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Unfermented and fermented rooibos teas (*Aspalathus linearis*) increase plasma total antioxidant capacity in healthy humans

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ABSTRACT

The aim of the study was to assess the effect of drinking rooibos tea (*Aspalathus linearis*) on total antioxidant capacity (TAC), lipid triacylglycerols, cholesterol and glycaemia plasma levels in humans. *In vitro*, unfermented rooibos tea displayed a 28% higher value of TRAP than did the fermented beverage. An acute intervention study, cross-over design, was performed, with 15 healthy volunteers who consumed 500 ml of either water, unfermented or fermented rooibos teas. Plasma antioxidant capacity increased significantly with both teas, reaching a peak at 1 h post-consumption (+6.6%, $p < 0.05$ fermented tea; +2.9%, $p < 0.01$ unfermented tea). No changes in triacylglycerols, cholesterol or uric acid were observed with any of the treatments. A transitory increase in glycaemia at 30 min was linked to glucose upload. The data show that rooibos teas represent a source of dietary antioxidants in humans.

1. Introduction

Rooibos tea is a herbal tea produced from the leaves of the indigenous South African plant, *Aspalathus linearis*. It has traditionally been used as a folk remedy to treat asthma, colic disorders, allergies and dermatological problems (McKay & Blumberg, 2007). Traditional rooibos tea is produced by fermentation of the leaves and stems to obtain a red-brown infusion. In contrast, the unfermented rooibos tea (green rooibos) keeps oxidation to a minimum, better preserving antioxidant properties. Rooibos tea is a good source of unusual polyphenolic compounds. Aspalathin and nothofagin, C-linked glycosides of the flavones, apigenin and luteolin, and four eriodictyol-C-glycoside isomers constitute the major flavonoids in rooibos tea. Fermentation results in a reduction in the levels of these compounds as they are oxidised to polymeric brown products and the flavone-C-glycosides, orientin and vitexin (Joubert, 1996; Krafczy & Glomb, 2008; Stalmach, Mullen, Pecorari, Serafini, & Crozier, 2009).

Studies performed with *in vitro* systems and animal models have shown that rooibos extracts possess antioxidant potential, antimutagenic and hepatoprotective properties and immuno-modulating effects (Joubert, Gelderblom, Louw, & de Beer, 2008). Bioavailability studies have been performed on animal models to correlate circulating metabolites to the *in vivo* beneficial effects observed. After absorption, flavonoids are predominantly metabolized in the colon and liver. Phase II biotransformations in the liver include glucuronidation, sulfation or methylation of the phenolic hydroxyl groups. Flavonoids not absorbed in the upper gastrointestinal tract are degraded by bacteria in the colon, with hydrolysis of conjugates and glycosides and ring fission of the aglycones to phenolic acids, followed by reabsorption (Crozier, Jaganath, & Clifford, 2009). Following acute ingestion of rooibos teas (same as this study) by humans, eight metabolites were detected in 0–24 h urine. These were *O*-linked methyl, sulphate and glucuronide metabolites of aspalathin and an eriodictyol-*O*-sulphate. The main compound excreted was an *O*-methyl-aspalathin-*O*-glucuronide, following ingestion of the unfermented drink and eriodictyl-*O*-sulphate, after ingestion of the fermented tea. The timing of excretion indicated that the aspalathin metabolites were absorbed in the small intestine and that absorption of the eriodictyol-*O*-sulphate occurred principally in the large intestine. However, overall levels of metabolites excreted in urine of <0.3% of intake indicate that the dihydrochalcone and flavone C-glycosides in rooibos teas are not readily absorbed and have a very restricted bioavailability (Stalmach et al., 2009).

In contrast to the vast literature on the *in vivo* properties of green and black tea (Rietveld & Wiseman, 2003), only a few studies have investigated the potential protective effects of rooibos tea consumption in human subjects. These have focused on iron status

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(Bree, Kruger, Jerling, & Oosthuizen, 2005; Hesseling, Klopper, & Van Heerden, 1979), antihistaminic effects (Hesseling & Joubert, 1982), and benefits regarding dermatological diseases (Shindo & Kato, 1991). There are no reports on the potential *in vivo* antioxidant properties of rooibos teas. Hence, in the present study we evaluated the *in vivo* antioxidant properties of fermented and unfermented rooibos teas in healthy humans and related these to the *in vitro* antioxidant capacity of the beverages.

2. Materials and methods

2.1. Chemicals

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Fluka (Italy). R-Phycoerythrin (R-PE) was from Europa Bioproducts Ltd.; 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemical (Germany). Phosphate-buffered saline tablets were from Sigma (St. Louis, MO USA). High-purity water was obtained using an Alpha-Q system (Millipore, Marlborough, MA).

2.2. Beverages

Bottles containing 500 ml of unfermented and fermented "ready to drink" rooibos teas were kindly provided by Beverage Partners Worldwide (Zürich, Switzerland). Beverages were formulated with 1.5 g/l of rooibos extract powder. Phenolic composition of the beverages has previously been described by Stalmach et al. (2009).

2.3. Human intervention study

The feeding protocol was approved by the local ethical committee. All procedures involving human subjects comply with the Declaration of Helsinki, as revised in 2000. All participants in the study gave their written consent. Fifteen healthy volunteers were selected on the basis of the following criteria: non-smoking, body mass index between 18 and 25 kg/m^2, normolipidaemic, taking no antioxidant supplements and not on any medication. Physical characteristics of the participants and baseline values of the biomarkers studied are described in Table 1. For two days prior to each feeding study, the subjects followed a low antioxidant diet by avoiding foods known to be high in antioxidants (all fresh fruit and vegetables and their products, such as tea, coffee, fruit juices and wine) and dietary antioxidant supplements. Dietary records were kept to check that subjects abstained from eating food rich in antioxidants.

The study followed a cross-over design; on the day of the intervention, after an overnight fast, subjects were randomized into three groups and received 500 ml of water (Group A), or 500 ml of fermented rooibos tea (Group B), or 500 ml of unfermented rooibos tea (Group C). Venous blood samples were collected at different time points (0, 0.5, 1, 2 and 5 h post-ingestion). Blood was collected in EDTA- and/or heparin-tubes and centrifuged immediately at 1300 *g* at 4 °C for 15 min, after which the plasma was separated and stored at −80 °C. After two weeks of wash out, experiments were repeated swapping the treatments until the subjects received all the different beverages.

Table 1
Physical characteristics and fasting biomarker profiles at baseline of the subjects ($n = 15$) participating in the study (means ± S.D.).

Biomarker	
Age (years)	33 ± 5
BMI (kg/m^2)	20.9 ± 1.8
Glucose (mg/dl)	87 ± 8
Triacylglycerols (mg/dl)	43 ± 11
Total cholesterol (mg/dl)	171 ± 24
Uric acid (μmol/l)	242 ± 72
TRAP (μmol/l)	1044 ± 85

2.4. Analytical procedures

The total radical-trapping antioxidant potential assay (TRAP) was used to measure the "chain-breaking" total antioxidant capacity (TAC) of plasma and teas. The method is based on the measurement of the fluorescence decay of the protein, R-Phycoerythrin (R-PE), induced by the peroxyl radicals obtained by thermal decomposition of the azo compound ABAP. Briefly, 50 μl of diluted sample were added to 75 μl of phosphate-buffered saline (pH 7.4), 15 μl of diluted R-PE (fc 4.30×10^{-3} μg μl^{-1}) and 60 μl of ABAP (fc 7.5 mM); the reaction kinetic at 38 °C was recorded for 60 min (λ_{ex} = 495 nm, λ_{em} = 570 nm) by a Tecan GENios Standard fluorescent plate reader spectrometer (Tecan Italia s.r.l, Segrate, MI). The length of the lag-phase, automatically calculated, was used to assess TRAP values, expressed as μmol/l for rooibos extracts and plasma. The protection afforded by the sample is reflected in a lag-phase directly related to the amount and activity of antioxidants contained on it (Ghiselli, Serafini, Maiani, Azzini, & Ferro-Luzzi, 1995). The assay allows evaluation of changes in TAC over a wide interval of values due to dietary interventions (Serafini & Del Río, 2004).

Plasma glucose, total cholesterol, triacylglycerols, and urate were measured using commercial kits (Sentinel Diagnostics, Milan-Italy).

2.5. Statistical analyses

All data were checked for normal distribution with use of the Shapiro–Wilk's test. The variables were considered as Gaussian, following the transformation to natural logarithms, before statistical analysis. The effect of rooibos teas on selected biomarkers, compared to the control group, was tested by analysis of covariance (ANCOVA) after adjusting for baseline values (T0), followed by Tukey's HSD multiple rank test. The effect of treatments at different time points compared to baseline values was tested with a paired t-test, as described by Serafini et al. (2002).

3. Results

3.1. In vitro total antioxidant capacity (TAC) of rooibos teas

Unfermented rooibos tea displays a 28% higher *in vitro* antioxidant capacity than does fermented rooibos tea, measured as chain-breaking antioxidant activity (TRAP) (5.23 ± 0.80 mmol/l and 4.07 ± 0.29 mmol/l, respectively). Fig. 1 illustrates TRAP values, expressed as mmol trolox/l, for the rooibos beverages in comparison with reference values from the literature for commercially available teas and fruit juices obtained with the same methodology (Pellegrini et al., 2003). Both rooibos teas possess a lower chain-breaking antioxidant potential than do green and black teas but higher than commercially available instant tea.

3.2. Human intervention study

No statistical changes were observed in plasma triacylglycerols and cholesterol levels after consumption of the teas (Table 2). Glycaemia did not change in the control group (Table 2) but increased significantly 30 min after the ingestion of fermented (+32.0% with respect to baseline; $p < 0.001$) and unfermented

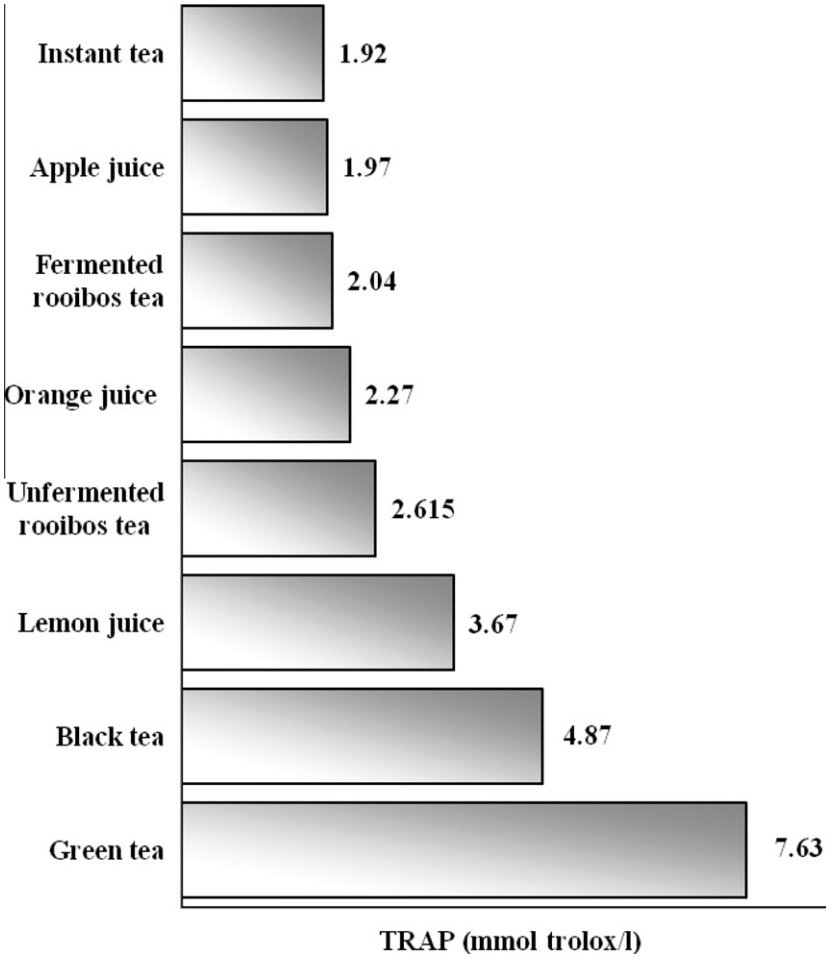

Fig. 1. Comparison of TRAP values of the unfermented and fermented rooibos teas with reference values for commercial teas and fruit juices.

Table 2
Plasma concentrations of biochemical parameters, expressed as mean values ± standard error, before and 0.5, 1, 2 and 5 h after the ingestion of water, fermented rooibos and unfermented rooibos teas (n = 15).

Biomarker	Time (h)	Water	Unfermented rooibos tea	Fermented rooibos tea
Triacylglycerols (mg/dl)	T0	43 ± 3	44 ± 3	41 ± 3
	T0.5	40 ± 3	42 ± 3	40 ± 3
	T1	37 ± 3	44 ± 4	38 ± 3
	T2	37 ± 4	39 ± 3	39 ± 4
	T5	39 ± 4	43 ± 3	44 ± 3
Cholesterol (mg/dl)	T0	167 ± 7	173 ± 7	175 ± 6
	T0.5	169 ± 6	169 ± 7	173 ± 6
	T1	172 ± 6	172 ± 6	174 ± 6
	T2	173 ± 6	171 ± 6	179 ± 6
	T5	177 ± 7	176 ± 7	177 ± 6
Glycaemia (mg/dl)	T0	85 ± 2	87 ± 2	89 ± 3
	T0.5	82 ± 2	106 ± 4[a]	118 ± 4[a]
	T1	84 ± 2	82 ± 5	84 ± 5
	T2	83 ± 2	79 ± 2	80 ± 2
	T5	83 ± 2	82 ± 2	84 ± 2
Uric acid (μmol/l)	T0	244 ± 20	245 ± 19	238 ± 18
	T0.5	236 ± 17	254 ± 18	244 ± 17
	T1	232 ± 19	253 ± 18	232 ± 17
	T2	231 ± 19	239 ± 19	235 ± 18
	T5	245 ± 18	240 ± 17	237 ± 21

[a] Statistically different with respect to control group (water); $p < 0.001$ by ANCOVA.

(+21.6% with respect to baseline; $p < 0.001$) rooibos teas. No changes in plasma uric acid were detected during the entire period of observation, for all the treatments (Table 2).

As expected, there were no changes in plasma TRAP values after drinking water (Table 3). The ingestion of fermented rooibos tea displayed a clear antioxidant effect *in vivo*: plasma TRAP increased slightly after 30 min (+4.8%) reaching a statistically significant peak after 1 h (+6.6%; $p < 0.05$ compared to baseline and $p < 0.05$ compared to control), started to decline at 2 h (+4.9%; $p < 0.05$ compared to baseline) and returned to baseline values after 5 h (+2.2%). Consumption of the unfermented rooibos tea increased plasma chain-breaking antioxidant potential, with TRAP values increasing after 30 min (+1.7%) and attained significantly higher values after 1 h (+2.9%; $p < 0.01$ with respect to control) and 2 h (+2.7%; $p < 0.05$ with respect to control) before returning to baseline values after 5 h (Table 3).

Table 3
Plasma TRAP (μmol/l) expressed as mean values ± standard error before and 0.5, 1, 2 and 5 h after the ingestion of water, fermented rooibos and unfermented rooibos teas (n = 15).

Time (h)	Water	Unfermented rooibos	Fermented rooibos
T0	1026 ± 28	1073 ± 17	1035 ± 19
T0.5	1028 ± 23	1093 ± 25	1083 ± 28
T1	1020 ± 25	1105 ± 26[c]	1100 ± 25[a,b]
T2	1039 ± 29	1103 ± 28[b]	1084 ± 26[a]
T5	1034 ± 28	1062 ± 28	1054 ± 18

[a] $p < 0.05$ Statistically different with respect to baseline; by paired t-test.
[b] $p < 0.05$. Statistically different respect to control group (water); by ANCOVA.
[c] $p < 0.01$ Statistically different with respect to control group (water); by ANCOVA.

4. Discussion

The data obtained in this study show, for the first time, that both fermented and unfermented rooibos teas are able to boost plasma antioxidant defences in humans. The *in vitro* results from the present study show that unfermented rooibos tea has a higher antioxidant potency than has fermented rooibos tea; the decrease on the antioxidant capacity has been linked to the fermentation process, where a decline in total polyphenol content occurs with the oxidation of aspalathin and nothofagin (Joubert, Winterton, Britz, & Ferreira, 2004). Results of a previous study from our group have revealed that the levels of the dihydrochalcones, aspalathin and nothofagin, were *ca.* Ten fold lower in the fermented tea than in the unfermented drink (Stalmach et al., 2009).

The two rooibos teas exhibited a lower antioxidant potential than did green and black tea infusions but higher than commercially available instant tea. The same order of potency has been obtained with other radical-scavenging assays as in the DPPH$^{\cdot}$ method (Stanley et al., 2001; Von Gadow, Joubert, & Hansmann, 1997;) and ABTS$^{+\cdot}$ assay (Schulz, Joubert, & Schütze, 2003). This lower *in vitro* TAC is linked to the different phenolic contents of these beverages compared to the *Camellia sinensis* teas and, together with the low bioavailability of these compounds (Stalmach et al., 2009), explains the modest but significant *in vivo* effect observed. Variables, such as absorption, inter-individual variability, bioavailability and mechanism of redox endogenous regulation, play an important role in defining the absorption and the efficiency of dietary antioxidants *in vivo*. The *in vitro* values need to be considered as a potentiality of the food to deliver redox-active ingredients in the circulation but this needs to be supported by experimental evidence in humans.

In order to attempt to correlate the observed increases in plasma TAC (Table 3) with the absorption of the flavonoids in rooibos tea, a bioavailability study was previously performed, focused on the quantification of aspalathin and nothofagin metabolites in plasma and urine after ingestion of unfermented and fermented rooibos teas *(*Stalmach et al., 2009*).* No flavonoids or their metabolites were detected in plasma but urine contained *O*-linked methyl, sulphate and glucuronide metabolites of aspalathin and an eriodictyol-*O*-sulphate. The main compound excreted was an *O*-methyl-aspalathin-*O*-glucuronide, following ingestion of the unfermented drink, and eriodictyol-*O*-sulphate, after ingestion of the fermented tea. The timing of excretion indicated that the aspalathin metabolites were absorbed in the small intestine and that absorption of the eridictyol-*O*-sulphate occurred principally in the large intestine. Total 0–24 h excretion of metabolites corresponded to 0.09% and 0.22% of the flavonoid content of the ingested fermented and unfermented teas, demonstrating the low bioavailability of the dihydrochalcone and flavone C-glycosides in the teas (Stalmach et al., 2009).

The absence of detectable quantities of metabolites of dihydrochalcone and flavanone C-glycosides in plasma most probably reflects their rapid removal from circulation. On the basis of the data obtained in the bioavailability study, we cannot explain the *in vivo* antioxidant effect observed after ingestion of the rooibos teas (Table 2). This is not unusual in intervention studies with antioxidant-rich foods (Serafini et al., 2003, 2009), where most of the investigations showing an increase in plasma antioxidant defences, measured as TAC, have failed to identify the components responsible, raising questions as to the direct involvement of flavonoid metabolites as antioxidant molecules *in vivo*.

Lotito and Frei (2004) suggested that the increase of plasma TAC induced by acute ingestion of apple was the consequence of a metabolic effect on urate levels, due to the fructose content of the apples. This proposal is not in keeping with a recent report that evidenced how fructose loading (75 g) was unable to modify TAC plasma levels in healthy humans and patients with type 2 diabetes (Faure, Polge, Monneret, Favier, & Halimi, 2008) nor is it in agreement with data obtained in the current study where a significant increase in plasma TAC occurred after drinking rooibos teas (Table 3) without concomitant statistically significant increases in plasma uric acid levels (Table 2).

In conclusion, for the first time, it has been shown, with healthy humans, that acute ingestion of both fermented and unfermented rooibos teas induced an increase in plasma antioxidant defences, measured as TAC. However, further intervention trials with humans are needed in order investigate the effects of more long-term consumption on TAC to support our findings suggesting that the rooibos teas, like traditional green and black teas, are a useful supplementary source of dietary antioxidants.

Conflict of interest statement

None declared.

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