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Synergism of plant-derived polyphenols in adipogenesis: Perspectives and implications

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ABSTRACT

Dietary polyphenols may exert their pharmacological effect via synergistic interactions with multiple targets. Putative effects of polyphenols in the management of obesity should be primarily evaluated in adipose tissue and consequently in well-documented cell model. We used *Hibiscus sabdariffa* (HS), a widely recognised medicinal plant, as a source of polyphenols with a number of salutary effects previously reported. We present here the full characterisation of bioactive components of HS aqueous extracts and document their effects in a model of adipogenesis from 3T3-L1 cells and in hypertrophic and insulin-resistant adipocytes. Aqueous extracts were up to 100 times more efficient in inhibiting triglyceride accumulation when devoid of fibre and polysaccharides. Significant differences were also observed in reactive oxygen species generation and adipokine secretion. We also found that, when polyphenols were fractionated and isolated, the benefits of the whole extract were greater than the sum of its parts, which indicated a previously unnoticed synergism. In conclusion, polyphenols have interactive and complementary effects, which suggest a possible application in the management of complex diseases and efforts to isolate individual components might be irrelevant for clinical medicine and/or human nutrition.

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Introduction

Obesity-associated metabolic, oxidative and inflammatory disturbances are a growing epidemic and are associated with at least six of the top ten causes of death (McGeer and McGeer 2004). Adipocytes store excess energy, but when overloaded they become resistant to insulin, which compromises their ability to accumulate lipids and facilitates alterations in structure and metabolism in remote tissues, such as the pancreas, liver and muscle (Yu and Zhu 2004; Jernas et al. 2006; Rull et al. 2010). Excessive oxidation in adipose cells is common and triggers cellular stress (Furukawa et al. 2004; Yeop Han et al. 2010). The resulting sequence of events remains poorly understood in humans but tends to self-perpetuate if untreated. Initially, there is a complex process of cellular adaptation, monitored by tissue-resident macrophages. When failure and malfunction become extreme, a chronic inflammatory response is unleashed (Rull et al. 2010).

If assumptions are accurate, it is conceivable that antioxidant and/or anti-inflammatory therapies that act on adipose tissue may have potential benefits in the amelioration of obesity-related diseases. However, current available drugs have not been assayed yet. The only validated therapeutic measure consists of preventing hypertrophy in adipocytes via caloric restriction or increased caloric expenditure, but changes in lifestyle are difficult to achieve. Plant-derived polyphenols may provide a similar effect without restricting caloric intake (Lamming et al. 2004; Howitz and Sinclair 2008). Polyphenols are antioxidant and anti-inflammatory

Abbreviations: HS, *Hibiscus sabdariffa*; AHS, aqueous extract of *H. sabdariffa*; PEHS, phenolic extract of *H. sabdariffa*; FBS, foetal bovine serum; TNF-α, tumour necrosis factor-alpha; IGF1, insulin-like growth factor-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; IL-1a, interleukin-1 alpha; IL-1b, interleukin-1 beta; MCP-1, monocyte chemoattractant protein-1; IBMX, 3-isobutyl-1-methylxanthine.

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molecules that interact in humans with molecular targets involved in stress response pathways, and increased ingestion of dietary polyphenols could be helpful. However, plant-derived polyphenols are secondary metabolites that are synthesised in response to a major stress event; consequently, the expected amount of polyphenols in our commonly consumed food is very low. We reasoned that tropical plant-derived products could be a potential source of polyphenol concentrate and could be used to design dietary supplements. Recent data indicate that aqueous extracts of *Hibiscus sabdariffa* (HS) might ameliorate metabolic disturbances (Carvajal-Zarrabal et al. 2005; Alarcon-Aguilar et al. 2007; Kim et al. 2007), but human trials have been generally unsatisfactory, due to an incomplete characterisation of the essential bioactive components (Beltrán-Debón et al. 2009; Mozaffari-Khosravi et al. 2009; Kuriyan et al. 2010). In this study, we address this issue, document the effects of polyphenols on mouse adipocytes and provide data that support multi-target action in the same signalling cascades or response networks.

Materials and methods

Materials

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, crystal violet, Ascentis C18 preparative reverse phase column, formic acid, acetonitrile and ethanol were obtained from Sigma–Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium, calf serum, foetal bovine serum, and an antibiotic mixture (penicillin–streptomycin) were purchased from PAA Laboratories (Linz, Austria). Sodium pyruvate and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA). Polyvinyldifluoride (PVD) filters, 0.22 μm, were obtained from Millipore (Bedford, MA). AdipoRed™ Assay Reagent was obtained from Lonza (Walkersville, MD USA). The resin used for the preparative chromatography was Amberlite™ FPX66 (Rohm and Haas, Philadelphia, USA). The standards for the calibration curves, chlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-*O*-rutinoside, kaempferol 3-(*p*-coumaroylglucoside), quercetin, 4-hydroxycoumarin and delphinidin-3-sambubioside were purchased either from Fluka, Extrasynthese (Genay, France) or Polyphenols Laboratories (Hanaveien, Norway).

Methods of extraction and fractionation of polyphenols

Primary aqueous extract (AHS) was obtained from sun-dried calyces from plants harvested by investigators in Senegal with an approximate plant-to-extract ratio of 5:1 as previously described (Beltrán-Debón et al. 2009). The purified extract (PEHS) was prepared by removing fibre and polysaccharides by precipitation in 85% ethanol (v/v). Extracts were reconstituted in water at 170 mg/ml and loaded onto a 1.5 cm × 25 cm chromatography column containing Amberlite™ FPX66. The retained phenolic fraction was finally eluted with 95% ethanol and 0.01% trifluoroacetic acid, rotary evaporated and freeze-dried. Total phenolic content in AHS and PEHS was measured with the Folin–Ciocalteu method (Huang et al. 2005). To further characterise the bioactive components, PEHS was dissolved in distilled water to a concentration of 230 mg/ml, filtered through a 0.45 μm PVD filter and fractionated using a WellChrom preparative HPLC system (Merck-Knauer, Berlin, Germany). We used an Ascentis C18 preparative reverse phase column (10 μm, 25 cm × 21.2 mm), and elution was performed using acetonitrile as a mobile phase in a multistep linear gradient at room temperature with a flow rate of 19 ml/min. The preparative version of EuroChrom® software, version 3.01, was used for data acquisition and analysis. We obtained 35 fractions representing distinct combinations of components, which were identified and quantified. We then lyophilised the resulting fractions for assays described below.

Characterisation and quantification of polyphenols

Analysis was performed in a Rapid Resolution Liquid Chromatography 1200 (Agilent Technologies, Palo Alto, CA) using a Zorbax Eclipse Plus C_{18}, 4.6 mm × 150 mm, 1.8 μm column at room temperature with a flow rate of 0.5 ml/min and an injection volume of 10 μl (Rodríguez-Medina et al. 2009). The chromatographic system was coupled to a time-of-flight (TOF) mass spectrometer (MS) (Bruker Daltonic Bremen, Germany) that was equipped with an orthogonal electrospray interface (ESI; model G1607A from Agilent Technologies, Palo Alto, CA, USA) that operated in negative and positive modes of ionisation. Compound identification was made by comparing the retention times and mass spectra obtained by TOF-MS with those of authentic standards or interpreted according to previously obtained mass spectra. Quantification of the major compounds in AHS, PEHS and the isolated fractions was carried out using commercially available standards when available or previously reported structurally similar compounds (Fernandez-Arroyo et al., 2011).

In vitro experimental models

The 3T3-L1 preadipocytes were propagated and differentiated according to described procedures (Green and Kehinde 1975) (see also supporting information). Differential effects on adipogenesis were assayed by adding extracts and fractions in pre-designed concentrations to the media at the beginning of the induction period; these conditions were maintained until cells were harvested. The absence of cytotoxicity was ascertained by the crystal violet method. In all experiments, more than 90% of the cells were mature adipocytes after 8–10 days of incubation. For other experiments, we used hypertrophied, insulin-resistant adipocytes obtained by increasing the time of incubation (22 days) in 25 mM glucose (Yeop Han et al. 2010). In these cases, extracts and fractions were added at day 18 and allowed to incubate for 4 days before harvesting. We assessed triglyceride accumulation with AdipoRed™; extracts and fractions were added either at day 8 (mature adipocytes) or at day 18 (hypertrophied adipocytes) after induction and were incubated for 2 or 4 additional days, respectively. Fat droplets were analysed with a Nikon Eclipse TE 2000U fluorescence microscope controlled by NIS-Elements software.

Measurement of intracellular reactive oxygen species (ROS) and secreted adipokines

Measurements were performed on hypertrophied adipocytes in 25 mM glucose to assess the effect of proposed extracts. These extracts were added to adipocytes at day 18 after inducing differentiation, and incubation proceeded for four additional days under the same conditions. ROS generation was assessed with 2′,7′-dichlorodihydrofluorescein diacetate as described (Yeop Han et al. 2010), and fluorescence was measured in a multiwell plate reader (POLARstar Omega microplate) with excitation at 485 nm and emission at 520 nm. In separate experiments, several cytokines (leptin, tumour necrosis factor-alpha (TNF-α), insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), interleukin-1 alpha (IL-1a), interleukin-1 beta (IL-1b) and monocyte chemoattractant protein-1 (MCP-1)) were measured by ELISA (Signosis, Inc., Sunnyvale, CA, USA) in resulting supernatants following the manufacturer's instructions.

Statistical analyses

Values were represented as means ± standard deviation. Differences between two or more groups were compared using non-parametric tests and were considered statistically significant when $p < 0.05$. The means of quantitative variables were analysed using one-way ANOVA, the Student *t*-test for unpaired samples and Tukey test for multiple comparisons. All statistical analyses were performed with the Statistical Package for Social Science version 17.0 (SPSS, Chicago, IL, USA).

Results

Relative composition of candidate bioactive components

There were no major qualitative differences in polyphenol content between AHS and PEHS; therefore, the precipitation procedures used to remove other soluble material did not result in selective losses in benefit (Fig. 1A and B and Table 1). However, such manipulations resulted in immediately apparent quantitative differences and subsequent changes in relative contribution (Table 2). The phenolic content of AHS, as expressed in gallic acid equivalents, was significantly ($p < 0.001$) lower (2.26 ± 0.11 g/100 g) than that of PEHS (28.42 ± 0.33 g/100 g). Relative differences were observed in organic acids and all families of phenolic compounds: organic acid derivatives (hydroxycitric and hibiscus acids), anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside), phenolic acid derivatives (chlorogenic acid and 5-*O*-caffeoylshikimic acid), and several flavonol derivatives (quercetin, myricetin and kaempferol glycosides) (Table 2). PEHS was fractionated into 35 different fractions, and only fractions 6, 9 and 14 (Fig. 1C–E and Table 2) significantly inhibited adipogenesis at concentrations ranging from 10 to 40 μg/ml. For clarity, negative results for the remaining fractions are not shown. During the fractionation procedure and the subsequent concentration, significant peaks were additionally detected and identified in PEHS (hibiscus acid dimethyl ester, coumaroylquinic acid, leucoside (kaempferol-3-*O*-sambubioside)) and several unidentified compounds. At least six new identified peaks (numbers 32–37) appeared only in isolated fractions. The composition of these fractions differed notably. The major component of fraction 6 was delphinidin-3-sambubioside, fraction 9 contained cyanidin-3-sambubioside, chlorogenic acid and tetra-*O*-methyljeediflavanone as major components, and fraction 14 was especially rich in glycosylated flavonols, such as quercetin-3-sambubioside and myricetin-3-glucoside (Table 2).

Phenolic compounds inhibited adipogenic differentiation of 3T3-L1 cells: fibre and/or polysaccharides were either inactive or interfered in the model

We found that AHS significantly inhibited lipid accumulation and adipogenic differentiation of pre-adipocytes but only at concentrations ≥500 μg/ml, which are not likely to be achieved *in vivo*. However, the relative activity of PEHS was much higher and evident even at concentrations <10 μg/ml. Both extracts showed a significant reduction in the number of differentiated cells when compared with the control, as well as a dose-dependent response in the reduction in the accumulation of triglycerides (Fig. 2A–E). Cells differentiated in the presence of adipogenic agents plus 1000 μg/ml AHS or 40 μg/ml PEHS extracts accumulated 44.4% and 19.3% of triglyceride, respectively, as compared to levels normally observed

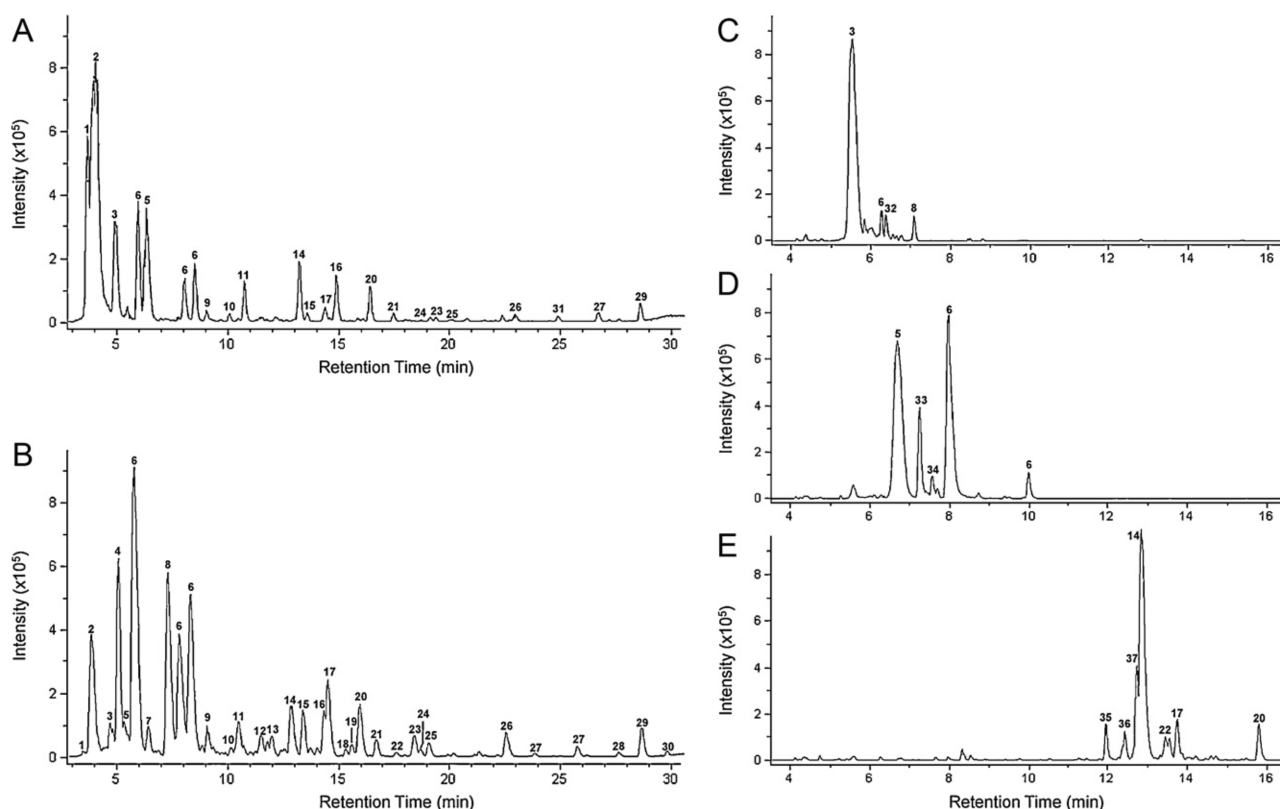

Fig. 1. Representative base peak chromatograms obtained by HPLC–ESI-TOF-MS of *Hibiscus sabdariffa* AHS (A) and PEHS (B) extracts at 50 mg/ml and 5 mg/ml, respectively. Qualitative differences between AHS and PEHS were deemed negligible, but quantitative differences were evident, especially with respect to the content of phenolic acids, anthocyanins and flavonols. Fractionation of PEHS, obtained through semipreparative liquid chromatography, showed that only a few fractions (C–E) were active in adipocytes.

Table 1
Relevant analytical data for components isolated in AHS and PEHS (see also Fig. 1). Note that peaks 31–37 were only identified after a further process of purification.

Peak number	Compound	Retention time (min)	Molecular formula	[M−H]⁻	UV–Vis (nm)
1	Hydroxycitric acid	4.35	$C_6H_8O_8$	207.0140	–
2	Hibiscus acid	4.72	$C_6H_6O_7$	189.0035	–
3	Delphinidin-3-sambubioside	5.50	$C_{26}H_{30}O_{16}$	595.1446	280, 520
4	Unknown	5.86	$C_8H_{12}O_8$	235.0461	278, 334
5	Cyanidin-3-sambubioside	6.11	$C_{26}H_{30}O_{15}$	579.1493	280, 520
6	Chlorogenic acid	6.52/8.41/8.92	$C_{16}H_{18}O_9$	353.0891	297, 324
7	Unknown	7.13	–	230.0127	272, 298
8	Hibiscus acid dimethyl esther	7.91	$C_8H_{10}O_7$	217.0354	292
9	Methyl digallate	9.62	$C_{15}H_{12}O_9$	335.0409	278
10	2-*O*-Trans-caffeoyl-hydroxicitric acid	10.60	$C_{15}H_{14}O_{11}$	369.0463	285, 330
11	Myricetin-3-arabinogalactoside	10.91	$C_{26}H_{28}O_{17}$	611.1254	260, 354
12	Coumaroylquinic acid	11.86	$C_{16}H_{18}O_8$	337.0929	310
13	Unknown	12.34	$C_{11}H_{11}NO_5$	236.0564	272
14	Quercetin-3-sambubioside	13.11	$C_{26}H_{28}O_{16}$	595.1309	345
15	Unknown	13.63	$C_{16}H_{20}O_{10}$	371.0984	278
16	Quercetin-3-rutinoside	14.50	$C_{27}H_{30}O_{16}$	609.1462	255, 353
17	5-*O*-Caffeoylshikimic acid	14.69	$C_{16}H_{16}O_8$	335.0768	296, 326
18	Leucoside (kaempferol-3-*O*-sambubioside)	15.44	$C_{26}H_{28}O_{15}$	579.1355	278, 505
19	Unknown	15.69	$C_{17}H_{22}O_{10}$	385.1140	270
20	Quercetin-3-glucoside	16.04	$C_{21}H_{20}O_{12}$	463.0873	253, 356
21	Kaempferol-3-*O*-rutinoside	16.71	$C_{27}H_{30}O_{15}$	593.1512	265, 350
22	Unknown	17.58	$C_{18}H_{22}O_9$	381.1191	278
23	Unknown	18.33	$C_{21}H_{30}O_{11}$	457.1715	–
24	Methyl-epigallocatechin	18.60	$C_{16}H_{16}O_7$	319.0823	268, 336
25	Unknown	18.93	–	503.1759	278
26	Myricetin	22.19	$C_{15}H_{10}O_8$	317.0298	372
27	*N*-Feruloyltyramine	23.40/25.19	$C_{18}H_{20}NO_4$	312.1234	286, 316
28	Unknown	26.94	–	307.0726	288, 353, 414
29	Quercetin	28.67	$C_{15}H_{10}O_7$	301.0339	253, 372
30	Unknown	29.89	$C_{18}H_{34}O_5$	329.2333	–
31	Prodelphinidin B3	24.82	$C_{30}H_{26}O_{14}$	609.1250	312
32	Tetra-*O*-methyljeediflavanone	4.30	$C_{34}H_{30}O_{11}$	613.1715	341
33	Unknown	6.11	$C_{26}H_{30}O_{16}$	597.1461	338
34	Caffeoylglucose	6.46	$C_{15}H_{18}O_9$	341.0878	314
35	Unknown	11.37	$C_{26}H_{44}O_{16}$	611.2557	277
36	Unknown	11.93	$C_{27}H_{32}O_{17}$	627.1567	278
37	Myricetin-3-glucoside	12.25	$C_{21}H_{20}O_{13}$	479.0831	355

in control cells. The extract concentration that led to 50% of inhibition of triglyceride accumulation (IC_{50}) was 799 ± 225 μg/ml for AHS and 9.1 ± 2.8 μg/ml for PEHS, respectively. This result revealed that PEHS was approximately 90–100 times more effective in reducing triglyceride accumulation. This difference was around 10 times higher than that expected for actual polyphenol concentrations, which indicated that the absence of polysaccharides and other soluble material improved the inhibition of triglyceride cellular uptake in this model.

Anti-adipogenic activity of polyphenols was no longer conserved in most isolated fractions

Only 3 of 35 polyphenolic fractions (numbers 6, 9 and 14; Fig. 1C–E) significantly inhibited adipogenesis. The efficacy of these fractions was dose-dependent; fraction 14 was the most active throughout the dosing range (Fig. 2F). None of these fractions at 40 μg/ml achieved the effectiveness that was obtained with the total mixture of polyphenols (PEHS). Changes in activity with combinations of these fractions at different concentrations were negligible. None of the binary combinations (20 or 30 μg/ml of each fraction) achieved higher activities than the individual fractions. Moreover, the strong inhibitory action of fraction 14 was maintained when using pairs 6/14 and 9/14. Finally, the combination of three of the fractions (20 μg/ml of each) also failed to improve the anti-adipogenic activity, as compared with both isolated fractions and combinations. Taking all results into account, the presence of all polyphenols was necessary in order to achieve maximum efficacy, and the relative proportion was potentially a relevant factor.

Phenolic compounds were active in mature as well as hypertrophied and insulin-resistant adipocytes

When extracts were assayed in mature adipocytes, the addition of AHS did not significantly affect triglyceride content, even at 1000 μg/ml, but 40 μg/ml of PEHS decreased triglyceride content by 20–30% when assayed at 40 μg/ml (Supporting Information; Fig. 2). These novel findings prompted the exploration of the effects of PEHS in a cell model of adipocyte hypertrophy in the context of insulin resistance, similar to that observed in the adipose tissue of obese patients (Yu and Zhu 2004; Jernas et al. 2006; Takahashi et al. 2008; Yeop Han et al. 2010). Surprisingly, we found significant effects with AHS and that both AHS and PEHS were more efficient at reducing triglyceride accumulation in insulin-resistant adipocytes than in mature adipocytes (1000 μg/ml AHS: 19% mean values reduction; 40 μg/ml PEHS: 38% mean values reduction) (Fig. 3). It appeared that the PEHS-mediated reduction in triglyceride accumulation was significantly higher during the adipogenesis process than in mature or hypertrophic adipocytes but we found a differential response with both extracts. The generation of endogenous ROS was not affected by AHS (Fig. 4A and B), but the effect of PEHS was significant and dose-dependent, achieving a 30% reduction, which indicated that the removed material might have deleterious effects on either the diffusibility or the intrinsic antioxidant activity of phenolic compounds. These deleterious effects were not observed with their putative anti-inflammatory properties. We observed that both extracts at the tested concentrations significantly decreased the amount of secreted adipokines with respect to controls (Fig. 4C). This result indicated that the effect of fibre and/or saccharides could be specific. For most of the adipokines assayed,

Table 2
Quantitative data in ppm (m/m) for major components found in extracts and fractions with significant biological activity.

Peak number	Compound	Quantification technique	AHS	PEHS	Fraction 6	Fraction 9	Fraction 14
1	Hydroxycitric acid	MS-TOF (*m/z* 207)	8288.0 ± 397.6	–	–	–	–
2	Hibiscus acid	MS-TOF (*m/z* 189)	31,122.0 ± 1128.4	128,134.2 ± 9486.9	–	–	–
3	Delphinidin-3-sambubioside	DAD-UV (520 nm)	2701.2 ± 165.6	207,315.5 ± 1807.6	415,227.9 ± 18,558.3	–	–
5	Cyanidin-3-sambubioside	DAD-UV (520 nm)	1939.2 ± 39.3	87,143.1 ± 393.1	–	193,354.1 ± 3119.0	–
6	Chlorogenic acid	DAD-UV (325 nm)	5720.0 ± 39.4	106,469.9 ± 1182.0	5381.5 ± 173.9	137,219.1 ± 1425.8	–
8	Hibiscus acid dimethyl ester	MS-TOF (*m/z* 217)		–	5300.5 ± 325.9	–	–
9	Methyl digallate	DAD-UV (270 nm)	–	2802.3 ± 46.6	–	–	–
11	Myricetin-3-arabinogalactose	DAD-UV (350 nm)	57.3 ± 2.5	4755.5 ± 53.8	–	–	–
12	Coumaroylquinic acid	DAD-UV (310 nm)	–	772.9 ± 10.4	–	–	–
14	Quercetin-3-sambubioside	DAD-UV (350 nm)	304.0 ± 5.9	7673.8 ± 34.6	–	–	113,130.2 ± 2356.4
16	Quercetin-3-rutinoside	DAD-UV (350 nm)	495.7 ± 4.3	4953.2 ± 47.9	–	–	–
17	5-*O*-Caffeoylshikimic acid	DAD-UV (325 nm)	171.5 ± 6.9	3526.7 ± 49.2	–	–	7312.7 ± 168.9
18	Leucoside	DAD-UV (350 nm)	–	1123.0 ± 25.4	–	–	–
20	Quercetin-3-glucoside	DAD-UV (350 nm)	143.7 ± 2.2	3071.6 ± 15.9	–	–	12,789.1 ± 268.6
21	Kaempferol-3-*O*-rutinoside	DAD-UV (350 nm)	91.9 ± 2.3	2185.5 ± 15.5	–	–	–
24	Methyl-epigallocatechin	DAD-UV (270 nm)	–	310.9 ± 5.5	–	–	–
26	Myricetin	DAD-UV (370 nm)	–	4765.9 ± 49.1	–	–	–
27	*N*-Feruloyltyramine	DAD-UV (325 nm)	99.0 ± 1.8	867.9 ± 8.6	–	–	–
29	Quercetin	DAD-UV (370 nm)	121.2 ± 2.0	5795.2 ± 61.7	–	–	–
31	Prodelphinidin B3	DAD-UV (310 nm)	1839.2 ± 25.3	327.0 ± 2.9	–	–	–
32	Tetra-*O*-methyljeediflavanone	DAD-UV (350 nm)			13,515.7 ± 125.8	–	–
34	Caffeoylglucose	MS-TOF (*m/z* 341)			–	2902.1 ± 67.5	–
37	Myricetin-3-glucoside	DAD-UV (350 nm)			–	–	29,383.6 ± 1245.1

both extracts showed the same quantitative efficacy. A differential response, higher for PEHS than for AHS, was only observed with the secretion of leptin and MCP-1.

Discussion

The efficacy of phytotherapy is currently under intense debate (Wagner 2011). To firmly establish beneficial effects that may yield valuable nutritional advice or dietary supplements, human studies demand (Mozaffari-Khosravi et al. 2009; Kuriyan et al. 2010) full chemical characterisation of the source of polyphenols and the effect of further manipulation in the relative composition. Our novel findings suggested that the previous removal of fibre and polysaccharides, which represent up to 60% of the total weight of soluble extracts (Müller and Franz 1992), might significantly increase the activity of a polyphenolic mixture. Adipogenesis was substantially inhibited by a standardised *Hibiscus sabdariffa* extract, and the effect of the full extract was higher than the sum of its parts, which provided further evidence that a combination of bioactive components was superior to isolated constituents. In essence, the assayed extracts are a complex mixture of anthocyanins, organic acids, phenolic acids and flavonols (Rodríguez-Medina et al. 2009). Nevertheless, our results revealed that some compounds can have a higher contribution to the observed effects, which suggested the importance of relative composition and that different formulations might yield different outcomes. For instance, the putative hypolipidemic effects of polyphenolic mixtures have been mainly associated with the presence of organic acids (Carvajal-Zarrabal et al. 2005). In contrast, the described effects of PEHS in adipogenesis were obtained in a scenario in which the proportion of phenolic compounds was higher than that of organic acids. From data obtained from isolated fractions, it could be concluded that glycosylated flavonols were the most active compounds amongst individual components, but this finding seemed irrelevant when compared to the action of the full mixture. Although other isolated phenolic compounds (apigenin, epigallocatechin gallate, resveratrol and quercetin) have also shown to inhibit adipogenesis in 3T3-L1 adipocytes in similar concentrations (10–50 μg/ml, Lin et al. 2005; Bandyopadhyay et al. 2006; Yang et al. 2008), cytotoxicity was readily observed. This point is extremely important because plant-derived polyphenols are a complex mixture that interacts with numerous endogenous molecular targets in humans but are surprisingly safe even at high doses (Corson and Crews 2007; Goel et al. 2008; Efferth and Koch 2011).

Another novel finding was that PEHS also actively diminished triglyceride accumulation in mature and even insulin-resistant hypertrophied adipocytes, which suggested an induction in the lipolysis rate (Yu and Zhu 2004; Takahashi et al. 2008). This effect could be important in the management of metabolic disturbances because the uninhibited release of fatty acids from hypertrophied adipocytes might lead to systemic lipotoxicity and insulin resistance (Unger 1995). It was also established that excess triglyceride accumulation in adipocytes generated an excess of ROS that triggered inflammation (Yeop Han et al. 2010). Although the differential abilities observed between AHS and PEHS deserve further consideration, PEHS clearly possesses antioxidative and anti-inflammatory actions in mature and hypertrophied adipocytes. These properties might also have therapeutic implications because these are important processes in 3T3-L1 adipocytes that are directly related to the accumulation of fat and with potential regulation via JNK/NF-κB pathways as described (Furukawa et al. 2004; Takahashi et al. 2008; Yeop Han et al. 2010). Once again we highlight the importance of a particular formulation of phenolic compounds because PEHS was particularly active in inhibiting the secretion of leptin and MCP-1, which are important adipokines

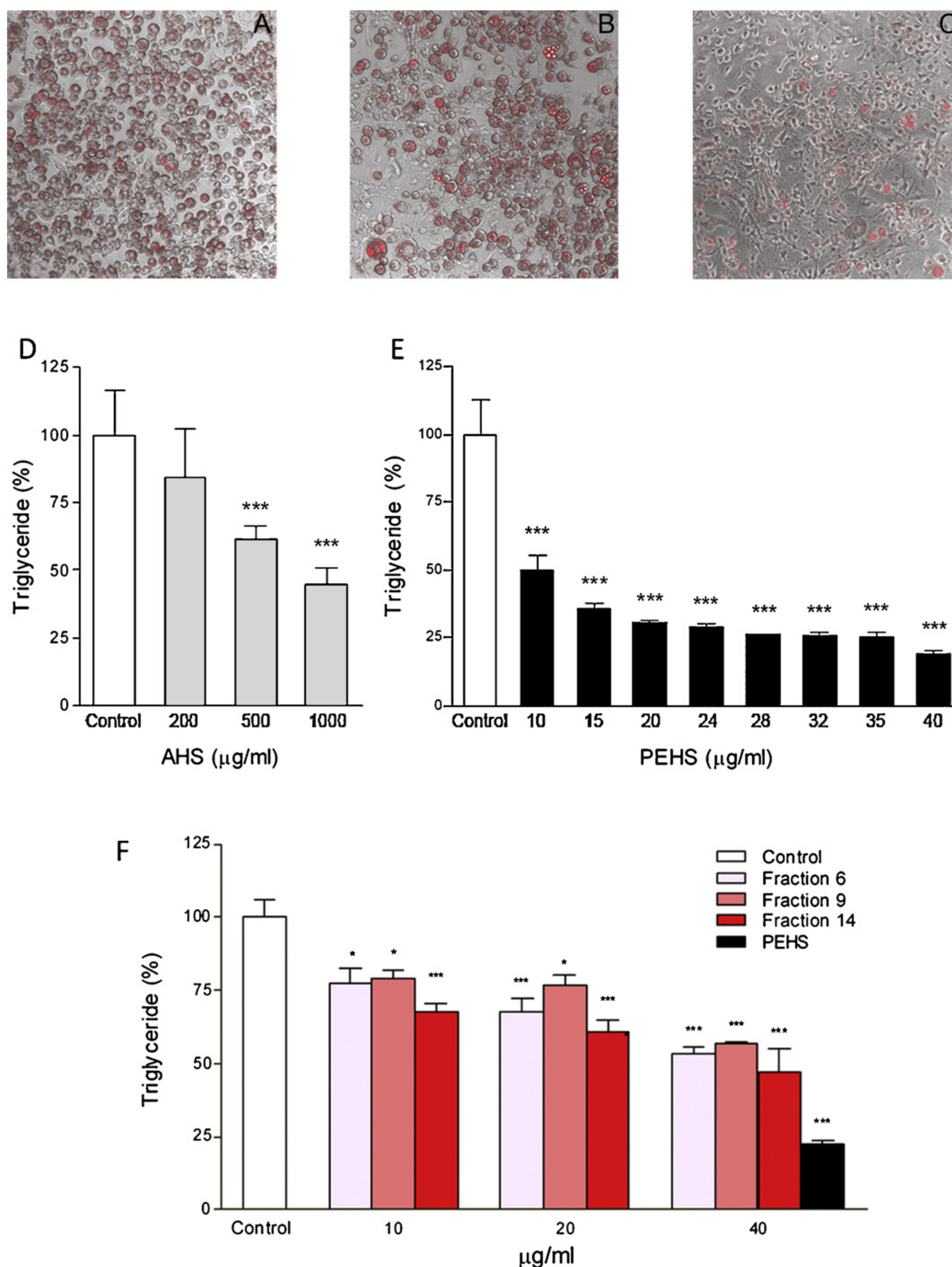

Fig. 2. Polyphenols from *Hibiscus sabdariffa* significantly inhibited the accumulation of triglycerides in 3T3-L1 preadipocytes and programmed adipogenesis. Representative microphotographs of cells stained with AdipoRed™ (treatment groups: control, 1000 μg/ml AHS or 40 μg/ml PEHS; A–C), demonstrate that the number of mature adipocytes was substantially decreased in cells treated with extracts. The quantitative effects of different doses on the final content of triglyceride are also shown (D–E). The effect of selected fractions in the accumulation of triglycerides in 3T3-L1 preadipocytes is shown in (F). Cell viability was unaffected even at higher concentrations. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control.

that regulate the migration of non-resident macrophages to the adipose tissue and overall systemic metabolism (Furukawa et al. 2004). This anti-inflammatory effect has also been observed with resveratrol or alpha lipoic acid (Szkudelska et al. 2009; Prieto-Hontoria et al. 2011).

The apparently additive, synergistic or even antagonistic action of each polyphenol and the intrinsic complications of understanding adipocyte metabolism without knowledge of genetic and proteomic kinetics impede efforts to describe the possible biological reactions and metabolic networks involved. Polyphenol reaction and diffusion rates also confound these efforts. Moreover, the process of transformation and regeneration in these particular cells, and the effects that we described strongly suggested the presence of repeat-pattern mechanisms (Gierer and Meinhardt 1972), which is a self-organising, self-repairing, reaction–diffusion system (Turing 1990).

We speculate that this relationship can be applied to every combination of polyphenols with medicinal effects, but at least two major points should be highlighted. Leptin production correlates positively with insulin resistance, fat mass and adipocyte volume in response to metabolic stress (Frederich et al. 1995). The observed benefit with PEHS was comparatively higher than that observed

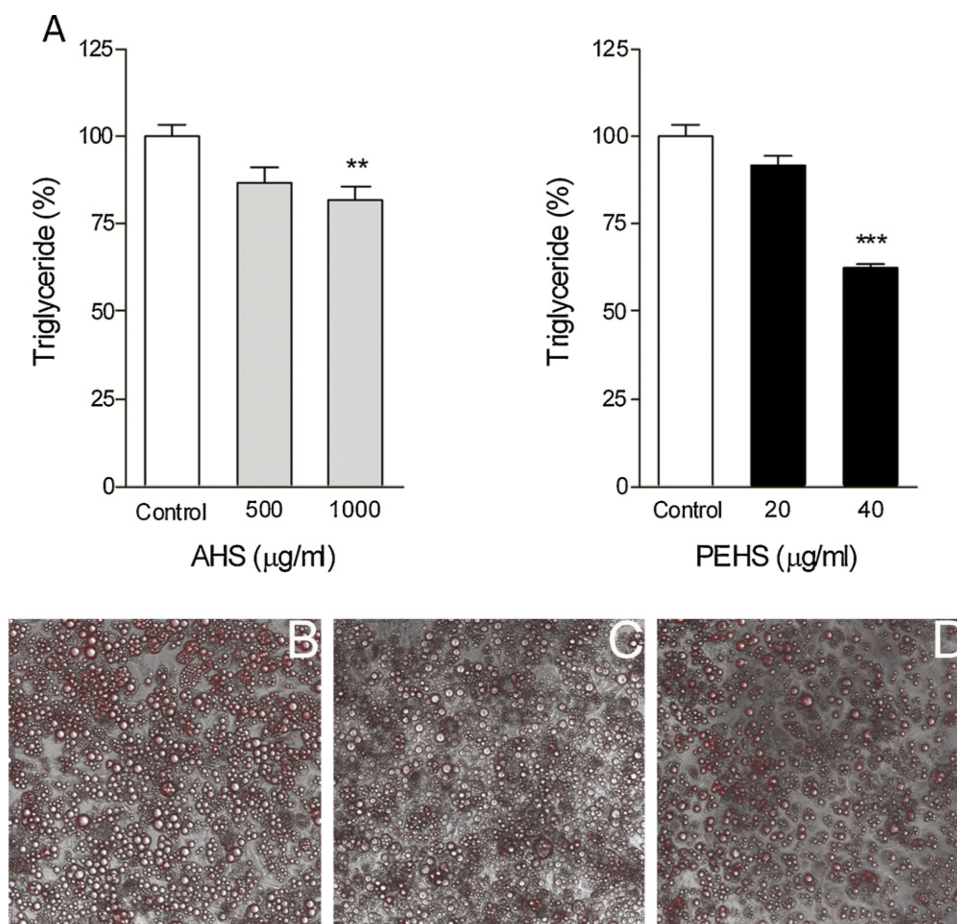

Fig. 3. Polyphenols from *Hibiscus sabdariffa* significantly decreased the accumulation of triglycerides in 3T3-L1 hypertrophied/insulin-resistant adipocytes (A). Effects are shown for AHS (500–1000 μg/ml) (left panel) and PEHS (20–40 μg/ml) (right panel). (B)–(D) are representative microphotographs of cells stained with AdipoRed™; treatment groups: control, 1000 μg/ml AHS or 40 μg/ml PEHS, respectively. The number of fat droplets per cell was decreased in the experiments with PEHS. Cell viability was unaffected even at higher concentrations. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control.

with triglyceride accumulation, which suggested that leptin gene expression was a possible pharmacologic target, although this is effect obviously modulated by actions on a variety of other targets (Frederich et al. 1995; Rupnick et al. 2002; Tilg and Moschen 2006; Samuni et al. 2010). Results with MCP-1 excretion were expected. We have previously reported in humans that these polyphenolic extracts significantly reduce the concentration of circulating MCP-1, which is relevant because MCP-1 has been proposed as a biomarker and therapeutic target in the management of obesity and its related complications (Beltrán-Debón et al. 2009).

We should express caution, however, when interpreting *in vitro* data to actual actions of polyphenols in the body, especially if no data have been collected regarding the action of physiological metabolites of tested polyphenols on the same cell systems. First, the intestinal flora is likely to metabolise some of these compounds. Once the glucoside cleaved, the released aglycone is subjected to the action of specific enzymes in the wall of the small intestine leading to glucuronide, sulphated, and methylated metabolites, which may reach their target tissues and organs. Obviously, our *in vitro* assays do not take into account the *in vivo* bioavailability issue and can lead to false positive interpretations. To date, there is no data about human or animal studies on metabolites deriving from HS polyphenols. Although some bioavailability studies on phenolic acids, anthocyanins and flavonols are available, these are contained in very different food matrixes what may radically modify the interaction amongst these compounds in the gastrointestinal tract and therefore their absorption. Whilst human absorption and bioavailability studies have revealed that phenolic acids and anthocyanins can be retrieved in plasma and urine in their intact form after food consumption (Paredes-Lopez et al. 2010; Williamson et al. 2011), flavonols and flavanols seem to be found in various forms, free or conjugated with glucuronide, sulphate or methyl groups (Day et al. 2001; Williamson et al. 2011).

Because in studies with green tea polyphenols, the metabolites mostly had reduced biological activity, it might be tempting to suggest that conversion of HS polyphenols into less-active metabolites would compromise the cellular effects denoted in the present study. In some systems, however, polyphenols-derived metabolites were found to have the equivalent or even greater activity than the parental polyphenols (Lambert et al. 2007). In the present study, PEHS contains up to thirty identifiable phenolic compounds by HPLC–MS. Accepting that absorption of many of these compounds is negligible; still several of them will be conjugated and will interact with multiple metabolic targets. Thus, to study the effects of all possible metabolites becomes an enormous and fascinating target, which will be undoubtedly matter for future research.

In conclusion, we propose that this particular formulation of polyphenols should be assayed in clinical trials because of its observed regulation of adipogenesis, its regulation of oxidative stress signalling pathways in mature and/or hypertrophied adipocytes and its subsequent ability to alter the expression of adipokines. In regards to the preparation of phytopharmaceuticals or dietary supplements, we propose that the separation of soluble material other than polyphenols prior to use will increase

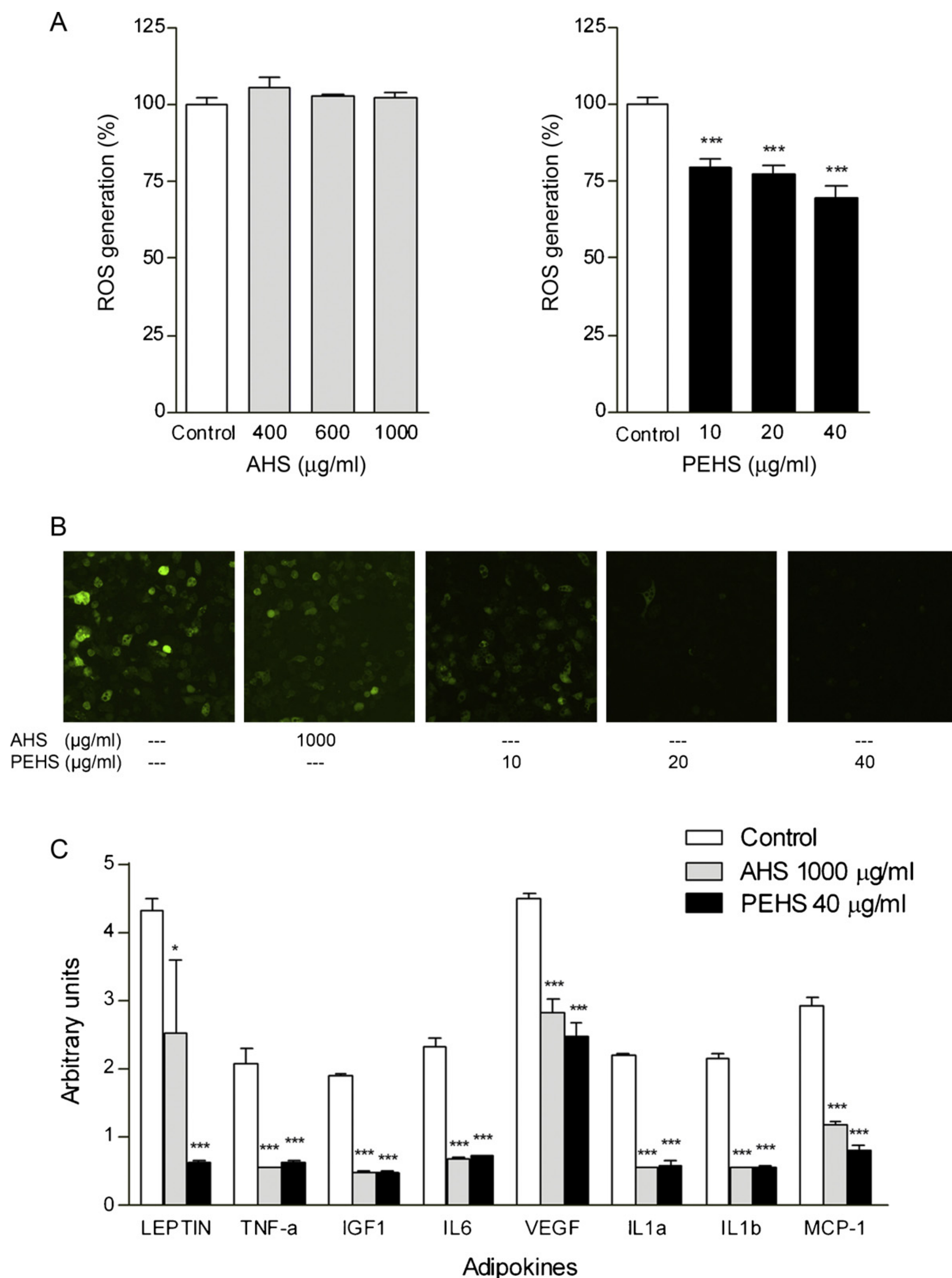

Fig. 4. The phenolic fraction from *Hibiscus sabdariffa* inhibited ROS generation and modulated the concentration of secreted adipokines in hypertrophied 3T3–L1 adipocytes (A). Cells were incubated with different doses of AHS (400–1000 μg/ml) (left panel) or PEHS (10–40 μg/ml) (right panel) and ROS generation was measured by H_2DCFDA labelling. Whereas PEHS affected ROS generation in a dose-dependent manner, AHS did not. Representative fluorescence microphotographs of cells stained with H_2DCFDA are shown in (B). Although the secretion of assayed adipokines was efficiently decreased with both extracts, a differential response was obtained with leptin and MCP-1 (C). $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control.

the possibility of safely obtaining synergistic effects. Although bioavailability and safety issues require further studies, we have demonstrated the potential pharmacological and therapeutic superiority of a combination of polyphenols with respect to their individual components. The existence of synergistic efficacy of binary combinations of compounds has been evaluated and verified by Berenbaum's isobole method (Berenbaum 1989; Wagner 2011). Unfortunately, this method cannot be applied in our case at

this stage due to the complexity of the polyphenol mixture. It is possible that the benefits of polyphenols are the result of mere additive effects or simple combinatory actions, but the data suggest a synergistic effect at least in the sense of that described by Mark Twain: "a bonus achieved when things work together harmoniously".

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2011.12.001.

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