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Unfermented Rooibos Tea: Quantitative Characterization of Flavonoids by HPLC−UV and Determination of the Total Antioxidant Activity

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Unfermented rooibos originates from the leaves and the stems of the indigenous South African plant, *Aspalathus linearis*, and it has been reported to have a higher content of flavonoids compared to that of fermented rooibos. The HPLC/UV method developed in our laboratory for the analysis of the fermented rooibos was applied to the quantitative characterization of the major flavonoids present in the unfermented rooibos. Main compounds determined were aspalathin (49.92 ± 0.80 mg/g), isoorientin (3.57 ± 0.18 mg/g), orientin (2.336 ± 0. 049 mg/g), and rutin (1.69 ± 0.14 mg/g), followed in order by isovitexin, vitexin, isoquercitrin and hyperoside, quercetin, luteolin and chrysoeryol. The identity of detected flavonoids was confirmed by comparing their retention times and UV spectra with those of corresponding standards. The total antioxidant activity (TAA) of the tea infusions was measured by the $ABTS^{\bullet+}$ radical cation decolorization assay. The TAA of unfermented rooibos (0.8 Trolox meq/g) resulted 2-fold higher than that of the fermented rooibos. When compared with different water infusions of *Camellia sinensis* (green and black tea), this TAA value was about 50% lower.

KEYWORDS: Rooibos; *Aspalathus linearis; Camellia sinensis*; flavonoids; HPLC−UV; TAA; total polyphenols

INTRODUCTION

Rooibos (*Aspalathus linearis*) is a leguminous shrub native to the mountainous areas of the northwestern Cape Province in South Africa (*1*). Its leaves and fine stems are used for the production of unfermented and fermented rooibos tea.

Fermented rooibos tea is reported to have different biological properties, such as calming digestive disorders and various stomach problems, reducing nervous tension, and alleviating allergies (*1*). It is also used for topical treatment of dermatological diseases, such as Behcet's disease and photosensitive dermatitis (*2*).

Rooibos tea infusions are reported to have a good antioxidative activity (*3*), which can be attributed to the presence of the polyphenolic fraction.

Different flavonoids have been identified and quantified in fermented rooibos tea by HPLC−UV (*4*). However, few data on the content of these compounds in unfermented Rooibos are available (*5*). The aim of this study was the quantitative determination of the different flavonoid compounds by LC-UV-DAD.

In addition, the total antioxidant activity (TAA), and the total polyphenol content of fermented and unfermented rooibos (*Aspalathus linearis*) were determined and compared with the values of green and black tea (*Camellia sinensis*).

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MATERIALS AND METHODS

Chemicals. Rutin, orientin, isoorientin, vitexin, isovitexin, isoquercitrin, luteolin, and chrysoeriol were purchased from Extrasynthese (Genay, France). Quercetin was purchased from Sigma-Aldrich (Steinheim, Germany) and aspalathin was from Leuven Bioproducts (Belgium). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich Chemical Co., Gillingham, UK) was used as the antioxidant standard. $ABTS^{\bullet+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], gallic acid, and Folin Ciocalteu's phenol reagent were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile were HPLC grade from Merck (Darmstadt, Germany). Standards were dissolved in methanol (1 mg/mL) and stored at −20 °C.

Water Extraction of Polyphenol Fraction from the Different Types of Tea. Standard grade unfermented and fermented rooibos were obtained from Rooibos LTD-BPK (Clanwilliam, South Africa) and from Nutritea (Cedaberg, South Africa). Standard grade green and black tea were from Lipton (London, UK).

The aqueous extracts were obtained by pouring 60 mL of hot (90 °C) distilled water on 1 g of tea and steeping it for 10 min. The infusions were cooled to room temperature, filtered through filter paper, made up to volume (100 mL) with distilled water, and centrifuged at 4000 rpm for 10 min. The solutions were stored at −20 °C until time of analysis.

Chromatographic Conditions. HPLC separations were performed using a Waters 625 LC system (Milford, MA) connected with a Waters model 996 photodiode array detector, equipped with a Rheodyne injector (loop 50 μL) and a Millennium workstation (Waters). Acquisition was set at 255 and 287 nm (spectral acquisition in the range 200−400 nm).

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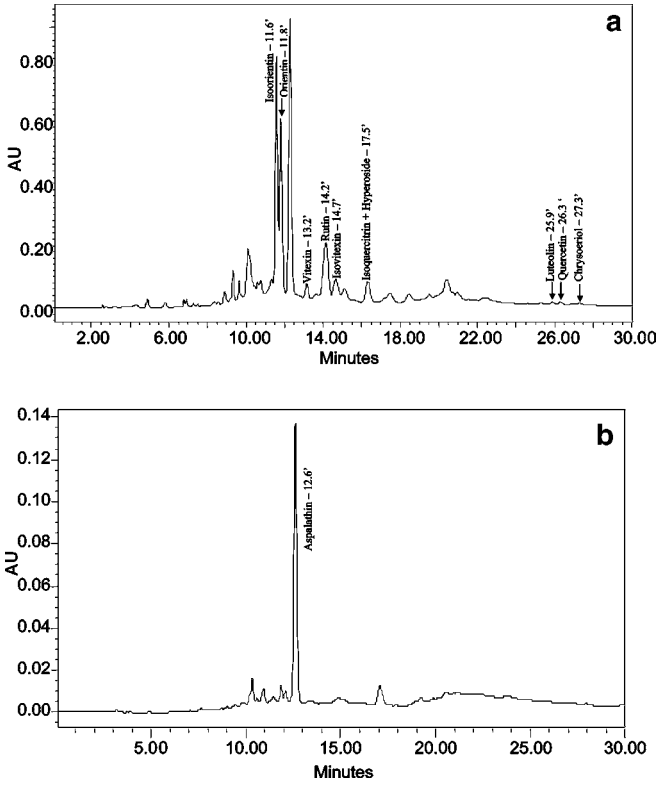

Figure 1. Typical HPLC chromatogram of unfermented rooibos tea aqueous infusion, injected volume 50 μL, at $\lambda = 255$ nm (**a**) and 287 nm (**b**).

Total Antioxidant Activity (TAA). The total antioxidant activity (TAA) of the tea infusions was measured by the $ABTS^{\bullet+}$ radical cation decolorisation assay, according to Miller and Rice-Evans (*6*).

$ABTS^{\bullet+}$ radical cation was prepared by reacting 10 mL of 2 mM ABTS water solution with 100 μL of 70 mM potassium persulfate, and the mixture was allowed to stand in the dark at room temperature for 12 h before use. Prior to the assay, the solution was diluted in ethanol to give an absorbance at 734 nm of 0.70 ± 0.02 in a 1-cm cuvette, and equilibrated at 30 °C.

A 5 mM stock solution of Trolox was prepared in ethanol and stored at −20 °C for 6 months. The working Trolox solutions had a concentration between 0.3 and 2.0 mM; these concentrations were obtained by diluting with ethanol the stock solution before each determination.

One milliliter of ethanolic $ABTS^{\bullet+}$ radical cation solution was added to aliquots of 10 μL of different Trolox standard solutions, or tea infusions, stirred for 30 s, and the absorbance was read at 734 nm.

Total Polyphenols. The determination of the total polyphenol content was carried out according to Singleton and Rossi *(7)*. Results were expressed as gallic acid equivalent per 1 g of dry leaf.

RESULTS AND DISCUSSION

Typical HPLC−UV chromatograms of an aqueous infusion of unfermented rooibos are shown in **Figure 1** at 255 nm (a) and 287 nm (b). Flavonoids extracted from rooibos were identified by comparison of their retention times and UV spectra with those of the reference standards (*4*). To determine the content of the flavonoids, calibration curves were prepared in the range from 0.1 to 20 μg/mL. Absorbance at $\lambda = 255$ nm (except for aspalathin at $\lambda = 287$ nm) increased linearly for all standards over the indicated concentration range (**Table 1**). An example of a calibration curve is given in **Figure 2**. The limit of detection was 50 ng/mL.

The levels of flavonoids present in unfermented and fermented rooibos were obtained by duplicate analysis, and the results are shown in **Table 2**. The main compounds determined in the unfermented rooibos were aspalathin, isoorientin, orientin, and rutin, followed in the order by isovitexin, vitexin, isoquercitrin and hyperoside, quercetin, luteolin, and chrysoeryol. These polyphenols were more abundant in unfermented rooibos than in fermented; particularly, the levels of aspalathin were almost 50 times higher. Indeed, the fermentation process involves an extensive degradation of aspalathin, which is oxidized to dihydro-*iso*-orientin (*8*). The *C*-glycosyl-flavones isoorientin,

Table 1. Equations of Calibration Curves Obtained for Flavonoid Standards

standard	calibration curves	R^2
isoorientin	$y = 114717.21 - 3449.87$	0.9996
orientin	$y = 128712.2 - 1867.26$	0.9998
aspalathin	$y = 128135.42 - 1801.99$	0.9999
vitexin	$y = 73829.04 + 1749.33$	0.9998
rutin	$y = 126398.88 - 2313.31$	0.9996
isovitexin	$y = 108817.5 + 3265.76$	0.9996
isoquercitrin	$y = 214867.99 - 10549.77$	0.9999
luteolin	$y = 417490.79 - 31020.85$	0.9999
quercetin	$y = 193998.33 - 28748.59$	0.9995
chrysoeriol	$y = 299429.5 - 3219.82$	0.9999

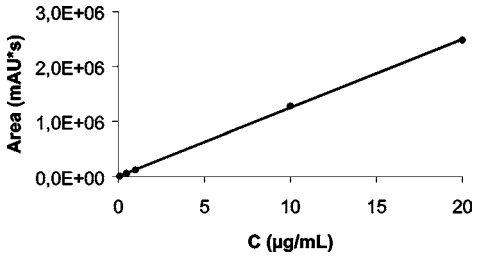

Figure 2. Calibration curve of the aspalathin standard, injected in the range from 0.1 to 20 μg/mL.

Table 2. Flavonoids Detected in Unfermented and Fermented Rooibos Aqueous Extract (mg/g ± SD)

compound	unfermented rooibos	fermented rooibos[a]
isoorientin	3.57 ± 0.18	0.833 ± 0.007
orientin	2.336 ± 0.049	1.003 ± 0.010
aspalathin	49.92 ± 0.80	1.234 ± 0.010
vitexin	0.504 ± 0.002	0.330 ± 0.002
rutin	1.69 ± 0.14	1.269 ± 0.006
isovitexin	0.659 ± 0.005	0.265 ± 0.002
isoquercitrin and hyperoside	0.326 ± 0.006	0.429 ± 0.002
luteolin	0.022 ± 0.002	0.029 ± 0.001
quercetin	0.042 ± 0.006	0.107 ± 0.002
chrysoeriol	0.0079 ± 0.0004	0.022 ± 0.001
total	59.08 ± 0.59	5.521 ± 0.055

[a] Values regarding the fermented Rooibos refer to our previous paper (*4*).

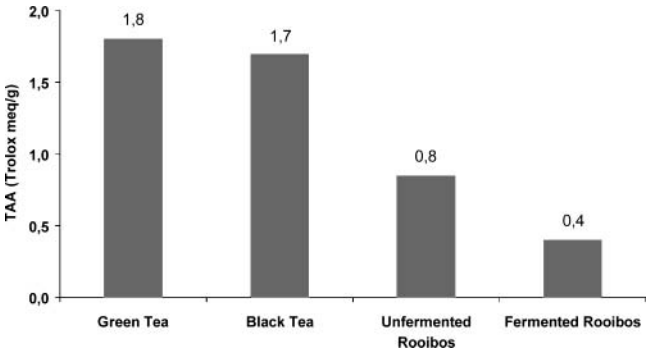

Figure 3. Total antioxidant activity of different types of tea.

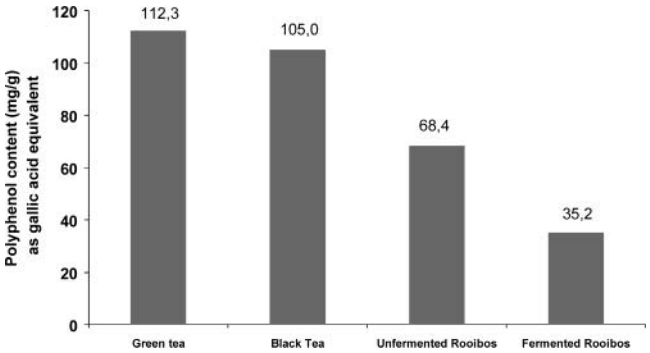

Figure 4. Total polyphenol content of different types of tea.

orientin, vitexin, and isovitexin are degraded at a lower extent. The main flavonol-glycoside rutin is partly converted to the aglycone quercetin, as evidenced by its increased level in fermented rooibos. As shown in **Figure 3**, the antioxidant activity of the water infusion of the unfermented rooibos (0.8 Trolox meq/g) was 2-fold higher than that of the fermented one. When compared with different water infusions of *Camellia sinensis* (green and black tea), obtained in the same conditions, this TAA value was about 50% lower. A similar trend for the antioxidant activity of these teas using the DPPH radical scavenging method was found by Von Gadow et al. (*3*) and is in good agreement with the total polyphenols content of the examined infusions (**Figure 4**).

In conclusion, unfermented rooibos tea is characterized by an higher content of polyphenols (particularly, aspalathin) and, similarly to green tea from *Camellia sinensis*, it displays a more elevated antioxidant activity in comparison to the fermented product.

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