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# Continuous administration of polyphenols from aqueous rooibos (*Aspalathus linearis*) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice

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A R T I C L E I N F O

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*Aspalathus linearis*
Hepatic steatosis
Hyperlipidemia
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Macrophage recruitment
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A B S T R A C T

The incidence of obesity and related metabolic diseases is increasing globally. Current medical treatments often fail to halt the progress of such disturbances, and plant-derived polyphenols are increasingly being investigated as a possible way to provide safe and effective complementary therapy. Rooibos (*Aspalathus linearis*) is a rich source of polyphenols without caloric and/or stimulant components. We have tentatively characterized 25 phenolic compounds in rooibos extract and studied the effects of continuous aqueous rooibos extract consumption in mice. The effects of this extract, which contained 25% w/w of total polyphenol content, were negligible in animals with no metabolic disturbance but were significant in hyperlipemic mice, especially in those in which energy intake was increased via a Western-type diet that increased the risk of developing metabolic complications. In these mice, we found hypolipemiant activity when given rooibos extract, with significant reductions in serum cholesterol, triglyceride and free fatty acid concentrations. Additionally, we found changes in adipocyte size and number as well as complete prevention of dietary-induced hepatic steatosis. These effects were not related to changes in insulin resistance. Among other possible mechanisms, we present data indicating that the activation of AMP-activated protein kinase (AMPK) and the resulting regulation of cellular energy homeostasis may play a significant role in these effects of rooibos extract. Our findings suggest that adding polyphenols to the daily diet is likely to help in the overall management of metabolic diseases.

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## Introduction

Oxidation and inflammation are major pathogenic factors involved in metabolic disorders in which excessive energy consumption plays a major role. These disorders include obesity, liver steatosis, type 2 diabetes, dyslipidemia and probably cancer, which exhibit increases in morbidity and mortality rates not only in Western societies but also throughout the world (http://www.cdc.gov). Caloric intake is a major contributor to these diseases, but the type of diet consumed also plays a role. It has been known for decades that a diet rich in fruits, vegetables and olive oil may help to reduce cardiovascular and metabolic complications, but the components of these foods that confer health benefits are currently unknown (Hu 2003). Recent interest has been focused on the biological activities of plant-derived phenolic compounds, but safety and efficacy issues remain unresolved in humans. For instance, it has already been documented that polyphenols from green tea or wine may confer a cardiovascular protective effect (Feillet-Coudray et al. 2009; Li et al. 2004), and other medicinal plants are currently being considered for anti-inflammatory purposes (Beltran-Debon et al. 2010).

Rooibos (*Aspalathus linearis*) (Fig. 1) grows naturally in certain areas of South Africa (Joubert et al. 2008) and is widely used to

*Abbreviations:* AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; CD, chow diet; C/EBP, CCAAT/enhancer-binding protein; DAD, diode array detector (analyzer); eWAT, epididymal white adipose tissue; FASN, fatty acid synthase; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; HF, high-fat, high-cholesterol diet; HPLC, high performance liquid chromatography; iWAT, inguinal white adipose tissue; LDL, low density lipoprotein; LDLr$^{-/-}$, low density lipoprotein receptor knock-out mice; MCP-1, monocyte chemoattractant protein-1; MS, mass spectrometry; TOF, time of flight (analyzer); UV, ultraviolet; VLDL, very low density lipoprotein.

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**Fig. 1.** Rooibos plant (*Aspalathus linearis*).

prepare infusions that represent a rich source of polyphenols without caloric value (Krafczyk and Glomb 2008). Moreover, *in vitro* and *in vivo* studies have demonstrated that rooibos has significant bioactivity, with described effects of preventing lipid oxidation and oxidative stress (Fukasawa et al. 2009; Ulicna et al. 2006), inflammation (Baba et al. 2009), hyperglycemia (Kawano et al. 2009) and chemically induced liver damage (Ulicna et al. 2003). In this study, we have characterized phenolic compounds in rooibos extract and found that the continuous administration of this extract has no effect in metabolically normal mice. However, we found a significant metabolic modulatory effect of rooibos extract administration in low-density lipoprotein receptor (LDLr) deficient mice, a model for metabolic disturbances that can be further exacerbated by feeding the animals a Western-type diet (Rodríguez-Sanabria et al. 2010).

## Materials and methods

### *Identification of phenolic compounds*

Rooibos plant material was kindly provided by Arend Redelinghuys (Rooibos BPK, Ltd., South Africa). Samples were prepared in an aqueous solution at a concentration of 1000 ppm; they were then filtered and injected into the HPLC system, an Agilent 1200-series RRLC system (Agilent Technologies, Palo Alto, CA, USA) that was equipped with a binary pump and DAD. Injected samples (10 μl) were passed through a Zorbax Eclipse Plus $C_{18}$ (4.6 mm × 150 mm, 1.8 μm) column at room temperature at a constant flow of 0.5 ml/min. A solution of 1% formic acid in water/acetonitrile 90:10 v/v and acetonitrile were used as mobile phases A and B, respectively, and programmed as follows: gradient elution from 5% to 20% B for 20 min, from 20% to 40% B for 5 min, from 40% to 5% B for 5 min, and an isocratic solution of 5% B for 5 min.

TOF-MS was conducted using a microTOF™ (Bruker Daltonik GmbH, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an electrospray ionization (ESI) interface. Parameters for analysis were set using the negative ion mode with a capillary voltage of 4500 V, a dry gas temperature of 200 °C, a dry gas flow of 7.0 l/min, a nebulizer pressure of 1.5 bar and a spectra rate of 1 Hz. The spectra were acquired over a mass range of 50–1000 *m*/*z*. All operations were controlled by DataAnalysis 3.4 software (Bruker Daltonik), which provided a list of possible elemental formulas using the GenerateMolecularFormula™ editor.

UV–visible spectrophotometry delimited the class of phenolic compounds, and the accurate mass measurements on the TOF-MS enabled us to identify the compounds in the extract. Finally, the fragmentation pattern obtained in the MS/MS experiments performed with an Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik) confirmed the proposed structures. The ion trap scanned at a 50–1000 *m*/*z* range with a gradient optimized for a negative ionization polarity. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +3.5 kV; dry gas temperature, 300 °C; dry gas flow, 7 l/min; and nebulizer pressure, 1.5 bar.

The total phenolic content of the rooibos extract was determined by colorimetric assay using Folin–Ciocalteu reagent (Singleton and Rossi 1965). The absorbance of the solution was measured at a wavelength of 725 nm in a Spectronic Genesys™ 5 spectrophotometer.

### *Animal experimental models*

Studies on C57BL/6J male mice showed a lack of toxicity with the continuous administration of aqueous rooibos extract, and we found no effect (for clarity, data are not shown because changes were negligible). To test our hypothesis, we decided to use male $LDLr^{-/-}$ mice as a model of intense metabolic alterations that in some aspects resemble the human metabolic syndrome (Rodríguez-Sanabria et al. 2010). They were the progeny of animals obtained from the Jackson Laboratory in a C57BL/6J background. The number of animals used in the experiment ($n = 32$) was determined based on previous data (Joven et al. 2007). Animals were housed under a 12-h light/dark cycle at a constant temperature of 25 °C with free access to water and a commercial mouse diet (14% Protein Rodent Maintenance Diet, Harlan, Barcelona, Spain). At 10 weeks of age, animals with equivalent body weights were randomly assigned to two dietary study groups. The chow diet group (CD, $n = 16$) continued to receive the same maintenance diet (3% fat and 0.03% cholesterol, w/w), and the second group ($n = 16$) was fed a high-fat, high-cholesterol diet (HF, 20% fat and 0.25% cholesterol, w/w). Each dietary group was divided into two treatment groups ($n = 8$). One of these treatment groups received tap water as a unique liquid source (control), while the other group received a rooibos extract (10 g/l). To prevent possible degradation, fresh extracts were prepared every two days. The material was weighted, boiled in tap water, cooled down to room temperature and filtered. The animals had free access to liquid sources and food; body weight and food intake were measured weekly. Treatments were maintained over 14 weeks, after which the animals were sacrificed under xylazine–ketamine anesthesia. All procedures were performed in accordance with institutional guidelines.

### *Cell culture*

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone, 3-isobutyl-1-methylxanthine, insulin and crystal violet were obtained from

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Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, calf serum, fetal bovine serum, and antibiotic mixtures (penicillin–streptomycin) were purchased from PAA Laboratories (GmbH, Linz, Austria). Sodium pyruvate and trypsin-EDTA were obtained from the Invitrogen Co. (Carlsbad, CA, USA). The AdipoRed™ Assay Reagent was purchased from Lonza (Walkersville, MD, USA).

The 3T3-L1 pre-adipocyte line, maintained at low passage number, was grown at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 1% pyruvate, 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were subcultured before reaching a confluence of 80%. Pre-adipocytes were differentiated using standard protocols (Maslowska et al. 2006). The cells were harvested 10 days after the initiation of differentiation, at which time 90% of cells were mature adipocytes with accumulated fat droplets (as determined by microscopic evaluation). To test the effects of rooibos on adipogenesis, rooibos extract was added to the medium at a final concentration of 600 μg/ml. Extracts were prepared with distilled water, filtered through a 0.22-μm filter and lyophilized. Cytotoxicity was measured by the crystal violet method as previously described (Ishiyama et al. 1996). Lipid content was measured using the Adipored assay following the manufacturer's protocol.

### Sample collection and laboratory measurements

Blood and tissue samples were obtained and processed as previously described (Rull et al. 2007). Serum cholesterol, triglycerides, free fatty acids and bilirubin concentrations and AST activity were determined using an automatic analyzer Synchron LXi 725 system (Beckman Coulter, IZASA, Barcelona, Spain). A glucose tolerance test was performed as described elsewhere (Rull et al. 2007).

### Histology

Liver steatosis was qualitatively evaluated on a scale of 0–3, where 0 represented an absence of steatosis and 3 indicated a major grade of steatosis (>66%). Immunohistochemistry and measurements of the area of adipocytes were performed as described elsewhere (Rull et al. 2007).

### FPLC lipoprotein fractionation

Plasma lipoproteins were separated using a Bio-Rad BioLogic DuoFlow 10 system (Bio-Rad, Spain). Plasma pooled samples from each group (100 μl) were injected into a Superose 6/300 GL column (GE Healthcare Europe GmbH, Barcelona, Spain), and 500-μl fractions were collected. Cholesterol and triglycerides in each fraction were determined as described above.

### Western blot analysis

Liver and eWAT tissues were weighted and homogenized in 5 ml/g of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 1 mM Pefabloc and 1% phosphatase inhibitor cocktail no. 2, Sigma–Aldrich Inc., Steinheim, Germany) using the Precellys 24 system (Bertin Technologies, France). Samples were delipidated using a methanol/diethyl ether (3:7) extraction. Proteins were quantified using a 2D Quant kit (GE Healthcare, Piscataway, NJ, USA) and electrophoresed in NuPAGE 4–12% Bis–Tris gradient polyacrylamide gels (Invitrogen, Barcelona, Spain). MES was used for AMPK, pAMPK, C/EBPβ and actin electrophoresis, while MOPS buffer (Invitrogen) was used for FASN. Proteins were transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Detection antibodies were rabbit anti-AMPK (2532, Cell Signaling Tech., Danvers, MA, USA), rabbit anti-pAMPK (2531, Cell Signaling Tech.), rabbit anti-C/EBPβ (Δ198, St. Cruz Biotech., Heidelberg, Germany), rabbit anti-FAS (3180, Cell Signaling Tech.) and rabbit anti-actin (H-300, St. Cruz Biotech.). The secondary antibody was goat anti-rabbit-HRP (Dako, Glostrup, Denmark). Chemiluminescent detection was performed using the ECL Advance Western Blotting Detection kit (Amersham, GE Healthcare, Barcelona, Spain), and membranes were analyzed in a VersaDoc system (Bio-Rad, Spain).

### Statistical analyses

Values are represented as mean ± SEM. Differences between groups were compared using non-parametric tests and were considered statistically significant when $p < 0.05$. All statistical analyses were carried out using the Statistical Package for Social Sciences version 15.0 (SPSS, Chicago, IL, USA).

## Results

### Rooibos extract is composed of multiple distinct phenolic compounds

A total of 25 phenolic compounds were successfully separated and identified with a gradient optimized for negative ionization polarity in less than 30 min. The base peak chromatogram obtained using an ESI-TOF mass spectrometer and a three-dimensional chromatogram showed a correlation between retention time, $m/z$, and intensity, as depicted in Fig. 2A and B, respectively. Identified and characterized compounds of each peak are summarized in Table 1. The average total polyphenol content, as evaluated by the Folin–Ciocalteu assay, was 252.07 ± 8.01 mg/g of rooibos tea extract, expressed as the equivalent of caffeic acid.

### Effects on body weight and food intake were apparently contradictory and evident after a short period of treatment

The extract is a complex mixture of polyphenolic compounds (Fig. 2 and Table 1) in which the quantitatively important components are orientin, isoorientin, vitexin, aspalathin, rutin and quercetin (and derivatives). The continuous ingestion of such a mixture under the study conditions did not exhibit toxic effects. Metabolic effects, however, were clearly dependent upon the type of diet consumed, even in a mouse model that was already metabolically compromised. This was immediately evident in the amount of body weight gain and food intake observed in the animals. Surprisingly, when mice were fed a high-fat, high-cholesterol diet, the rooibos extract increased the consumption of food, and a subsequent increase in body weight was observed (Fig. 3A). Conversely, when fed a chow diet, the rooibos extract had the opposite effect; the mice did not gain body weight after 4–7 weeks of treatment. Such an effect was not due to a generally decreased food intake (Fig. 3B). These diet-conditioned effects were further analyzed by weighting individual organs and tissues (Table 2). Notably, under both diets, the weight of the pancreas was significantly higher when supplemented with the rooibos polyphenolic mixture. There were also trends towards lower muscle weight and higher brown adipose tissue (BAT) weight. However, the two diets elicited opposite effects in other tissues. The rooibos extract decreased liver weight and increased the weight of epididymal and inguinal white adipose tissue (WAT) in mice fed CD. However, when fed a HF diet, liver weight increased and WAT weight decreased significantly, indicating that the effect of rooibos is sensitive to the characteristics and function of the analyzed metabolic tissue.

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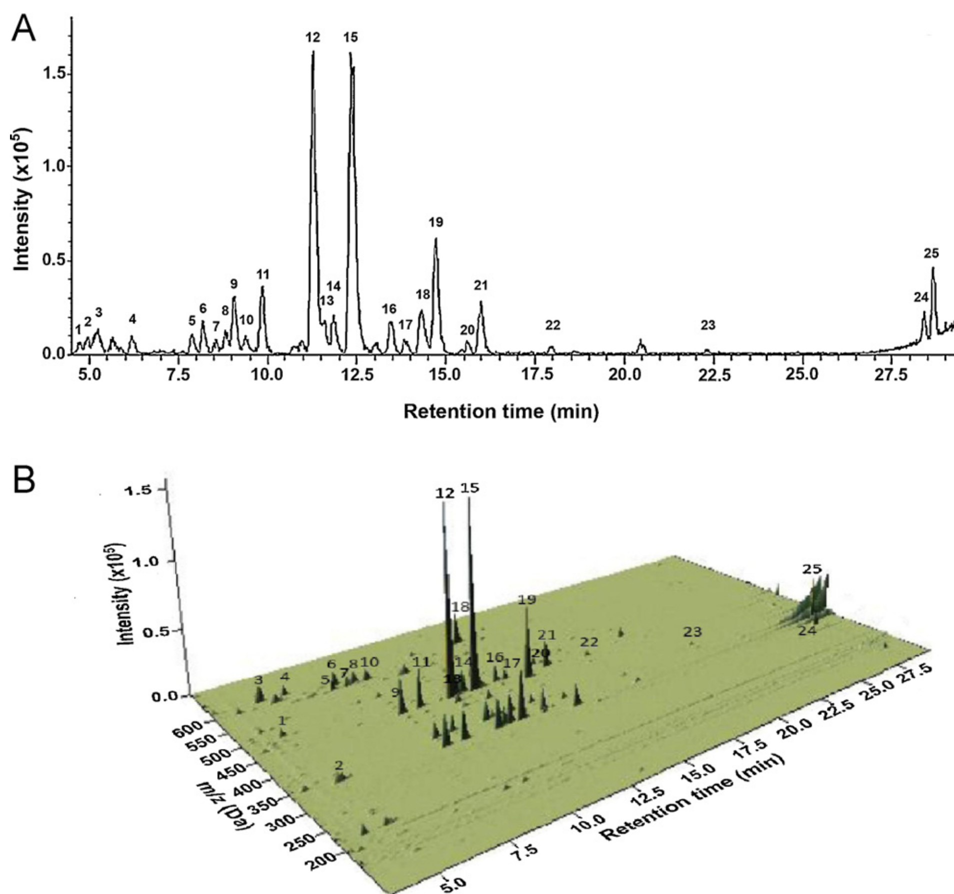


**Fig. 2.** The ESI-microTOF base peak chromatogram of rooibos extract obtained using negative ion mode (A). A three-dimensional chromatogram of the rooibos extract (B).

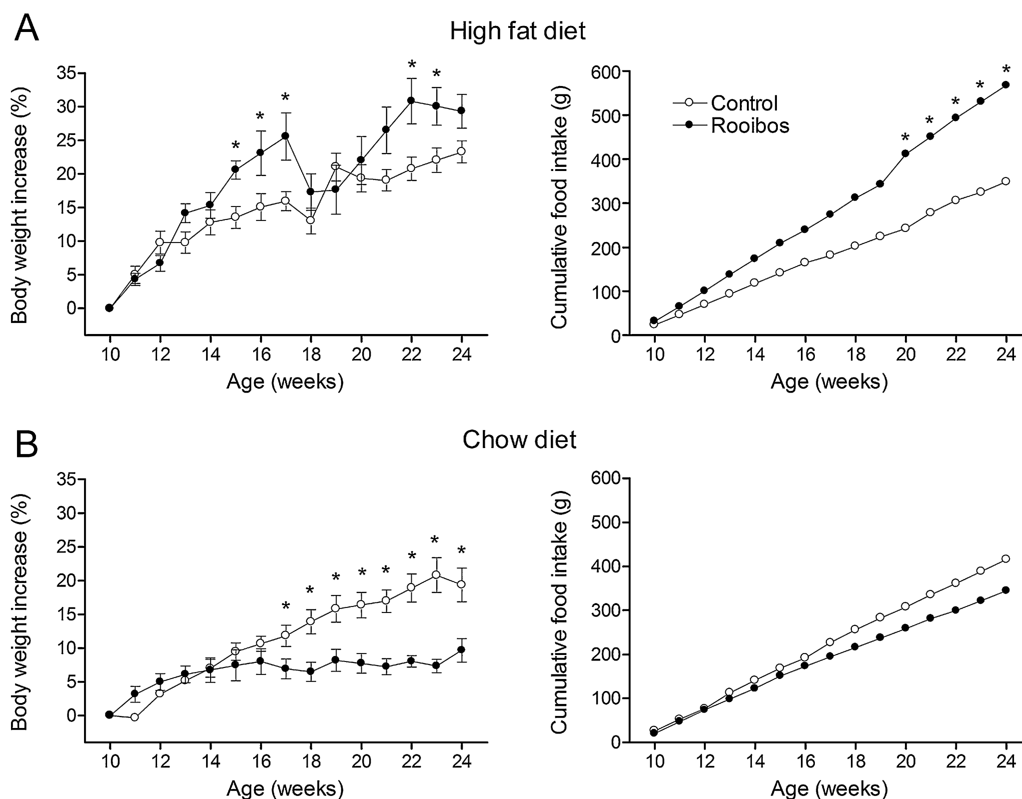


**Fig. 3.** Body weight gain and cumulative food intake in mice fed either HF (A) or CD (B). Open circles represent the control group and closed circles the group supplemented with aqueous rooibos extract. $^{*}p < 0.05$ for differences between groups.

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**Table 1**
Mass spectral data obtained in the rooibos tea extract and identified by ESI-microTOF-IT.

| Compound | RT | Molecular formula | *m/z* experimental | *m/z* calculated | UV (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|
| 1 | 4.79 | $C_{22}H_{21}O_{13}$ | 493.0968 | 493.0988 | 278 | 475, 403, 373, 385, 355 | Patuletin 7-glucoside |
| 2 | 4.99 | $C_{15}H_{15}O_9$ | 339.0710 | 339.0722 | 257<br>284<br>314(sh) | 249, 219 | Esculin |
| 3 | 5.30 | $C_{27}H_{31}O_{16}$ | 611.1600 | 611.1618 | 284 | 521, 491, 401, 371 | Safflomin A |
| 4 | 6.22 | $C_{27}H_{29}O_{16}$ | 609.1446 | 609.1461 | 261<br>341 | 519, 489 | Quercetin-3-*O*-robinobioside |
| 5 | 7.91 | $C_{26}H_{27}O_{15}$ | 579.1359 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 441, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-β-arabinopyranosylorientin |
| 6 | 8.20 | $C_{27}H_{29}O_{15}$ | 593.1496 | 593.1512 | 271<br>333 | 575, 503, 473, 413, 383, 353 | Vicenin-2 |
| 7 | 8.58 | $C_{26}H_{27}O_{15}$ | 579.1356 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 429, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 8 | 8.85 | $C_{26}H_{27}O_{15}$ | 579.1353 | 579.1355 | 271<br>341 | 559, 519, 489, 459, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 9 | 9.10 | $C_{21}H_{21}O_{11}$ | 449.1088 | 449.1089 | 287 | 359, 329 | (*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 10 | 9.40 | $C_{26}H_{27}O_{15}$ | 579.1365 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 429, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 11 | 9.89 | $C_{21}H_{21}O_{11}$ | 449.1072 | 449.1089 | 287 | 359, 329 | (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 12 | 11.27 | $C_{21}H_{19}O_{11}$ | 447.0923 | 447.0933 | 269<br>348 | 429, 411, 357, 327 | Isoorientin |
| 13 | 11.63 | $C_{21}H_{21}O_{11}$ | 449.1055 | 449.1089 | 285 | 359, 329 | (*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 14 | 11.88 | $C_{21}H_{21}O_{11}$ | 449.1069 | 449.1089 | 286 | 359, 329 | (*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 15 | 12.34 | $C_{21}H_{19}O_{11}$ | 447.0939 | 447.0933 | 254<br>267(sh)<br>349 | 357, 327 | Orientin |
| 16 | 13.43 | $C_{21}H_{23}O_{11}$ | 451.1253 | 451.1246 | 280 | 361, 331 | Aspalathin |
| 17 | 13.83 | $C_{21}H_{21}O_{11}$ | 449.1100 | 449.1089 | 282 | 359, 329, 285 | Aspalalinin |
| 18 | 14.32 | $C_{27}H_{29}O_{16}$ | 609.1465 | 609.1461 | 267<br>348 | 301 | Rutin |
| 19 | 14.74 | $C_{21}H_{19}O_{10}$ | 431.1002 | 431.0984 | 270<br>336 | 413, 341, 311 | Vitexin/isovitexin |
| 20 | 15.59 | $C_{21}H_{19}O_{12}$ | 463.0897 | 463.0882 | 276 | 301, 341, 371 | Quercetin-3-*O*-glucoside/galactoside |
| 21 | 15.97 | $C_{21}H_{19}O_{11}$ | 447.0921 | 447.0933 | 270<br>342 | 285 | Luteolin-7-*O*-glucoside |
| 22 | 17.93 | $C_{21}H_{23}O_{10}$ | 435.1287 | 435.1297 | 283 | 345, 315 | Nothofagin |
| 23 | 22.29 | $C_{20}H_{25}O_6$ | 361.1633 | 361.1657 | 280 | 346, 331 | Secoisolariciresinol |
| 24 | 28.37 | $C_{15}H_9O_6$ | 285.0386 | 285.0405 | 241<br>269<br>335 | 151, 107 | Luteolin |
| 25 | 28.60 | $C_{15}H_9O_7$ | 301.0335 | 301.0354 | 254<br>271(sh)<br>371 | 179, 151 | Quercetin |

*Note*: sh = shoulder.

### *Effects of aqueous rooibos extract in hepatic and adipose tissue*

We examined liver histology and found that dietary-induced liver steatosis was completely prevented by the rooibos extract (Fig. 4A) and that this was associated with a significant decrease in macrophage recruitment as assessed by F4/80 immunostaining (Fig. 4B). In mice fed CD, the degree of steatosis was significantly lower, and the effect of the rooibos extract was not appreciable. Further, we did not find significant alterations in serum biomarkers of liver injury, mainly bilirubin and AST, in any of the conditions assayed (Fig. 4C).

**Table 2**
The course of individual weights of organs and selected tissues (mg/g body weight) were dependent on the type of diet (#) and on treatment with rooibos extract (*). (*,# $p < 0.05$; **,## $p < 0.01$, denote differences respect to the corresponding group).

| | Chow diet | | High fat diet | |
|---|---|---|---|---|
| | Control | Rooibos | Control | Rooibos |
| Liver | 42.2 ± 1.4 | 38.9 ± 1.7 | 41.9 ± 1.3 | 46.5 ± 0.7*,## |
| Pancreas | 9.4 ± 0.8 | 13.6 ± 0.4** | 9.5 ± 0.9 | 13.9 ± 0.6** |
| Muscle | 13.5 ± 0.3 | 12.8 ± 0.3 | 13.4 ± 0.4 | 12.1 ± 0.2* |
| Epididimal WAT | 13.5 ± 1.1 | 15.6 ± 2.3 | 18.5 ± 1.9 | 14.5 ± 1.7 |
| Inguinal WAT | 11.8 ± 0.8 | 15.9 ± 0.2** | 15.1 ± 1.0# | 12.7 ± 0.9# |
| BAT | 2.5 ± 0.2 | 3.2 ± 0.6 | 2.1 ± 0.2 | 2.4 ± 0.1# |

We also assessed the size and number of adipocytes in adipose tissue. As expected, the adipocyte area was increased in control mice fed HF relative to mice fed CD. Despite a significant trend towards lower WAT weight in response to rooibos treatment, the differences in adipocyte size and number were not significant. However, the opposite effect was observed in mice fed CD (Fig. 5A), where rooibos supplementation resulted in a significant increase in the adipocyte area of epididymal WAT. The large variability in adipocyte size in inguinal WAT prevented the difference between the groups in adipocyte area in this tissue from reaching significance (data not shown). To further assess this effect, we used an

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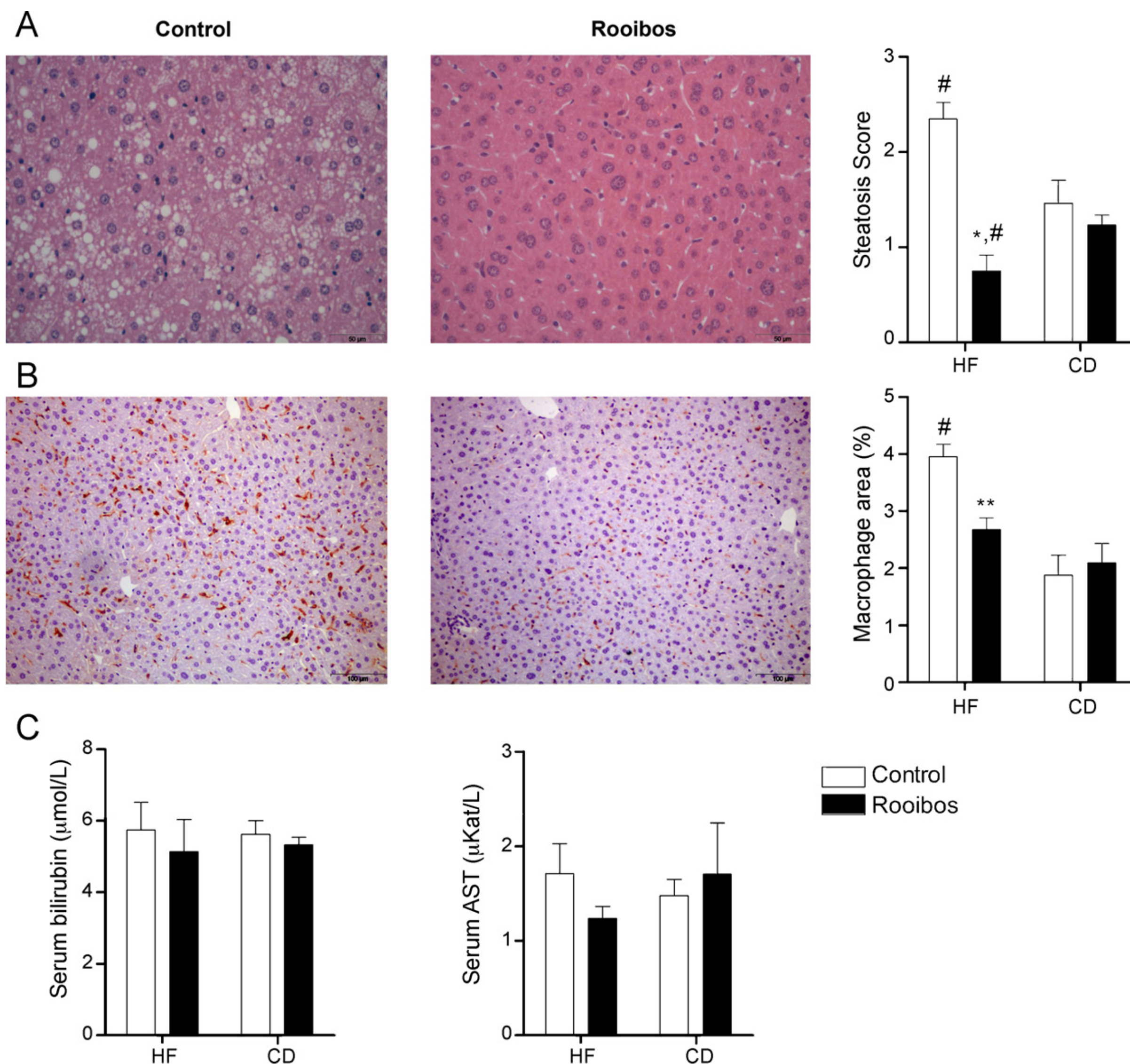


**Fig. 4.** Representative microphotographs illustrating the effect of rooibos extract consumption in livers of mice fed HF, decreasing steatosis (A) and macrophage infiltration (B) without affecting serum biomarkers of liver injury (C). $^{*}p<0.05$; $^{**}p<0.01$ for differences between control and rooibos treatment for the same dietary group; $^{\#}p<0.05$ for differences between dietary groups.

*in vitro* model with cultured 3T3-L1 mouse adipocytes. We found no effect of rooibos on differentiation from pre-adipocytes, but in mature adipocytes, which readily accumulate large intracellular fat droplets, rooibos extract significantly inhibited the accumulation of triglycerides in a dose-dependent manner as assessed by AdipoRed staining (Fig. 5B) without affecting cell viability (Fig. 5C and D).

*Other metabolic effects and possible mechanisms*

Despite such obvious metabolic effects, these were not related to changes in insulin resistance as assayed by glucose tolerance tests (Fig. 6). Effects of rooibos on glucose metabolism are therefore unlikely, but we found significant changes in lipoprotein metabolism associated with rooibos extract consumption. Specifically, rooibos extract had marked hypolipidemic effects in this particular model. This finding was limited to mice fed a HF diet and consisted of significant reductions in serum cholesterol, triglycerides and free fatty acids (Fig. 7A). As observed in the FPLC distribution, the reduction in cholesterol was evident in all lipoprotein particles, while the reduction in triglycerides was limited to very low density lipoproteins (VLDL) (Fig. 7B).

Taken together, our results suggest that possible molecular targets of the polyphenols contained in rooibos extract are mainly located in the liver and adipose tissue, and we assessed the expression of relevant candidate proteins in both of these tissues. We found that rooibos extract significantly activated the AMP-activated protein kinase (AMPK) in the liver but not in the eWAT, and this effect was not dependent on the type of diet. Similarly, the expression of CCAAT/enhancer-binding protein (C/EBPβ) was higher in the eWAT of rooibos-treated animals. Again, this effect was not affected by diet and was not evident in the liver. Finally, there was a trend towards lower expression of fatty acid synthase (FASN) in the liver, but the difference was not significant. Conversely, FASN expression was increased in adipose tissue, but this was only evident in the eWAT of mice fed CD under rooibos treatment (Fig. 8).

## Discussion

Our data indicate that the continuous administration of polyphenolic compounds may play a complementary role in the treatment of metabolic diseases. Although a preventive effect can-

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**Fig. 5.** The rooibos extract increased the adipocyte size of animals fed on CD (A, representative microphotographs for eWAT). 3T3-L1 adipogenesis was analyzed using the AdipoRed stain (B, adipocytes treated with 600 μg/ml of rooibos extract), showing dose-dependent anti-adipogenic activity of rooibos extract (C). No toxic effects were observed at the assayed concentrations (D). Adipocyte area differences are marked with # between dietary groups and with * between treatment groups for the same diet (both $p < 0.05$). For cellular experiments, differences between groups are marked with * ($p < 0.05$).

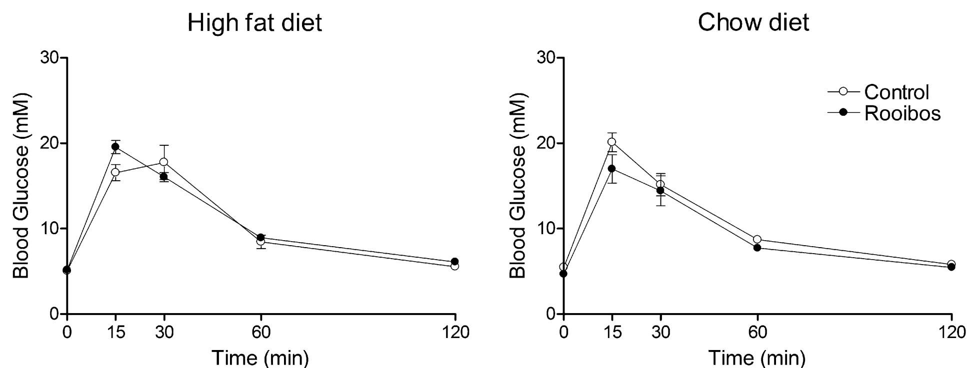


**Fig. 6.** The glucose tolerance tests showed no differences derived from diet type or rooibos extract supplementation.

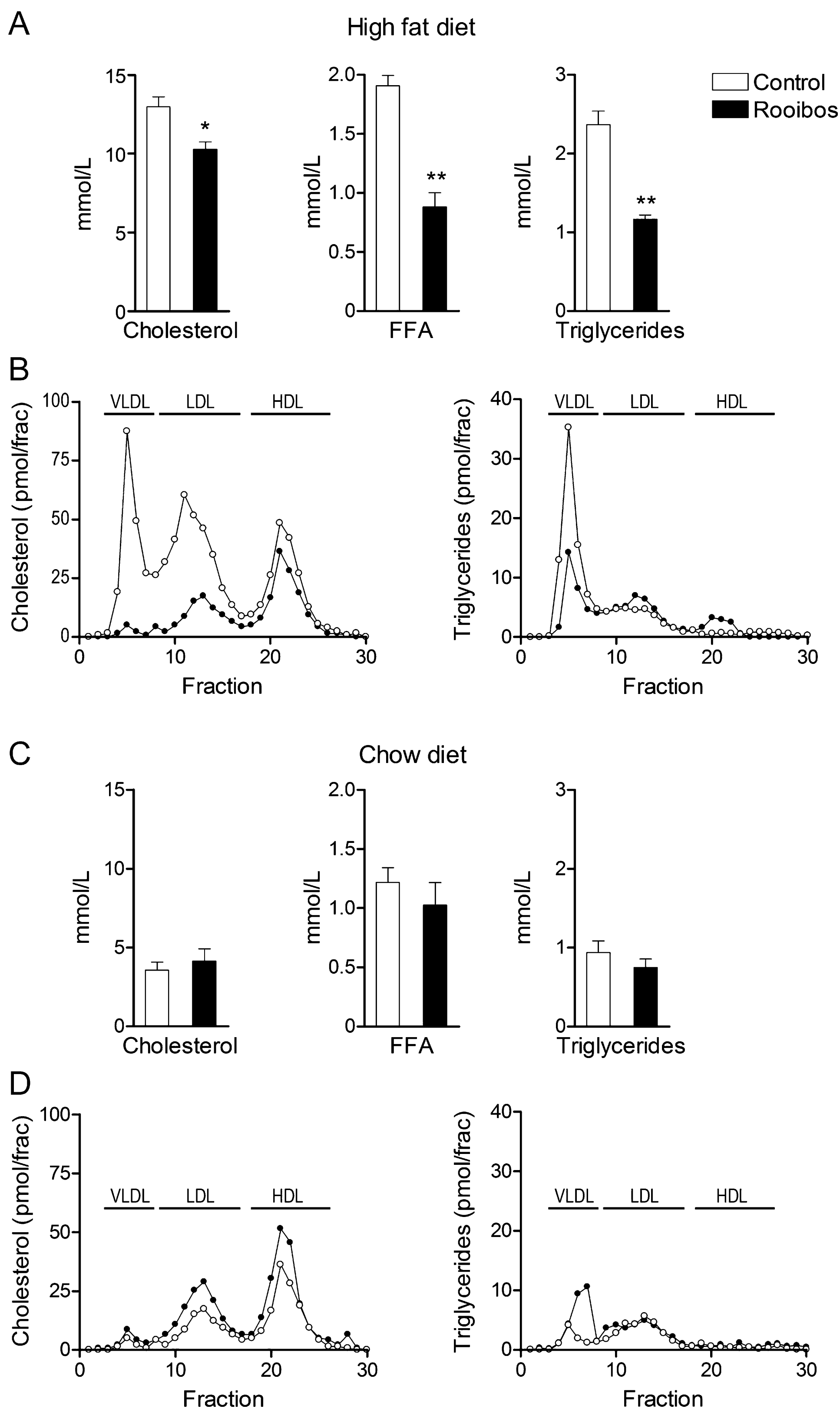


**Fig. 7.** The rooibos extract elicited a hypolipemic effect in mice fed on HF (A) with significant changes in lipoprotein distribution (B). Such effects were not observed in the CD dietary group (C and D). Open bars and circles represent controls, and closed bars and circles represent rooibos-treated animals. *$p < 0.05$ and **$p < 0.01$ for differences between control and rooibos treatment.

not be ruled out from our design, this seems unlikely because none of the beneficial effects were observed in metabolically normal mice. In this particular hyperlipemic model, which involves well-documented metabolic disturbances, we found that rooibos extract significantly affected body weight, the development of liver steatosis, the accumulation of triglycerides in adipocytes and the course of hyperlipidemia. These effects were not related to changes in insulin resistance and were most evident in animals fed

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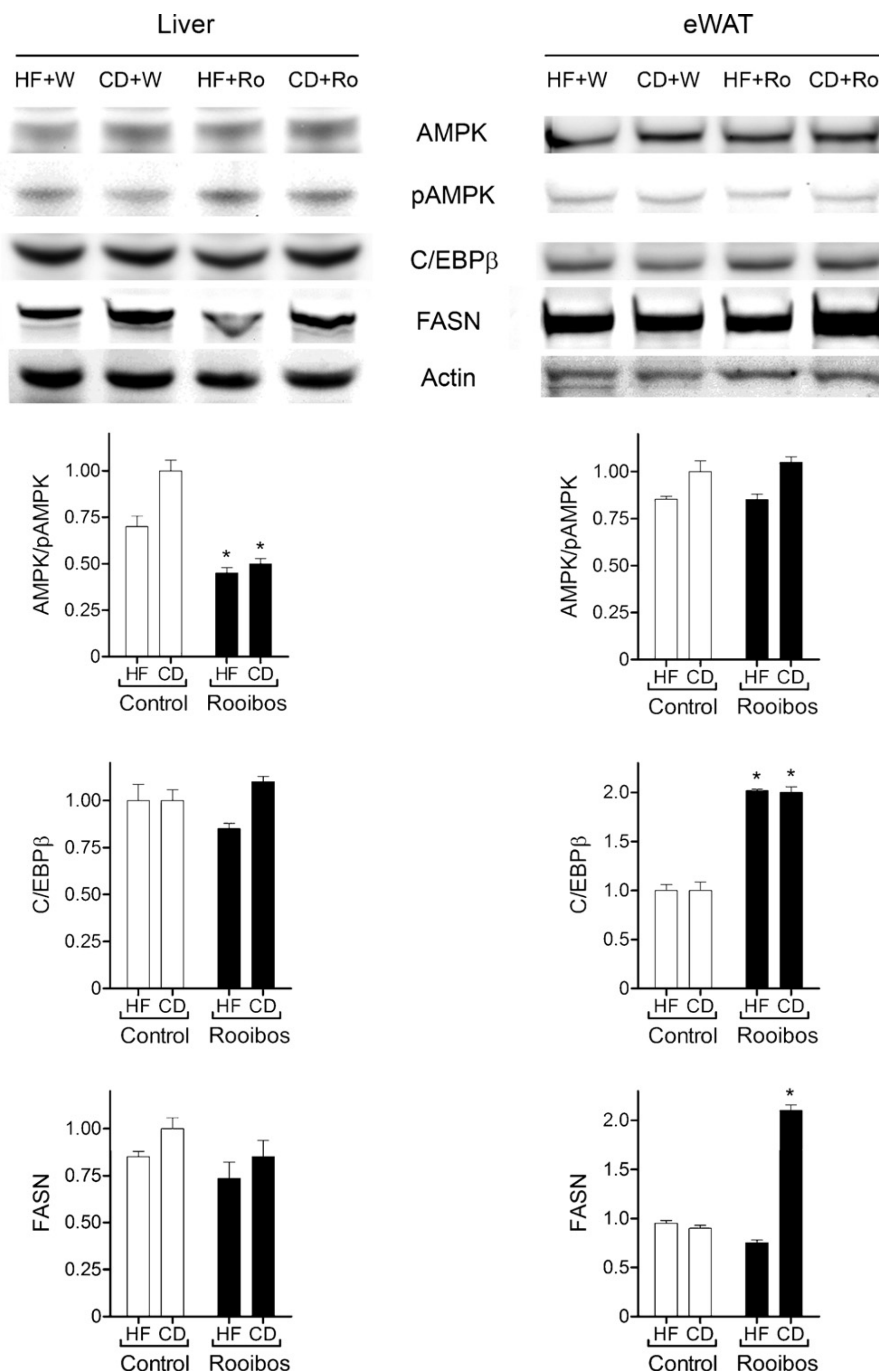


**Fig. 8.** The different expression of candidate proteins in the liver (left) and epididymal WAT (right) from all groups studied. The rooibos extract activated AMPK in both dietary groups by increasing pAMPK expression in the liver. In the eWAT, rooibos affected mainly C/EBPβ protein expression in both dietary groups, but the increased expression of FASN was only evident in the CD dietary group. $^{*}p < 0.05$ for differences between control and rooibos treatment.

a Western-type diet. That plants, and particularly fruits and vegetables, may benefit human health is widely recognized (Joshipura et al. 2001), but the role of dietary polyphenols, although suggestive, remains unclear. There is currently little serious effort to develop plant-derived molecules for therapeutic purposes. There is certainly no enthusiasm for promoting the ingestion of complex mixtures of polyphenols and testing their possible health benefits in humans, probably because patentability is clearly limited. In addition, polyphenols seem to interact with numerous targets and multiple deregulated signaling pathways (Johnson et al. 2010), raising doubts regarding their safety and ease of administration. For instance, resveratrol modulates more than twenty enzymes and receptors (Baur and Sinclair 2006). Green tea polyphenols are also an example of this notion (Khan et al. 2006; Johnson et al. 2010), and curcumin, a much simpler compound, may influence more than 60 molecular targets (Goel et al. 2008). Due to the recent increasing interest in the consumption of natural products, certain beverages containing polyphenols are widely distributed, proving

*ex juvantibus* that they are generally nontoxic and may provide a wide range of health benefits in humans. One of these examples is rooibos, which is now consumed in Western societies as a plant infusion and therefore as a complex mixture of molecules. Most of its components are recognized as having antioxidant properties (Bramati et al. 2002), although it is unlikely that *in vivo* antioxidant effects explain the alleged health benefits. On the other hand, it is documented that the polyphenols contained in the rooibos extract, mainly aspalathin, orientin and rutin, are effectively absorbed (Boyle et al. 2000; Courts and Williamson 2009; Kreuz et al. 2008; Li et al. 2008).

The effects we describe are certainly the result of intense modulation of the overall management of cellular energy. It is interesting to note that these effects are negligible in normal mice and only evident in a model in which metabolism is seriously disturbed (Rodríguez-Sanabria et al. 2010). Moreover, these effects are more intense in mice fed a clearly unhealthy diet similar to that recently adopted by humans. Our findings regarding the body weight and food intake of animals supplemented with aqueous rooibos extract may seem counterintuitive. Our interpretation is based on the fact that the liver and the WAT are the two primary lipid storage organs in mammals. Although obesity is characterized by an increase in adipocyte number and/or size (White and Stephens 2009), in the absence of aberrant diets, rooibos extract promotes lipid accumulation in adipose tissue and probably breaks down stored fat in the liver, thus preventing lipotoxicity in this non-adipose organ. Rooibos extract, in the presence of excessive caloric intake provided by a high-fat diet, protects the liver from lipid storage without increasing the accumulation of fat in adipose tissue. The effects we describe in the liver may be relevant because liver steatosis is highly prevalent in adults and children and may progress to more serious conditions (Alkhouri et al. 2009).

Obviously, impaired fat absorption as an explanation for such a net reduction in fat storage cannot be ruled out based on our data, but our findings clearly suggest that rooibos extract may elicit similar effects to those described for resveratrol, i.e., increasing metabolic rate and mitochondrial biogenesis (Marques et al. 2009). Interestingly, we did not observe a hypoglycemic effect or a change in insulin sensitivity as previously described in diabetic mice (Kawano et al. 2009), but we partially confirmed the hepatoprotective and anti-lipogenic effects reported in rats and other mouse models (Ulicna et al. 2003; Hoffmann-Bohm et al. 1992; Janbaz et al. 2002; Odbayar et al. 2006). It is also possible that the previously reported anti-inflammatory effects of rooibos extract (Baba et al. 2009) may play a role in the significant decrease in macrophage recruitment that we observed. Moreover, polyphenols selectively decrease macrophage chemotaxis via a reduction in MCP-1 expression (Beltran-Debon et al. 2010).

Additionally, we found a strong hypolipidemic effect that, again, was only observed in mice fed a HF diet, which may partially confirm the hypocholesterolemic effect observed in similar models (Ziaee et al. 2009). It is of note, however, that we also observed a significant decrease in serum triglyceride and free fatty acid concentrations, highlighting an overall effect on the management of fat by both the liver and adipose tissue. In this regard, we found significant activation of AMPK in the liver but not in adipose tissue. The activation of this master metabolic regulating enzyme is relevant and a novel finding. Activated AMPK switches cells from active ATP consumption (e.g., fatty acid and cholesterol biosynthesis) to active ATP production (e.g., fatty acid and glucose oxidation) (Carling 2004). Recent studies in mice deficient in the catalytic subunit of AMPK may also explain the responses of FASN and C/EBP in this model and suggest that AMPK is the central target for the metabolic effects of polyphenols (Um et al. 2010).

Our data also blaze new trails to further investigate a more general mechanism of action for ingested polyphenols. For instance, it is extremely suggestive of the recent finding that autophagy regulates adipose mass and differentiation in mice (Singh et al. 2009), a phenomenon that enables adaptation to stress, maintains metabolism and promotes cellular viability as well as fitness (Sasaki et al. 2010). Polyphenols have well documented antioxidant activities and provide other therapeutic targets (Ulrich-Merzenich et al. 2009). We confirm in this study previous findings suggesting more beneficial effects of the whole extract than, probably, individual, isolated compounds. We also support a change in paradigm in this field and the search for possible synergies may constitute an appropriate research effort (Wagner and Ulrich-Merzenich 2009).

We add Rooibos extract and its metabolic effects to such effort and suggest the combination with other bioactive extracts in order to attenuate the metabolic abnormalities associated to human diseases.

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## References

Alkhouri, N., Dixon, L.J., Feldstein, A.E., 2009. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. Expert. Rev. Gastroenterol. Hepatol. 3, 445–451.

Baba, H., Ohtsuka, Y., Haruna, H., Lee, T., Nagata, S., Maeda, M., Yamashiro, Y., Shimizu, T., 2009. Studies of anti-inflammatory effects of Rooibos tea in rats. Pediatr. Int. 51, 700–704.

Baur, J.A., Sinclair, D.A., 2006. Therapeutic potential of resveratrol: the in vivo evidence. Nat. Rev. Drug Discov. 5, 493–506.

Beltran-Debon, R., Alonso-Villaverde, C., Aragones, G., Rodriguez-Medina, I., Rull, A., Micol, V., Segura-Carretero, A., Fernandez-Gutierrez, A., Camps, J., Joven, J., 2010. The aqueous extract of *Hibiscus sabdariffa* calices modulates the production of monocyte chemoattractant protein-1 in humans. Phytomedicine 17, 186–191.

Boyle, S.P., Dobson, V.L., Duthie, S.J., Hinselwood, D.C., Kyle, J.A., Collins, A.R., 2000. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. Eur. J. Clin. Nutr. 54, 774–782.

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P., Pietta, P., 2002. Quantitative characterization of flavonoid compounds in Rooibos tea (*Aspalathus linearis*) by LC-UV/DAD. J. Agric. Food Chem. 50, 5513–5519.

Carling, D., 2004. The AMP-activated protein kinase cascade – a unifying system for energy control. Trends Biochem. Sci. 29, 18–24.

Courts, F.L., Williamson, G., 2009. The C-glycosyl flavonoid, aspalathin, is absorbed, methylated and glucuronidated intact in humans. Mol. Nutr. Food Res. 53, 1104–1111.

Feillet-Coudray, C., Sutra, T., Fouret, G., Ramos, J., Wrutniak-Cabello, C., Cabello, G., Cristol, J.P., Coudray, C., 2009. Oxidative stress in rats fed a high-fat high-sucrose diet and preventive effect of polyphenols: involvement of mitochondrial and NAD(P)H oxidase systems. Free Radic. Biol. Med. 46, 624–632.

Fukasawa, R., Kanda, A., Hara, S., 2009. Anti-oxidative effects of rooibos tea extract on autoxidation and thermal oxidation of lipids. J. Oleo Sci. 58, 275–283.

Goel, A., Kunnumakkara, A.B., Aggarwal, B.B., 2008. Curcumin as "Curecumin": from kitchen to clinic. Biochem. Pharmacol. 75, 787–809.

Hoffmann-Bohm, K., Lotter, H., Seligmann, O., Wagner, H., 1992. Antihepatotoxic C-glycosylflavones from the leaves of *Allophyllus edulis* var. edulis and gracilis. Planta Med. 58, 544–548.

Hu, F.B., 2003. The Mediterranean diet and mortality-olive oil and beyond. N. Engl. J. Med. 348, 2595–2596.

Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., Ueno, K., 1996. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol. Pharm. Bull. 19, 1518–1520.

Janbaz, K.H., Saeed, S.A., Gilani, A.H., 2002. Protective effect of rutin on paracetamol- and $CCl_4$-induced hepatotoxicity in rodents. Fitoterapia 73, 557–563.

Johnson, J.J., Bailey, H.H., Mukhtar, H., 2010. Green tea polyphenols for prostate cancer chemoprevention: a translational perspective. Phytomedicine 17, 3–13.
Joshipura, K.J., Hu, F.B., Manson, J.E., Stampfer, M.J., Rimm, E.B., Speizer, F.E., Colditz, G., Ascherio, A., Rosner, B., Spiegelman, D., Willett, W.C., 2001. The effect of fruit and vegetable intake on risk for coronary heart disease. Ann. Intern. Med. 134, 1106–1114.
Joubert, E., Gelderblom, W.C., Louw, A., de Beer, D., 2008. South African herbal teas: *Aspalathus linearis*, Cyclopia spp. and Athrixia phylicoides – a review. J. Ethnopharmacol. 119, 376–412.
Joven, J., Rull, A., Ferre, N., Escola-Gil, J.C., Marsillach, J., Coll, B., Alonso-Villaverde, C., Aragones, G., Claria, J., Camps, J., 2007. The results in rodent models of atherosclerosis are not interchangeable: the influence of diet and strain. Atherosclerosis 195, e85–92.
Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y., Yagasaki, K., 2009. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. Phytomedicine 16, 437–443.
Khan, N., Afaq, F., Saleem, M., Ahmad, N., Mukhtar, H., 2006. Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. Cancer Res. 66, 2500–2505.
Krafczyk, N., Glomb, M.A., 2008. Characterization of phenolic compounds in rooibos tea. J. Agric. Food Chem. 56, 3368–3376.
Kreuz, S., Joubert, E., Waldmann, K.H., Ternes, W., 2008. Aspalathin, a flavonoid in *Aspalathus linearis* (rooibos), is absorbed by pig intestine as a C-glycoside. Nutr. Res. 28, 690–701.
Li, D., Wang, Q., Yuan, Z.F., Zhang, L., Xu, L., Cui, Y., Duan, K., 2008. Pharmacokinetics and tissue distribution study of orientin in rat by liquid chromatography. J. Pharm. Biomed. Anal. 47, 429–434.
Li, Y.M., Zhang, X.G., Zhou, H.L., Chen, S.H., Zhang, Y., Yu, C.H., 2004. Effects of tea polyphenols on hepatic fibrosis in rats with alcoholic liver disease. Hepatobiliary Pancreat. Dis. Int. 3, 577–579.
Marques, F.Z., Markus, M.A., Morris, B.J., 2009. Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. Int. J. Biochem. Cell Biol. 41, 2125–2128.
Maslowska, M., Legakis, H., Assadi, F., Cianflone, K., 2006. Targeting the signaling pathway of acylation stimulating protein. J. Lipid Res. 47, 643–652.
Odbayar, T.O., Badamhand, D., Kimura, T., Takashi, Y., Tsushida, T., Ide, T., 2006. Comparative studies of some phenolic compounds (quercetin, rutin, and ferulic acid) affecting hepatic fatty acid synthesis in mice. J. Agric. Food Chem. 54, 8261–8265.
Rodríguez-Sanabria, F., Rull, A., Aragonès, G., Beltrán-Debón, R., Alonso-Villaverde, C., Camps, J., Joven, J., 2010. Differential response of two models of genetically modified mice fed with high fat and cholesterol diets: relationship to the study of non-alcoholic steatohepatitis. Mol. Cell Biochem. 343, 59–66.
Rull, A., Escola-Gil, J.C., Julve, J., Rotllan, N., Calpe-Berdiel, L., Coll, B., Aragones, G., Marsillach, J., Alonso-Villaverde, C., Camps, J., Blanco-Vaca, F., Joven, J., 2007. Deficiency in monocyte chemoattractant protein-1 modifies lipid and glucose metabolism. Exp. Mol. Pathol. 83, 361–366.
Sasaki, M., Miyakoshi, M., Sato, Y., Nakanuma, Y., 2010. Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab. Invest. 90, 835–843.
Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A.M., Luu, Y.K., Tang, Y., Pessin, J.E., Schwartz, G.J., Czaja, M.J., 2009. Autophagy regulates adipose mass and differentiation in mice. J. Clin. Invest. 119, 3329–3339.
Singleton, Y.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.
Ulicna, O., Greksak, M., Vancova, O., Zlatos, L., Galbavy, S., Bozek, P., Nakano, M., 2003. Hepatoprotective effect of rooibos tea (*Aspalathus linearis*) on CCl4-induced liver damage in rats. Physiol. Res. 52, 461–466.
Ulicna, O., Vancova, O., Bozek, P., Carsky, J., Sebekova, K., Boor, P., Nakano, M., Greksak, M., 2006. Rooibos tea (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. Physiol. Res. 55, 157–164.
Ulrich-Merzenich, G., Zeitler, H., Vetter, H., Kraft, K., 2009. Synergy research: vitamins and secondary plant components in the maintenance of the redox-homeostasis and in cell signaling. Phytomedicine 16, 2–16.
Um, J.H., Park, S.J., Kang, H., Yang, S., Foretz, M., McBurney, M.W., Kim, M.K., Viollet, B., Chung, J.H., 2010. AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. Diabetes 59, 554–563.
Wagner, H., Ulrich-Merzenich, G., 2009. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16, 97–110.
White, U.A., Stephens, J.M., 2009. Transcriptional factors that promote formation of white adipose tissue. Mol. Cell Endocrinol. 318, 10–14.
Ziaee, A., Zamansoltani, F., Nassiri-Asl, M., Abbasi, E., 2009. Effects of rutin on lipid profile in hypercholesterolaemic rats. Basic Clin. Pharmacol. Toxicol. 104, 253–258.

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