DOI:10.1021/jf901417k

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Antioxidant Activity of the Dihydrochalcones Aspalathin and Nothofagin and Their Corresponding Flavones in Relation to Other Rooibos (*Aspalathus linearis*) Flavonoids, Epigallocatechin Gallate, and Trolox

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The antioxidant activity of rooibos flavonoids, including the dihydrochalcones aspalathin and nothofagin and their corresponding flavone glycosides, was evaluated using the ABTS radical cation, metal chelating, and Fe(II)-induced microsomal lipid peroxidation assays. Epigallocatechin gallate (EGCG) and Trolox were used as reference standards. Optimized geometric conformers of aspalathin and nothofagin, in addition to calculated physicochemical properties, were considered to explain interaction with the microsomal membrane structure and thus relative potency of the dihydrochalcones. The most potent radical scavengers were aspalathin (IC_{50} = 3.33 μM) and EGCG (IC_{50} = 3.46 μM), followed by quercetin (IC_{50} = 3.60 μM) and nothofagin (IC_{50} = 4.04 μM). The least effective radical scavengers were isovitexin (IC_{50} = 1224 μM) and vitexin (IC_{50} > 2131 μM). Quercetin (IC_{50} = 17.5 μM) and EGCG (IC_{50} = 22.3 μM) were the most effective inhibitors of lipid peroxidation. Aspalathin (IC_{50} = 50.2 μM) and catechin (IC_{50} = 53.3 μM) displayed similar potencies. Nothofagin (IC_{50} = 1388 μM) was almost as ineffective as its flavone glycoside analogues.

KEYWORDS: Rooibos; *Aspalathus linearis*; aspalathin; nothofagin; optimized geometric conformers; lipid peroxidation; metal chelation; herbal tea

INTRODUCTION

A recent review describes the phenolic composition and health-promoting properties of rooibos (*Aspalathus linearis*) (*1*), a plant endemic to South Africa with a long history of traditional use as an herbal tea. Rooibos flavonoids and their antioxidant properties are considered in many cases to be the underlying basis for the bioactivity of this herbal tea.

Aspalathin (2′,3,4,4′,6′-pentahydroxy-3′-*C*-*β*-D-glucopyranosyldihydrochalcone), a compound unique to rooibos, its flavone analogues orientin and iso-orientin, respectively, the 8-*C* and 6-*C*-*β*-D-glucopyranoside derivatives of luteolin, and the flavonol rutin are the major monomeric flavonoids in aqueous extracts prepared from fermented rooibos. The relatively large quantities of aspalathin present in the dried aerial parts of the rooibos plant (4.5−9.3%) have led to the production of extracts from green ("unfermented") rooibos for the food, beverage, and cosmetic industries (*1*). Nothofagin, the 3-deoxy analogue of aspalathin, is present in much lower quantities. Its flavone analogues comprise the regioisomers vitexin and isovitexin, respectively the 8-*C*- and 6-*C*-*β*-D-glucopyranoside derivatives of apigenin. Other rooibos flavonoids of interest include luteolin, chrysoeriol, quercetin, isoquercitrin, and hyperoside (*1*).

The unique combination of dihydrochalcones and their corresponding flavone analogues lends itself to comparative structure−activity studies. Previous comparative studies on a number of rooibos flavonoids demonstrated comparable DPPH and superoxide anion radical scavenging abilities (*2*, *3*) for aspalathin to quercetin and luteolin. The latter compounds were, however, substantially more effective as inhibitors of linoleic acid oxidation in the *β*-carotene bleaching method (*2*). Krafczyk et al. (*4*) demonstrated weaker ABTS radical cation scavenging activity and inhibition of LDL oxidation for nothofagin than for aspalathin, whereas nothofagin was not effective in Fremy's salt assay. For greater understanding of the potential antioxidant activity of rooibos flavonoids in vivo, inhibition of Fe(II)-induced microsomal lipid peroxidation was chosen as a model for oxidation in a membrane system. Because free radical scavenging and metal chelation could contribute to their antioxidant activity in this system, simple assays, namely, the ABTS radical cation assay and

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 Published on Web 07/09/2009

the change in λ_{max}, were employed to determine these properties. For better comprehension of the interaction of the dihydrochalcones with the membrane, their minimum energy conformations were calculated and physicochemical parameters, among others log *P* and polar surface area as predictors of the relative affinity of the flavonoids to interact with the membrane, were also taken into account.

MATERIALS AND METHODS

Chemicals. Aspalathin and nothofagin were isolated from unfermented rooibos to a purity of >95% at the PROMEC Unit. Purity was confirmed by HPLC and MS for nothofagin, as well as by NMR for aspalathin. Green label grade flavonoids, that is, luteolin, chrysoeriol, orientin, iso-orientin (as homoorientin), vitexin, isovitexin, isoquercitrin (quercetin-3-*O*-*β*-D-glucopyranoside), and hyperoside (quercetin-3-*O*-*β*-D-galactopyranoside) were obtained from Extrasynthese (Genay, France). Quercetin (as quercetin dihydrate; ≥98%), rutin (>95%), (+)-catechin (>96%), epigallocatechin gallate (EGCG; >95%), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid (TBA), and solvents (analytical grade) were obtained from Sigma-Aldrich, Cape Town, South Africa. Chemical structures of the various flavonoids used in this study are shown in **Figure 1**.

Calculation of Physicochemical Properties. Predicted log *P* (C.log *P*), polar surface area (PSA), number of hydrogen bond acceptors, number of hydrogen bond donors, and freely rotatable bonds of each compound were obtained from ChemSpider (www.chemspider.com), which uses Advanced Chemistry Development (ACD/Laboratories) software. ChemSketch MOL files of the structures of aspalathin and nothofagin were imported into ChemSpider and their properties calculated.

Conformational Analysis of Aspalathin and Nothofagin. A systematic conformational search was carried out via Monte Carlo random searching in the SYBYL 8.1 program (Tripos International, St. Louis, MO) using MMFF94 molecular mechanics force-field calculation. An energy cutoff of 5 kcal/mol was used to generate a wide window of conformers in the Boltzmann population. Further geometrical optimization was carried out in the Gaussian 03 program (*5*) using the hybrid density functional theory (DFT) method at the B3LYP/6-31G** level at 298 K. Harmonic vibrational frequencies were calculated at the same level to confirm the stability of the conformers. Finally, conformational analysis was performed by using Gibbs free energies. Optimized geometries of the lowest energy conformers of aspalathin and nothofagin in the gas phase at the B3LYP/6-31G** level were drawn with CS ChemDraw (CambridgeSoft).

ABTS$^{\bullet+}$ Decolorization Assay. The ABTS$^{\bullet+}$ assay (*6*) was performed with minor modifications. The radical cation was generated by mixing ABTS (7 mM) and potassium persulfate (2.45 mM) in water and incubating in the dark for 12−16 h prior to use. The ABTS$^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm (30 °C). The flavonoid solution (50 μL), dissolved in DMSO, was added to the diluted ABTS$^{\bullet+}$ solution (1 mL) and incubated at 30 °C for 4 min, and the absorbance was measured at 734 nm. Sample and reaction blanks were included in each assay, and determinations were conducted in triplicate. Trolox was used as a reference ABTS$^{\bullet+}$ scavenger. The percentage scavenging of ABTS$^{\bullet+}$ was calculated and expressed as an IC_{50} value.

Inhibition of Fe(II)-Induced Microsomal Lipid Peroxidation. Rat liver microsomes were prepared from male Fisher (F344) rats (200−300 g) (*7*). Lipid peroxidation was performed according to the method of Yen and Hsieh (*8*) with slight modifications. The reaction mixture consisted of microsomes (1 mg of protein/mL in 0.2 M potassium phosphate buffer; pH 7.4), flavonoids dissolved in DMSO (100 μL), and ferrous sulfate (500 μM; 200 μL) in a total volume of 1 mL. Sample blanks included incubations with each flavonoid tested, DMSO, and the buffer, in the absence of microsomes. The mixtures were incubated for 1 h at 37 °C and the reaction was terminated with ice-cold 10% (m/v) trichloroacetic acid (TCA) solution (2 mL) containing butylated hydroxytoluene (BHT, 0.01% m/v) and ethylenediaminetetraacetic acid (EDTA, 0.1% m/v). Samples were centrifuged, 2 mL of the supernatant was mixed with 2 mL of a 0.67% (m/v) thiobarbituric acid (TBA) solution, and the thiobarbituric acid reactive substances (TBARS) were measured at 532 nm after 20 min

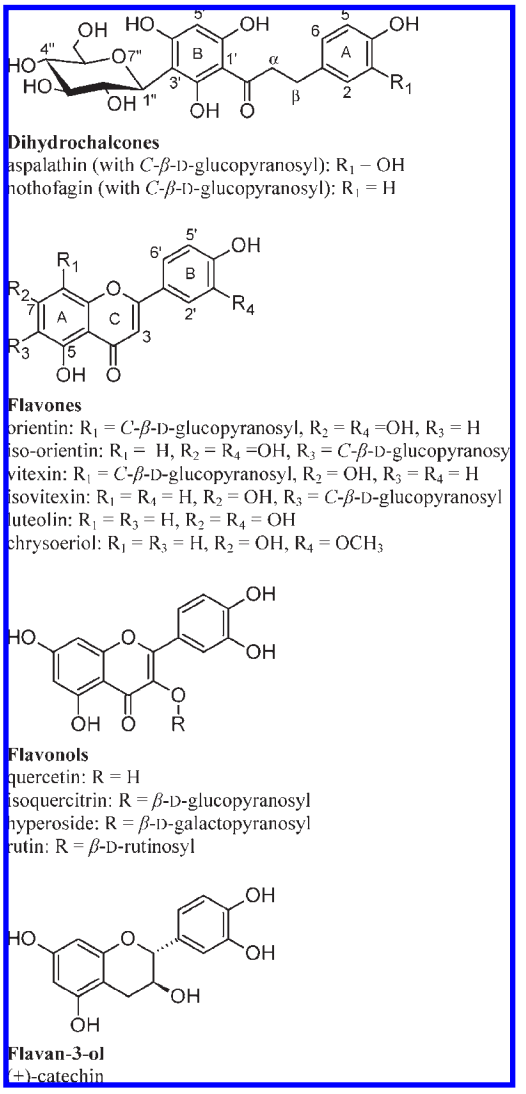

Figure 1. Chemical structures of rooibos flavonoids with ring labeling and atom numbering of dihydrochalcones and other flavonoids indicated.

of incubation at 90 °C. The percentage inhibition of TBARS formation relative to the positive control was calculated by

$$[(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ and A_{sample} refer to the corrected absorption readings for the positive control and sample, respectively. The assay was conducted in triplicate, and the IC_{50} values were calculated from the means.

UV−Vis Spectroscopy of Flavonoid Metal Complexes. The iron chelating potentials of flavonoids were compared according to the method described by Moridani et al. (*9*). A shift in the Fe(II)-induced absorption bands after addition of the chelator EDTA can be ascribed to the formation of a complex between the phenolic functional groups of the compounds and Fe(II). The absorption spectrum of each flavonoid (25 μM) was recorded in potassium phosphate buffer (10 mM; pH 7.4) between 200 and 700 nm before and 5 min after the addition of ferrous sulfate (50 μM). The reversibility (absorption shift) of the flavonoid−Fe(II) complex was monitored by the addition of EDTA (1.25 mM). Molar concentrations indicate the concentration in the reaction mixture.

Calculation of IC_{50} Values. The IC_{50} values were calculated on the basis of the best fit for dose−response data of the compounds according to different models (4LP, 3PLFB and 3PLFT, 3LPFBoth), using GraphPad Prism software (version 5.00 for Windows) (GraphPad Software, Inc., La Jolla, CA). The appropriate model was selected on the basis of the smallest fitting error. In a number of cases where the smallest fitting error resulted in gross underestimation of the IC_{50} value, manual calculation of

the IC_{50} value, based on the plot of the data according to the model, was done. In such cases 95% confidence levels are not available.

RESULTS

Physicochemical Properties. All rooibos flavonoids have log *P* values of < 5, but the glycosides violate Lipinski's "Rule of 5" in terms of the number of hydrogen bond acceptors and hydrogen bond donors. In addition, the PSA values for aspalathin, nothofagin, and rutin are >140 Å, whereas their numbers of freely rotatable bonds, as well as those of orientin and iso-orientin, are >10 (**Table 1**).

Conformation of Aspalathin and Nothofagin. Seventy-two and 57 conformers were derived for aspalathin and nothofagin, respectively. The numbers of conformers with an energy window of 5 kcal/mol were 35 for aspalathin and 29 for nothofagin. The latter conformers were then geometrically optimized at 298 K using the hybrid DFT method at the B3LYP/6-31G** level, and 29 and 25, respectively, were relocated as stable conformers of aspalathin and nothofagin. The populations of the optimized geometries of the 10 lowest energy conformers of aspalathin and nothofagin were 39.8, 18.8, 14.4, 7.1, 5.9, 3.5, 2.3, 1.6, 1.1, and 1.0%, and 28.6, 20.7, 11.2, 5.5, 4.5, 4.5, 4.0, 3.5, 3.1, and 3.1%, respectively. The key dihedral angles of the three lowest energy conformers of aspalathin (A1−A3) and nothogafin (N1−N3) (**Figure 2**) are listed in **Table 2**.

Table 1. Physicochemical Properties of Rooibos Flavonoids

compound	HBA[a]	HBD[b]	C.log *P*[c]	FRB[d]	PSA[e]
aspalathin	11	9	2.07	15	208
nothofagin	10	8	2.68	14	188
orientin/iso-orientin	11	8	1.58	11	109
vitexin/isovitexin	10	7	1.28	10	100
luteolin	6	4	2.40	5	63
chrysoeriol	6	3	1.81	5	63
rutin	16	10	1.76	16	156
isoquercitrin	12	8	1.75	12	119
hyperoside	12	8	1.75	12	119
quercetin	7	5	2.08	6	72
(+)-catechin	6	5	0.49	6	55

[a] HBA (<10), hydrogen bond acceptors. [b] HBD (<5), hydrogen bond donors. [c] C. log *P* (<5), calculated octanol−water partition coefficient. [d] FRB (≤10), free rotatable bonds. [e] PSA (<140 Å^2), polar surface area. Numbers in parentheses indicate limits for good absorption.

$ABTS^{\bullet+}$ Decolorization Assay. The most potent radical scavengers (**Table 3**) were aspalathin (IC_{50} = 3.33 μM) and EGCG (IC_{50} = 3.46 μM), followed by quercetin (IC_{50} = 3.60 μM) and nothofagin (IC_{50} = 4.04 μM). The 95% confidence intervals showed that the potency within the pairs aspalathin and EGCG, EGCG and quercetin, and quercetin and nothofagin was not significantly different ($P \geq 0.05$). (+)-Catechin (IC_{50} = 6.49 μM) was less potent than EGCG. Iso-orientin (IC_{50} = 11.25 μM), orientin (IC_{50} = 11.43 μM), and their aglycone luteolin (IC_{50} = 10.82 μM) were equipotent. However, they were less potent than aspalathin, but substantially more potent than chrysoeriol (IC_{50} = 21.54 μM). The flavones isovitexin (IC_{50} = 1223.77 μM) and vitexin ($IC_{50} \gg 2313$ μM), in contrast to their dihydrochalcone analogue, nothofagin, had very low potency as radical scavengers. The flavonol glycosides hyperoside, rutin, and isoquercitrin were less potent than their aglycone, quercetin, with values of 8.55, 10.47, and 12.89 μM, respectively. The overall potency of the compounds in scavenging $ABTS^{\bullet+}$, in descending order, is: aspalathin ≥ EGCG ≥ quercetin ≥ nothofagin > (+)-catechin > hyperoside > rutin ≥ luteolin ≥ iso-orientin ≥ Trolox ≥ orientin > isoquercitrin ≫ chrysoeriol ≫ isovitexin ≫ vitexin.

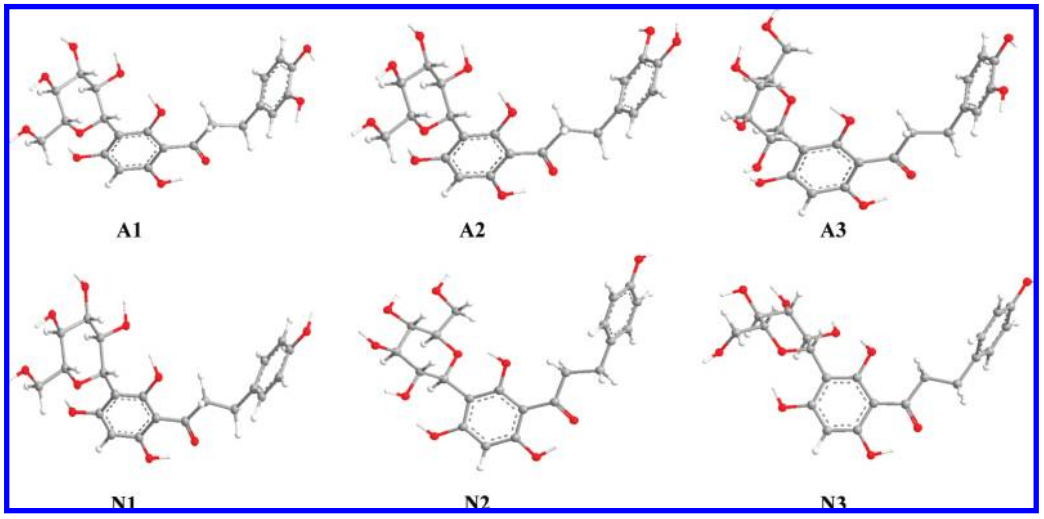

Figure 2. Calculated three minimum-energy conformers for aspalathin (A1−A3) and nothofagin (N1−N3). C-2′(O−H)−C-2″ (O) distances: 1.795, 1.794, and 1.837 Å for A1−A3; 1.797, 1.833, and 1.804 Å for N1−N3. C-4′(O−H)−C-1″ (O) distances: 1.769, 1.767, and 1.723 Å for A1−A3; 1.766, 1.722, and 1.760 Å for N1−N3.

Table 2. Key Dihedral Angles (Degrees) of the Three Lowest Energy Conformers of Aspalathin (A1−A3) and Nothofagin (N1−N3)

	C2−C1−Cβ−Cα	C1−Cβ−Cα−CO	Cβ−Cα−CO−C1′	Cα−CO−C1′−C2′	C2′−C3′−C1″−O7″
A1	89	−179	−180	0	−153
A2	−93	178	180	−1	−153
A3	90	−179	179	1	27
N1	−95	179	180	−1	−153
N2	−97	178	−179	−1	27
N3	−95	179	179	0	−152

Table 3. Antioxidant Properties of Rooibos Flavonoids and Reference Compounds EGCG and Trolox

	inhibition of Fe(II)-induced lipid peroxidation			$ABTS^{\bullet+}$ scavenging			metal chelation (shift in band I absorption)	
compound	model[a]	IC_{50} (μM)	95% confidence level	model[a]	IC_{50} (μM)	95% confidence level	+ Fe^{2+} (nm)	+ EDTA (% recovery)
aspalathin	4LP	50.2	45.6−55.3	3LPFT	3.33	3.13−3.56	0	0
nothofagin	3LPFT	1388	1255−1534	3LPFBoth	4.04	3.57−4.58	0	0
iso-orientin	3LPFBoth	480.7	394.7−585.5	3LPFT	11.25	10.71−11.82	25	95
orientin	3LPFBoth	137.9	123.1−154.5	4LP	11.43	10.99−11.88	27	96
isovitexin	4LP	1689	1606−1777	3LPFT	1224[b]	−[c]	0	0
vitexin	4LP	>2323[d]	−	4LP	>2313[d]	−	0	0
luteolin	3LPFT	185.9	165.5−208.9	4LP	10.82	10.16−11.52	26	93
chrysoeriol	3LPFBoth	217.7	185.2−256.0	4LP	21.54[b]	−	0	0
rutin	3LPFBoth	240.1	211.6−272.5	4LP	10.47	9.75−11.26	23	98
isoquercitrin	3LPFBoth	111.3	102.8−120.6	3LPFT	12.89	12.10−13.72	19	92
hyperoside	3LPFT	283.2	265.7−301.8	3LPFB	8.55[b]	−	5	89
quercetin	4LP	17.5	16.9−18.1	3LPFT	3.60	3.46−3.74	46	71
(+)-catechin	4LP	53.3	48.6−58.6	3LPFT	6.49	6.02−6.99	0	0
EGCG	4LP	22.3	20.6−24.0	3LPFT	3.46	3.00−3.99	0	0
Trolox	3LPFBoth	133.2	99.4−178.5	3LPFT	11.37	10.70−12.10	nd[e]	nd

[a] GraphPad Prism model used for curve fitting, calculation of IC_{50} values, and 95% confidence levels. [b] IC_{50} value manually estimated from fitted curve. [c] No 95% confidence levels available. [d] Inhibition did not reach 50%; value indicates concentration at maximum inhibition obtained. [e] Not determined.

The graphical plots of the data provide more information on the behavior of the compounds. The dose−response curves for the dihydrochalcones, their corresponding flavones, and luteolin are shown in **Figure 3A**,**B**. Aspalathin, nothofagin, luteolin, orientin, and iso-orientin reached ≥96% maximum efficacy. For isovitexin and vitexin the maximum plateaus of their sigmoidal dose−response curve were at ca. 52 and 37% scavenging, respectively.

Iron Chelation. None of the compounds evaluated could produce a band II shift as given by the A ring absorption (250−320 nm) (**Table 3**). Of the compounds having band I absorption, no band I shifts (320−420 nm) were observed for chrysoeriol, vitexin, and isovitexin. Chelation of Fe(II) by quercetin, rutin, isoquercitrin, and hyperoside caused band shifts in the band I region of 46, 23, 19, and 5 nm, respectively, whereas those for luteolin, orientin, and iso-orientin ranged from 25 to 27 nm (**Table 3**). Addition of EDTA reversed >90% of these shifts, except in the case of quercetin, for which only 71% of the compound was recovered from the complex.

Inhibition of Fe(II)-Induced Microsomal Lipid Peroxidation. The models used for curve fitting, IC_{50} values, and 95% confidence limits are summarized in **Table 3**. Of the rooibos flavonoids, quercetin was the most potent at 17.5 μM, with the green tea (*Camellia sinensis*) flavan-3-ol, EGCG (22.3 μM), slightly less potent. Aspalathin (50.2 μM) and (+)-catechin (53.3 μM) displayed similar protective effects against Fe(II)-induced lipid peroxidation. Orientin (137.9 μM) and iso-orientin (480.7 μM) were markedly less potent than their corresponding dihydrochalcone, aspalathin. Orientin showed more protection than the flavone aglycones, luteolin (185.9 μM) and chrysoeriol (218 μM). The group of compounds with the lowest potency included nothofagin (1388 μM) and its flavone analogues, vitexin (≫2313 μM) and isovitexin (1689 μM). Within the flavonol series, the glycosylated flavonols isoquercitrin (111.3 μM), rutin (240.0 μM), and hyperoside (283.0 μM) were substantially less potent than their aglycone, quercetin. Trolox (133.0 μM) had a potency similar to that of orientin. The overall potency of the rooibos flavonoids, EGCG, and Trolox as inhibitors of Fe(II)-induced microsomal lipid peroxidation, in decreasing order, was quercetin > EGCG > aspalathin ≥ (+)-catechin > isoquercitrin > Trolox ≥ orientin > luteolin > chrysoeriol > rutin > hyperoside ≫ iso-orientin ≫ nothofagin > isovitexin ≫ vitexin.

The dose−response curves for the dihydrochalcones, their corresponding flavones, and luteolin are shown in **Figures 3C**,**D**. Of interest is that the maximum efficacy of aspalathin, orientin, iso-orientin, and luteolin varied within a narrow range of 94−98%. Although the efficacy of nothofagin approached 95%, its corresponding flavones were markedly less effective, with isovitexin reaching a maximum of 79%, whereas vitexin leveled off at 46%.

DISCUSSION

The antioxidant activity of flavonoids has received much attention in the past decade or more due to their perceived beneficial health effects. During this period global consumption of rooibos as an herbal tea has grown substantially (*1*). It contributes to the daily intake of a range of flavonoids, some of which, namely, rutin and quercetin, are abundantly present in a number of food sources. Other rooibos flavonoids, namely, the dihydrochalcones aspalathin and nothofagin, are not part of the diet, except when ingested in a rooibos infusion or food products containing rooibos extracts (*10*). Even as a $C_6-C_3-C_6$ group of natural products, dihydrochalcones are rare.

The radical scavenging ability of flavonoids in an aqueous environment has been well established as an indication of their potential to protect against oxidative damage. Differences in the radical scavenging properties of flavonoids will exist when utilizing different assays representing the aqueous and/or lipid environment of the cell, with the latter more representative of in vivo conditions. When using rat liver microsomes as a model for a biomembrane, planarity and hydrophobicity of the flavonoids will determine their partitioning into the lipid bilayer and access of the lipid core of the membrane. In a membrane environment, interaction with protein constituents could also affect the radical scavenging properties. Secondary antioxidant activity, namely, metal chelation, is also important when the protective effect of flavonoids in a membrane environment is considered. Because of the importance of cellular membrane integrity in cell survival, the present study addressed the free radical scavenging and metal chelating abilities of rooibos flavonoids at physiological pH as factors in their ability to protect membranes against lipid peroxidation.

Before consideration of the antioxidant activity of the rooibos flavonoids in a membrane environment, their activity in an aqueous medium, as affected by structural characteristics, will first receive attention. On the basis of their behavior as radical scavengers in an aqueous medium, the flavan-3-ols, flavonols, and flavones can be broadly divided into two groups, which greatly differed according to potency. The compounds either

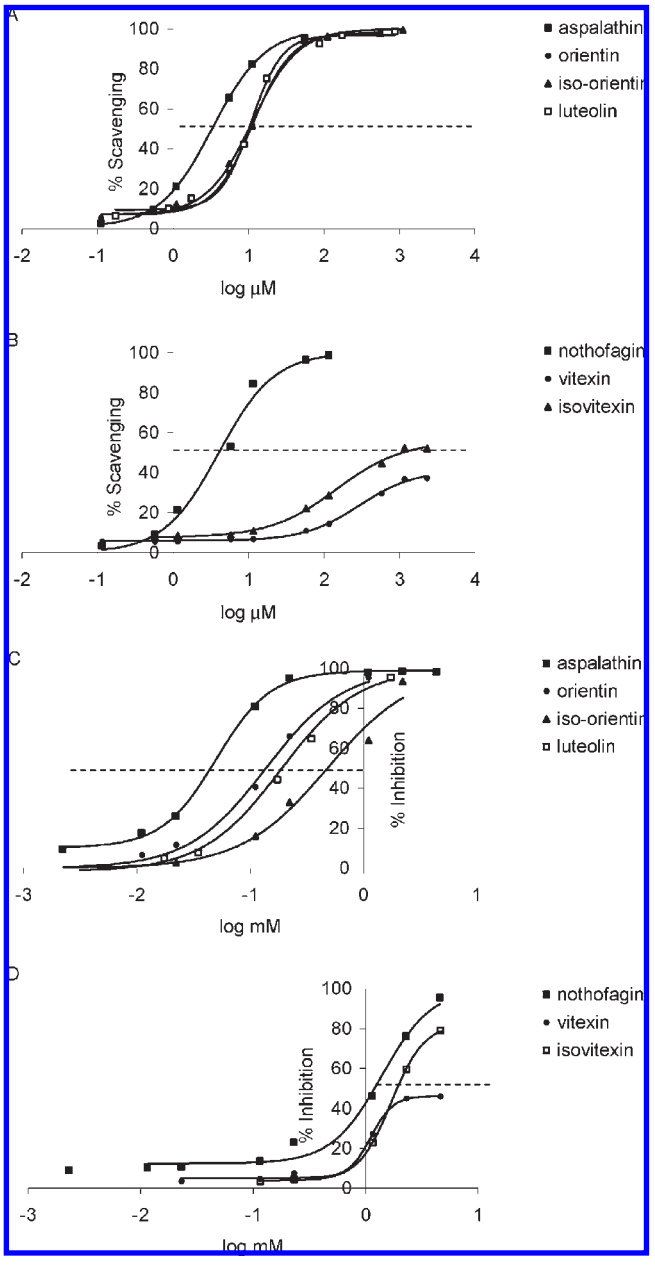

Figure 3. Dose−response curves for aspalathin, nothofagin, their corresponding flavones, and luteolin in the $ABTS^{\bullet+}$ scavenging (**A**, **B**) and Fe(II)-induced microsomal lipid peroxidation (**C**, **D**) assays.

fulfill all (e.g., quercetin) or at least two (e.g., (+)-catechin, luteolin, quercetin glycosides) of the structural requirements for radical scavenging (*11*). The presence of a catechol functionality confers the capability to form a semiquinone, stabilized by H-bonding (*12*), resulting in the substantially higher potency of orientin and iso-orientin compared to vitexin and isovitexin, which lack the catechol functionality. Although electron donation by the 3′-OCH_3 group of chrysoeriol reduces the bond dissociation energy (BDE) of the adjacent OH group, thus increasing its activity (*13*), it was not as potent as luteolin with the *o*-dihydroxy B-ring arrangement. A previous study showed both chrysoeriol and vitexin to be poor radical scavengers, with chrysoeriol being more effective than vitexin in scavenging the superoxide anion radical, but less effective in scavenging the DPPH radical (*3*).

Whereas iso-orientin and orientin had similar radical scavenging activities, their 3′-deoxy analogues, isovitexin and vitexin, differed greatly in potency and efficacy. This suggests that the structural and conformational differences of these two compounds due to the linkage position of the glucose moiety are important. The lower potency of vitexin compared to isovitexin may be explained by the difference in rate constant for hydrogen/deuterium (H/D) exchange. Zhang and Brodbelt (*14*) demonstrated that both compounds undergo at most one H/D exchange and at a very slow rate, with the rate constant for vitexin slightly less than that for isovitexin.

Glycosylation of the 3-OH of quercetin resulted in the expected decrease of activity demonstrated in numerous previous studies. The present study showed that the relative potencies of the glycosides depended on the specific carbohydrate moiety. Introduction of the carbohydrate moiety not only occupies the hydroxy group that is preferentially subjected to hydrogen abstraction of quercetin (*15*), but the bulky moiety additionally resulted in a twisted ring conformation, reducing the coplanarity of the B and C rings, hence inhibiting extended electron delocalization (*16*). The deviation from coplanarity of quercetin is 1.2°, and it increases to 22.1° for isoquercitrin and to 25.6° for hyperoside (*16*). Although the torsion angle for hyperoside was slightly larger, it was more potent as a radical scavenger than isoquercitrin, suggesting that the properties of the sugar itself are important. Hyperoside was also shown to be more effective than isoquercitrin in inhibiting Cu(II)-induced oxidation of human low-density lipoprotein (*17*). On the basis of the findings of Zhang and Brodbelt (*14*) for flavonoid glycosides, the difference in conformation of the sugar moieties of hyperoside and isoquercitrin may be responsible for the difference in potency. This is true if the hydroxy groups of the galactosyl moiety are spatially close to the deprotonation site (4′-OH) and/or the negative charge from C-4′*O* migrates to the C-4 keto group, which would bring the charge in close proximity to the C-3-*O* sugar unit, thus promoting H/D exchange and enhancing radical stabilization. Conformational studies are, however, required to confirm this hypothesis.

The relative potencies of luteolin and (+)-catechin indicated that the 3-OH group of (+)-catechin are more important for radical scavenging than either the 2,3-olefinic bond, 4-keto group, or planarity. It is reasonable to argue that the absence of coplanarity of the B and C rings compromises the electron delocalization capacity and thus the stability of the flavonoid phenoxy radical. However, despite the B and C rings of (+)-catechin being almost perpendicular to each other and the luteolin radical adopting a planar structure (*18*, *19*), which allows complete conjugation and enhanced stabilization, it was less effective than (+)-catechin in scavenging the ABTS radical cation. Comparative radical scavenging activity data of these flavonoids show a dependency on the type of radical, with (+)-catechin being more effective in scavenging the superoxide anion radical (*3*) and $ABTS^{\bullet+}$, whereas luteolin is more effective in scavenging DPPH (*3*) and galvinoxy (*12*) radicals.

The dihydrochalcones aspalathin and nothofagin are devoid of an extended conjugated system for electron delocalization and radical stabilization, yet both were potent radical scavengers. The C-2′ and C-6′ hydroxy groups, as well as the keto−enol tautomerism of the carbonyl and α-methylene groups (*20*, *21*), are important contributing factors to the antioxidant activity of dihydrochalcones. The obvious difference between aspalathin and nothofagin is the presence of the catechol-type A ring of aspalathin (**Figure 1**). Kozlowski et al. (*22*), studying a series of chalcones, including the dihydrochalcone phloretin, confirmed the lower BDEs for the A-ring catechol moiety. In the absence of the catechol moiety, the OH groups at C-2′ and C-6′ (B ring) have similar, but lower, BDEs than the C-4′ OH and are therefore more important in terms of hydrogen donation. A remarkable feature

of the conformational distribution of both aspalathin and nothofagin (**Table 2**) is the hydrogen bonding between the C-2′ and C-4′ hydroxy groups (B ring) and, respectively, the C-2″ hydroxy group and the ethereal oxygen of the glucopyranosyl moiety. The feasibility of the indicated hydrogen bonding is supported by the spatial proximity of the appropriate groups, varying between 1.722 and 1.837 Å (**Figure 2**). Notably, these hydrogen bonds are only evident in conformers possessing a C-2′−C-3′−C-1″−O-7″ dihedral angle of −153°. These types of conformers occupy 81 and 60.5% of the conformational itineraries of aspalathin (A1, A2, A4−A7, and A9) and nothofagin (N1, N3, N6−N8, and N10), respectively. Owing to the effect of hydrogen bonding on the BDEs of the phenolic groups, such a difference may compensate in an undefined manner for the enhanced susceptibility of oxygen radical formation at the catechol-type A ring of aspalathin, compared to the 4-hydroxyphenyl A ring of nothofagin, to eventually explain the very similar radical scavenging properties of these dihydroxychalcone analogues in an aqueous environment.

Evaluation of the antioxidant activity of polyphenols based on radical scavenging, measured in a homogeneous solution ($ABTS^{\bullet+}$ decolorization assay), is too simplistic when the inhibition of lipid peroxidation is considered. Several factors govern the efficacy in a membrane environment, for example, physicochemical properties of the compounds, access to the lipid bilayer, and positioning at the membrane interphase. When inhibition of lipid peroxidation and radical scavenging were compared, the same relative order of potency for the rooibos flavonoids was not observed. On the basis of C.log *P* values alone, all rooibos flavonoids should be able to access the lipid bilayer core of the microsomal membranes. However, to various extents other physicochemical properties of rooibos flavonoids exceed the limits set for good membrane permeability (*23*, *24*), signifying that they would preferentially be located at the lipid/aqueous interphase or in the aqueous medium. Luteolin, an aglycone, for example, showed a broad distribution in an artificial membrane, but with distribution biased toward the aqueous phase. With the addition of a sugar moiety the molecule acquires a very hydrophilic center, which faces the aqueous environment and thus broadens the distribution pattern (*25*).

Planarity not only favors the stability of the phenoxy radical, thus enhancing radical scavenging ability, but also enhances flavonoid intercalation into the organized structures of the phospholipids within the membrane (*26*). Pawlikowska-Pawlęga et al. (*27*) reported that quercetin influenced only the polar region of the bilayer of human erythrocyte membranes, protecting the surface against peroxidation and leaving the hydrophobic core of the membrane unchanged. Considering quercetin, isoquercitrin, and hyperoside, the degree of planarity of the molecules, their radical scavenging ability, and metal chelation are three important factors that may play a role in their relative potency. The planar quercetin molecule offered the most protection in the microsomal lipid peroxidation system, followed by isoquercitrin, which deviated less from coplanarity than hyperoside (*16*). Isoquercitrin was also more effective as a metal scavenger than hyperoside, which will contribute to its potency in the Fe(II)-induced microsomal lipid peroxidation system. Rutin was found to be both a better metal chelator and a better radical scavenger than isoquercitrin, yet it was less effective than isoquercitrin against lipid peroxidation, which is a clear indication that other factors such as interaction with membranal protein constituents will also play a role.

In the lipid peroxidation assay, orientin and iso-orientin differed greatly in potency, with iso-orientin being substantially less effective than orientin. This suggests that the position of the glucose moiety is of critical importance in a membrane environment. A similar relative order of potency was obtained for orientin and iso-orientin in another study using microsomes as the membrane source (*28*). Greater membrane intercalation for luteolin having a relatively planar structure and higher hydrophobicity than its corresponding glycoside, orientin, is to be expected. However, orientin was slightly more effective than luteolin in protecting against microsomal lipid peroxidation. This finding is contrary to the relative order of potency for luteolin and orientin obtained by Mora et al. (*28*). However, in the latter study cysteine, in addition to iron ions, was added, which would mean the involvement of ROS such as hydroxyl radicals (*29*). The predisposition of orientin relative to luteolin to locate itself at the membrane−water interphase seems to be pivotal. Metal ions can be "trapped" before they are able to penetrate the lipid bilayer to initiate oxidation. Scavenging of peroxy radicals at the membrane−water interphase is possible as vertical fluctuations of the lipid alkyl chains toward the membrane surface occur (*30*). Further investigation of this group of compounds is required.

Most notable is the very poor potency of nothofagin in protecting membrane lipids against peroxidation, despite being a good radical scavenger. The physiochemical properties of nothofagin and aspalathin most likely to determine their accessibility to the lipid bilayer are very similar, and the slight differences in these properties between the two compounds would in fact rather favor nothofagin, yet it displayed almost no activity relative to aspalathin. The orientation of the A ring differs between aspalathin and nothofagin, with their C-2−C-1−C-β−C-α dihedral angles of 89° and −95° for A1 and N1, respectively. In both cases the orientation of the sugar moiety is the same. This gives aspalathin a more "open" structure, improving the accessibility of the catechol A ring to interaction with the polar heads of the lipid bilayers, where radical scavenging could be exercised at the membrane interphase.

Although no metal chelation was demonstrated for aspalathin under the present experimental conditions, it possesses two of the three possible binding sites for metal ions by flavonoids, namely, the catechol A ring, as well as the C-4 keto group and the C-2′ hydroxy group of the B ring. Metal chelation could play a significant role in the relative antioxidant potency of nothofagin and aspalathin in a membrane environment. It must be assumed that the assay employed in the present study underestimates the metal chelating ability of the flavonoids.

Notwithstanding the poor bioavailability of aspalathin, the occurrence of the unconjugated form in the urine of pigs (*31*) after ingestion of an aspalathin-enriched rooibos extract confirms the importance of this flavonoid as an antioxidant in the diet. The antioxidant activity of the original molecule ingested when drinking rooibos herbal tea thus remains relevant. However, because most of the aspalathin absorbed is conjugated at various OH groups (*31*, *32*), which take part in radical scavenging, the in vivo antioxidant activity of aspalathin is expected to depend on the balance between nonconjugated and conjugated forms. Information obtained with conformational analysis could support future studies on aspalathin bioavailability and position of conjugation of its metabolites.

Supporting Information Available: Figures of the optimized geometries of the minimum-energy conformers of aspalathin and nothofagin in the gas phase at the B3LYP/6-31G** level. Tables of thermodynamic parameters and conformational analysis of aspalathin and nothofagin, optimized *Z*-matrices of the lowest energy conformers of aspalathin and nothofagin in the gas phase, and frequencies of the lowest energy conformers of aspalathin at

the B3LYP/6-31G** level in the gas phase. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received April 29, 2009. Revised manuscript received June 25, 2009. Accepted June 26, 2009. Funding was provided by the National Research Foundation of South Africa (Grant FA2004051100014), Cancer Association of South Africa, and South African Rooibos Council. This work was also supported in part by the U.S. Department of Agriculture, Agricultural Research Service Specific Cooperative Agreement 58-6048-0009. Data of the antioxidant activity assays for this paper represent part of an M.Sc. thesis in chemistry by P.W.S., completed in 2007. A poster on preliminary antioxidant activity data for aspalathin and nothofagin was presented at the 1st International Conference on Polyphenols and Health, held November 18−21, 2003, in Vichy, France.