPO alone or together with the aqueous rooibos extract significantly lowered the level of CDs while the MDA level was not affected by any of the treatment. In the liver, CD levels were also unaffected by either chronic supplementation of rooibos extract, RPO or their combination. Hepatic MDA did not respond to chronic feeding of either aqueous rooibos extract or RPO, but the combined feeding significantly lowered the MDA level, showing that the combination of the extracts elicits a protective antioxidant effect, a pointer to a positive interaction of the two extracts. The main endogenous enzymatic antioxidants are CAT, SOD, GPx and GR. These enzymes have different cellular localization and are responsible for reduction of different reactive oxygen species. SOD catalyses the dis-mutation of superoxide radicals to oxygen and hydrogen peroxide (H₂O₂) which is degraded by CAT to oxygen and water. GPx and GR are important enzymes of the glutathione defense system. GPx protects membrane lipids, protein and nucleic acids by being responsible for removal of a wide range of peroxides, ranging from organic hydro-peroxides to H₂O₂ using GSH as a co-substrate while GR regenerates GSH from GSSG at the expense of NADPH. Determination of the activity of antioxidant enzymes as a marker of protective role of chronic feeding of the rooibos extract, RPO or their combination, did not show any major effect in our study. In the erythrocytes, we observed a significant increase only in the activity of CAT as a result of combined feeding of the rooibos extract and RPO, while the other three enzymes (SOD, GPx and GR) remained unchanged by any of the feeding protocols. In the liver, SOD and GPx activities were not affected by any of the treatments; CAT activity was increased by chronic feeding of the rooibos extract alone or in combination with RPO while the combination treatment also increased the GR activity. Evidence from literature has shown that the effect of the consumption of polyphenol- and other antioxidant-rich diets on the activity of antioxidant enzymes remain contradictory. Some authors have reported no increase after consumption of a polyphenol-rich diet containing red wine, green tea, as well as fruits and vegetables in humans (Van der Gaag et al., 2000; Van den Berg et al., 2001; Young et al., 2002), while others have reported an increase in erythrocyte GPx and GR after consumption of a grape skin extract diet for one week (Young et al., 2000). Rat feeding experiments revealed that, green tea leaves caused an increase in SOD and CAT activity (Lin et al., 1998), thyme oil and thymol prevented age-induced decline in GPx and SOD in

ageing rat brain (Youdim and Deans, 2000), lycopene fed to young female rats increased erythrocyte activity of SOD, GPx and GR (Breinholt et al., 2000), while gavage of natural flavonoids to female rats decreased the activity of CAT, GPx and GR (Breinholt et al., 1999). These studies show that the responses of the antioxidant enzymes do not follow set patterns, but could be diet-, tissue- and species-specific (Crawford et al., 2000).

Reduced glutathione (GSH) is the major and most important intracellular thiol antioxidant in living organisms and plays important roles in a myriad of cellular functions, such as maintenance of reduced protein thiols and coordinating cellular antioxidant defence, including detoxification of hydrogen- and lipid peroxides, as well as direct non-enzymatic scavenging of free radicals, such as hydroxyl and superoxide radicals. Measuring the GSH level or its oxidized form (GSSG) is a universally accepted method of detecting oxidative stress and the GSH/GSSG ratio can serve as a good indicator of systemic oxidative status and disease risk. Results from this study showed that while blood GSSG remain similar across all treatment groups, chronic feeding of rooibos alone or in combination with RPO for 22 weeks significantly increased the GSH level in the blood, and consequently GSH/GSSG ratio was also significantly increased in the rooibos alone and combination group. Feeding RPO alone did not show any significant effect on either GSH level or the GSH/GSSG ratio. The increased GSH level may be ascribed to the ability of flavonoids in rooibos to increase GSH synthesis by up-regulating the mRNA expression of γ -glutamylcysteine synthetase (γ -GCS), the rate limiting enzyme in the GSH biosynthetic pathway, since previous studies have shown that polyphenolic compounds from plants increased the γ -GCS activity and GSH contents (Jeon et al., 2003; Chen et al., 2004; Moskaug et al., 2005) and the rooibos extract has a significantly higher total polyphenol content when compared to the RPO in the current study. In the liver, feeding rooibos, RPO or their combination for 22 weeks did not show any effect on the GSH level, however GSSG level was significantly reduced in the rooibos alone or the combination group resulting in an increased GSH/GSSG ratio in this groups. This observation may be as a result of the ability of the polyphenolic substances in rooibos to directly scavenge free radicals and prevent oxidation of GSH to GSSG thereby improving the redox status of the hepatic cells.

In conclusion, the present results suggest that chronic feeding of RPO alone to rats for 22 weeks neither induced any hepatic or renal toxic effect nor influence the antioxidant/oxidant balance (redox status) in the blood and liver of the rats significantly. Our results also show that chronic feeding of rooibos extract alone for 22 weeks did not induce any adverse hepatic or renal effect, but modulate the antioxidant/oxidant balance by increasing CAT, GSH (liver) and GSH/GSSG ratio (blood and liver). This study showed that feeding rooibos and RPO together for 22 weeks did not induce any deleterious effect on the liver and kidney, the combination treatment however improved the glutathione redox status (GSH and GSH/GSSG) in the blood and liver of the rats. We ascribed these observations to the polyphenolic compounds present in rooibos, since the magnitude of these effects was similar to what was obtained when rooibos was fed alone and RPO alone was ineffective. Also feeding rooibos and RPO together significantly increased plasma total polyphenol and antioxidant capacity measured as TEAC and decreased hepatic MDA even when rooibos or RPO were ineffective on this biomarker, however because of the magnitude of these effects, we could not ascribe the combine effects to a synergistic interaction between the water-soluble bioactive compounds in rooibos and the lipid soluble components in RPO. It is pertinent to say that since this study was conducted in apparently healthy rats, future studies should explore supplementation of these extracts in rats under extensive pathology or those that are pre- or post- exposed to an oxidative stress inducer to fully understand the mechanisms behind these observed effects.

Acknowledgements

Fermented rooibos (superior grade) plant material used in the study was a generous gift from Mr Arend Redelinghuys, Rooibos Limited, Clanwilliam, South Africa. RPO used is a gift from Prof Jacques Van Rooyen. The authors gratefully acknowledged the staffs of the Primate Unit, Stellenbosch University (Tygerberg Campus), South Africa for care and the maintenance of the animals, and Mr Fanie Rautenbach for technical assistance. Funding from the Cape Peninsula University of Technology is graciously acknowledged.

Conflict of Interest and Funding: The authors declare no competing financial interest.

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