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Honey flavonoids as protection agents against oxidative damage to human red blood cells

Manuela Blasa, Manila Candiracci, Augusto Accorsi, Maria Piera Piacentini, Elena Piatti *

Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino, Via Saffi 2, 61029 Urbino, Italy

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Abstract

Honey phenol extracts separated on the base of their hydrophobicity were evaluated for the antioxidant content and for the ability to inhibit oxidative damage induced by radical species generated in the water phase or in the membrane of human erythrocytes. The water and ether fractions obtained from crude methanol extract of honey exhibited a phenolic content of 5.33 and 2.62 mg caffeic acid equivalents/100 g honey, respectively. These values correlate well with those of total antioxidant power, as assessed by FRAP assay (37.67 vs. 10.65 μmol/100 g honey). Flavonoid contents were 2.57 and 1.64 mg catechin equivalents/100 g honey for ether and water fractions, respectively. Although both honey fractions protect erythrocytes against 2,2′-azobis(2-amidinopropane)dihydrochloride-induced lysis, only the ether fraction was found to be active in inhibiting hemolysis but not methemoglobin and ferrylhemoglobin formation caused by H_2O_2. In addition, the ether fraction prevents *tert*-butylhydroperoxide-induced lipid peroxidation in whole erythrocytes and in isolated membranes. The significant antioxidant effect against damages induced by both water-soluble and hydrophobic exogenous oxidants suggests that the ether fraction, owing to its lipophilic character, can interact with red blood cell membrane, and the protective effect can be associated with the binding of the flavonoids to the membrane. On the other hand, the water fraction is more hydrophilic than ether fraction and it acts only from the outside of the membrane by scavenging the radicals before they attack the erythrocyte membrane.
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1. Introduction

Numerous epidemiological studies have shown that the Mediterranean diet is associated with some of the world's highest adult life expectancy rates and among the lowest rates of coronary heart disease, certain cancers and some other diet-related chronic diseases (Spencer, El Mohsen, & Rice-Evans, 2004; Trichoupoulou & Vassilopoulou, 2000). The key to this diet is that it is rich in antioxidants which protect vital cell components from oxidative damage and slow the aging process by neutralizing free radicals (Brandi, 1992; Havsteen, 1983). Among natural food antioxidants, polyphenols are ubiquitously distributed in the vegetable kingdom as plant secondary metabolites (Paganga & Rice-Evans, 1997). They have been the subject of several studies of biological effects that are mostly ascribed to their antioxidant capacity, free radical-scavenging power and chelation of redox-active metal ions (Hanasaki, Ogawa, & Fukui, 1994; Morel et al., 1993). Natural polyphenols can range from simple molecules to highly polymerised compounds, with flavonoids representing the most common and widely distributed subgroup.

Unlike other components of the Mediterranean diet, namely fruits, vegetables, wine and olive oil, that have been intensively studied (Kalt, Forney, Martin, & Prior, 1999; Manna, Galletti, Cucciolla, Montedoro, & Zappia, 1999; Tedesco et al., 2000), honey is relatively unknown for its antioxidant properties. Recently, Gheldof and co-workers have demonstrated that various kinds of antioxidant components in honey may play important roles in a combinative

* Corresponding author. Tel.: +39 722 305242; fax: +39 722 305324.
E-mail address: e.piatti@uniurb.it (E. Piatti).

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or synergistic contribution to its total antioxidant activity (Gheldof, Wang, & Engeseth, 2002). These components have been separated on the basis of their solubility, and their relative contribution to the total antioxidant activity of honey has been determined.

In a previous work, we evaluated antioxidant activity of two representative Italian raw honeys, namely *Millefiori* and *Acacia*. They displayed a phenolic content ranging from 12.5 to 17.5 CAE mg/100 g and from 3 to 11 CAE mg/100 g in *Millefiori* and *Acacia*, respectively. Concerning total flavonoids, the content ranged from 1.23 to 2.93 CE mg/100 g in *Millefiori* and from 0.45 to 1.01 CE mg/100 g in *Acacia*. The results demonstrated a strong correlation between honey colour and antioxidant power, with darker and more crystallized honeys having stronger antioxidant activity than lighter and transparent honeys (Blasa et al., 2006).

In the present study, raw multifloral honey, collected over the Marche region (central Italy), was used. Phenols were absorbed on an Amberlite XAD-2 column and eluted by methanol. The eluate was vacuum-dried, dissolved with water and partitioned in ethyl ether to fractionate the polar and non-polar compounds (Ferreres et al., 1994).

For the *in vitro* characterization of antioxidant propensities, the use of a single test system is widely discouraged, since the mechanisms of reactions involving antioxidants are complex. Therefore, after measuring the total antioxidant content of honey extract fractions by the FRAP assay, we determined protection vs. damage caused by oxidants on red blood cells (RBCs). The peroxyl radical-scavenger activity was tested by the inhibition of RBC hemolysis induced by 2,2′-azobis-(amidinopropane)dihydrochloride (AAPH) which generates such radicals, attacking from the outside of the membrane (Miki, Tamai, Mino, Yamamoto, & Niki, 1987). The antioxidant efficiency against oxidative stress induced by hydroperoxides was also estimated, utilising water-soluble hydrogen peroxide (H_2O_2) and the lipophilic *tert*-butylhydroperoxide (*t*-BOOH).

The aim of the work was to assess and compare antioxidant activities of honey phenol extracts, separated by their hydrophobicity. Available literature indicates that no previous studies have been reported on antioxidant properties of polyphenols of italian honeys. Thus, to our knowledge, this is the first report of such an *in vitro* study.

2. Material and methods

2.1. Chemicals and honey sample

Butylated hydroxytoluene (BHT), Folin & Ciocalteu's phenol reagent, *tert*-butyl hydroperoxide (*t*-BOOH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) and Amberlite XAD-2 resin were purchased from Sigma Chemical Co. (St. Louis, MO). Wako Chemicals GmbH (Neuss, Germany) was the firm from which we bought 2,2′-azobis-(amidinopropane)dihydrochloride (AAPH). All other reagents were analytical grade.

Unprocessed multifloral honey was locally obtained from Associazione Marchigiana Apicoltori (A.M.A., Marche, Italy) with guarantee of authenticity and known history. The sample was harvested in 2004 and stored in the darkness at room temperature to minimize any alterations.

2.2. Preparation of phenolic extracts of the honey

A procedure for recovery of flavonoids from the whole raw honey, using nonionic polystyrene resin (Amberlite XAD-2), was used (Ferreres et al., 1994). Briefly, an acidified honey solution (50 g in 250 ml of water, pH 2.1) was poured into a column previously packed with XAD-2 (25×1.8 cm). A 300 ml volume of acidified water was used to wash out the sugars and polar compounds. Before the phenolic compounds were collected, the column was washed with 300 ml of neutral water. Then, phenolics were eluted with 300 ml of methanol, which were concentrated by a rotary evaporator (40 °C), and dissolved in 5 ml of water. The concentrated methanol extract was re-extracted by diethyl ether to separate flavonoids on the basis of their hydrophobicity, according to Gheldof et al. (2002). The colourless more hydrophobic ether fraction was treated with anhydrous Na_2SO_4 for 30 min, then filtered, concentrated again, dissolved in DMSO and thereafter termed EtF. The yellow-coloured water-phase fraction after ether extraction, more hydrophilic than EtF, was designated WF. Both fractions were stored at –80 °C prior to further analysis.

2.3. Preparation of erythrocytes and induction of oxidative stress

Human blood from healthy volunteers was kindly provided by the "Centro Trasfusionale" ("Blood Transfusion Centre") of the local hospital. Erythrocytes were obtained by consecutive centrifugations and washing in cold phosphate-buffered saline (PBS, 125 mM NaCl, 10 mM sodium phosphate, pH 7.4) to remove plasma, platelets and buffy coat. Conditions chosen to induce RBC oxidative lysis were those usually reported in the literature (Ko, Hsiao, & Kuo, 1997): AAPH or H_2O_2 were added to the cell suspension (about 10%, v/v) at final concentrations of 50 or 20 mM, respectively, and the extent of hemolysis was evaluated spectrophotometrically according to Ko et al. (1997). Hemoglobin (Hb) oxidation was induced by incubating RBC suspension (10% v/v) with 1 mM H_2O_2 and the concentrations of the oxidation products were calculated by equations, as reported by Ko et al. (1997). Incubations with H_2O_2 were carried out in the presence of 1 mM sodium azide, added 5 min before the addition of H_2O_2 to inhibit catalase. Malondialdehyde (MDA) formation was determined as a measure of lipid peroxidation after 10% RBCs (v/v) were incubated in the presence of 1 mM *t*-BOOH, according to Cesquini, Torsoni, Stoppa, and Ogo (2003). The concentration of MDA was measured by the method already described (Beuge & Aust, 1978).

In all experiments, RBCs were resuspended in PBS and the honey extracts, at various concentrations, were added immediately before the oxidative compounds. Where specified, RBCs were preincubated at 37 °C for 30 min in the presence of EtF and WF, and to avoid a direct interaction between the polyphenols and the oxidant source, the medium was changed. Incubations were carried out at 37 °C under continuous shaking in the dark for the time indicated.

Ghosts preparation and protein determination were performed as already described (Repka & Hebbel, 1991). Lipid peroxidation in RBC ghosts (1 mg/ml) was induced by 1 mM *t*-BOOH plus 50 μM Fe(II) at 37 °C for 30 min, after a preincubation of 50 min in the presence of honey extracts.

Samples were analyzed in four replications.

2.4. Determination of the total content of polyphenols and flavonoids

The concentrations of total phenolics and flavonoids in EtF and WF were determined according to Singleton, Orthofer, and Lamuela-Raventos (1999) and to Kim, Jeong, and Lee (2003), respectively. Total phenolics were expressed as mg caffeic acid equivalents (CAE)/100 g honey and total flavonoids as mg catechin equivalents (CE)/100 g, as already reported (Blasa et al., 2006). Samples were analyzed in four replications.

2.5. Determination of the total antioxidant power (FRAP assay)

The ferric reducing/antioxidant powers (FRAP) of EtF and WF were estimated as reported by Blasa et al. (2006). Results were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$ used as the standard solution. Adequate dilution was needed if the FRAP value measured was above the linear range of the standard curve. All tested samples were replicated four times.

2.6. Statistical analysis

Results are presented as mean values ± standard deviation. Statistical analyses between experimental results are based on the Student's *t* test. Significant differences were statistically considered at the level of $P < 0.05$.

3. Results and discussion

As shown in Table 1, the water fraction (WF) showed higher polyphenol content than did the ether fraction (EtF) (5.33 and 2.62 CAE mg/100 g honey, respectively), although its flavonoid concentration was 1.6-fold lower than that of EtF (1.64 vs. 2.57 CE mg/100 g honey). A higher polyphenol amount in WF may be due to phenolics with high molecular weight or present as glycosidic derivatives, since polymerization and glycosylation make these molecules more water-soluble. Indeed, as reported by Ferreres et al. (1994), ether extraction is an extra step in the purification of honey flavonoids, leaving (in the aqueous phase) darker phenolic polymers and contaminant sugars that give the extract a brown pigmentation and a syrup texture.

Table 1
Content of total phenol and flavonoid, and total antioxidant power in honey fractions

	Total polyphenols (CAE mg)	Total flavonoids (CE mg)	FRAP (μmol)
Ether fraction (EtF)	2.62 ± 0.46	2.57 ± 0.64	10.7 ± 1.92
Water fraction (WF)	5.33 ± 1.20	1.64 ± 0.19	37.7 ± 5.31

Data are referred to 100 g of honey and expressed as mean values ± standard deviation ($n = 4$).

Since antioxidant and antiradical properties of an extract have mainly been assigned to phenolic compounds (Beretta, Granata, Ferrero, Orioli, & Maffei Facino, 2005; Soares, Dinis, Cunha, & Almeida, 1997), it is expected that the effectiveness of a honey fraction is related to its phenolic content. FRAP assay has been widely used to directly test "total antioxidant power" of several extracts of foods and plants and is based on the ability of the analyte to reduce the Fe(III)/Fe(II) couple (Benzie & Strain, 1999). As shown in Table 1, WF possessed a much higher FRAP value than EtF (37.7 vs. 10.7 μmol/100 g honey) and these values correlate well with those of total polyphenol concentrations. Hence, the polyphenols present in WF, as glycosides and polymers, mostly contributed to the total antioxidant activity.

A factor limiting the antioxidant activity of a molecule in the cell is its localization or access to different cell components. The lipid-soluble antioxidants can protect the hydrophobic part of the cell membrane and the more hydrophilic molecules can act in the aqueous environment. Particularly, in the experiments of Saija et al. (1995), a fundamental requisite for the expression of antioxidant activity of a compound clearly appears to be, together with redox properties, the ability to interact with biomembranes. Therefore, since the hydrophobicity of a molecule correlates with its association with the membrane, honey phenolic extracts could exert their antioxidant effects according to their hydrophobicity.

Peroxyl radicals generated by AAPH may attack the erythrocyte from the outside of the membrane and the extent of hemolysis is proportional to their amount (Miki et al., 1987). As shown in Fig. 1a, both the WF and EtF were very efficient (beginning from 2.5 μg flavonoids/ml of incubation) in protecting the cells from lysis induced by peroxyl radicals (Fig. 1a). In agreement with published results (Niki et al., 1988), the WF, like water-soluble radical-scavengers, may efficiently scavenge the peroxyl radicals in the medium before they attack the erythrocytes, thus protecting them from lysis. On the other hand, the

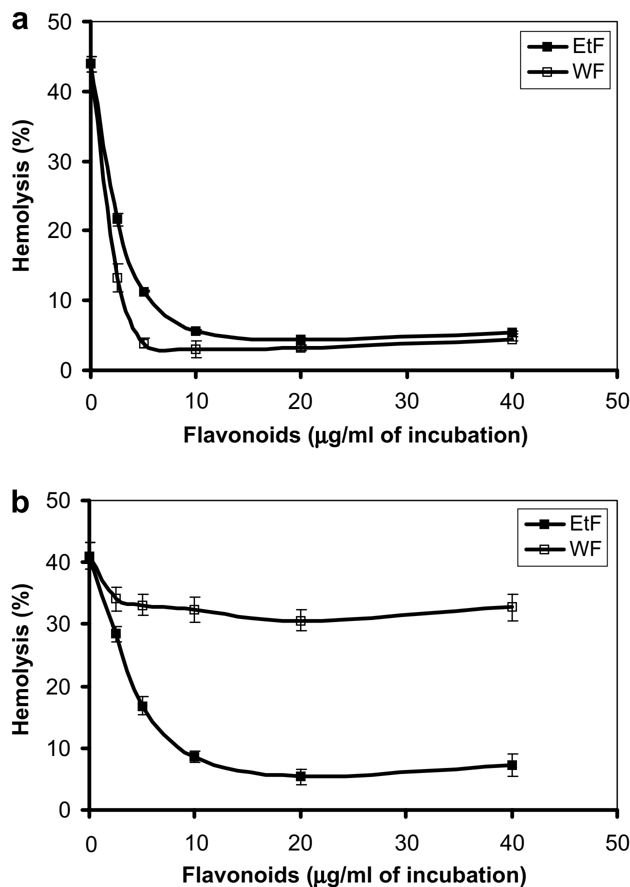

Fig. 1. Protective effect of flavonoids contained in the ether (EtF) and water (WF) fractions of honey on AAPH-induced hemolysis in human RBC suspensions (Ht 10%). The fractions, at the concentrations indicated, were present during a 3 h incubation simultaneously with (a) or prior to (b) 50 mM AAPH. Percentages of hemolysis are reported as means ± SD ($n = 4$).

EtF, like lipid-soluble chain-breaking antioxidants which are located in lipophilic regions of the membranes, may predominantly scavenge radicals within this environment, besides directly interacting with AAPH in the medium. These observations were confirmed by results obtained when the honey extracts were added to RBCs and then removed by washing, prior to the oxidative treatment. Indeed, as shown in Fig. 1b, no protection was observed in cells pretreated with WF before they were challenged with AAPH. On the contrary, owing to preincubation of the erythrocytes with EtF, the extent of protection remained almost the same.

Hydrogen peroxide, which crosses the RBC membrane and acts on the intracellular moiety, forms ferryl radical or hydroxyl radical by interacting with hemoglobin and initiates a series of reactions, resulting in RBC lysis (Van den Berg, Op den Kamp, Lubin, Roelofsen, & Kuypers, 1992). As expected, in our experiments no significant protection was observed when the erythrocyte suspensions were treated with a high H_2O_2 concentration (20 mM) in the presence of different WF amounts (ranging from 2.5 to 40 µg flavonoids/ml of incubation) (Fig. 2), whereas EtF protected the cells from the lysis in a concentration-dependent manner. The low protective effect of EtF on H_2O_2-induced hemolysis versus that induced by AAPH (compare Figs. 1a and 2) may be due, at least in part, to the non-interaction of flavonoids with the H_2O_2 in the medium.

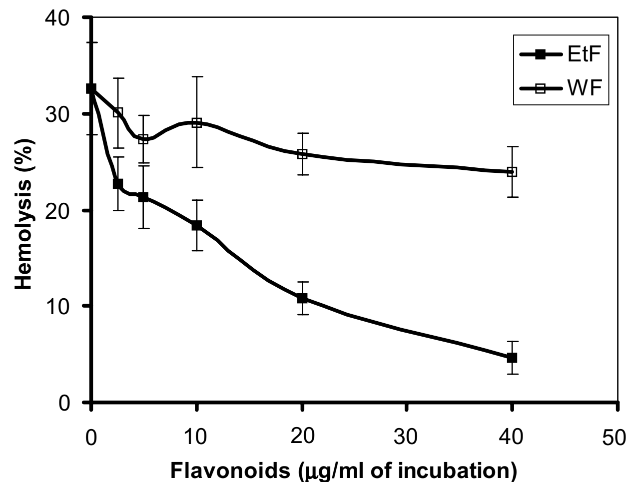

Fig. 2. Protective effect of various amounts (2.5–40 µg/ml of incubation) of flavonoids contained in the ether (EtF) and water (WF) fractions of honey on the hemolysis of human RBC suspensions (Ht 10%) induced by 20 mM H_2O_2. Percentages of hemolysis are reported as means ± SD ($n = 4$).

We continued our attempt to elucidate the protective role of honey extracts by treating RBCs with H_2O_2 levels that do not induce hemolysis (Chen, Sorette, Chiu, & Clark, 1991). We investigated the effects of flavonoids on prehemolytic changes (Hb oxidation and lipoperoxidation) that result from moderate oxidative stress. In these experiments, the exposure to 1 mM H_2O_2 for 15 or 30 min (no difference) yielded extensive Hb oxidation, and the methemoglobin (metHb) and ferrylhemoglobin (ferrylHb) formation is shown in Table 2. Both WF and EtF did not influence Hb oxidation, indicating that they did not interfere with the generation of primary radicals. The extent of lipid peroxidation, reflected by the generation of MDA, was determined by treatment with *t*-BOOH. This compound, unlike H_2O_2, does not interfere with the analytical method (Chen et al., 1991). As shown in Fig. 3, EtF prevented the MDA production in a concentration-dependent manner, whereas WF had minimal effect on the lipid peroxidation.

Table 2
Effect of honey fractions on hemoglobin oxidation products generated by treatment with 1 mM H_2O_2 for 30 min

	MetHb (mmol/ml)	FerrylHb (mmol/ml)
No addition	32.4 ± 2.11	0.18 ± 0.18
H_2O_2 1 mM	88.3 ± 1.65	14.2 ± 1.28
H_2O_2 1 mM + EtF 2.5 µg/ml	83.9 ± 1.63	15.2 ± 1.74
H_2O_2 1 mM + EtF 40 µg/ml	96 ± 13	16 ± 2.13
H_2O_2 1 mM + WF 2.5 µg/ml	86.7 ± 6.84	14.8 ± 0.5
H_2O_2 1 mM + WF 40 µg/ml	99.6 ± 5.2	15.6 ± 1.74

Values are expressed as means ± standard deviation ($n = 4$).

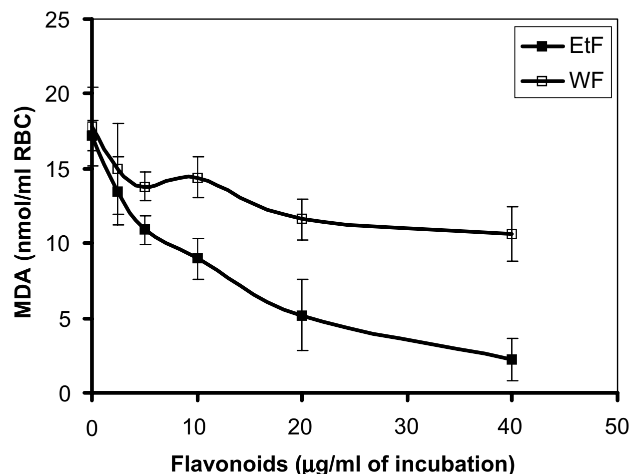

Fig. 3. Protective effect of various amounts (2.5–40 µg/ml of incubation) of flavonoids contained in the ether (EtF) and water (WF) fractions of honey on the lipid peroxidation of human RBC suspensions (Ht 10%) induced by 1 mM *t*-BOOH. Results are means ± SD ($n = 4$).

In isolated erythrocyte membranes, we observed similar behaviour, since the antioxidant effect occurred efficiently only for EtF, with an IC_{50} of 3 µg flavonoids/ml of incubation (Fig. 4). These data are consistent with the results obtained by others regarding the effect of lipid-soluble antioxidants on RBCs (Davies & Goldberg, 1987; Miki et al., 1987; Van den Berg et al., 1991). They indicate that, owing to their higher degree of liposolubility, the compounds present in EtF could be strongly incorporated into the membranes and act as scavengers of radicals in the RBC membrane. By contrast, the phenolic molecules of the WF, being preferentially localized in the aqueous phase, have less access to radicals generated from H_2O_2 and *t*-BOOH at the membrane level.

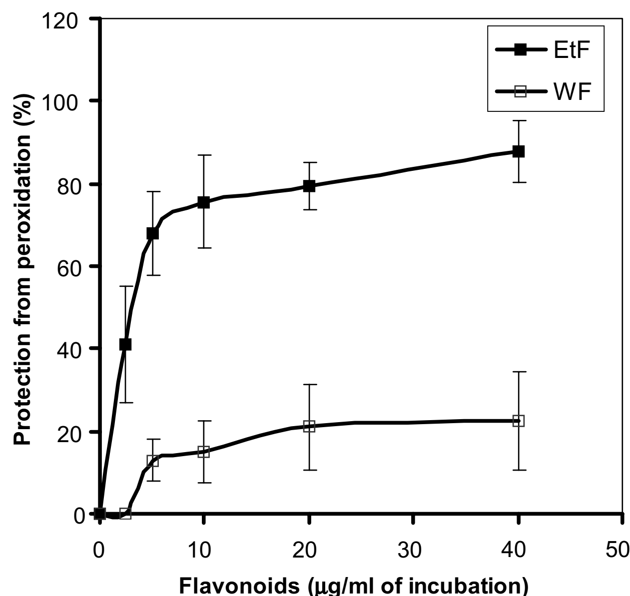

Fig. 4. Protective effect of various amounts (2.5–40 µg/ml of incubation) of flavonoids contained in the ether (EtF) and water (WF) fractions of honey on the lipid peroxidation of human ghost membranes (1 mg protein /ml) induced by 1 mM *t*-BOOH plus 50 µM Fe(II). The protection percentages are means ± SD ($n = 4$).

4. Conclusion

Our results indicate that the antioxidant activity is located in both the ether and the water fractions, indicating that the flavonoids of honey may be available to various compartments of the human body where they may exert different physiological effects. The water fraction, particularly enriched in polyphenols, possesses the highest total antioxidant power and may function as primary antioxidant by directly reducing the formation of peroxyl radical generated by AAPH. However, the ether fraction shows a more potent antioxidant activity against RBC hemolysis and lipid peroxidation induced by radical species. This may be due to the higher degree of liposolubility of the flavonoids extracted by ethyl ether, which could be strongly incorporated into the membrane and act as antioxidants against radicals generated in the lipophilic phase.

Although the compounds responsible for the antioxidant activity of the tested extracts have not yet been isolated (experiments are in progress), it will be useful to further analyze these fractions in order to identify the active molecules. These results support the hypothesis that flavonoids contribute greatly to the nutritional value of honey; therefore more investigations are highly recommended to elucidate the potential use of honey as a rich source of natural antioxidant phenolics.

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