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Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage

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ABSTRACT

The aim of the present work was to analyze the phenolic extracts from two monofloral Cuban honeys for their *in vitro* total antioxidant capacity, phenolic compounds content and free radical scavenging activity. The phenolic extracts, rich in lipophilic compounds, were tested further for their ability to inhibit AAPH-induced oxidative damage (hemolysis, lipid peroxidation and cytosolic depletion of reduced glutathione and decrease of superoxide dismutase activity) in erythrocytes. Results indicate an important total antioxidant capacity measured by TEAC and ORAC assays, as well as a relevant radical scavenging activity performed by EPR. Moreover, 13 phenolic compounds were identified using HPLC–LC/MS with quercetin as the most abundant flavonoid. The results also show that both extracts were able to inhibit erythrocytes oxidative damage, and that this may likely be due to their incorporation into cell membranes and their ability to cross it and reach the cytosol. In fact, flavonoid uptake by erythrocytes was further confirmed by testing quercetin, which efficiently incorporated into erythrocytes. Overall, this study indicates that honey contains relevant antioxidant compounds responsible, at least in part, for its biological activity and that uptake of its flavonoids may provide defense and promote cell functions in erythrocytes.

1. Introduction

In the long human tradition, honey has been used not only as a nutrient but also as a medicine. During the past decade, the use of honey for therapeutic purposes has been re-evaluated in a more scientific setting and several properties have been identified (Dias et al., 2008). They include antibacterial (Estevinho et al., 2008; Gomes et al., 2010), antifungal (Feás and Estevinho, 2011) and anti-inflammatory effects as well as stimulation of wound and burn healing (Alvarez-Suarez et al., 2010a). Honey also displays a significant antioxidant activity; recent studies demonstrate a strong correlation between the content of phenolic compounds in honeys from various floral sources and their antioxidant capacity (Gheldof and Engeseth, 2002; Blasa et al., 2006; Alvarez-Suarez et al., 2010b; Morais et al., 2011).

Flavonoids are the major functional components of honey and may significantly contribute to its total antioxidant activity and beneficial effects in human health (Hung et al., 2004). One of the potential benefits of flavonoids is to stabilize cell membranes by reducing lipid peroxidation and scavenging free radicals (Chaudhuri et al., 2007). In addition, data from *in vitro* experiments have recently revealed that the antioxidant properties of flavonoids could lie in their localization in lipoprotein domains and cell membranes, which generally serve as targets for lipid peroxidation, suggesting a protective interaction of flavonoids with lipid

Abbreviations: AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EPR, electron paramagnetic resonance; GSH, reduced glutathione; HO·, hydroxyl radical; PE, phenolic extract; ORAC, oxygen radical absorbance capacity; RBCs, red blood cells; SD, standard deviation; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric reactive substances; TEAC, trolox equivalent antioxidant capacity; TE, trolox equivalents; XOD, xanthine oxidase; % RSC, percentage of radical scavenging activity.

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bilayers. In fact, certain flavonoids may incorporate into the hydrophobic core of the membrane bilayer, causing a reduction in membrane fluidity and membrane stability (Arora et al., 2000; Chaudhuri et al., 2007). This reduced membrane fluidity may limit diffusion of free radicals and improve the antioxidant effectiveness of flavonoids.

The aim of the present work was to study the antioxidant activity and the protective effect against oxidative damage in red blood cells (RBCs) of two monofloral Cuban honey phenolic extracts (PE). Ether fractions obtained after methanol extraction were analyzed for phenolic contents and ability to scavenge different free radicals. The protective capacity of honey-phenolics against AAPH-induced hemolysis in human erythrocytes was studied. In the same way, the capacity to protect RBCs against cytosolic reduced glutathione (GSH) and superoxide dismutase (SOD) depletion as well as lipid peroxidation were investigated. Finally, since quercetin was found to be the most abundant flavonoid in honey PE, its intracellular accumulation in human RBCs was studied to determine the capacity of this flavonoid to cross the erythrocyte membrane, and to protect the cell against oxidation.

2. Materials and methods

2.1. Biological materials

Two different monofloral honeys were obtained from the National Center of Apiculture Research of Cuba. The floral sources and the number of samples analyzed were *Christmas vine* (*Turbina corymbosa* (L.) Raf, 10 samples, Convolvulaceae family) and *Linen vine* (*Gouania polygama* (Jack) Urb, 10 samples, Rhamnaceae family). Physico-chemical tests were carried out according to the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) (AOAC, 1990) and the botanical origin of samples was confirmed by the traditional qualitative microscopic analysis (Louveaux et al., 1978).

2.2. Chemicals

All chemicals and solvents were of analytical grade. 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), bovine serum albumin (BSA) and fluorescein were purchased from Fluka Chemie (Buchs, Switzerland). 2,2′-azo-bis(2-methylpropionamidine dihydrochloride (AAPH), 1,1,3,3-tetraethoxypropane (TEP), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethylsulfoxide (DMSO), 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB), nitroblue tetrazolium (NBT), and quercetin were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Enzo Life Science AG (Lausen, Switzerland) and was used without further purification (no residual signals were detected in DMPO water solutions).

2.3. Preparation of phenolic extracts of the honey

Phenolic compounds in honey were recovered as previously reported (Ferreres et al., 1991). Honey samples (50 g each) were mixed with five parts of acidified water (pH 2 with HCl) until completely fluid and filtered through cotton to remove solid particles. The filtrate was then passed through a column (25 × 2 cm) of Amberlite XAD-2 resin (pore size = 9 nm, particle size = 0.3–1.2 mm). Sugars and polar compounds were eluted with acidified water (350 mL) (*fraction 1*), the column was washed with 300 mL of neutral water (*fraction 2*), and phenolic compounds further eluted with methanol (600 mL). The methanol phase was concentrated under vacuum at 40 °C, suspended in water (5 mL) and extracted three times with diethyl ether (5 mL each). The ether layers were collected, evaporated under vacuum, and redissolved in methanol (*fraction 3*). Hesperetin was used as internal standard. Each honey sample was fractionated and analyzed in triplicate.

2.4. HPLC-DAD–ESI-MS/MS analysis of honey phenolics

Fraction 3, which was expected to contain the lipid-soluble phenolics, as previously reported (Gheldof and Engeseth, 2002; Blasa et al., 2007; Alvarez-Suarez et al., 2010c), was analyzed by HPLC-DAD–ESI-MS/MS. To this purpose, the concentrated ether layers were redissolved in methanol/H_2O (50:50 v/v) to prevent the elution of the compounds in the elution front.

Analyses were performed on a Hewlett–Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a C-18 LiChroCART column, (Merck, Darmstadt, Germany) (RP-18e, 250 mm × 4 mm, 5 μm), thermostatted at 35 °C. Solvents used were: (A) H_2O/formic acid (99:1, v/v) and (B) methanol/isopropanol (90:10, v/v), and the following gradient program was established: from 10% to 30% B over 20 min, from 30% to 40% over 10 min, from 40% to 60% B over 10 min, from 60% to 80% over 5 min and then isocratic for 5 min set at 1 mL/min of flow rate. Identification of honey phenolics was carried out by comparing retention time and spectral characteristics of unknown analytes with standards. Spectroscopic data from all peaks were accumulated at 290 nm for phenolic acids and 360 nm for flavonoids. Individual phenolic acids were quantified using a calibration curve of the corresponding standard compound, flavonoids were quantified using a quercetin calibration curve and expressed in terms of quercetin equivalents.

The mass spectrometer analysis was carried out on a Finnigan LCQ (San Jose, CA) equipped with an ESI system and an ion trap mass analyser, which was controlled by the LCQ Xcalibur software. Nitrogen was used as both auxiliary and sheath gas at flow rates of 6 and 1.2 L/min, respectively. The capillary voltage was 10 kV and the capillary temperature 225 °C. MS spectra were acquired in the negative and positive ionization modes between *m/z* 100 and 800. The MS detector was programmed to perform a series of two consecutive scans: a full scan and an MS–MS scan of the most abundant ion in the first scan, using normalized collision energy of 50%.

2.5. HPLC-DAD analysis of quercetin taken up by human RBCs

The extra- and intracellular distribution of quercetin was determined as previously reported (Fiorani et al., 2006). Briefly, after incubation of RBCs suspensions with quercetin (30 μg/mL for 45 min), samples were centrifuged at 1500*g* for 5 min and the supernatants were collected. The packed RBCs were washed three times with PBS. Then, the packed cells (200 μL) were resuspended with 1 volume of PBS and lysed with hypotonic buffer. Quercetin in samples (extracellular milieu and RBC lysate) was extracted three times with ethyl acetate (400 μL). All samples were taken to dryness by rotary evaporation, redissolved in DMSO and diluted with methanol/H_2O (50:50, v/v) just before HPLC analysis. The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of a Waters 600 controller, a Waters 996 DAD set at absorbance of 360 nm. Analysis was performed on a 15 cm × 4.6 mm Supelcosil™ C-18 column. Solvents A (0.1% formic acid) and B (acetonitrile) were run at a flow rate of 1 mL/min. The running gradient was adjusted to 15% B (2 min), increasing to 25% B (5 min), 35% B (10 min), 55% B (5 min), and then 100% B (10 min), followed by a re-equilibration at 15% B (15 min). The flow rate was 1 mL/min. Retention time of quercetin was about 21.34 min. Identification of quercetin was carried out by comparing retention time and spectral characteristics with a commercial standard. Calibration curve of quercetin was linear within the range investigated (0.5–110 μM).

2.6. Total antioxidant capacity (TAC)

2.6.1. Trolox-equivalent antioxidant capacity assay (TEAC)

This assay was carried out in combination with a flow injection analysis system (Bompadre et al., 2004). Briefly, samples appropriately diluted (10 μL) were injected into a serpentine-knotted reaction coil and allowed to react with the $ABTS^{\cdot+}$ working solution pumped into the coil at a flow rate of 1.2 mL/min. The linearity range of the Trolox (0.03–2 mM) calibration curve was used. The results are expressed as micromoles of Trolox equivalents per gram of honey (μmol TE/g of honey).

2.6.2. Oxygen radical absorbance capacity assay (ORAC)

The ORAC assay was applied as described previously (Gillespie et al., 2007). The reaction was carried out in PBS (75 mM, pH 7.0) in a 96-well plate. Samples were suitably diluted in PBS. Each well contained in a final volume of 200 μL assay solution, 150 μL of fluorescein (0.08 μM) and 25 μL of appropriately diluted PE. The reaction solution was preincubated for 10 min at 37 °C and then 25 μL of AAPH (150 mM) were added. The plate was shaken automatically for 3 s and the fluorescence was measured every 2 min for 120 min with emission and excitation wavelengths of 485 and 530 nm, respectively, using a microplate fluorescence reader (Synergy™ Multi-Detection Microplate Reader, Bio-Tek®, Instruments, Inc., USA) that was maintained at 37 °C. A blank sample and six calibration solutions of Trolox (2.25–100 μM) were also tested. The ORAC values were calculated as area under the curve and expressed as μmol TE/g of honey. Methanol was used in the blank and standard measurements.

2.6.3. $DPPH^{\cdot}$ scavenging activity

This assay was performed using EPR spectroscopy as previously reported (de la Rosa et al., 2011). The reaction mixture was prepared by mixing 100 μL of 500 μM DPPH (in methanol), 75 μL of PE (125 μg/mL final concentrations) and 25 μL of methanol. This reaction mixture was immediately transferred into a capillary tube and the spectrum was recorded after 10 min on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) operating at X-Band equipped with an XL microwave frequency counter, with the following settings: frequency 9.78 GHz, power 25 mW, modulation amplitude 2 G (Gauss), gain 5×10^5, field width 100 G, time constant 0.64 ms, scan time 21 s. The decrease in the signal

amplitude in the test samples correlates with their radical scavenging activity. The height of the second peak was measured and the percentage of radical scavenging activity (% RSC) was then calculated according to equation (1):

$$\%\ \mathrm{RSC} = 100 - [(\text{EPR signal of the sample})/(\text{EPR signal of the control})] \times 100 \qquad (1)$$

2.6.4. Hydroxyl radical scavenging activity

Electron paramagnetic resonance (EPR) spectroscopy, in combination with the spin-trapping technique, was used to investigate the HO· scavenging activities of honey PE. Highly reactive and transient radicals, like the hydroxyl radical, can be detected only after reaction with a diamagnetic molecule, such as the spin trap DMPO. This latter compound easily undergoes an addition reaction with HO· to form a more persistent paramagnetic species, the 'spin-adduct' DMPO-OH, characterized by a four line spectrum with a 1:2:2:1 pattern and hyperfine splitting constants $a_N = a_H^\beta = 14.85$ G (Buettner, 1987).

The HO· scavenging ability of PE was examined by their addition to the hydroxyl radical-generating Fenton system following the procedure previously described (Henriques et al., 2006). EPR spectra were recorded with the following settings: frequency 9.78 GHz, power 25 mW, modulation amplitude 0.5 G (Gauss), gain 5×10^5, field width 100 G, time constant 0.64 ms, scan time 21 s. Briefly, 28 μL of appropriately diluted PE in methanol (5 μg/mL, final concentration) were added to 65 μL of 0.1 M DMPO (in water) in an eppendorf tube and gently mixed by pipetting. To this reaction solution, 2 μL of 0.06 M H_2O_2 were added followed by 5 μL of freshly prepared and nitrogen-flushed 0.5 mM $FeSO_4$ to initiate the reaction. The reaction mixture was immediately transferred into a sample tube and measurements were taken 90 s after $FeSO_4$ addition. The DMPO-OH adduct was observed together with traces of another adduct, the DMPO-CH_2OH, characterized by the following hyperfine splitting: a_N 15.8 and a_H 22.3 (Buettner, 1987). This latter adduct arises from the trapping of the CH_2OH radical generated from the Fenton system when methanol is present. As control, the EPR spectrum obtained from a reaction mixture containing DMPO, H_2O_2 and $FeSO_4$ (positive control) was used and the corresponding signal amplitude was compared to those of the DMPO-OH adducts recorded in the presence of PE. In order to check for the absence of radical species in the samples, a negative control was prepared by simply mixing DMPO and PE solutions. The height of the first peak was recorded and the% RSC was then calculated according to Eq. (1).

2.7. Protective effects of honey phenolic extracts against free radical damage in RBCs

2.7.1. Blood collection and preparation of erythrocytes suspensions

RBCs were obtained from healthy non-smoker adult volunteers after informed consent. Erythrocytes from heparinized blood were obtained by consecutive centrifugations at 1000*g* for 10 min at 10 °C. After removal of plasma and buffy coat, RBCs were washed once with cool 0.9% NaCl solution, then three times with PBS containing 150 mM NaCl, 1.9 mM Na_2HPO_4 and 8.1 mM NaH_2PO_4, pH 7.4 at 4 °C and finally resuspended in PBS to obtain cell suspensions at 8% hematocrit.

2.7.2. Hemolysis assays

PE or quercetin were initially solubilized in a small amount of DMSO and further diluted in PBS. From these serial dilutions, the DMSO final concentration exceeded 1%. Control samples were performed in the presence of DMSO under the same experimental conditions. The protective capacity of PE or quercetin against hemolysis induced by oxidants was evaluated in two tests in parallel, as follows: (I) The RBCs were preincubated at 37 °C for 45 min in the presence of PE (10–80 μg/mL, final concentration) or quercetin (30 μg/mL, final concentration), and then the oxidant (AAPH) was added. (II) After incubation with PE or quercetin and before adding the oxidizing agent, the cells were centrifuged, washed and resuspended in PBS, to eliminate any interaction between polyphenols remaining in the extracellular medium and the oxidant.

In both groups (I, II) the conditions chosen to induce RBCs oxidative lysis were similar to those reported elsewhere (Cesquini et al., 2003). AAPH was added to the cell suspension at a final concentration of 50 mM, this reaction mixture was shaken gently during incubation at 37 °C. The control (RBCs + PBS), hemolysis control (RBCs + AAPH) and phenolic protection against AAPH-induced hemolysis (RBCs + PE or quercetin + AAPH) were recorded after 1, 3 and 5 h of RBCs incubation. Reference values were determined using the same volume of RBC in a hypotonic buffer (5 mM phosphate buffer at pH 7.4, 100% hemolysis). The extent of hemolysis was determined spectrophotometrically at 545 nm. The hemolysis percentage was calculated using the equation:

$$\%\ \mathrm{Hemolysis} = \mathrm{Absorbance\ of\ sample\ supernatant}/\mathrm{Reference\ value} \times 100$$

At each time point, hemolysis was analyzed in four replicates and results expressed as % hemolysis.

2.7.3. Determination of reduced glutathione (GSH)

The intracellular concentration of GSH in both experiments was determined with the DTNB assay as described previously (Ven den Berg et al., 1996). After centrifugation of RBC mixtures (2 mL), 0.6 mL of water was added to the erythrocyte pellets in order to complete the hemolysis. Then, 0.6 mL of the lysate was precipitated by addition of 0.6 mL metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.2 g EDTA (disodium salt and 30 g NaCl in 100 mL water). After 5 min, the protein precipitate was isolated from the remaining solution by centrifugation at 18,000*g* for 10 min. The supernatant (0.45 mL) was then combined with 0.45 mL of 300 mM Na_2HPO_4, and the absorbance at 412 nm was read against a blank consisting of 0.45 mL solution plus 0.45 mL water. Subsequently, 100 μL DTNB solution (20 mg DTNB in 100 mL of 1% citrate solution) were added to the blank and to the sample. The absorbance of the sample was read against the blank at 412 nm. The hemoglobin content (Hb) in an aliquot of lysate (prior to precipitation with TCA) was determined by the Drabkin method (Drabkin and Austin, 1935). The GSH values are expressed as μmoL/g Hb.

2.7.4. Determination of superoxide dismutase (SOD) activity

This procedure is a variation of the classical NBT method (Spitz and Oberley, 1989). The RBCs mixture (100 μL) was centrifuged at 1000*g* for 2 min and the supernatant was removed. Then, 35 μL of ice water was added to induce total erythrocytes lyses. Afterwards, a SOD kit from Randox Laboratories was used to measure SOD activity. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliun chloride (INT) to form a red formazan dye. SOD activity was estimated by the degree of inhibition of this reaction. The calculation was performed according to the Randox instructions, and SOD activity is expressed as U/mg Hb.

2.7.5. RBCs ghost membrane preparation

To induce complete hemolysis, packed RBCs were resuspended in 20 times their volume (1:20) in 5 mM hypotonic phosphate buffer at pH 7.4 and the mixture was kept at 4 °C for 30 min. The solution was then centrifuged at 3800*g* for 20 min at 8 °C, the supernatant was discarded and the pellet resuspended again in 2.5 mM hypotonic phosphate buffer (2.2 mM EDTA, 1:20) pH 8. The procedure was repeated and 1.25 mM phosphate buffer pH 8 was added in the last step, to complete the hemolysis. After centrifugation at 13,500 rpm for 20 min at 8 °C, RBCs ghost membranes in the pellet appeared completely clear, hence they were resuspended in PBS buffer plus 2.2 mM EDTA (1:5) and stored at −80 °C until analysis. Membrane protein was estimated by the Lowry method (Lowry et al., 1951).

2.7.6. Susceptibility of ghost membrane lipids to peroxidation (TBARS assay)

The solutions containing RBCs ghost membranes were thawed; storage buffer was removed after centrifugation at 1800*g* for 20 min at 8 °C, and the pellet washed twice in 0.9% NaCl. The packed ghost membranes were then resuspended in 0.9% NaCl. TBARS formation was determined as a measure of lipid peroxidation after RBCs ghost membranes exposure to the oxidant using a modified thiobarbituric acid (TBA) assay (Buege and Aust, 1978). In this procedure, 2 mL of TBA–TCA–HCl (0.375% w/v TBA, 15% w/v TCA, 0.2 M HCl) was added to 0.6 mL of sample containing 0.3 mM BHT to prevent the possible peroxidation of ghost membrane lipids during the TBA assay. The samples were heated for 20 min at 95 °C followed by cooling at room temperature and centrifugation (3500 rpm). The absorbance of the supernatant was measured at 535 nm. A standard curve of TEP was used to quantify the amount of substances that react with TBA.

2.8. Statistical analysis

Data were subjected to one-way analysis of variance for mean comparison, and inter-honey significant differences were calculated according to HSD Tukey's multiple-range test. Correlations were calculated on a honey mean basis according to Pearson's test. Differences of $p < 0.05$ were considered to be statistically significant. Statistical analysis was performed using STATISTICA software (Statsoft Inc., Tulsa, OK). The mean of three analyses was used and the results and data are reported as mean ± standard deviation (SD).

3. Results and discussion

3.1. Analysis of phenolic compounds by HPLC-DAD–ESI-MS/MS

Linen vine and *Christmas vine* honeys were selected for this study based on their relatively high content of antioxidant compounds, TAC and radical scavenging activity (Alvarez-Suarez et al., 2010b,c, 2011). In the analyzed PE, twelve chromatographic peaks were identified. Table 1 summarizes the peak data obtained in the HPLC-DAS–MS analyses, together with the identities of the compounds established on the basis of their UV–Vis and mass spectra, and on their chromatographic profile compared to external standards when available. The table also shows the quantification of these compounds in the PE from the different samples of the two honeys. *Linen vine* extracts have a higher polyphenol content than *Christmas vine* ones, as previously reported (Alvarez-Suarez et al.,

Table 1
Rt, UV, MS spectrum (MS, $[M-H]^-$; MS^2 $[M-H]^-$) and total content from the different phenolic compounds identified in the two honeys analyzed.

Peak	HPLC-DAD-ESI-MS/MS Identification Data					Phenolic compounds content (μg/100 g of honey)	
	Phenolic compound	t_R (min)	UV (nm)	$[M-H]^-$	MS^2 $[M-H]^-$	*Linen vine* ($n = 10$)	*Christmas vine* ($n = 10$)
1	Phloroglucinol	4.84	255	n.a	n.a	28.17 ± 3.10	n.d
2	Vanillic acid	5.35	260, 294	n.a	n.a	31.27 ± 3.03	17.24 ± 3.63
3	Caffeic acid	7.12	298sh, 324	n.a	n.a	37.63 ± 3.53	24.43 ± 4.54
4	Syringic acid	8.25	274	n.a	n.a	27.18 ± 4.19	n.d
5	*p*-Coumaric acid	11.10	232, 310	n.a	n.a	47.81 ± 3.28	37.53 ± 5.20
6	Ferulic acid	13.35	296, 322	n.a	n.a	58.21 ± 2.74	25.28 ± 4.26
7	Unknown	23.50	260, 354, 387sh	241	213	n.q	n.q
8	Quercetin-*O*- rhamnoside	28.45	256, 268sh, 296sh, 350	447	301	36.38 ± 2.79	n.d
9	Quercetin	28.50	255, 300sh, 370	301	–	52.59 ± 4.43	32.24 ± 3.20
10	Kaempferol-7–*O*-rhamnoside	33.15	263, 320sh, 367	431	285	35.13 ± 3.23	21.24 ± 1.42
11	Kaempferol	33.95	266, 320sh, 366	285	267	39.68 ± 4.38	29.39 ± 4.27
12	Isorhamnetin	34.35	255, 267sh, 301sh, 370	315	300	37.93 ± 5.22	31.25 ± 2.83
13	8-Methoxykaempferol	35.20	268, 292sh, 340	315	285	31.42 ± 2.75	22.37 ± 2.57
Total phenolic contents						463.43 ± 19.53	240.92 ± 15.20

n.a, Compounds were not analyzed by mass spectrometry. Values expressed as means (μg/100 g of honey) ± SD. n.d, not detected. n.q, not quantified but identified in both honeys. Each sample was analyzed in triplicate.

2010b,c). The phenolic compounds detected in these samples were identified as phloroglucinol (peak 1), phenolic acids (peaks 2–6) and flavonoids (peaks 8–13). All the identified flavonoids belong to the group of flavonols.

A total of four peaks were assigned to flavonol aglycones (Table 1). Quercetin (peak 9), kaempferol (peak 11), and isorhamnetin (peak 12) are compounds widely reported in honey. Peak 13 showed a molecular ion $[M-H]^-$ at *m/z* 315, releasing a major MS^2 fragment at *m/z* 285 attributed to the possible loss of a methoxy group (−30 amu), which allowed to identify it as a methoxy derivative of kaempferol. Its UV–Vis spectrum and retention time were consistent with 8-methoxykaempferol in our spectra library. The presence of methoxykaempferol has been previously documented in the honeys here studied and in other honey types (Fiorani et al., 2006; Alvarez-Suarez et al., 2010c). Two compounds (peaks 8 and 10) were identified as flavonol glycosides. Compound 8 was detected only in *Linen vine* honey, which was also the richest one in flavonoids. This compound showed a molecular ion at *m/z* 447 $[M-H]^-$ releasing a MS^2 fragment at *m/z* 301 (quercetin). The loss of a fragment with −146 amu and the fact that its UV–Vis spectrum was not modified in the UV region, thus discarding the presence of a *p*-coumaroyl substituent, allowed the assignment of the compound as a quercetin-rhamnoside. Compound **10**, detected in both honey types, showed a negative molecular ion at *m/z* 431 $[M-H]^-$ and released an MS^2 fragment at *m/z* 285 (kaempferol) from the loss of a rhamnose residue (−146 amu). Its UV–Vis spectrum showed a maximum wavelength in band I at 367 nm, indicating that the hydroxyl in position 3 of the aglycone was free (Mabry et al., 1970). A compound with these same characteristics, identified as kaempferol-7-*O*-rhamnoside, was found by Truchado et al. (2009) in Acacia honey and previously reported in *Linen vine* and *Christmas vine* honeys (Alvarez-Suarez et al., 2010c).

Nine compounds were present in all samples: vanillic, caffeic, *p*-coumaric and ferulic acids, and the flavonols quercetin, isorhamnetin, kaempferol, methoxykaempferol and kaempferol-7-*O*-rhamnoside. Among the phenolic acids, the hydroxycinnamic acids, i.e., caffeic acid (peak 3), *p*-coumaric acid (peak 5) and ferulic acid (peak 6) were predominant. These compounds were previously reported as predominant in these honeys (Alvarez-Suarez et al., 2010a). Among the flavonoids, quercetin was found in highest concentration compared to others.

Another relevant compound (peak 7) present in all honey samples could not be identified, however it does not seem to correspond to a phenolic compound. It displayed a UV spectrum with λ_{max} at 354 nm and a molecular ion $[M-H]^-$ at *m/z* 241 that released a major fragment at *m/z* 213 (loss of C=O −28 amu). A compound with similar characteristics was previously reported by Truchado et al. (2009) who suggested a possible floral marker of Chestnut honey. The compound was not identified by those authors, but it was tentatively assigned as a possible intermediate in tryptophan metabolism pathway. The same compound was also previously reported by our group in these honeys (Alvarez-Suarez et al., 2010c), but no further contribution to its identification could be made and, thus, it remains unidentified.

3.2. Total antioxidant capacity and radical scavenging activity of phenolic extracts

Many methods have been proposed to evaluate the antioxidant activity, or capacity, of phenolic compounds and phenolic-rich extracts. These methods have been classified according to the mechanism of radical deactivation (hydrogen atom transfer, ORAC, or electron transfer, TEAC and DPPH), according to the physiological relevance of the free radical, or according to the competitive or direct approach of the reaction (Prior et al., 2005). Therefore, TAC in PE was analyzed by four methods based on their different radical scavenging principle (DPPH, ABTS and ORAC assay), widely used as screening methods for the antioxidant activity in food and beverages, including honey (Alvarez-Suarez et al., 2009). Moreover, the ability to scavenge specific active oxygen species ($HO^{\cdot}$) was also studied by EPR.

As shown in Table 2, PE from *Linen vine* had the highest TAC values (ABTS and ORAC) and a significant correlation was found between values in both assays ($r = 0.9382$, $p < 0.001$). In addition, $DPPH^{\cdot}$ scavenging activity was measured using EPR spectroscopy. The PE from *Linen vine* honey was a more effective $DPPH^{\cdot}$ scavenger than *Christmas vine* extract (Table 2). Phenolics from *Linen vine* scavenged almost 58.40 ± 6.65% of $DPPH^{\cdot}$ radicals in 10 min whereas *Christmas vine* 38.15 ± 5.18%. The $DPPH^{\cdot}$ scavenging activity of quercetin, the principal flavonoid in both honeys, was also analyzed, obtaining percentage of radical scavenging activity (% RSC) values of 30.25 ± 3.01% at a concentration of 7.5 μg/mL. This concentration was selected based on the potential of quercetin to decrease the EPR signal intensity, which at higher quercetin concentrations was suppressed almost completely within 10 min. An important point to note is that higher concentrations of the extracts had to be used in this assay than in the ORAC and ABTS assays, probably due to the intrinsic characteristics of the test and the lower ability of the compounds analyzed to react with the $DPPH^{\cdot}$ (Alvarez-Suarez et al., 2009).

Table 2
Total antioxidant and radical scavenging activities of the phenolic extracts after fractionation with Amberlite XDA-2 resin.

Honey source	Phenolic extract			
	Total antioxidant capacity (μmol of TE/g)*		% RSC	
	ORAC	TEAC	DPPH	OH·
Linen vine (n = 10)	3.85 ± 0.08[a]	1.08 ± 0.05[a]	58.40 ± 6.65[a]	49.73 ± 7.21[a]
Christmas vine (n = 10)	0.94 ± 0.05[b]	0.31 ± 0.01[b]	38.15 ± 5.18[b]	19.14 ± 3.36[b]

% RSC, percentage of radical scavenging capacity; TE, Trolox equivalents; OH·, hydroxyl radical.
* Values expressed as means ± SD. Mean values within a column having different letter are significantly different by Tukey's multiple range test ($p < 0.05$). Each sample was analyzed in triplicate.

The HO· radical was generated from hydrogen peroxide by the Fe(II)-catalyzed Fenton reaction and determined by the EPR-spin trapping technique. PE were very effective in scavenging the HO· radical, although their ability varied depending on the composition of the extract. Thus, phenolics from *Linen vine* honey were almost 2.5-times better HO· scavengers than *Christmas vine*'s (Table 2). A significant ($p < 0.05$) decrease in the DMPO-OH signal amplitude was observed for *Linen vine* extracts. At a concentration of 5 μg/mL the samples of *Linen vine* and *Christmas vine* showed 49.73 ± 7.21% and 19.14 ± 3.36% RSC respectively, while quercetin, at the same concentration, showed 61.37% RSC. It is known that phenolics possess relevant radical scavenging activity, which would support their putative important role in the radical scavenging properties of honey (Henriques et al., 2006).

3.3. Phenolic fractions from honey protect human RBC against free radical damage

3.3.1. Hemolysis assay

Various studies have recently investigated the potential protective effects of phenolics against RBCs oxidative damage (Ferrali et al., 1997; Fiorani et al., 2002, 2006; Pawlikowska-Pawlęga et al., 2003; Fiorani and Accorsi, 2005; Blasa et al., 2007; Suwalsky et al., 2007; Tulipani et al., 2011; Mendes et al., 2011; Valente et al., 2011), suggesting a possible interaction of flavonoids within cell membranes, which generally serve as targets for lipid peroxidation (Ferrali et al., 1997). A factor limiting the antioxidant activity of a molecule in the cell is its localization or the access to different cell components. Once compartmentalized into the cell membranes, antioxidant compounds, such as polyphenols, seem to exert a significant inhibition of lipid peroxidation, and, at the same time, enhance membrane integrity against several chemical and physical stress conditions (Pawlikowska-Pawlęga et al., 2003). RBCs are the most abundant cells in the human body and are especially susceptible to oxidation due to their high lipid content, their rich oxygen supply and their content of transition metals such as iron and copper (Delmas-Beauvieux et al., 1995). Oxidative damage of RBCs membrane generally involves lipid peroxidation, which may cause its malfunctioning by altering its fluidity and the membrane-bound enzyme and receptor functions; this has been proposed as a general mechanism leading to RBCs hemolysis involved in cell injury and death. In this study, the capacities of PE from two monofloral Cuban honeys were initially evaluated for their efficacy to protect human RBCs against hemolysis induced by the oxidant AAPH. Fig. 1 shows human RBCs hemolysis induced by AAPH and the effect of the addition of PE in experiment I. RBCs incubated at 37 °C as an 8% suspension in PBS were stable with little hemolysis within 5 h (6.5 ± 1.1%). When AAPH was added to the RBCs suspension, a time-dependent hemolysis was observed (58 ± 4.07% after 1 h to 94 ± 4.18% after 5 h). The fact that hemolysis is delayed suggests that endogenous antioxidants in the erythrocytes such as glutathione, ascorbate and enzymes, such as catalase and superoxide dismutase, can efficiently quench radicals to protect the cell against free radical-induced hemolysis (Delmas-Beauvieux et al., 1995). Moreover, when the cells were incubated with either 1% DMSO or the highest concentration of each phenolic fraction in the absence of AAPH, hemolysis was maintained at a background level similar to control (data not shown). Both PE significantly protected erythrocyte membranes from hemolysis induced by AAPH in a concentration- and time-dependent manner, with IC_{50} values (concentration of PE that causes 50% inhibition of hemolysis) after 5 h of incubation of 24.68 ± 6.73 μg/mL for *Linen vine* extracts and 62.56 ± 12.07 μg/mL for *Christmas vine* extracts, respectively. Further, a dose-dependent correlation could be demonstrated between lag times for both PE.

Since lipid-soluble antioxidants are expected to protect the hydrophobic part of the cell membrane, honey PE should exert their antioxidant effects because of their hydrophobicity (Blasa

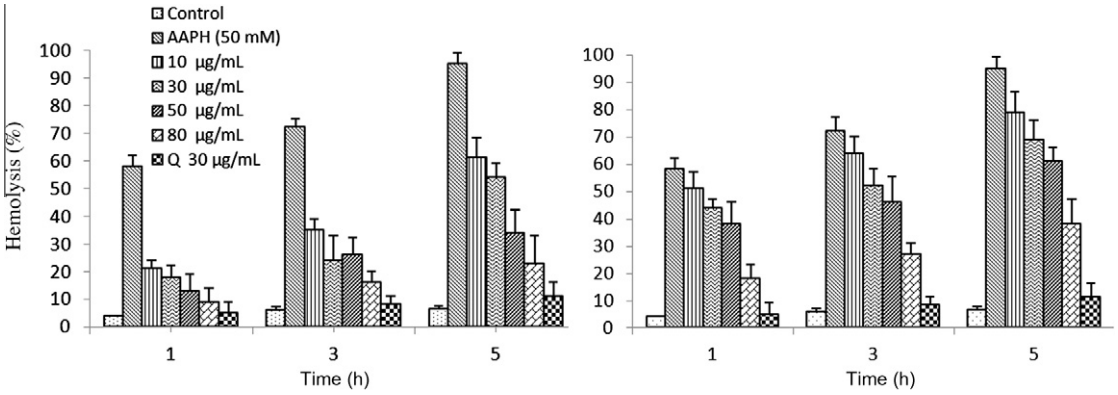

Fig. 1. Protective effect of phenolic fraction of honey (PE) (A, *Liven vine*; B, *Christmas vine*) on AAPH-induced hemolysis in human RBCs suspensions (Ht 8%) in experiment I. The RBCs were preincubated at 37 °C for 45 min in the presence of phenolic extract (10–80 μg/mL, final concentration) or quercetin (30 μg/mL, final concentration), and then the oxidant was added. Percentages of hemolysis are reported as mean ± SD of three experiments. *Significant difference with control group ($p < 0.05$); # Significant difference with AAPH group ($p < 0.05$). Error bars refer to the biological variability.

et al., 2007). Therefore, parallel to the previous assay, another experiment was conducted in order to evaluate the affinity and interaction of the studied phenolics with the cell membrane. With this aim, after 45 min of incubation with the PE and before adding the oxidizing agent, the cells were centrifuged, washed to eliminate phenolics not incorporated in the cells and re-suspended in PBS; then AAPH was added to the medium. No significant differences were found between experiments I and II (data not shown), signifying that phenolics could be incorporated inside cell membranes. This latter observation suggests that the anti-hemolytic effects of PE are dependent on their content of ether-soluble flavonoids and their affinity with cell membranes (Blasa et al., 2007). Hydrophobic flavonoids might incorporate into the hydrophobic core of the membrane bilayer, causing a reduction in membrane fluidity and membrane stability (Chen and Deuster, 2009). Peroxyl radicals generated by AAPH may attack the RBCs from the outside of the membrane and the extent of hemolysis is proportional to their amount (Miki et al., 1987). Therefore, PE, like lipid-soluble chain-breaking antioxidants located in lipophilic regions of the membranes, may predominantly scavenge radicals within this environment, besides directly interacting with AAPH in the medium. This reasoning is supported by the results obtained when the PE were added to RBCs suspension and then the medium was changed prior to the oxidative treatment, and are in agreement with previous reported results (Blasa et al., 2007).

In experiment I, quercetin showed an important capacity to protect RBCs against hemolysis induced by AAPH. The percentage of inhibition of AAPH-induced hemolysis after 5 h of incubation was found to be 88.29 ± 7.23% (Fig. 1), whereas in experiment II the anti-hemolytic capacity of quercetin remained almost the same (data not shown). Previous studies demonstrated that this compound possesses an important anti-hemolytic capacity (Ferrali et al., 1997; Pawlikowska-Pawlęga et al., 2003; Chen and Deuster, 2009). Quercetin, a very hydrophobic molecule, would have selectively partitioned into hydrophobic regions (Chaudhuri et al., 2007) or altered lipid-protein components of the RBCs membrane (Pawlikowska-Pawlęga et al., 2003). These properties may explain the efficacy against hemolysis shown by quercetin in this study.

3.3.2. Antioxidant enzymes (GSH and SOD)

GSH is one of the primary low-molecular weight antioxidants that protect cells by converting reactive oxygen species into less harmful molecules. Thus, one way of protection against oxidative hemolysis is preventing GSH depletion (Dröge, 2002). Table 3 shows the effect of honey PE on AAPH-induced changes in GSH content of RBCs in experiment I. In the control group, the normal basal GSH level in the RBCs was 4.47 ± 0.03 μmol/g Hb with no significant changes after 5 h of incubation. When RBCs suspension was incubated with AAPH (50 mM), a significant ($p < 0.05$) time-dependent consumption of cytosolic GSH content was produced, decreasing to 1.46 ± 0.28 μmol/g Hb after 1 h of incubation with AAPH, and to 0.32 ± 0.01 μmol/g Hb after 5 h. Both PE significantly protected RBCs against depletion of intracellular GSH content. Again, no statistical differences were observed when the two types of experiments were performed (I and II) (data not shown). However, significant differences ($p < 0.05$) between GSH values in cells treated with PE from the two honey types were found, with the best results obtained for the *Linen vine* extracts. The protection of different flavonoid-rich extracts against GSH depletion in different cells has been previously reported (Hofmann et al., 2006; Schaefer et al., 2006), but at this regard no studies have been done using phenolics from honey.

SOD is widely distributed in all cells and is also present in high amounts in erythrocytes. This enzyme protects cells by dismutation of the highly reactive superoxide anion ($O_2^{-\cdot}$) to a less reactive species, H_2O_2 (Ko et al., 1997). Table 3 shows the effect of PE on AAPH-induced changes in SOD activity in RBCs in experiment I. Both extracts were very efficient in modulating AAPH-related decline in SOD activity. SOD activity in the control group decreased during incubation by 20% compared to baseline time. When the oxidant AAPH was added to RBCs, a time-dependent decline in SOD activity was found, with significant differences ($p < 0.05$) compared to the control group, causing a decrease from 443 ± 35 U/mg Hb after 1 h to 84 ± 4.67 U/mg Hb after 5 h incubation. In this case, incubation with both extracts prevented AAPH-induced decline in RBCs SOD activity, with *Linen vine* extracts being the most active. No statistical differences were observed between experiments I and II (data not shown). These results are in agreement with those of Yang et al. (2006) who reported protection by an extract rich in flavonoids against the decline of SOD activity in RBCs previously treated with AAPH.

Quercetin also exhibited an important capacity to protect against cytosolic GSH depletion and to decrease SOD activity induced by AAPH (Table 3), as a consequence of its incorporation in human RBCs. Previously, Fiorani et al. (2001) reported that quercetin prevents glutathione depletion induced by dehydroascorbic acid in rabbit RBCs. Our findings agree with these latter data indicating that quercetin, besides being located at the polar–unpolar interface of the membrane (Chaudhuri et al., 2007), can be incorporated inside RBCs. Here, it is able to bind to some intracellular

Table 3
Effect of *Liven vine* and *Christmas vine* honey phenolic extracts on AAPH-induced depletion in GSH and SOD content of erythrocytes.

Experimental condition	GSH (μmol/ g Hb) Time (h)			SOD activity (U/mg Hb) Time (h)		
	1	3	5	1	3	5
Control	4.47 ± 0.03[#]	4.26 ± 0.12[#]	4. 12 ± 0.24[#]	984 ± 58[#]	835 ± 57[#]	783 ± 42[#]
+AAPH	1.46 ± 0.28[*]	0.85 ± 0.03[*]	0.32 ± 0.01[*]	443 ± 35[*]	164 ± 24[*]	84 ± 4.67[*]
+10 μg/mL Lv Ext	2.87 ± 0.36[*,#]	1.63 ± 0.31[*]	0.58 ± 0.07[*]	536 ± 26[*]	298 ± 23[*]	125 ± 18[*]
+30 μg/mL Lv Ext	3.18 ± 0.17[*,#]	2.44 ± 0.03[*,#]	0.78 ± 0.04[*]	618 ± 38[*,#]	386 ± 31[*,#]	224 ± 27[*,#]
+50 μg/mL Lv Ext	3.63 ± 0.21[*,#]	2.96 ± 0.27[*,#]	1.38 ± 0.27[*,#]	787 ± 31[*,#]	417 ± 37[*,#]	237 ± 24[*,#]
+80 μg/mL Lv Ext	4.12 ± 0.11[#]	3.23 ± 0.10[*,#]	1.78 ± 0.34[*,#]	823 ± 38[#]	544 ± 39[*,#]	285 ± 31[*,#]
+10 μg/mL Cv Ext	2.14 ± 0.03[*]	1.03 ± 0.20[*]	0.53 ± 0.02[*]	481 ± 42[*]	187 ± 12[*]	96 ± 8[*]
+30 μg/mL Cv Ext	2.74 ± 0.22[*,#]	1.87 ± 0.11[*]	0.62 ± 0.03[*]	579 ± 46[*,#]	283 ± 23[*]	113 ± 12[*]
+50 μg/mL Cv Ext	3.11 ± 0.02[*,#]	2.15 ± 0.28[*,#]	1.03 ± 0.12[*,#]	604 ± 52[*,#]	351 ± 27[*,#]	184 ± 10[*]
+80 μg/mL Cv Ext	3.47 ± 0.12[*,#]	2.43 ± 0.23[*,#]	1.18 ± 0.10[*,#]	723 ± 50[*,#]	472 ± 21[*,#]	223 ± 26[*,#]
Quercetin 100 μM	4.23 ± 0.21[*,#]	3.53 ± 0.10[*,#]	3.17 ± 0.21[*,#]	897 ± 43[#]	615 ± 52[*,#]	551 ± 43[*,#]

Since statistically significant differences were not found between experiments I and II, the table presents only the results of experiment I. The RBCs were preincubated at 37 °C for 45 min in the presence of the phenolic extracts at different concentrations (10–80 μg/mL), and then the oxidant AAPH (50 mM) was added. Values of GSH content and SOD activity are expressed as the mean ± SD of three experiments.

[*] Significant difference with control group ($p < 0.05$).

[#] Significant difference with AAPH group ($p < 0.05$). *Liven vine* phenolic extract (Lv Ext), *Christmas vine* phenolic extract (Cv Ext).

component, retaining its biologically active form and is quantitatively consumed upon intra/extracellular oxidant supplementation (Fiorani et al., 2001).

3.3.3. Protection of lipid membranes against peroxidation

To gain further insights into the protective effect of honey extracts against AAPH-induced oxidative damage in RBCs, the extent of lipid peroxidation was assessed by measuring the formation of thiobarbituric reactive substances (TBARS), closely related to malondialdehyde formation, a well-known carbonyl product of oxidative lipid damage (Esterbauer et al., 1991). Fig. 2 shows TBARS values in experiment I. The TBARS levels in control group were stable after 5 h of incubation. As expected, TBARS values significantly increased after 5 h of incubation with AAPH when compared to the control ($p < 0.05$). When RBCs were incubated with the highest concentration of PE (80 μg/mL), in the absence of AAPH, TBARS values remained at a background level similar to the control (data not shown). However, when RBCs suspension was pretreated with each of the PE, the oxidative effects of AAPH were significantly concentration-dependent decreased ($p < 0.05$). Treatment with both extracts, at the highest concentration after 5 h incubation, lead to a significant reduction in TBARS values of 79.39 ± 6.1% and 61.81 ± 5.1% for *Liven vine* and *Christmas vine* PE, respectively. As for the previous results presented here, no statistically significant differences were found between experiments I and II (data not shown), while the difference between the two honeys PE remained, with *Linen vine* honey extract resulting the best. Various studies have reported the protective effect of many polyphenol-rich extracts against the damage of membrane lipids mediated by free radicals (Cesquini et al., 2003; Yang et al., 2006). Moreover, quercetin remarkably protected RBCs against AAPH-induced TBARS increase (Fig. 3). After 5 h of incubation, TBARS values were similar to values of the control group, and significant differences were not found between experiments I and II. The obtained results suggest that, owing to their high degree of lipophilicity, the compounds present in the extracts could be incorporated into membranes and act as scavengers of radicals in the lipophilic environment.

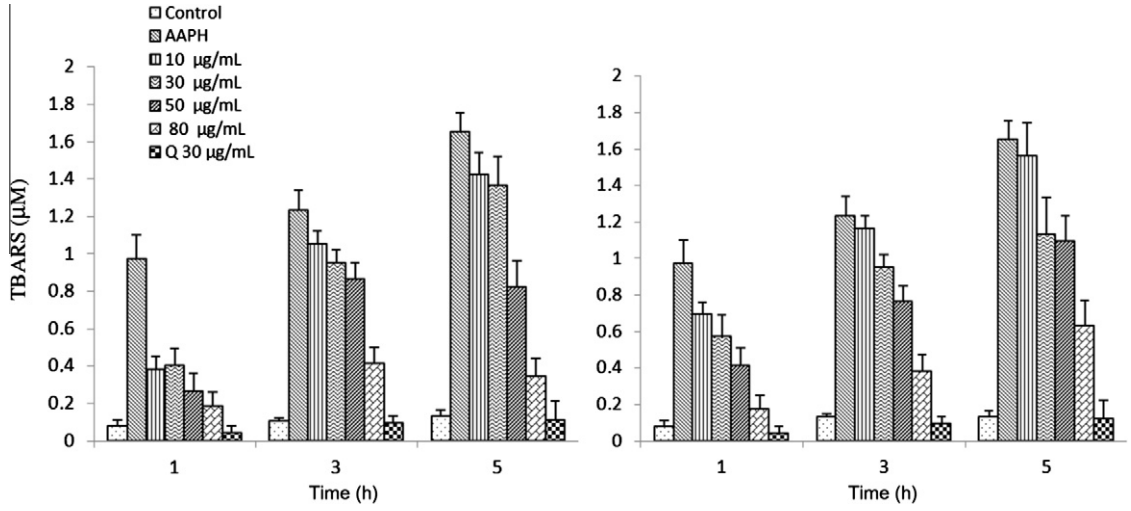

Fig. 2. Protective effect of phenolic fraction of honey (PE) (A, *Liven vine*; B, *Christmas vine*) on AAPH-induced lipid peroxidation in human RBCs suspensions (Ht 8%) in experiment I. The RBCs were preincubated at 37 °C for 45 min in the presence of phenolic extract (10–80 μg/mL, final concentration) or quercetin (30 μg/mL, final concentration), and then the oxidant was added. TBARS values are reported as mean ± SD of three experiments. *Significant difference with control group ($p < 0.05$); # Significant difference with AAPH group ($p < 0.05$). Error bars refer to the biological variability.

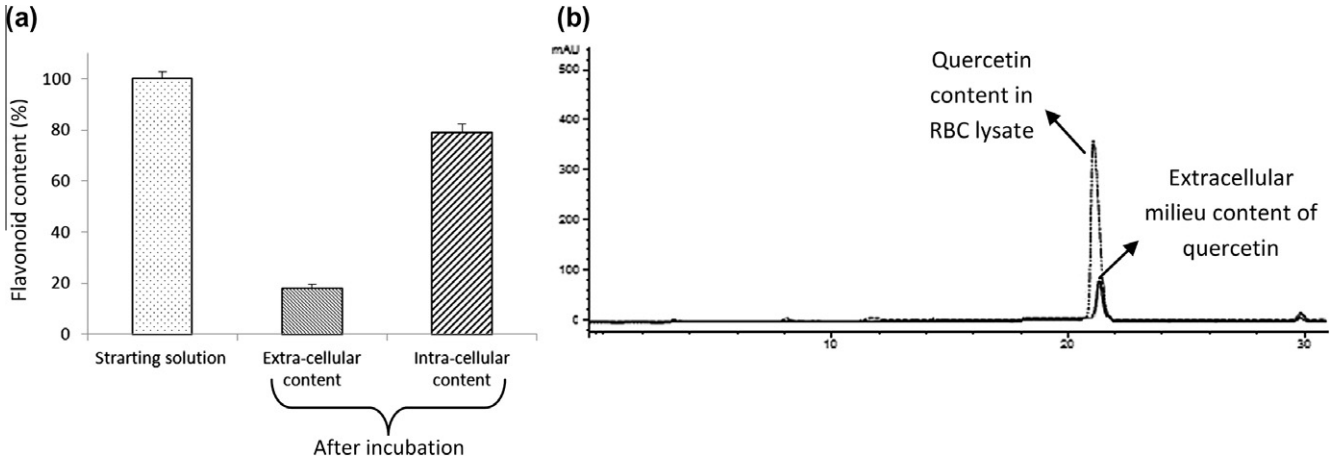

Fig. 3. Percent of quercetin uptaken by human RBCs. (A) Extra and RBC intracellular amounts after incubation with 30 μg/mL of quercetin for 45 min and (B) representative chromatograms of quercetin obtained after incubation. Data are expressed as percentage of the flavonoid recovered in RBC with regard to the total flavonoid content (extra and intracellular concentrations).

3.3.4. HPLC-DAD analysis of quercetin up take by human RBCs

Previous uptake studies employing PE from honeys showed an excellent uptake of these flavonoids in human RBCs (Fiorani et al., 2006). The capacity of quercetin uptake by RBCs was taken as an experimental model to confirm the capacity of flavonoids to interact with RBCs membrane for two main reasons. Firstly, quercetin was identified as one of the major components in the studied extracts and, secondly, different studies have reported its involvement in protection of RBCs membranes against oxidative damage (Ferrali et al., 1997; Pawlikowska-Pawlęga et al., 2003; Chaudhuri et al., 2007). Fig. 3 shows the extracellular quercetin content and the amount uptaken by RBCs (in %) (A), as well as representative chromatograms of quercetin obtained after incubation (B). The results indicate that quercetin is efficiently uptaken by RBCs and actually accumulates at levels related with the initial extracellular loading concentration. In the extracellular milieu, 17.86 ± 1.7% of quercetin after 45 min of incubation was recovered, whereas in RBCs lysate, 78.86 ± 4.6% was detected after incubation. Under this condition, approximately 23.64 ± 2.16 μg/ml of quercetin were incorporated by RBCs. The efficient uptake of quercetin by human RBCs was previously reported to be rapid (after only 5 min), since intracellular values of quercetin of up to 80% were recovered after incubation with this flavonoid (Ferrali et al., 1997; Fiorani et al., 2002, 2006). Similar results were previously obtained in another study in which it was found that quercetin is almost quantitatively uptaken by mouse RBCs after 60 min of exposure (Ferrali et al., 1997). These results indicate that some flavonoids may be uptaken by RBCs and incorporated into their structure providing defense and promoting cell functions. However, additional studies are required to fully evaluate the potential uptake by RBCs of the different flavonoids present in our samples.

4. Conclusion

In summary, honey contains relevant antioxidant compounds. In particular, the monofloral Cuban honeys here analyzed show relatively high concentrations of phenolic acids and flavonoids, which are responsible, at least in part, for their antioxidant activity. The obtained results show that an ether-soluble phenolic fraction of honey exhibits important radical scavenging activity and protection of RBCs against hemolysis and lipid peroxidation induced by free radicals, as well as protection against depletion of important intracellular antioxidant enzymes, such as SOD and GSH. The observations may suggest that highly liposoluble flavonoids contained in this fraction might be incorporated into the RBCs cell membrane and cross through this. Once within the cell, they can act as antioxidants against radicals generated in the lipophilic phase. Quercetin might also be able to incorporate into RBCs structures providing defense and promoting cell function. These results support the hypothesis that flavonoids contribute greatly to the functional value of honey.

Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgements

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