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Research Article

Structure–antioxidant relationship of flavonoids from fermented rooibos

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Rooibos tea (*Aspalathus linearis*) contains different bioactive phenolic compounds such as dihydrochalcones, flavonols, flavanones, flavones, and flavanols. Flavonoids isolated from rooibos were subjected to different *in vitro* assays: Trolox equivalent antioxidant activity, LDL oxidation and Fremy's salt assays to determine the total antioxidant activity (TAA). Assay results were compared, and the structure–antioxidant relationship was investigated. A decoupled LDL oxidation test was established with the objective of having an assay adapted more to an *in vivo* situation. The different *in vitro* methods were coupled offline to HPLC-DAD. Results from these coupled offline methods were compared to the TAA to assess the usefulness of the coupling setup.

Keywords: Fremy's salt / LDL-oxidation / Rooibos tea / Structure – antioxidant relationship / TEAC

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1 Introduction

Rooibos tea is made from the leaves and fine stems of *Aspalathus linearis.* Fermented rooibos tea is reported to have different biological properties, such as calming various stomage problems, reducing nervous tensions and alleviating allergies [1]. Polyphenols were isolated and identified from fermented and unfermented rooibos by multilayer countercurrent chromatography, *e.g.*, flavanone-*C*-glycosides ((*S*)- and (*R*)-eriodictyol-6-*C*-β-D-glycopyranoside, (*S*)-and (*R*)-eriodictyol-8-*C*-β-D-glycopyranoside); dihydrochalcones (aspalathin, nothofagin); flavone-*C*-glycosides (orientin, isoorientin, vitexin, and isovitexin); flavonol-*O*-glycosides (isoquercitrin, hyperoside, and rutin); flavonol (quercetin); and flavones (luteolin, chrysoeriol) [2]. The structures of these polyphenols are shown in Fig. 1.

The structure of a phenolic compound affects its antioxidant capacity (AC). More specifically, the antioxidant activity is influenced by the position and degree of hydroxylation of the ring structure. An *ortho*-dihydroxyl functional group at the B-ring of flavonoids is highly effective for scavenging free radicals. After interception of radicals flavonoids are oxidized to quinones. The antioxidant potential is increased by a 3-hydroxyl functional group and a 2,3-double bond conjugated to a 4-keto function at the flavonoid *C*-ring [3]. Flavanones and flavones are less active than their corresponding dihydrochalcones [4].

These differences in activities of flavonoids and phenolic acids were gathered from tests done in hydrophilic phases. In addition to this, the antioxidant activity against lipid radicals should also be addressed. In this case, three modes of inhibition need to be discussed: scavenging lipid alkoxyl and peroxyl radicals by acting as hydrogen donors, chelating metal ions *via* the *ortho*-dihydroxyl or α/β-hydroxycarbonyl structures, and the ability to regenerate tocopherol radicals [3]. It has been hypothesized that flavonoids might be localized near membrane surfaces, *e.g.*, the LDL membrane, scavenging aqueous radicals, and thus preventing the consumption of tocopherols, whereas α-tocopherol mainly acts as a chain-breaking lipid peroxyl radical scavenger within the LDL [5].

Previous work concerning the AC of rooibos extracts showed that unfermented or fermented rooibos tea had a high AC relative to Trolox® in the DPPH-assay and the β-carotene bleaching method [6]. Additionally, von Gandow *et al*. [7] researched the AC of aspalathin, the major flavonoid compound of unfermented rooibos, and other phenolic compounds of rooibos with the result that aspalathin inhibited the DPPH radical by 91.4%. Joubert *et al*. [8] investigated the antioxidant and pro-oxidant activity of aqueous extracts and crude phenolic fractions of rooibos,

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Abbreviations: AC, antioxidant capacity; **TEAC,** trolox equivalent antioxidant activity

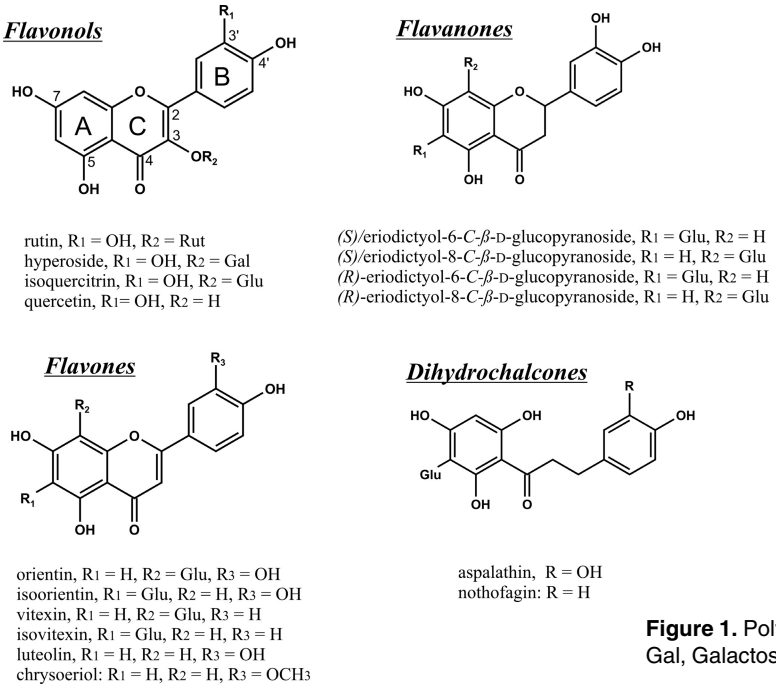

Figure 1. Polyphenols isolated from rooibos tea [2] (Rut, rutinose, Gal, Galactose, Glu, glucose).

using a linoleic acid–Tween buffer emulsion for lipid peroxidation and the deoxyribose degradation assay based on a Fenton reaction system. Contrary to expectations, the inhibitory effect of the ethyl acetate soluble fraction of fermented rooibos was significantly higher than that of unfermented rooibos.

Because of these conflicting results, the antioxidant potential of polyphenolic ingredients of rooibos in hydrophilic and lipophilic phases was reinvestigated using different *in vitro* methods: trolox equivalent antioxidant activity (TEAC) assay, Fremy's salt assay and LDL oxidation assay. An offline-HPLC screening method was developed to couple with the assay methods described. Similar to the method of Koleva *et al*. [9], this HPLC method was useful to evaluate the antioxidant activity of the ethyl acetate extract of rooibos.

2 Materials and methods

2.1 Chemicals

Chemicals of highest quality available were obtained from Roth (Karlsruhe, Germany) unless otherwise indicated. HPLC-grade methanol was from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich (Steinheim, Germany). Potassium nitrosodisulfonate (Fremy's salt) was purchased from Sigma (Taufkirchen, Germany).

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), quercetin dihydrate, potassium peroxodisulfate, copper(II) sulfate anhydrous, and heptafluorobutyric acid were purchased from Fluka (Taufkirchen, Germany). Natrium bromide was obtained from Riedel de Häen (Seelze, Germany).

2.2 Methods

All data are expressed as averages of triplicate experiments.

2.2.1 Extraction of rooibos tea and production of tea extract

Fermented rooibos tea from Bieduow Valley of South Africa was obtained from Ronnefeldt (Worpswede, Germany). Fermented rooibos (3 g) were extracted with 30 mL acetone/water (7:3 v/v) at 5°C for 24 h under argon atmosphere, and decanted. Acetone was removed under reduced pressure. The residual H_2O phase was successively extracted with diethyl ether (10 mL) and ethyl acetate (10 mL). From the extracts solvents were removed under reduced pressure.

Unfermented rooibos (100 g) from Ronnefeldt (Worpswede, Germany) was extracted with 1 L of water. After filtration the solution was freeze-dried. Yield of aspalathin in tea extract was 53 mg/g.

2.2.2 Trolox equivalent antioxidant capacity (TEAC) assay

This assay was performed on a Jasco UV–Visible spectrophotometer (Gross-Umstadt, Germany), based on the method of Re *et al*. [10]. $ABTS^{\bullet+}$ radical cation was produced by reacting 7 mM ABTS and 140 mM potassium peroxodisulfate at 8°C in the dark for 16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm after 2.5 min. Individual fractions

collected from analytical HPLC separation were evaporated by vacuum centrifugation, redissolved in 100 μL of ethanol, then subjected to the TEAC assay.

For determination of the TEAC value Trolox standards of final concentration 20–140 μM in ethanol were prepared and assayed under the same conditions. TEAC values of samples were calculated based on the inhibition exerted by standard Trolox® solutions.

2.2.3 Fremy's salt assay

The Fremy's salt assay was carried out according to Rösch *et al*. [11]. Isolated substances or fractions of polyphenols were dissolved in methanol. The solutions of the compounds were allowed to react with an equal volume of a solution of Fremy's salt (1 mM in phosphate buffer 0.2 M, pH 7.4). ESR spectrum of Fremy's radical was obtained after 20 min, after which the reaction was complete. Signal intensity was obtained by integration, and the AC was calculated by comparison with a control reaction with methanol. Spectra were obtained on a Miniscope MS 100 spectrometer (Magnettech, Berlin, Germany) in a 50 μL micropipet. The microwave power and modulation amplitude were set at 10 dB and 1500 mG, respectively.

2.2.4 Isolation of LDL and oxidation

Human LDL (d = 1.006–1.063 g/mL) was isolated from fasting plasma collected in EDTA according to the method of Tiedink *et al*. [12], using a Sorvall ultracentrifuge RC M 150 GX (rotor S 150-AT) (Kendo Laboratory Products, Hanau, Germany). Then subjected to dialysis for 24 h at 4°C three times against 1 L of 0.01 M PBS, 0.0027 M KCl, and 0.138 M NaCl, pH 7.4. LDL solution was flushed with N_2, stored at 4°C. Protein content was measured according to the method of Bradford *et al*. [13], using bovine serum albumin as standard.

LDL obtained from dialysis (0.05 mg protein/mL) was oxidized in TRIS buffer (1 mM, 0.9% NaCl, pH 7.4) in the presence of 70 μM $CuSO_4$. Absorption was measured at 234 nm according to Esterbauer *et al*. [14]. Solutions of isolated substances and of individual fractions from HPLC-DAD separation were added to LDL solution before addition of $CuSO_4$.

2.2.5 Decoupled LDL oxidation assay

Two-hundred microliter of LDL (0.05 mg/mL), diluted with PBS buffer (pH 7.4) were added to antioxidant solutions of aspalathin and isoquercitrin (20 μM) and incubated at 37°C for 2, 8, and 12 h. After dialysis against PBS buffer the LDL oxidation was observed as described above.

2.2.6 HPLC-DAD screening assay

A Jasco (Gross-Umstadt, Germany) quaternary gradient unit PU 2080, with degasser DG 2080-54, autosampler AS 2055, column oven (Jasco Jetstream II) and multiwavelength detector MD 2015 coupled to an Advantec fraction collector CHF122SB (Tokyo, Japan) was used. Chromatographic separations were performed on stainless steel columns (VYDAC CRT. #218TP54, 250 × 4.0 mm, RP 18, 5 μm, Hesperia, CA) using a flow rate of 1.0 mL/min. The mobile phase used was water (solvent A) and MeOH/water (7:3 v/v, solvent B). To both solvents (A and B), 0.6 mL/L heptafluorobutyric acid (HFBA) was added. The column temperature was 25°C. The effluent was monitored at 280 nm and 350 nm. *Method A*: sample (ethyl acetate extract: 2.1 mg/50 μL) was injected at 10% B, the gradient then changed linear to 30% B in 40 min, to 70% B in 60 min, to 100% B in 2 min, and held at 100% B for 8 min. 65 fractions of 2 mL were collected in 130 min. *Method B*: ethyl acetate extract (420 μg/10 μL) was injected at 10% B, the gradient then changed to 30% B in 25 min, to 70% B in 40 min, to 100% B in 5 min, and held at 100% B for 15 min. Fractions with eluted material (substances **1**–**9**, Fig. 6) were collected and evaporated. Residues were redissolved in 500 μL of TRIS buffer. One-fifth was used for subsequent LDL oxidation.

3 Results and discussion

3.1 Structure antioxidant relationship

3.1.1 TEAC and Fremy's salt assay

Authentic polyphenol compounds were previously isolated from rooibos by multilayer countercurrent chromatography and preparative HPLC [2]. Quercetin, isoquercitrin, hyperoside, and rutin have the necessary prerequisites for a compound with high AC: a 2,3-double bond and a 4-oxo-functional group at the C-ring conjugating the A and B aromatic ring system. The TEAC value of quercetin was 2.70 ± 0.13 mM (Fig. 1, Table 1). Isoquercitrin, rutin, and hyperoside showed only half of the antioxidant activity of quercetin. These results clearly demonstrate the importance of a free hydroxyl group at position 3 on the C-ring. The TEAC value of quercetin was similar to that obtained by Re *et al*. [10]. The Fremy's salt assay gave an AC (mol Fremy's salt/mol antioxidant) of 4.48 ± 0.03 for quercetin (Table 1). Theoretically, quercetin has five possible scavenging groups, two hydroxyl groups at the A-ring, two hydroxyl groups at the B-ring and one hydroxyl group at position 3 of the C-ring. However, the *meta*-configuration of the A-ring hydroxyl substituents does not favor efficient quenching of radicals. Accordingly, quercetin was able to reduce at least four radicals, based on the AC-value of Fremy's salt assay. Values of isoquercitrin and hyperoside are in the same range, but slightly less active than quercetin (Table 1). AC of rutin (3.70 ± 0.07) was lower than the AC of isoquercitrin and hyperoside (Table 1). Thus, a sugar unit greater than a monosaccharide at position 3 of the aglycon significantly reduced the AC.

Removal of the 3-OH group at the C-ring, as in flavones, decreased the antioxidant activity (TEAC) to a value of

Table 1. TEAC and AC (mol Fremy's salt/mol antioxidant) of isolated compounds from rooibos

Compounds	Free OH-substituents	Glycosylated position	TEAC (mM) ($n = 3$)	AC (mol Fremy's salt/mol) ($n = 3$)
Dihydrochalcones				
Aspalathin	3, 4, 2′, 4′, 6′	5′-Glu	2.62 ± 0.07	4.81 ± 0.03
Nothofagin	4, 2′, 4′, 6′	5′-Glu	2.06 ± 0.14	0.02 ± 0.01
Flavanones				
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	5, 7, 3′, 4′	6-Glu	0.88 ± 0.08	2.67 ± 0.19
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	5, 7, 3′, 4′	6-Glu	1.04 ± 0.02	3.13 ± 0.22
Flavones				
Orientin	5, 7, 3′, 4′	8-Glu	1.47 ± 0.04	3.48 ± 0.03
Isoorientin	5, 7, 3′, 4′	6-Glu	1.54 ± 0.05	3.00 ± 0.03
Vitexin	5, 7, 4′	8-Glu	0.86 ± 0.03	1.57 ± 0.10
Isovitexin	5, 7, 4′	6-Glu	0.81 ± 0.04	1.58 ± 0.14
Flavonols				
Rutin	5, 7, 3′, 4′	3-α-L-Rha-1 → 6-β-D-glu	1.20 ± 0.07	3.70 ± 0.07
Isoquercitrin	5, 7, 3′, 4′	3-Glu	1.23 ± 0.05	4.08 ± 0.11
Hyperoside	5, 7, 3′, 4′	3-Gal	1.33 ± 0.08	4.21 ± 0.02
Quercetin	3, 5, 7, 3′, 4′	–	2.70 ± 0.13	4.48 ± 0.03
Tea extract[a)]	–	–	3.20 ± 0.01	17.65 ± 1.12

a) Relative to aspalathin; glu, glucose; rha, rhamnose; gal, galactose.

1.47 ± 0.04 mM (orientin) and 1.54 ± 0.05 mM (isoorientin) (Table 1). The antioxidant effect of these two flavone-*C*-glycosides is based on the 3′,4′-dihydroxy substituents at the B-ring, and the 2,3-double bond adjacent to the 4-oxo-function at the C-ring. Orientin and isoorientin gave a degradation of Fremy's salt in the same range (orientin AC 3.48 ± 0.03; isoorientin 3.00 ± 0.03).

Vitexin and isovitexin contain only one hydroxyl substituent (position 4′ at the B-ring) in comparison to orientin/isoorientin. The absence of an *ortho*-diphenolic structure further reduced the ability to scavenge free radicals to 0.86 ± 0.03 mM (vitexin) and 0.81 ± 0.04 mM (isovitexin) in TEAC assay (Table 1). Consequently, this also resulted in lower AC values (1.57 ± 0.10 for vitexin and 1.58 ± 0.14 for isovitexin).

The (*R*)- and (*S*)-eriodictyol-6-*C*-β-D-glycopyranosides lack the 2,3-double bond at the C-ring resulting in reduced TEAC-value of 0.88 ± 0.08 mM and 1.04 ± 0.02 mM, respectively (Table 1). The low antioxidant activity of these flavanones was paralleled by significantly reduced AC values in comparison to orientin and isoorientin.

The highest potency to scavenge free radicals ($ABTS^{\bullet+}$ or Fremy's salt) were found for the dihydrochalcone aspalathin and especially for the tea extract, which was standardized to the same amount of aspalathin (1 nmol) but of course the tea extract contained many further antioxidants. According to previous work, dihydrochalcones are more active against free radicals than their corresponding flavones, chalcones, and flavanones [4]. In the TEAC assay, nothofagin, which has only one hydroxyl group at the B-ring gave less activity in comparison to aspalathin, as expected. In the Fremy's salt assay the scavenging activity of nothofagin was completely lost.

3.1.2 LDL oxidation assay

The oxidation of LDL can be used as a model to investigate the efficacy of flavonoids as chain breaking antioxidants. Radicals are formed during oxidation of lipids in the presence of Cu^{2+}. Flavonoids quench these radicals to give phenoxyl radicals, which are stabilized by delocalization of unpaired electrons within the aromatic ring structure.

Isolated pure substances (1 nmol) of rooibos ((*R*)- and (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside; aspalathin; nothofagin; orientin; isoorientin; isoquercitrin) and tea extract (standardized to 1 nmol aspalathin) were introduced into the LDL-oxidation system. *Lag*-time shifts were obtained in the following order: isoquercitrin (9.6 h) > aspalathin (6.2 h), tea extract (6.2 h) > nothofagin (4.3 h) > isoorientin (3.8 h) > orientin (2.7 h) > (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside (2.1 h) > control (1.7 h) (Fig. 2). The higher the lag-time shift relative to the control the more potent the structure is as an antioxidant in this experiment. Due to the fact that quercetin was not soluble in the media used for the LDL oxidation test, the *lag*-time shift of this aglycone could not be determined. The *lag*-time shift of the tea extract (6.2 h) was identical to *lag*-time shift of aspalathin (6.2 h). The tea extract contained 1 nmol aspalathin and other antioxidant compounds from rooibos. Therefore, in theory the *lag*-time shift of the tea extract experiment should be considerably higher than that of aspalathin as

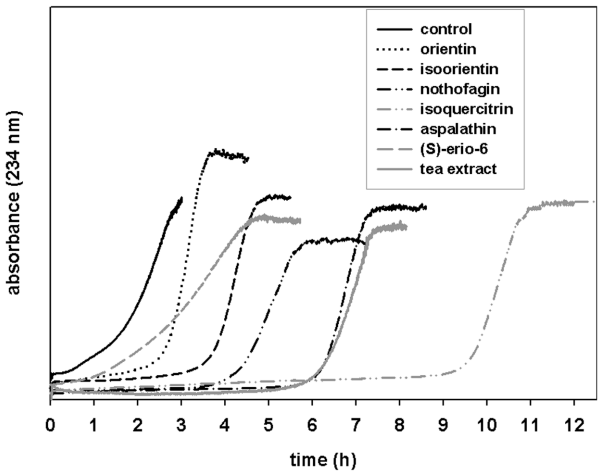

Figure 2. LDL oxidation assay in presence of Cu^{2+}. Lag-time shift was induced by 1 nmol of the following substances. (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside (2.1 h); aspalathin (6.2 h); orientin (2.7 h); isoorientin (3.8 h); nothofagin (4.3 h); isoquercitrin (9.6 h); tea extract (standardized to 1 nmol aspalathin) (6.2 h). *Lag*-phase of control was 1.4 h.

anticipated from the TEAC system. On the other hand, possible reactions of aspalathin with other ingredients during the very long time of oxidation should be taken into consideration. The tea extract from rooibos consisted of high molecular weight brown products. These browning substances are able to accelerate the oxidation of aspalathin to further brown products under neutral and aerated conditions [15]. Therefore, the antioxidant potential of the tea extract during the oxidation process might be reduced. The *lag*-time shift of aspalathin was longer than *lag*-time shift of nothofagin. The same difference in antioxidant activity was recorded in the TEAC/Fremy's salt assay for the two dihydrochalcones (Table 1). *Lag*-time of orientin and isoorientin was higher than that of (*S*)-eriodictyol-6-*C*-β-D-glycopyranoside, which was also anticipated. However, in contrast to TEAC/Fremy's salt assay there was a significant difference in the antioxidant potential of isoorientin (3.8 h) compared to orientin (2.7 h) (Fig. 2). This suggests that the position of the sugar attachment had an influence on the efficacy of these flavones.

In contrast to expectations, isoquercitrin gave a *lag*-time shift of 9.6 h. The antioxidant potential of isoquercitrin was only increased in this cupper-induced assay. Recent studies have shown that flavonoids are able to complex metal ions [16, 17]. Isoquercitrin has three possible binding sites for copper ions: one at the 3′,4′-dihydroxyl functional groups at the B-ring, and two because of the 3 and 5 *O*-substituents next to the 4-carbonyl group (Fig. 3) [18]. Obviously, the response of isoquercitrin is mainly due to its chelating activity. The LDL oxidation assay has frequently been used to assess the AC of potent compounds *in vivo*. However, the living system can be described as almost depleted from free metal ions. Therefore, to overcome the disadvantages of an

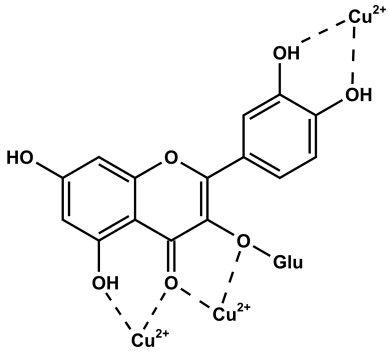

Figure 3. Binding sites of Cu^{2+} on isoquercitrin [18].

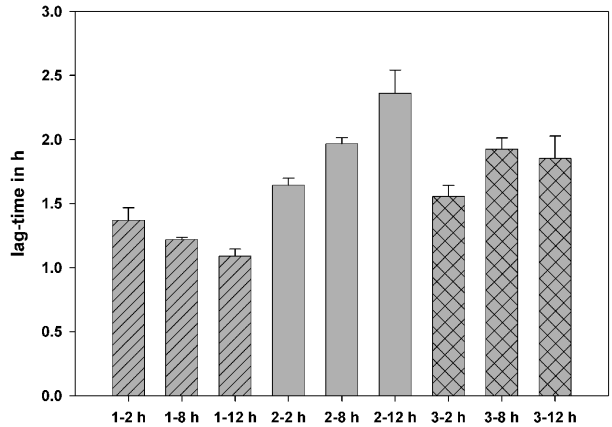

Figure 4. Decoupled LDL oxidation assay: LDL was incubated with antioxidants for 2, 8, and 12 h in absence of Cu^{2+}: (1) control; (2) aspalathin; and (3) isoquercitrin. After dialysis LDL oxidation was induced by Cu^{2+}.

in vitro system that uses high metal concentrations, a modified LDL oxidation assay was developed.

3.1.3 Decoupled LDL oxidation assay

To adapt the established *in vitro* LDL oxidation assay more closely to *in vivo* situation a decoupled LDL oxidation experiment was designed. The LDL solutions were pre-incubated with isoquercitrin and aspalathin, because these were the two most effective antioxidants in the *in vitro* LDL oxidation assay (Fig. 2). After different incubation times (2, 8, and 12 h) dialysis of LDL was performed against PBS–buffer and the LDLs were subsequently introduced into the Cu^{2+}-induced LDL oxidation. Thus, copper ions do not get in contact with the flavonoids, and possible chelating effects are excluded. Antioxidative chelating effects are strictly decoupled from antioxidative radical scavenging effects. On the other hand, during pre-incubation the antioxidants aspalathin and isoquercitrin enhance the antioxidant status of the LDL and thereby positively influence the *lag*-time during Cu^{2+} induced oxidation. Figure 4 presents the results from the decoupled LDL oxidation experiment. The control experiment gave a decrease in lag time during the

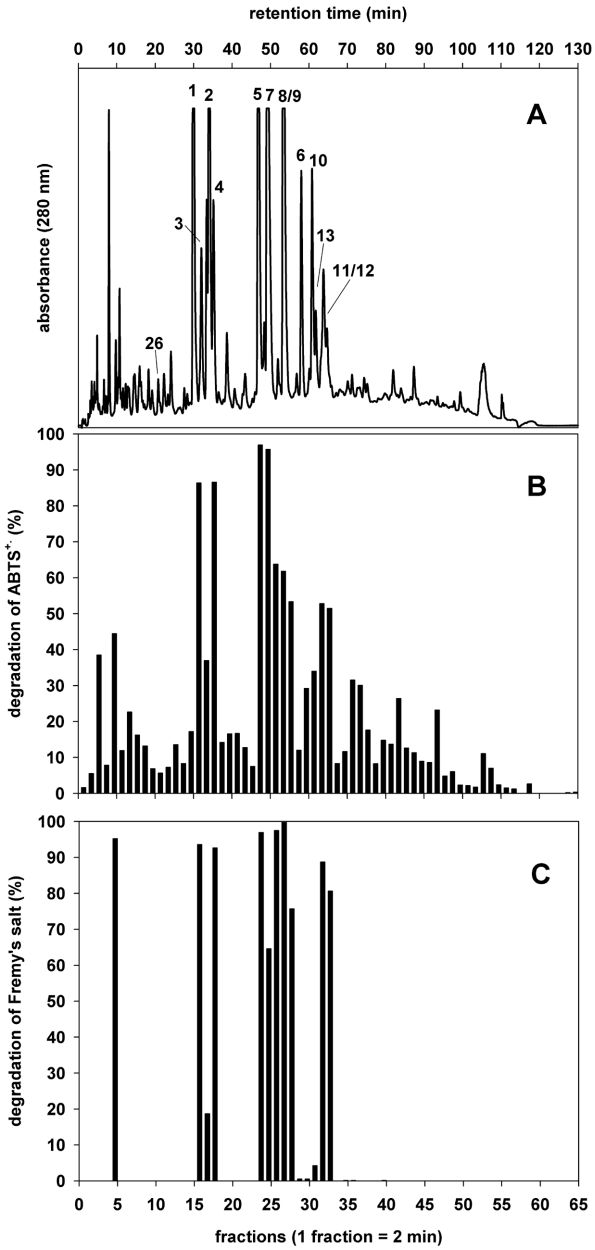

Figure 5. (A): HPLC-DAD chromatogram of ethyl acetate extract from fermented rooibos tea at $\lambda = 280$ nm. Retention times are given in brackets. (**1**): (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside (29.9 min); (**3**): (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside (33.3 min); (**2**): (R)-eriodictyol-6-*C*-β-D-glucopyranoside (34.1 min); (**4**) (R)-eriodictyol-8-*C*-β-D-glucopyranoside (35.1 min); (**5**) aspalathin (46.8 min); (**7**) orientin (49.2 min); (**8/9**) isoorientin/vitexin (53.2 min); (**6**) nothofagin (58.0 min); (**10**) isovitexin (60.8 min); (**13**) rutin (61.7 min); (**12**) hyperoside (63.7 min); (**11**) isoquercitrin (64.7 min); (B) degradation of ABTS•+ by collected fractions. (C) Degradation of Fremy's salt.

period of incubation, as expected. Autoxidation during pre-incubation proceeded with time and decreased the antioxidant status of LDLs.

The lag-time shift of LDLs that were in contact with aspalathin was prolonged with pre-incubation time in comparison to the control experiments. The highest *lag*-time was reached with 12 h pre-incubation. A time dependent *lag*-time shift was not observed with isoquercitrin. In the case of isoquercitrin a maximum LDL–lag time was reached with 8 h pre-incubation at levels lower than that of aspalathin. Thus, the antioxidative property of aspalathin appears to be based mainly on excellent radical scavenging. Isoquercitrin shows a mixed action of weaker radical scavenging and excellent metal chelating activity.

3.2 Screening assays

Results from the TEAC, Fremy's salt, and LDL-oxidation assays for the isolated polyphenols were used to explore the validity of an offline HPLC screening method. Rooibos extract was first separated by HPLC-DAD. After removal of solvent by vacuum centrifugation, fractions collected were subjected to the described antioxidant assays.

3.2.1 TEAC and Fremy's salt assay of the ethyl acetate extract

HPLC-DAD chromatogram (absorbance 280 nm, method A) and degradation of radicals (ABTS•+, Fremy's salt) are shown in Fig. 5 for the ethyl acetate extract.

The highest degradation of ABTS•+ was obtained with fraction 24 (46–48 min), which contained aspalathin, followed by fraction 25 (which contained orientin, 48–50 min) and fraction 16 and 18 (containing the *(S)*- and *(R)*-eriodictyol-6-*C*-β-D-glucopyranosides, 30–32 min and 34–36 min, respectively) (85–97%). The fractions containing hyperoside (fraction 32, 62–64 min) and isoquercitrin (fraction 33, 64–66 min) also induced a relatively high degradation of ABTS•+ (50%). The absorbance maxima of *(S)*- and *(R)*-eriodictyol-6-*C*-β-D-glucopyranosides and aspalathin are 280 nm, whereas for orientin, isoquercitrin and hyperoside it is located at 350 nm.

Because of high variation in peak intensities the molar extinction coefficient of the substances in a mixture needs be considered in order to relate UV absorbance with the absolute AC of single compounds. However, in screening assay of plant extracts with unknown constituents, it is complex or impossible to calculate the extinction coefficients for each substance. Thus, the offline HPLC analysis can only give information on potential antioxidative material eluting within specific time frames in the chromatographic run, which can then be subjected to further structural elucidation. For further characterization of eluting material, the ABTS•+ degradation/HPLC-DAD peak area ratios were calculated for each peak (Table 2).

Based on their structure and the results from TEAC assay, the antioxidant capacities for the compounds monitored at 280 nm were expected to be in following order: aspalathin > nothofagin > (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside,

Table 2. Ratio of ABTS$^{\bullet+}$ degradation/HPLC-DAD peak area[a)] of flavonoids included in ethyl acetate extract

Flavonoids			
Compound (280 nm)	Ratio[a)]	Compound (350 nm)	Ratio[a)]
Aspalathin	9.53×10^{-5}	Orientin	6.13×10^{-5}
Nothofagin	5.52×10^{-5}	Isoorientin/vitexin	4.93×10^{-5}
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside[a)]	9.36×10^{-5}	Isovitexin/rutin	3.50×10^{-5}
		Hyperoside[a)]	7.94×10^{-5}
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside[a)]	10.22×10^{-5}	Isoquercitrin[a)]	7.74×10^{-5}

a) Fraction co-eluted with further components.

(*R*)-eriodictyol-6-*C*-β-D-gluco-pyranoside. Fractions containing only a single substance provided the anticipated results (aspalathin ratio: 9.53×10^{-5} > nothofagin ratio: $5.52 \cdot 10^{-5}$) (Table 2). However, this approach is limited. The *(S)*- and *(R)*-eriodictyol-6-*C*-β-D-glycopyranosides coeluted with *(S)*- and *(R)*-eriodictyol-8-*C*-β-D-glycopyranosides in fractions 16–18. While the ABTS$^{\bullet+}$ UV absorbance ratio ($9.36 \cdot 10^{-5}$ (**1** + **3**) and 10.22×10^{-5} (**2** + **4**) suggested the compounds are as effective as aspalathin, the assays for the isolated structures verified significant differences (Table 2).

On the basis of results from the TEAC-assay antioxidant capacities for the compounds monitored at 350 nm were expected to be in the following order: hyperoside, isoquercitrin > orientin, isoorientin > vitexin, isovitexin. The expectation was achieved with fractions having only one major compound (hyperoside ratio: 7.94×10^{-5}, isoquercitrin ratio: 7.74×10^{-5} > orientin ratio: 6.13×10^{-5}). For fractions with further coeluting major compounds, the ratios were not directly comparable with the fractions of hyperoside, isoquercitrin and orientin, and should be considered only in part. For example, fraction 27 (52–54 min), containing isoorientin and vitexin, gave a ratio ABTS$^{\bullet+}$-degradation/HPLC-peak area of 4.93×10^{-5}. Isovitexin and rutin were in fraction 31 (60–62 min) and gave a lower ratio (3.50×10^{-5}) compared to fraction 27 (Fig. 5, Table 2).

Fractions of the ethyl acetate extract that induced an ABTS$^{\bullet+}$ degradation over 40% also showed a Fremy's salt degradation in the range of 65–100% (Figs. 5B and C). In relating the offline HPLC screening with Fremy's salt assay it was not possible to see differences between the various flavonoid types. Nothofagin (fraction 30) gave a very low degradation of Fremy's salt (4%), much lower than in ABTS$^{\bullet+}$-assay (29%). This was not unexpected. The same phenomenon was observed in the TEAC and Fremy's salt assays for the isolated nothofagin, as described above.

3.2.2 LDL-oxidation screening assay of the ethyl acetate extract

The ethyl acetate extract was separated by HPLC-DAD (method B). Selected fractions containing substances **1**–**9** were subjected to the LDL oxidation assay (Fig. 6). The highest response was shown by fractions which included more than one antioxidant (**2**–**4**; **8**/**9**), as expected. Lag-time shift of the aspalathin fraction was higher (3.0 h) than that of the nothofagin fraction (1.8 h). Orientin and aspalathin both facilitated a similar response, which was higher than that of (*S*)-eriodictyol-6-*C*-β-D-glycopyranoside.

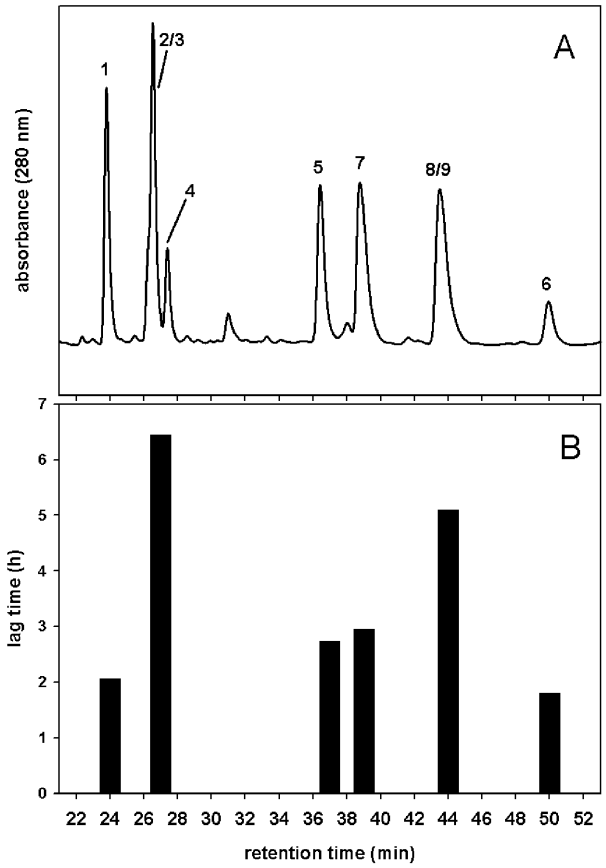

Figure 6. (A) Separation of ethyl acetate extract by HPLC; (**1**) (*S*)-eriodictyol-6-*C*-β-D-glycopyranoside (24.1 min) (**3**): (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside (26.9 min); (**2**) (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside (26.9 min); (**4**) (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside (27.8 min); (**5**) aspalathin (37.2 min); (**7**) orientin (39.6 min); (**8/9**) isoorientin/vitexin (44.4 min); (**6**) nothofagin (51.1 min); collected fractions were introduced into the LDL oxidation system. *Lag*-time shift of each fraction is presented in picture (B). *Lag*-time shift of control reaction without the addition of polyphenols was 1.6 h.

4 Concluding remarks

All assays performed in this study showed that fermented rooibos tea contains highly effective antioxidant compounds. The TEAC assay provides AC data relative to Trolox while the Fremy's salt assay relates to the absolute number of reduced radicals.

The decoupled LDL oxidation experiment is very useful to dissect the various mechanistic pathways of antioxidative action, *i.e.*, it differentiates between the radical scavenging and the metal chelating properties of a compound. The decoupled assay therefore may be a more appropriate estimate of the efficiency of an antioxidant *in vivo*. Isoquercitrin preferentially protects the LDLs by complexing metal ions. On the other hand, the antioxidant mechanism of aspalathin is based on scavenging of radicals. As protection against radicals as *e.g.*, reactive oxygen species is a key issue *in vivo*, the determination of the total antioxidant activity (TAA) of flavonoids *in vitro* should therefore separate between these two modes of action during the oxidation process. The results obtained emphasize the dihydrochalcone aspalathin as a structure more effective than the flavonol isoquercitrin. However, studies on the bioavailability of chalcones from food sources are limited.

The $ABTS^{\bullet+}$ degradation and the LDL oxidation assays can be used to screen for antioxidants in plant extracts in an HPLC-offline mode. Both are more sensitive than the Fremy's salt assay (Figs. 5 and 6). Even based on classification of compounds only by their UV maxima, the calculation of the $ABTS^{\bullet+}$-degradation/peak area ratio in the HPLC-DAD offline screening method was useful to estimate the relative AC of eluting material. However for screening extracts of plant material, the $ABTS^{\bullet+}$ assay should be preferred. The LDL oxidation is a far more time-consuming test in comparison to the $ABTS^{\bullet+}$ assay.

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