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Antioxidant and Pro-oxidant Activities of Aqueous Extracts and Crude Polyphenolic Fractions of Rooibos (*Aspalathus linearis*)

ELIZABETH JOUBERT,*,† PAULA WINTERTON,# TREVOR J. BRITZ,# AND WENTZEL C. A. GELDERBLOM§

ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa; Department of Food Science, Stellenbosch University, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa; and PROMEC Unit, Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa

Unfermented rooibos tea is known to contain higher levels of total polyphenols and flavonoids than its fermented counterpart, making it the obvious choice for the preparation of flavonoid-enriched fractions. Evaluation of aqueous extracts and crude polyphenolic fractions of unfermented and fermented rooibos showed anti- and/or pro-oxidant activities, using a linoleic acid−Tween−buffer emulsion for lipid peroxidation and the deoxyribose degradation assay, based on a Fenton reaction model system containing $FeCl_3$−EDTA and H_2O_2 for the generation of hydroxyl radicals. Except for the ethyl acetate fraction, with the highest total polyphenol (TP) content and offering the least protection presumably due to pro-oxidant activity, the inhibition of lipid peroxidation by the samples correlated moderately with their TP content in a linear relationship ($r = 0.896$, $P < 0.01$). Using the deoxyribose degradation assay, the pro-oxidant activity of the aqueous extracts and their crude polymeric fractions (0.1 mg/mL in the reaction mixture) was linear with respect to their dihydrochalcone (aspalathin and nothofagin) ($r = 0.977$, $P = 0.023$) and flavonoid ($r = 0.971$, $P = 0.029$) content. Pro-oxidant activity was demonstrated for pure aspalathin. Using the same assay, but with ascorbate added to regenerate Fe^{3+} to Fe^{2+}, the aqueous extract and crude polymeric fraction of fermented rooibos displayed hydroxyl radical scavenging activity. Fermentation (i.e., oxidation) of rooibos decreased the pro-oxidant activity of aqueous extracts, which was contributed to a decrease in their dihydrochalcone content. The in vitro pro-oxidant activity displayed by flavonoid-enriched fractions of rooibos demonstrates that one must be aware of the potential adverse biological properties of potent antioxidant extracts utilized as dietary supplements.

KEYWORDS: ***Aspalathus linearis*; aspalathin; nothofagin; dihydrochalcone; lipid peroxidation; Fenton reaction; pro-oxidant; hydroxyl radical scavenging**

INTRODUCTION

Polyphenol-enriched plant extracts are used by the functional food industry as antioxidants to enhance the apparent health-promoting properties of food products. Selective extraction and/or fractionation is often used to enhance the antioxidant potency of extracts. The plant, *Aspalathus linearis*, traditionally used in "fermented" (oxidized) form as a herbal tea, known as rooibos, is considered to be a good source of the dihydrochalcone aspalathin (**Figure 1**). Aspalathin and its dehydroxy analogue, nothofagin (**Figure 1**), are the major flavonoids of unfermented rooibos (*1*), comprising in some instances as much as 9.3 and 1.03% of the plant material (dry basis), respectively (*2*). In vitro studies showed that fermentation significantly decreases the antioxidant activity of rooibos (*3*, *4*). Less than 7% of the aspalathin content is retained after fermentation (*1*). This resulted in a demand for green (unfermented) rooibos as herbal tea and as source material for the preparation of antioxidant-enriched extracts, especially in terms of aspalathin for the global nutraceutical and cosmetic industries.

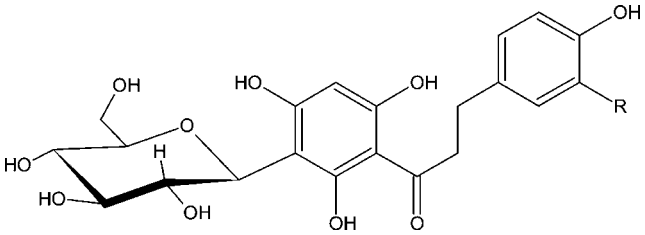

Figure 1. Structure of aspalathin (R = OH) and nothofagin (R = H), dihydrochalcones found in rooibos (*A. linearis*).

Aspalathin contributes ~43% of the total antioxidant capacity of aqueous extracts of unfermented rooibos (*2*). It is of comparable radical scavenging potency to the well-known

* Corresponding author (telephone +27 21 809 3444; fax +27 21 809 3400; e-mail JoubertL@arc.agric.za)
† ARC Infruitec-Nietvoorbij.
# Stellenbosch University.
§ PROMEC Unit, MRC.

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flavonoid antioxidants, quercetin and epigallocatechin gallate (*5*−*7*). Its potency is determined by the 3,4-dihydroxyl arrangement of the B ring, the 2′,6′-dihydroxyacetophenone group (*8*), and the keto−enol transformation of the carbonyl group that stabilizes the radical after hydrogen abstraction (*9*). Other flavonoids present in low quantities comprise orientin, isoorientin, vitexin, isovitexin, quercetin, luteolin, isoquercitrin, rutin, chrysoeriol (*10*), (+)-catechin, procyanidin B1 (*11*), and hyperoside (*12*).

The beneficial effects of plant antioxidants mainly focus on their protective role against excessive oxidative damage induced by reactive oxygen species (ROS) (*13*). However, from a health perspective one must be aware that potent antioxidants can also display pro-oxidant activities, leading to oxidative damage of cellular components (*14*, *15*). Apart from the destructive properties toward cellular components, ROS also play an important role in many normal cell functions and are key mediators of apoptosis, which eliminates cancer cells (*16*). The antioxidant/pro-oxidant properties of flavonoids could therefore be important in determining the fate of a cell, and the biological response could either be beneficial or deleterious, depending of the oxidative status prevailing within a cell (*17*).

Studies carried out using aqueous rooibos extracts demonstrated beneficial effects related to its phenolic composition and antioxidant activity. Chronic administration of rooibos tea to rats suppressed age-related accumulation of lipid peroxides in several regions of the brain (*18*). Rooibos tea increased the antioxidant status of rat liver in vivo by reducing the levels of oxidized glutathione and increasing the levels of reduced glutathione (*19*). It restored the CoQ_9H_2 and α-tocopherol concentration and inhibited lipid peroxidation in the liver of CCl_4-treated rats (*20*).

In recent studies the in vitro superoxide anion radical and α,α-diphenyl-β-picrylhydrazyl radical scavenging abilities of rooibos extracts, crude polyphenolic fractions, and flavonoids (*7*), as well as the effect of processing on the scavenging of these radicals (*4*), were evaluated. In an attempt to gain better understanding of the antioxidant behavior of these extracts and crude polyphenolic fractions, their ability to inhibit linoleic acid peroxidation was evaluated in the present study. Because several of the rooibos flavonoids fulfill the structural requirements for pro-oxidant activity (*21*), the potency of the extracts and fractions as pro-oxidants, as well as the role of processing in modulating the pro-oxidant potency, was evaluated in terms of their ability to enhance oxidative breakdown of deoxyribose, using a Fenton system.

MATERIALS AND METHODS

Chemicals. Sigma Chemical Co., St. Louis, MO, supplied linoleic acid (purity > 99%), polyethylene sorbitan monolaurate (Tween 20), 2-deoxy-*o*-ribose, (+)-catechin, and butylated hydroxytoluene (BHT), whereas Aldrich Chemical Co., Milwaukee, WI; Riedel-de Haen, Seelze-Hannover, Germany; and Merck, Bellville, South Africa, supplied 2-thiobarbituric acid (TBA), gallic acid, and Folin−Ciocalteu's phenol reagent, respectively. Orientin, iso-orientin, vitexin, luteolin, chrysoeriol, and isoquercitrin were purchased from Carl Roth GmbH, Karlsruhe, Germany. Rutin, quercetin, and isovitexin were purchased from Extrasynthese, Genay, France. Aspalathin [ASP; purity > 95% as determined by ^1H and ^{13}C NMR (*10*)], crude ASP (C-ASP; 46%), and tannin (TAN), isolated from unfermented rooibos (*22*), were kindly supplied by Dr. Daneel Ferreira, National Center for Natural Products Research, School of Pharmacy, University of Mississippi, USA. Nothofagin (purity > 95% as determined by MS) was supplied by Petra Snijman, PROMEC Unit, MRC, Tygerberg, South Africa.

All other reagents were of analytical reagent (AR) grade, except for HPLC grade methanol. Deionized carbon and RO treated water (conductivity level ~ 0.1 μs/cm) was obtained with a Modulab water purification system (Separations, Cape Town, South Africa). Additional purification was done with a Milli-Q academic system (Microsep, Bellville, South Africa) to obtain extrapure water for HPLC. Glassware was soaked overnight in a 0.3% (m/v) EDTA solution, followed by thorough rinsing with purified water before use to remove traces of iron.

Preparation of Plant Material. Processed rooibos (fermented; Choice grade; 35 kg bulk packaging), for preparation of the aqueous extract and its fractions, was supplied by a major processing company. The unfermented rooibos was prepared by drying plant material (~10 kg) harvested from a 4-year-old plantation (M. Bergh, Zeekoeivlei, Clanwilliam, South Africa) for 30 h at 40 °C in an experimental forced-air circulation drying tunnel. The plant material was not shredded prior to drying to prevent enzymatic oxidation. Both the fermented and unfermented tea materials were pulverized with a Retch mill (1.0 mm sieve) and stored in airtight containers at room temperature in the dark until used.

To study the effect of rooibos processing on pro-oxidant activity, seven samples (~1 kg each) from each of the following processing stages were randomly collected at a commercial processor: before fermentation (unfermented); directly after fermentation (followed by tunnel-drying at 40 °C), after sun-drying; and after steam pasteurization of sieved material as described by Standley et al. (*4*).

Preparation of Aqueous Extracts and Crude Polyphenolic Fractions. Aqueous extracts of fermented (AQF) and unfermented (AQU) rooibos and their respective crude polyphenolic fractions, comprising mainly monomeric (EAU and EAF) and polymeric (CPU and CPF) compounds, were prepared as described by Joubert et al. (*7*). Preparation entailed exhaustive extraction of the aqueous extracts with ethyl acetate to remove most of the monomeric flavonoids, leaving mainly the polymeric compounds in the aqueous layer. Samples of the different processing stages were subjected to aqueous extraction (*4*), and aliquots, after gravimetric determination of the soluble solids concentration, were stored at −20 °C until analyses.

Total Polyphenol (TP) and Flavonoid Content. The TP content of the samples, determined in duplicate with the Folin−Ciocalteu assay (scaled down to a 5 mL final volume) (*23*), was quantified in terms of gallic acid. Determination of individual flavonoids of the samples was conducted by HPLC (*1*), utilizing a Waters LC Module Plus system with automated injection (10 μL), a PDA 2996 detector, and Millenium 32 version 4 software for data acquisition and system control. The dihydrochalcones and flavone/flavonols were detected at 288 and 255 nm, respectively. A calibration curve for each compound, except for isoquercitrin and rutin which coeluted, was prepared close to the concentrations expected in the samples. Isoquercitrin and rutin were quantified in terms of quercetin. Analyses were carried out in duplicate.

Inhibition of Linoleic Acid Peroxidation. The assay was conducted according to the method of Lingnert et al. (*24*), determining the conjugated dienes at 234 nm. The emulsion mixture, comprising linoleic acid (10 mM), Tween 20 (0.5%; v/v), and potassium phosphate buffer (0.1 M; pH 6.5), was sonicated for 1 min at power level 8 (Virsonic 60 sonicator, The Virtis Co., Inc., Gardiner, NY). An aliquot (0.1 mL) containing 0.1 mg of the test sample was added to 2 mL of the emulsion in separate test tubes and vortexed (Vortex-2 Genie, Scientific Industries Inc., Bohemia, NY) for 5 s. AQU, AQF, CPU, CPF, and TAN were dissolved in purified water, whereas EAU, EAF, and C-ASP were dissolved in methanol. Two different solvent controls and sample blanks without linoleic acid were included. The sample blanks compensated for any interference due to absorbance at 234 nm. The emulsions, covered with Parafilm, were incubated in the dark at 40 °C. Aliquots (0.2 mL), removed periodically over a 21 h period, were solubilized in methanol (2 mL) and then further diluted with 60% methanol−water (6 mL) before the absorbance (as indication of conjugated dienes) was determined. Four replicate ($n = 4$) experiments were carried out, and absorbance measurements were done in duplicate. The end-point data at 14 and 21 h were used to calculate percent inhibition: $(A_C - A_{AO})/A_C \times 100$, where A_C and A_{AO} are the absorbance of control and test samples, respectively.

Oxidative Degradation of Deoxyribose in a Fenton System. The pro-oxidant ability of the samples, including ASP, was evaluated in

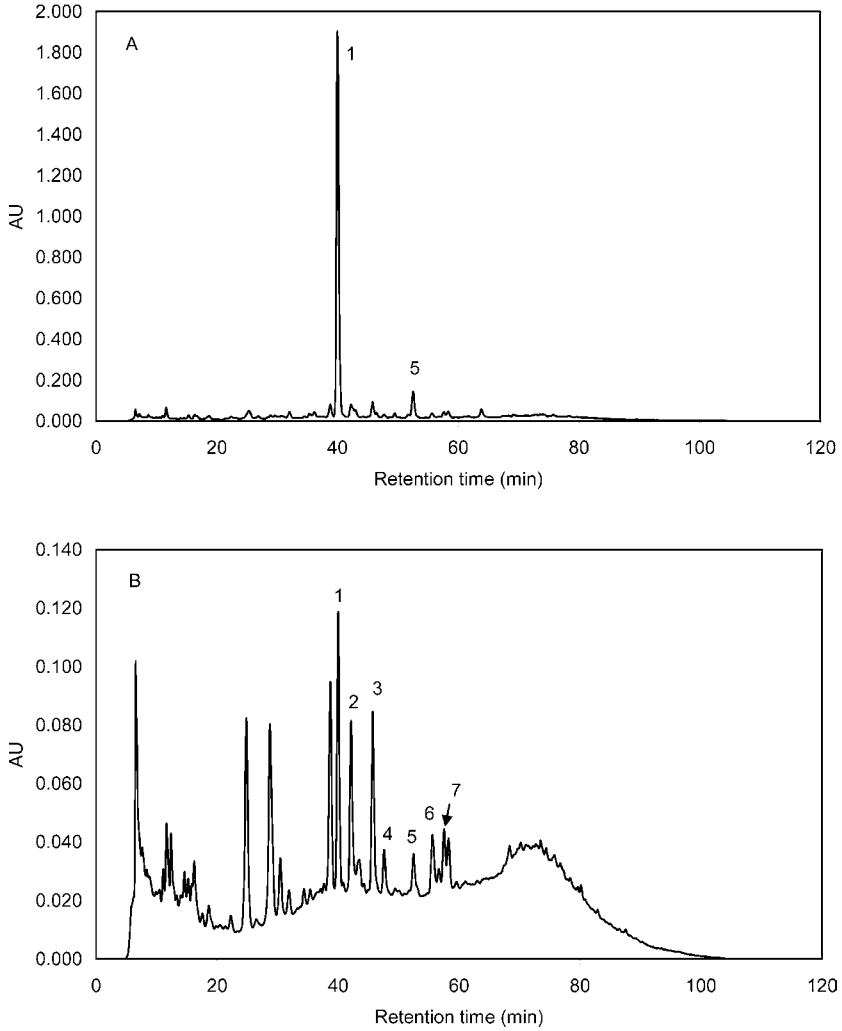

Figure 2. Typical HPLC reversed-phase chromatograms of (**a**) AQU and (**b**) AQF at 288 nm. The major identified compounds are indicated on the chromatograms: aspalathin (1), orientin (2), iso-orientin (3), vitexin (4), nothofagin (5), isovitexin (6), isoquercitin + rutin (7). Notation is as in **Table 2**.

terms of their ability to enhance the oxidative degradation of deoxyribose through regeneration of Fe^{3+} to Fe^{2+}. A Fenton reaction model system containing $FeCl_3$−EDTA and H_2O_2 was used (*25*, *26*). The final reaction mixture (1.2 mL), which contained the test sample, deoxyribose (2.8 mM), H_2O_2 (2.8 mM), KH_2PO_4−K_2HPO_4 buffer (20 mM, pH 7.4), $FeCl_3$ (25 μM), and EDTA (100 μM), was incubated at 37 °C for 1 h. EDTA and $FeCl_3$ were premixed just prior to addition to the reaction mixture. The extent of deoxyribose degradation was determined by adding 1.0 mL of TBA solution [1.0% (m/v) TBA dissolved in 0.05 M NaOH] and 1.0 mL of 2.8% (m/v) trichloroacetic acid to the reaction mixture, followed by incubation at 100 °C for 15 min in a water bath. The absorbance was measured spectrophotometrically at 532 nm and the amount of thiobarbituric acid reactive substances (TBARS) calculated as malondialdehyde (MDA) equivalents using a molar extinction coefficient of 159 000 (*27*). Controls without the test samples and sample blanks without deoxyribose were incubated to compensate for substances other than TBARS absorbing.

AQU, AQF, CPU, CPF, EAU, TAN, and C-ASP were tested at equal soluble solid concentrations of 0.1 and 1.0 mg/mL. Similarly, EAF, C-ASP, and ASP were compared to EAU, but using methanol as solvent. In this case methanol was also added to the control. The aqueous extracts of rooibos obtained from the different processing stages were tested at equal soluble solid and total polyphenol contents of 0.5 and 0.15 mg/mL, respectively. To demonstrate that the rooibos soluble solids (AQU, AQF, CPU, CPF, EAU, and EAF) could also scavenge $^{\bullet}OH$, thereby mainly acting as antioxidants (*26*), ascorbate (100 μM) was added to the $FeCl_3$−EDTA/H_2O_2/deoxyribose reaction mixture to recycle Fe^{3+}. Testing was carried out over the concentration range of 0.1−2 mg/mL. Methanol was once again included for EAF and EAU. Three replicates ($n = 3$) of all experiments were carried out, and absorbance measurements were done in duplicate.

The spectrophotometric readings of the different assays were conducted on a Beckman DU 65 UV−vis spectrophotometer (Beckman, Cape Town, South Africa), using a quartz cuvette (1 cm).

Statistical Analysis. The means were subjected to one-way ANOVA, and Student's *t*-LSD test (SAS release version 6.12) was calculated at the 5% level to compare treatment means. The Shapiro−Wilk's test was performed to test for non-normality. End-point data for linoleic acid peroxidation (percent inhibition) were compared for statistical differences between samples. Regression analysis was carried out on the data describing the change in conjugated diene formation with time. When intercepts and gradients did not differ significantly, data were pooled and a single line was fitted. Correlation coefficients between phenolic content and inhibition of linoleic acid peroxidation, as well as formation of TBARS, were calculated to identify major contributors to antioxidant and pro-oxidant effects.

RESULTS

Phenolic Composition. Typical HPLC chromatograms for AQU and AQF at 288 nm are depicted in **Figure 2**. EAU, prepared from unfermented rooibos, contained the highest total polyphenol and flavonoid contents (**Table 1**). Aspalathin was the major monomeric flavonoid of CPU, AQF, EAU, and C-ASP, comprising 5.3, 12.3, 54.7, and 46.1% of the soluble solids, while contributing as much as 14.7, 28.5, 73.7, and 86.8%

Table 1. Phenolic Composition of Rooibos Aqueous Extracts, Crude Polymeric Fractions, and Tannin

sample[a]	TP[b,c]	TF[b,c]	DHC[b,c]	flav[b,c]	aspal/TP[d]	aspal[b,c]	nothof[b,c]	orien[b,c]	iso-orien[b,c]	vitex[b,c]	isovitex[b,c]	isoq + rutin[b,c]	other[b,c]
AQF	34.25	2.96	0.78	2.08	1.63	0.61	0.17	0.76	0.85	0.17	0.11	0.11	0.08
AQU	39.30	15.22	13.38	1.85	28.45	12.29	1.08	0.59	0.81	0.13	0.17	0.16	trace
CPF	31.62	2.17	0.47	1.71	1.35	0.47	nd	0.54	0.76	0.12	0.21	0.09	trace
CPU	32.92	7.27	5.60	1.67	14.70	5.32	0.28	0.43	0.82	0.07	0.16	0.19	trace
EAF	55.82	18.26	5.75	12.51	5.93	3.64	2.11	5.53	3.01	1.88	1.49	0.60	trace
EAU	67.52	72.27	64.71	7.56	73.69	54.70	10.01	2.51	1.92	0.84	1.36	0.93	trace
C-ASP	48.30	66.26	63.94	2.32	86.82	46.10	17.84	nd	nd	nd	2.32	nd	nd
TAN	26.45	na	na	na	na	na	na	na	na	na	na	na	na

[a] Sample notation as in **Table 2**. [b] TP, total polyphenols [expressed as GAE; from Joubert et al. (*7*)]; TF, total flavonoids (summary of individual compounds as determined by HPLC); DHC, dihydrochalcones; flav, flavones and flavonols; aspal, aspalathin; nothof, nothofagin; orien, orientin; iso-orien, iso-orientin; vitex, vitexin; isovit, isovitexin; isoq + rutin, isoquercitrin + rutin; other, quercetin, luteolin, and chrysoeriol. [c] Results expressed as mass percentage of solids. [d] Aspalathin (expressed as GAE) as percent of TP. GAE conversion factor for aspalathin = 0.9096. [e] Not detected. [f] Not analyzed.

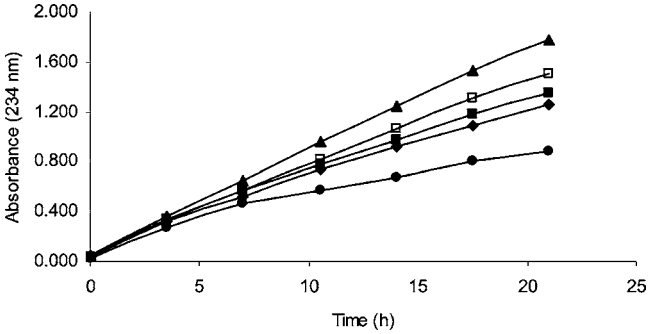

Figure 3. Time-dependent change in absorbance due to formation of conjugated dienes during linoleic acid peroxidation in a linoleic acid–Tween 20–phosphate buffer emulsion (pH 6.5) in the presence of rooibos extracts and polyphenolic fractions: AQF, AQU, and C-ASP combined (◆); CPU and CPF combined (■); controls with and without methanol (▲); EAU and TAN combined (□); EAF (●). Notation is as in **Table 2**.

Table 2. Inhibitory Effect of Aqueous Extracts and Crude Polyphenolic Fractions of Rooibos on Formation of Conjugated Dienes in a Linoleic Acid Emulsion

test sample	inhibition[a] (%)
EAF: ethyl acetate soluble fraction, fermented rooibos	48.05 ± 8.55d[b]
C-ASP: crude aspalathin	28.46 ± 10.67c
AQU: aqueous extract, unfermented rooibos	28.57 ± 17.62c
AQF: aqueous extract, fermented rooibos	28.04 ± 15.24c
CPF: crude polymeric fraction, fermented rooibos	23.49 ± 13.61bc
CPU: crude polymeric fraction, unfermented rooibos	22.59 ± 11.95bc
TAN: tannin	18.73 ± 11.21abc
EAU: ethyl acetate soluble fraction, unfermented rooibos	10.96 ± 4.50ab

[a] Inhibition after 21 h. [b] Means ± STD (number of replicates = 4) within the column followed by a different letter(s) differ significantly ($P < 0.05$).

of their total polyphenol content, respectively. Nothofagin was present in substantial quantities in AQU, EAF, EAU, and C-ASP, varying from 1.1 to 17.8% of the soluble solids. The total dihydrochalcone contents of C-ASP and EAU were 64.7 and 63.9%, respectively. Samples prepared from unfermented rooibos contained substantially more dihydrochalcones than their respective fermented counterparts. The highest levels of the flavone analogues of aspalathin (orientin and iso-orientin) and nothofagin (vitexin and isovitexin) were present in EAF and EAU, with the fraction prepared from fermented rooibos containing higher levels of these analogues than their unfermented rooibos counterparts. Only trace amounts of chrysoeriol, luteolin, and quercetin were detected in the samples, except for very low quantities in AQF.

In Vitro Inhibition of Lipid Peroxidation. The time-dependent inhibition of the formation of conjugated dienes from linoleic acid over a 21 h period in the two controls and samples is illustrated in **Figure 3**. The rate of lipid peroxidation did not differ between the control containing a small quantity of methanol and the control without methanol, and the data are therefore presented as a single line. Values obtained for C-ASP, AQU, and AQF, as well as CPF and CPU, did not differ significantly and were combined to represent two lines. No lag phase was present for any of the samples, but all inhibited lipid peroxidation to various degrees compared to the controls. The highest inhibition was obtained with EAF ($P < 0.05$), whereas EAU offered the least protection (**Table 2**). After 14 h, there was no significant difference between the aqueous extracts (AQU and AQF) and their respective crude polymeric fractions (CPU and CPF) (data not shown), but at 21 h they offered slightly better protection than CPU and CPF. When EAU was omitted from the sample set, the percent inhibition of the samples moderately correlated with their total polyphenol content in a linear relationship ($r = 0.896$, $P < 0.01$).

In Vitro Modulation of Oxidative Deoxyribose Breakdown. A preliminary test carried out with aqueous extracts, tested at 0.5 mg/mL increments in the reaction mixture, indicated that deoxyribose degradation is concentration dependent (data not shown). Maximum deoxyribose degradation was displayed at a soluble solid content of 0.5 mg/mL, whereas at ~>1.7 mg/mL net inhibition of deoxyribose degradation was obtained. The relative pro-oxidant potency of rooibos aqueous extracts, crude polyphenolic fractions, and tannin, determined at soluble solids concentrations of 0.1 and 1.0 mg/mL in the reaction mixture, is summarized in **Table 3**. At a dose level of 0.1 mg/mL all samples exhibited a pro-oxidant effect with the pro-oxidant potency decreasing in the following order: EAU > AQU > CPU > CPF ≥ TAN ≥ AQF ($P < 0.05$). The pro-oxidant potency of these samples at 0.1 mg/mL correlated with their dihydrochalcone content in a nonlinear relationship (lin x; $R^2 = 0.943$). By excluding EAU with its very high dihydrochalcone content, as well as TAN with no dihydrochalcones, from the dataset, significant linear relationships of TBARS with the dihydrochalcone ($r = 0.977$, $P = 0.023$) and total flavonoid ($r = 0.971$, $P = 0.029$) contents, but not with total polyphenol content ($P = 0.693$), were obtained. At 1.0 mg/mL only EAU and TAN significantly enhanced deoxyribose degradation; however, their effect was less than that at 0.1 mg/mL, especially for EAU.

The comparative pro-oxidant potency of EAU, EAF, C-ASP, and ASP relative to the control containing methanol is summarized in **Table 3**. The ability to degrade deoxyribose at 0.1

Table 3. Deoxyribose[a] Degradation in the Presence of Rooibos Aqueous Extracts, Crude Polyphenolic Fractions, Tannin, and Aspalathin

	TBARS[b] (μM MDA equiv)	
test sample[c]	0.1 mg/mL	1.0 mg/mL
control (with water)	3.48 ± 0.12f[d]	3.57 ± 1.04c[d]
AQU[e]	10.42 ± 0.04b	4.61 ± 0.71bc
AQF[e]	5.71 ± 0.13e	4.46 ± 0.53bc
CPU[e]	8.53 ± 0.04c	4.65 ± 0.59bc
CPF[e]	5.88 ± 0.04d	4.45 ± 1.06bc
EAU[e]	15.02 ± 0.10a	7.41 ± 1.08a
TAN[e]	5.74 ± 0.08ed	5.51 ± 0.71ab
control (with methanol added)	0.70 ± 0.01e	0.70 ± 0.01cd
EAF[f]	3.01 ± 0.45d	1.09 ± 0.04bc
EAU[f]	7.60 ± 1.45bc	1.50 ± 0.39ab
C-ASP[f]	8.50 ± 0.10b	1.52 ± 0.06ab
ASP: pure aspalathin[f]	9.50 ± 0.13a	2.06 ± 0.04a

[a] •OH generation in an $FeCl_3$–EDTA/H_2O_2 system in the absence of ascorbate. [b] Final concentration of soluble solids in the reaction mixture. [c] Notation as for **Table 2**. [d] Means ± standard deviation (number of replicates = 3) within a column followed by a different letter(s) differ significantly ($P < 0.05$). Test samples for each control were analyzed separately. Samples dissolved in water only should not be compared with samples containing a small quantity of methanol. [e] Test samples were dissolved in deionized water. [f] Test samples were dissolved in a small quantity of methanol.

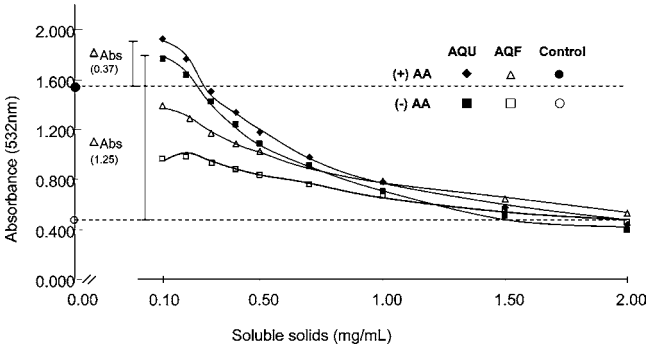

Figure 4. Dose-dependent effect of rooibos aqueous extracts on Fe^{3+}-EDTA/H_2O_2-induced deoxyribose degradation in the presence [control (●); AQU (◆); AQF (△)] and absence [control (○); AQU (■); AQF (□)] of ascorbate (AA). Notation is as in **Table 2**.

mg/mL decreased in the following order: ASP > C-ASP ≥ EAU > EAF ($P < 0.05$). A similar order was displayed at 1.0 mg/mL, but with substantially less pro-oxidant activity.

The dose-dependent deoxyribose degradation by AQU and AQF, tested both in the presence and in the absence of ascorbate for comparative purposes, is illustrated in **Figure 4**, using increase in absorbance as an indication of deoxyribose degradation. Similar to the previous experiment at fixed concentrations, the pro-oxidant behavior (in the absence of ascorbate) was substantially less marked for AQF compared to AQU, but ~1.5 mg/mL of AQU compared to ~1.8 mg/mL of AQF was needed to give net inhibition of deoxyribose degradation.

Addition of ascorbate to the reaction mixture effectively recycled Fe^{3+} to Fe^{2+}, indicated by the high absorbance value for the control. At 0.1 mg/mL of AQU deoxyribose degradation was significantly enhanced, giving a considerable increase in absorbance compared to the control (Δ Abs = 1.251), but to a lesser extent than when ascorbate was present (Δ Abs = 0.366) (**Figure 4**). When AQU reached a level of >0.3 mg/mL deoxyribose degradation was less than for the control, suggesting net scavenging of •OH. For AQF at all concentrations, deoxyribose degradation was less than for the control. Similar trends were obtained for CPU and CPF. Because methanol was required to solubilize EAF, it was also added to EAU for comparative purposes. Subsequently, addition of ascorbate had a small effect in the case of the control and samples (results not shown).

Table 4. Effect of Different Processing Stages of Rooibos on Deoxyribose[a] degradation

	TBARS (μM MDA equiv)	
processing stage	soluble solids[b] (0.5 mg/mL)	total polyphenols[b,c] (0.15 mg/mL)
control	4.55 ± 0.81d[d]c	4.55 ± 0.81c[d]c
unfermented	6.67 ± 0.39ac	8.17 ± 0.26a
fermented	5.64 ± 0.30bc	5.69 ± 0.26b
sun-dried	5.51 ± 0.04bc	5.65 ± 0.28b
steam pasteurized	5.52 ± 0.04bc	5.78 ± 0.26b

[a] •OH generation in an $FeCl_3$–EDTA/H_2O_2 system in the absence of ascorbate. [b] Final concentration in the reaction mixture. [c] Expressed as mg gallic acid equivalents (GAE). [d] Means ± standard deviation (number of replicates = 3) within a column followed by a different letter(s) differ significantly ($P < 0.05$).

The only processing stage that affected pro-oxidant activity was the fermentation step, resulting in decreased pro-oxidant activity (**Table 4**). Subsequent processing steps, that is, sun-drying and steam pasteurization, did not affect pro-oxidant activity of the aqueous extract. A similar trend was observed when the extracts were compared on the basis of equal TP content.

DISCUSSION

A range of flavonoid-enriched plant extracts is widely marketed for their antioxidant properties and the associated beneficial health properties (*28*). However, their use as dietary supplements should be considered with caution as they could also exhibit mutagenic and/or pro-oxidant effects (*14*, *15*). Conditions such as the availability of iron or the level of ascorbate may modulate the beneficial properties of these compounds in vivo (*14*).

Interaction of polyphenolic constituents with free radical species in different phases provides different perspectives on their antioxidant/pro-oxidant properties. Adverse biological effects could be obtained in in vitro systems over a wide range of concentration levels of the flavonoids depending on the specific assay or model system. The antioxidant activity of polyphenolic extracts of rooibos, previously screened for radical scavenging activity (*7*), was therefore also evaluated in a linoleic acid emulsion, to allow for the effect of partitioning of the compounds in a multiphase system, interphase transport, and surface accessibility (*29*−*32*) on their relative potency as antioxidants. Inhibition of lipid peroxidation showed that the relative efficacy of the different extracts and fractions depended on their total polyphenol content, and not the total or individual flavonoids. A similar effect was noticed in the DPPH and superoxide anion radical scavenging assays, where a decrease in TP was associated with a weaker scavenging potency of the samples (*7*). However, EAU, being the most potent radical scavenger of the different extracts and fractions (*7*) and with the highest total polyphenol content, had the lowest inhibitory effect on linoleic acid peroxidation. On the other hand, rooibos tannin, with the weakest radical scavenging potency (*7*) and lowest TP content, also displayed weak inhibition of lipid peroxidation. The crude polymeric fractions, CPU and CPF, containing <50% of the total polyphenols of EAU and shown

to be weak scavengers of DPPH and superoxide anion radicals (*7*), were more effective as inhibitors of linoleic acid peroxidation than EAU. These apparent discrepancies could be explained if pro-oxidation, due to the high concentration of total flavonoids, dihydrochalcones, and/or total polyphenols, is considered. Frankel et al. (*33*) showed that high concentrations of green tea extract resulted in pro-oxidation. C-ASP, with a substantially lower TP content than EAU, was a more effective inhibitor of lipid peroxidation, although their total dihydrochalcone contents were similar, indicating the contribution of other flavonoids in EAU to the resulting antioxidant/pro-oxidant effect.

Potent antioxidants, for example, epigallocatechin gallate (EGCG) (*34*, *35*) and gallic acid (*36*, *37*), display strong pro-oxidant activity in the deoxyribose degradation assay, due to their ability to reduce Fe^{3+} to Fe^{2+}. It was therefore to be expected that aspalathin, a potent antioxidant (*5*, *7*), having an antioxidant potency (radical scavenging ability) comparable to that of EGCG (*6*), would display pro-oxidant activity in this test system as was reported here for the first time. The structural attributes of a dihydrochalcone, for example, the presence of the 2′,6′-dihydroxyacetophenone group (*8*) and keto−enol tautomeric transformation between the carbonyl group and the α-methylene (*9*) conferring antioxidant activity, would, however, also be responsible for pro-oxidant activity in the presence of a transition metal (*21*, *38*). Nothofagin is structurally similar to aspalathin, except for the hydroxylation pattern of B ring (**Figure 1**), and could thus also be expected to contribute to pro-oxidant activity.

The pro-oxidant activity of the rooibos aqueous extracts, polyphenolic fractions, C-ASP, and TAN was subsequently investigated. Their ability to recycle Fe^{2+} in the absence of ascorbate in the so-called Fenton reaction was taken as an indication of pro-oxidant activity with the stimulation of deoxyribose degradation as endpoint (*39*, *40*). Because the total dihydrochalcone content of C-ASP and EAU, and not only their aspalathin, flavone, or TP contents, explained the similar pro-oxidant potencies obtained, it would also confirm that nothofagin also plays a major role. The higher levels of these dihydrochalcones in the aqueous extracts and fractions prepared from unfermented plant material would explain their increased pro-oxidative potency compared to their fermented counterparts.

Other phenolic compounds in the aqueous extracts, crude polymeric fractions, and TAN would either be inactive or contribute to pro-oxidant activity to a lesser degree. Rooibos tannin is of the procyanidin type, with (+)-catechin and (−)-epicatechin as chain-extending units (*22*). Hagerman et al. (*41*) reported only weak pro-oxidant activity for a procyanidin polymer with (−)-epicatechin subunits and (+)-catechin as a terminating unit. Quercetin, shown to be a highly potent pro-oxidant in this test system (*40*), is present in trace quantities in the test samples. Orientin, present in a substantial quantity in EAU and EAF, does not act as a pro-oxidant in the Fenton assay (*42*). Similarly, it could be expected that iso-orientin would not display pro-oxidant behavior. According to Duarte Silva et al. (*21*), the absence of the C-3 OH group is responsible for the low pro-oxidant potency. Rutin, another rooibos flavonoid, also exhibited very little pro-oxidative effects in the deoxyribose assay due to the glycosylation of the 3-OH group on the C ring (*21*).

When the dose-dependent pro-oxidant responses of the rooibos aqueous extracts and fractions (1.0 versus 0.1 mg/mL) are considered, their ability to either scavenge •OH or stimulate generation of •OH is of importance. In this regard the concentration and nature of the phenolic compounds would eventually determine the antioxidant and/or pro-oxidant potency of flavonoid-enriched extracts in a specific biological system. This became apparent in the present study when scavenging of •OH was still outweighed by the generation thereof at 1 mg/mL, resulting in a net pro-oxidant effect. The dose−response curves for AQF and AQU indicated that the concentration of soluble solids in the extract needs to be increased substantially before a net antioxidant effect could be obtained. The dihydrochalcone concentrations in these reaction mixtures were 31 and 445 μM, respectively. This concentration effect was also demonstrated for ascorbate concentrations, with a net antioxidant effect at concentrations >5 mM (*43*).

To confirm scavenging of •OH by the rooibos extracts, ascorbate was added to the reaction mixture as it increases the rate of •OH generation by reducing the Fe^{3+}-EDTA complex and maintaining a supply of Fe^{2+}-EDTA. Inhibition of deoxyribose degradation by an antioxidant under these conditions indicates scavenging of •OH (*44*). The stimulation of •OH formation is not additive to that obtained with ascorbate alone, and antioxidants take part in the redox cycling of Fe^{3+}, but at a much lower rate than ascorbate (*45*). Because AQF could not effectively compete with ascorbate in the redox recycling of Fe^{3+}, no pro-oxidant activity was displayed under these conditions. AQU with its higher dihydrochalcone content displayed pro-oxidant activity at low concentrations, whereas a net antioxidant effect was observed at >0.3 mg/mL (>89 μM dihydrochalcone). On the basis of rate constants Lee and Jang (*46*) found that aqueous and 75% ethanol extracts of rooibos (fermented), with substantially lower TP and flavonoid contents than the extracts and fractions used in the present study, showed poor scavenging of the hydroxyl radical. The decrease in deoxyribose was attributed to chelating of Fe^{3+}. This aspect needs to be clarified in the future.

The role of fermentation in modulating the pro-oxidant potency of rooibos was further investigated by monitoring the decrease in deoxyribose degradation in the absence of ascorbate by the aqueous extracts, prepared from a selection of plant material collected at different processing stages. Only the oxidation step (fermentation), initiated with shredding and bruising of the plant material, significantly decreased the pro-oxidant potency of the aqueous extract. It is attributed to the substantial loss in dihydrochalcone content with oxidation (*1*). Further changes in phenolic composition due to sun-drying as manifested by a darker product (*47*) did not affect pro-oxidant potency. Steam pasteurization conditions used by the industry also did not affect the pro-oxidant potency of rooibos, similar to previous findings for antioxidant and antimutagenic activities (*4*).

The generation of •OH by rooibos, in the presence of Fe^{3+}-EDTA at the physiological pH of 7.4, suggests that these preparations could stimulate oxidative damage by •OH in the presence of iron, especially under disease conditions that mobilize iron in the body. Oxidative DNA cleavage in the presence of transition metals such as Fe, as a result of pro-oxidant activity of polyphenols, has been reported (*35*). A recent study showed that EGCG exhibited pro-apoptotic effects in cancer cells in the presence of Fe^{2+} (*48*). It was suggested that the formation of hydroxyl radicals and the induction of apoptosis play an important role in the anticancer properties of EGCG. However, under certain conditions, some flavonoids could affect genotoxicity, depending on assay conditions and the dosage used. Under in vivo conditions, aspects such as their absorption and biotransformation also need to be considered (*21*). The

present in vitro study showed that a critical balance seems to exist between the pro-oxidant and antioxidant behaviors of rooibos extracts and polyphenolic fractions with the final concentration of the antioxidant constituents determining the biological outcome. The pro-oxidant activity of flavonoid-enriched rooibos extracts should be monitored in other in vitro biological systems as well as under in vivo conditions before being considered for therapeutical purposes. Studies on the bioavailability of rooibos flavonoids should provide more information on possible adverse effects.

ABBREVIATIONS USED

ASP, purified aspalathin; AQF, aqueous extract from fermented rooibos; AQU, aqueous extract from unfermented rooibos; C-ASP, crude aspalathin; CPF, crude polymeric fraction from fermented rooibos; CPU, crude polymeric fraction from unfermented rooibos; EAF, ethyl acetate soluble fraction from fermented rooibos; EAU, ethyl acetate soluble fraction from unfermented rooibos; TAN, tannin from unfermented rooibos; TP, total polyphenol content; BHT, butylated hydroxytoluene; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances.

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