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Antioxidant Activity of Uruguayan Propolis. In Vitro and Cellular Assays

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Supporting Information

ABSTRACT: The antioxidant capacity of propolis from the southern region of Uruguay was evaluated using in vitro as well as cellular assays. Free radical scavenging capacity was assessed by ORAC, obtaining values significantly higher than those of other natural products (8000 μmol Trolox equiv/g propolis). ORAC values correlated well with total polyphenol content (determined by Folin−Ciocalteu method) and UV absorption. Total polyphenol content (150 mg gallic acid equiv/g propolis) and flavonoids (45 mg quercetin equiv/g propolis) were similar to values reported for southern Brazilian (group 3) and Argentinean propolis. Flavonoid composition determined by RP-HPLC indicates a strong poplar-tree origin. Samples high in polyphenols efficiently inhibit low-density lipoprotein lipoperoxidation and tyrosine nitration. In addition, Uruguayan propolis was found to induce the expression of endothelial nitric oxide synthase and inhibit endothelial NADPH oxidase, suggesting a potential cardiovascular benefit by increasing nitric oxide bioavailability in the endothelium.

KEYWORDS: propolis, antioxidant activity, ORAC, polyphenols, flavonoids, nitric oxide, nitric oxide synthase, NADPH oxidase

INTRODUCTION

Propolis is a natural resinous product elaborated by honeybees (*Apis mellifera*) from different plant sources and used to seal holes in their honeycombs and protect the entrance against intruders (from the Greek "*pro*", in defense of, and "*polis*", city). Propolis has been used for centuries in folk medicine, and multiple pharmacological properties have been attributed to it including antibacterial, antiviral, antifungal, anti-inflammatory, anticancer, and antioxidant.[1−3] More recently, it is being used as a natural preservative in foods and beverages, in the preparation of healthy food, and in the cosmetic industry.[4,5]

Resins comprise approximately 50% of propolis and contain the most active compounds, a complex mixture of phenolic compounds including flavonoids (flavones, flavanones, flavonols, flavanonols) as well as aromatic carboxylic acids and esters.

The chemical composition of propolis depends on geographical region, thus, botanical origin, and also on the time and mode of collection. Analysis of phenolic resins revealed that propolis from Argentina[6] and southern Brazil (group 3[7]) have a poplar tree (*Populus* sp.) origin and is different from propolis from the southeastern or northeastern regions of Brazil, where *Hyptis divaricata* and *Baccharis dracunlifolia* are the main plant sources, respectively.[7,8] In addition, the accidental introduction of African *Apis mellifera scutellata* queen bees into Brazil over 50 years ago seems to mark a difference in the elaboration of this product.[9]

Brazilian propolis has been extensively studied in terms of chemical composition and biological activities and classified into 12 different groups.[7] In addition, a new type of propolis from northeastern Brazil, known as "red propolis", has been described[10] that does not fall into any of the 12 groups previously described by Park et al.,[7] nor does it share similarities with Venezuela or Cuban red propolis, the botanical origin of which is *Clusia* sp.[11,12] In contrast, Uruguayan propolis has been poorly analyzed, although its high quality is recognized among users. Serra Bonhevi et al.[13] first reported the bacteriostatic and radical scavenging activities of six samples of propolis from Uruguay, as well as identification of some phenolic compounds. A more detailed study based on HPLC-MS and NMR analysis of Uruguayan propolis chemical composition was performed by

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Kumazawa et al.[14]. They identified 18 flavonoids, 4 aromatic carboxylic acids, and 11 phenol acid esters. The major constituents were flavanones (pinocembrin, pinobanksin), flavonols (galangin), and flavones (chrysin).

Later, the same group compared the antioxidant activity of propolis from different origins, including a sample from Uruguay.[15] These authors as well as a recent study[16] utilized two assays of in vitro antioxidant capacity: inhibition of lipoperoxidation and free radical scavenging on 2,2-diphenyl-1-picrylhydrazyl (DPPH). There is a wide variety of methods to assess antioxidant capacity, each sharing advantages and disadvantages; unfortunately, there is not one simple method that can comprehensively and accurately determine antioxidant capacity. Recent reviews advise the evaluation of antioxidant capacity using different in vitro assays including radical scavenging by a competition method such as oxygen radical absorption capacity (ORAC) and inhibition of lipid peroxidation.[17−19] In addition, it is recommended that antioxidant capacity in culture cells and in vivo be pursued, methods that, although more expensive, include important aspects of the efficacy of an antioxidant compound such as cellular uptake, bioavailability, and capacity to elicit an antioxidant cellular response.[18] This is in line with the new definition of antioxidant, which is visualized not only as a good reducing compound (able to directly reduce the oxidant free radical) but also as a compound able to initiate a redox signaling cascade that finally reduces the oxidative cellular state.[20]

Here we study propolis from the southern region of Uruguay, not only evaluating the in vitro antioxidant capacity (free radical scavenging by ORAC, intrinsic reducing capacity by Folin, inhibition of lipid and protein oxidation) but also exploring the ability of this natural product to activate endogenous protecting systems at the cellular level, in line with the new concept of antioxidant.

MATERIALS AND METHODS

Chemicals. Sodium phosphate, sodium carbonate (Na_2CO_3), potassium bromide (KBr), fluorescein disodium salt (FL), tyrosine (Y), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, quercetin, 2,2′-azobis(2-amidinopropane) (ABAP), cupric chloride ($CuCl_2$), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), hydrogen peroxide (H_2O_2), manganese dioxide (MnO_2), NADPH, Amplex red reagent, horseradish peroxidase (HRP), Folin−Ciocalteu reagent, anti-eNOS antibody, anti-mouse HRP conjugate antibody, cell culture medium 199 (M199), fetal bovine serum (FBS), and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were of HPLC grade and were filtered (0.2 μm) before use.

Ethanolic Extracts Preparation. Propolis samples from the southern region of Uruguay were provided as raw material by the Uruguayan Beekeepers Association (SAU), collected in late spring/early summer, and stored at −20 °C in the dark until use. Propolis ethanolic extracts (EEP, 40 g/L) were prepared by adding 20 mL of 75% ethanol to 2 g of raw propolis previously milled. The suspension was heated to 50−60 °C for 30 min under agitation and then filtered. This procedure was repeated twice over each sample, and the collected extracts were combined to a final volume of 50.0 mL. EEP were gently bubbled with nitrogen and stored at room temperature in the dark. The UV absorption spectra were performed in a Cary 50 spectrophotometer (Varian, USA).

Total Polyphenols and Flavonoids Determination. The relative content in polyphenols was determined according to the Folin−Ciocalteu (FC) method.[21] Briefly, dilutions of EEP or gallic acid (standard) were mixed with FC reagent, and 10% Na_2CO_3 was added. Absorbance at 760 nm was measured in a Varioskan Flash microplate reader (Thermo Electron Corp.) after 2 h of incubation at room temperature. Flavonoid content was determined by mixing dilutions of EEP or quercetin (standard) with 5% Al_2Cl_3;[21] the mixture was left in the dark for 30 min, and the absorbance was measured at 425 nm in the microplate reader.

HPLC Analysis. EEP were injected in a HP 1050 HPLC-DAD equipped with a Luna C18 5 μm reverse phase HPLC column (Phenomenex, Torrence, CA) and eluted with a mobile phase containing water (solvent A) and methanol (solvent B). The gradient used was 30% B (0−15 min), 30−90% B (15−75 min), 90% B (75−95 min), and 90−30% B (95−105 min) at a flow rate of 1 mL/min. The chromatographic elution of phenolic compounds was followed at 254, 280, and 320 nm, and their UV spectra were recorded using a diode array detector. The compounds were identified as having the same retention time and UV spectra of selected standards from a homemade library.

ORAC Assay. The method was adapted from that of Davalos et al.[22] Briefly, different dilutions of EEP or Trolox (standard) were placed in a microplate containing 21 μM FL in 75 mM phosphate buffer, pH 7.4. The mixture was preincubated for 20 min at 37 °C, and then 19 mM ABAP was added. Fluorescence intensity (λ_{exc} = 485 nm, λ_{em} = 512 nm) was registered every 3 min for a period of 90 min in a Varioskan Flash microplate reader (Thermo Electron Corp.). The area under the curve (AUC) for the blank (FL + ABAP) and each concentration of Trolox was determined and plotted against Trolox concentration (μM) to obtain a calibration curve. Finally, the Trolox equivalent concentration for each sample of EEP was obtained from the calibration curve, and ORAC values were calculated using eq 1 and expressed as micromoles of Trolox equivalents (TE) per miligram of propolis.

$$\text{ORAC value} = \frac{\text{Trolox equivalent concentration}}{[\text{EEP}]\ (\text{mg/L})} \qquad (1)$$

Principal Component Analysis (PCA). HPLC-UV at λ = 280 nm data were converted to a *.csv file using the built-in program. The files were imported into Microsoft Excel files (Microsoft Corp.) and transferred to SPSS Statistics 13 (IBM Corp.). For all PCA studies, Pareto and/or mean-centering scaling methods were performed.

Inhibition of Low-Density Lipoprotein (LDL) Oxidation. LDL was purified from human plasma by ultracentrifugation in KBr gradient as reported previously.[23] LDL protein concentration was determined at 280 nm, ε_{280} = 1.05 mL/mg·cm^{-1}. Oxidation was induced by adding $CuSO_4$ (50 μM) to the LDL (0.05 mg protein/mL) in the absence or presence of different concentrations of EEP in 10 mM phosphate buffer, pH 7.4. LDL oxidation kinetics at 37 °C was followed by the formation of conjugated dienes at 234 nm in a Cary 50 spectrophotometer (Varian, USA) coupled to a Peltier thermoelectric device.

Inhibition of Tyrosine Nitration. Different concentrations of EEP and 0.1 mM tyrosine (Y) in 100 mM phosphate buffer and 0.1 mM DTPA, pH 7.4, were treated with 0.5 mM peroxynitrite (PN) to induce oxidation and thus nitrotyrosine (NO_2Y) formation.[24] Reaction products (NO_2Y and Y) were separated and quantified by reverse phase HPLC in a C18 column with acetonitrile 12%, trifluoroacetic acid 0.1%, and water as mobile phase and UV detection. Products were identified by comparison with standards. 3-Nitrotyrosine (NO_2Y) standard was prepared by first mixing 300 mg of tyrosine with acetic acid at room temperature until complete dissolution. The nitrating mix, consisting of 0.1 mL of concentrated HNO_3 and 0.06 mL of concentrated H_2SO_4, was added to 5 mL of tyrosine solution in an ice bath and stirred for 60 min at room temperature. A yellow solid appeared after incubation, which was filtered and washed with hexane. The solid was recrystallized in ethyl acetate and methanol. Peroxynitrite was synthesized from $NaNO_2$ and H_2O_2, as described previously.[25] Before each use, residual H_2O_2 was

removed with MnO_2, and peroxynitrite concentration was determined at 302 nm, ε_{302} = 1.67 mM^{-1} cm^{-1}.[25]

Endothelial Nitric Oxide Synthase (eNOS) Expression in Bovine Aortic Endothelial Cells (BAEC). BAEC were obtained from thoracic aortas donated by a local slaughterhouse. Cells were cultured in growth medium (M199 ± 10% FBS + 0.1 g/L streptomycin ± 0.1 g/L penicillin). For EEP assays, the same amount of cells corresponding to passages 6−13 was disposed in 60 mm diameter wells, and when confluence reached 90%, medium was replaced for one containing propolis stimuli in M199 + 0.4% FBS. Cells were grown at 37 °C in a 5% CO_2 atmosphere. After 16 h of incubation, cells were washed with PBS and harvested in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycolate, 0.1% SDS) containing protease inhibitors (aprotinin 10 μg/mL and PMSF 1 mM), and cell lysates were sonicated two times for 5 s each. Control cells were treated with ethanol (0.013%). Total protein was quantified with bicinconinic acid kit (Sigma-Aldrich), and 10 μg of protein sample was loaded in an SDS-PAGE 8% electrophoresis gel. Finally, a Western blot for eNOS was performed, using anti-eNOS (1:2000) as primary antibody and goat anti-rabbit IgG-peroxidase conjugated as secondary antibody, and visualized using the chemiluminescence reagent ECL Pierce.

NADPH Oxidase Activity Assay. This method was performed as described previously.[26] Rabbit aortic endothelial cells (RAEC, 1 × 10^6 cells grown on a 100 mm plate) obtained from a previously established selection-immortalized line were cultured in growth medium (F12 + 10% FBS + streptomycin 100 μM + penicillin 100 units/mL). After 40 h, culture medium was replaced by F12 with 0.1% FBS for 8 h, and then the propolis extract was added for a further 16 h. Control cells were treated with ethanol (0.013%). RAEC were disrupted by sonication in a buffer of 50 mM Tris, pH 7.4, 0.1 mM EDTA, and 0.1 mM EGTA containing protease inhibitors (aprotinin 10 μg/mL, leupeptin 10 μg/mL, and PMSF 1 mM). After centrifugation at 18000*g* for 15 min, supernatant was centrifuged at 100000*g* for 1 h, and the obtained pellet (enriched membrane fraction) was resuspended in the same buffer. NADPH oxidase-derived hydrogen peroxide was measured by incubation of membrane homogenates (15 μg of protein) with AmplexRed reagent (0.25 mM) and horseradish peroxidase (10 U/mL) in 50 mM Tris, pH 7.4, 0.1 mM EDTA, and 0.1 mM EGTA in the presence of NADPH (250 μM) for 30 min at 37 °C in the dark. Fluorescence was followed in a microplate reader (excitation/emission wavelengths, 550/590 nm) in a spectrofluorometer (SpectraMax M5, Molecular Devices).

Quantitative PCR. This method was performed as described previously.[26] RNA was isolated with an RNA SpinMini RNA isolation kit (GE Healthcare) and was converted to cDNA by incubation of 3 μg of mRNA, 25 ng/μL oligo-dT(12−18), 500 μM (each) dNTP, 5 μM dithiothreitol, and SuperScript II (Invitrogen) at 42 °C for 50 min. Quantitative PCR was performed with 150 ng of cDNA and SybrMastermix (Invitrogen) and was analyzed with Rotor-Gene 6000 software (Qiagen). Forward primers designed according to rabbit sequences were Nox1, CATCATGGAAGGAAGGAGA; Nox2, ATTTTTGTCAAGTGCCCCACG; Nox4, CCACAGACTTGGCTTTGGAT; and GAPDH, TCACCATCTTCCAGGAGCGA.

Rabbit Nox sequences were kindly provided by Dr. Bernard Lassegue (Emory University).

RESULTS

Table 1 shows the UV absorption coefficients ($E^{1\%}$), total polyphenol content, total flavonoid content, and ORAC values for all of the EEP assayed. The absorption maximum is observed at λ 290−295 nm, and the 10 samples analyzed can be clearly divided into two groups: one with high UV absorption ($E^{1\%}$average = 276, values close to those previously reported[15]) and another group with lower $E^{1\%}$ (average = 64). Samples from the first group (EEP 4, 7, 9, 10) had a pleasant odor and were pale brownish (amber), whereas the second group (EEP 1, 2, 3, 5, 6, 8) had less odor and a dark brown color. A good correlation was found between $E^{1\%}$ coefficients and total polyphenol or flavonoid contents (r^2 = 0.94). The UV absorption of the propolis extract could be an adequate rapid method to estimate the polyphenol content of the sample. An average value of 148 mg/g (gallic acid equiv) was obtained for samples EEP 4, 7, 9, and 10 (high UV absorption), which is in accordance with previous determinations.[15] It has to be mentioned that the Folin−Ciocalteu method quantified the amount of phenols with good reduction potential; therefore, it could be underestimating the total polyphenol content of the sample. In addition, the $AlCl_3$ complexation method at 425 nm is rather unspecific for total flavonoid content determination because only flavonoids with catecholic or specific hydroxylation on the B or C ring are detected.[27] Nevertheless, both methods are widely used, even by the official authorities, for the determination of total polyphenol and flavonoid contents in vegetables and derived products.

Table 1. Extinction Coefficient ($E^{1\%}$) at Maximum UV Absorbance, Total Content in Active Components, and ORAC Values Determined for Each Ethanolic Propolis Extract (EEP)[a]

EEP	$E^{1\%}$	[polyphenols] (mg/g propolis)	[flavonoids] (mg/g propolis)	ORAC (μmol TE/mg propolis)
1	105 ± 10	85 ± 6	22 ± 2	2.5 ± 0.6
2	39 ± 10	44 ± 3	11 ± 2	2.0 ± 0.4
3	73 ± 9	44 ± 2	13 ± 1	1.8 ± 0.2
4	**265 ± 7**	**141 ± 24**	**45 ± 4**	**7.5 ± 0.8**
5	34 ± 6	35 ± 1	4 ± 1	2.5 ± 0.3
6	38 ± 12	33 ± 3	7 ± 1	2.4 ± 0.2
7	**354 ± 16**	**176 ± 26**	**54 ± 3**	**9.0 ± 0.8**
8	91 ± 8	75 ± 3	22 ± 3	2.6 ± 0.4
9	**213 ± 17**	**128 ± 14**	**35 ± 3**	**7.1 ± 0.5**
10	**270 ± 21**	**146 ± 18**	**41 ± 2**	**8.0 ± 0.8**

[a] Results are shown as the mean ± SD (n = 3). Extracts with the highest polyphenol and flavonoid concentrations and ORAC values are shown in bold.

Uruguayan propolis samples showed very complex compositions; up to 47 different compounds were detected by HPLC analysis. The identification of some flavonoids and other phenolic compounds was carried out by direct RP-HPLC, and the absorption spectra resulting from diode array detection were used to distinguish peaks using comparison with authentic flavonoid standards. The identified constituents were quantified, and the results are summarized in Table 2. Those extracts with high polyphenol content revealed a composition rich in reduced flavones, that is, flavanones and much less aromatic acids and flavonols (see the Supporting Information). The flavone apigenin was identified in all of the studied samples. The flavonols galangin, fisetin, quercetin, and kaempferol, the flavanonol pinobanksin, the flavones luteolin and chrysin, and the flavanones naringin, pinocembrin, and hesperetin were identified. Aromatic acids such as coumaric and ferulic acids were also detected but in much lesser amounts. Our results are in agreement with the detailed analysis recently reported by Kumazawa et al.[14]

Table 2. Flavonoid and Aromatic Acid Constituents of Propolis Extracts Determined by HPLC

		mg/g propolis for EEP									
retention time (min)	compound	1	2	3	4	5	6	7	8	9	10
4.97	caffeic acid	ND[a]	ND	ND	**ND**	4.03	ND	**ND**	3.69	**ND**	**2.01**
5.19	ferulic acid	1.62	ND	ND	**ND**	ND	ND	**ND**	ND	**ND**	**ND**
35.93	naringin	ND	ND	ND	**ND**	ND	ND	**5.94**	1.55	**3.72**	**ND**
36.69	hesperetin	2.53	0.90	2.56	**5.10**	ND	ND	**ND**	ND	**ND**	**ND**
39.76	fisetin	2.86	1.11	2.20	**6.21**	0.73	ND	**5.95**	2.43	**4.48**	**ND**
44.97	luteolin	ND	ND	ND	**0.86**	ND	ND	**1.63**	0.19	**0.74**	**0.86**
46.43	quercetin	ND	0.53	ND	**0.79**	ND	ND	**0.94**	0.4	**0.57**	**0.71**
47.94	galangin	0.40	ND	ND	**ND**	ND	ND	**1.37**	ND	**ND**	**1.30**
49.31	pinobanskin	0.21	ND	0.62	**1.02**	ND	ND	**0.35**	1.16	**0.44**	**0.67**
50.92	kaempferol	0.60	ND	ND	**0.87**	ND	ND	**1.19**	ND	**1.15**	**0.99**
51.39	pinocembrin	5.01	1.60	ND	**1.26**	2.52	ND	**1.66**	0.82	**1.24**	**6.87**
51.63	CAPE[b]	ND	ND	ND	**6.87**	ND	ND	**11.21**	ND	**8.05**	**7.65**
56.35	galangin 3- methyl ether	16.15	7.87	6.19	**8.76**	5.24	0.94	**7.94**	ND	**4.52**	**ND**
58.32	apigenin	1.86	3.00	1.62	**4.35**	0.45	0.04	**5.91**	2.37	**3.66**	**3.99**
65.30	chrysin	4.39	2.03	0.35	**8.73**	0.92	ND	**8.16**	3.60	**5.44**	**8.94**
66.80	unknown	0.32	0.24	ND	**1.91**	ND	1.86	**3.07**	ND	**1.81**	**3.11**
78.07	techtochrysin	0.08	0.08	0.07	**0.04**	0.20	0.18	**ND**	0.06	**ND**	**ND**

[a] ND, not detected. [b] CAPE, caffeic acid phenethyl ester.

Caffeic acid phenethyl ester (CAPE), a phenolic compound with many reported pharmacological activities,[28,29] was detected only in the extracts high in polyphenols.

It is important to note that the ethanolic extracts from propolis mainly contained the flavonoids aglycones (in contrast with ethanolic extracts prepared from fruits and vegetables) because in the preparation of propolis by bees, they secrete β-glucosidase that hydrolyzes the glycosides initially collected from plant sources.

The antioxidant capacity of the ethanolic extracts was evaluated in vitro using the ORAC method. The oxidation of the probe (fluorescein) by a constant flux of peroxyl radicals (common radical formed in biological systems) is followed by loss of fluorescence in the absence and presence of the tested sample, and the capacity is assessed from the net area under the curve (AUC) (Figure 1A). The shape of the decay curve depends on the relative concentration and reactivity of each antioxidant compound. A calibration curve was performed with standard Trolox concentrations of 0−12 μM. The linearity between the net AUC and the concentration of each EEP sample was confirmed. Results were expressed as equivalents of Trolox (μmol TE/mg propolis) and are summarized in Table 1. A good linear correlation was found between the ORAC values and the total polyphenol content (r^2 = 0.90; Figure 1B).

To estimate the relative weight of each of the 47 detected polyphenols in the extracts, two PCA were performed. The propolis samples were classified in two groups, high and low ORAC, and submitted to PCA. Those samples with high ORAC values presented a much more complex composition than the extracts low in ORAC (Table 2 and the Supporting Information). The group with high ORAC needed only three components to include 100% of the observations, and 20 compounds displayed positive coefficients in these three components, showing a strong correlation to the compositions observed (see the Supporting Information). It is interesting to note that according to the PCA, compounds with a relatively low contribution to the total polyphenol content (less abundant) showed a high contribution to the antioxidant activity, indicated by relatively high coefficients (for example, quercetin). On the other hand, the low ORAC value group was expressed in five components to explain 100% of the events. Only six of the detected compounds have positive contributions (see the Supporting Information). Curiously, none of these compounds were in the group of high ORAC values, suggesting that the observed antioxidant activity of the propolis samples could be related to the presence of specific blends of polyphenolic compounds and not to a particular compound.

In addition to the ORAC index that estimates the content of antioxidants capable of scavenging radicals, the ability of these propolis samples to inhibit lipoperoxidation and protein oxidation was studied. Figure 2 shows the kinetic profile of copper-catalyzed LDL oxidation and the efficient protection exerted by EEP4 and EPP10 and, to a lesser extent, by EEP7 and EEP9. Extracts with low polyphenol content were not able to inhibit LDL oxidation at the concentrations assayed (exemplified in Figure 2 by EEP8). The oxidation of lipids is a classic free radical chain reaction with an initial phase during which the oxidant radical is formed, followed by a propagation phase when lipid peroxyl radicals oxidize nearby lipids and hydroperoxides accumulate; it ends when radical−radical termination reactions predominate. The inhibition of lipid oxidation can occur either at the initiation or at the propagation step, by rapid reduction of the intermediate lipid peroxyl radicals. The oxidation of proteins by nitroxidative agents to yield nitrotyrosine residues also involves a radical mechanism, but the radicals that participate are different from those in lipoperoxidation. Thus, we also investigated the ability of propolis to inhibit the reaction of tyrosine with peroxynitrite to form 3-nitrotyrosine. Again, the extracts high in polyphenols were better inhibitors of tyrosine nitration than the EEP low in polyphenols. However, the concentrations of EEP needed to protect tyrosine were 2 orders of magnitude higher than those needed for inhibition of LDL

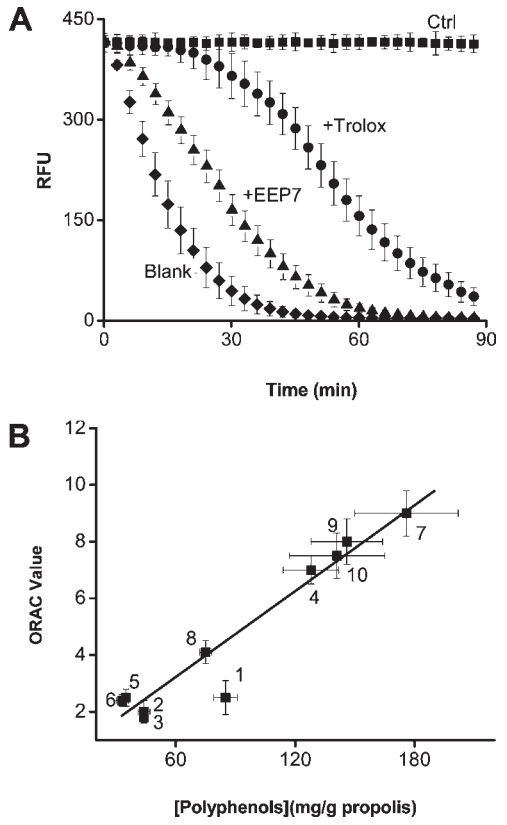

Figure 1. Antioxidant capacity of EEP determined by ORAC assay. (A) Time course of fluorescein (FL) reaction with ABAP in the absence (◆, blank) and presence of Trolox 12 μM (●) or EEP7 0.5 μg/mL (▲). Fluorescence in the absence of oxidant was stable during the assay time period as shown for control (■). Both Trolox and EEP prevented the ABAP-induced oxidation of FL in a concentration-dependent manner, but for figure clarity, only one concentration is shown. (B) Correlation between ORAC value (μM Trolox equiv/mg propolis) and polyphenols concentration (expressed as mg gallic acid equiv/g propolis). Results are the mean ± SD (n = 3).

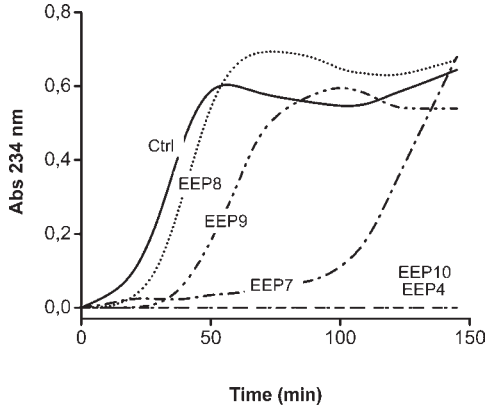

Figure 2. Inhibition of LDL oxidation by EEP. Oxidation of plasma-derived LDL was carried out by incubation with Cu^{2+} at 37 °C without (solid line) and with 0.4 μg/mL of each EEP sample (dashed lines). Conjugated diene formation was followed at 234 nm. Propolis prevented the Cu^{2+}-induced oxidation of LDL, affecting either lag or propagation phases. Samples with higher polyphenol concentration showed better antioxidant activity (see EEPs 4, 7, and 10).

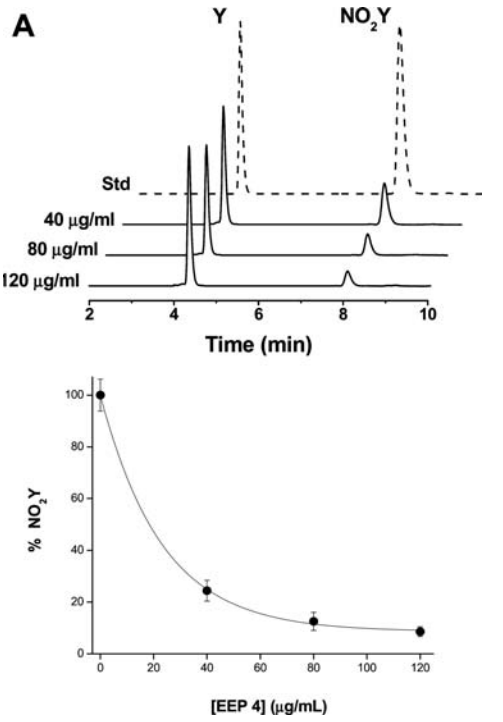

Figure 3. Inhibition of tyrosine oxidation by EEP. (A) Representative HPLC chromatogram for separation and quantification of 3-nitrotyrosine (NO_2Y) formed by peroxynitrite-induced tyrosine oxidation, in the presence of increasing concentrations of EEP4 (solid lines). NO_2Y formation was quantified by comparison with synthetic standards (dashed line). (B) NO_2Y formation dependence on propolis ethanolic extract concentration, yielding an IC_{50} = 20 μg/mL for EEP4.

oxidation (Figure 3), probably due to the high lipophilicity of the flavonoids and polyphenols, which leads to their accumulation in membranes.

Finally, the antioxidant effect of Uruguayan propolis at the cellular level was investigated on endothelial cells. Figure 4 shows that propolis were able to increase the expression of eNOS, the endothelial isoform of nitric oxide synthase, responsible for the synthesis of nitric oxide ($^{\bullet}NO$) in the endothelium that provokes vasodilation via activation of soluble guanylate cyclase (sGC). On the other hand, the same extract was able to decrease endothelial NADPH oxidase activity and promoted reduced mRNA expression of Nox4 isoform, whereas no statistical changes were observed for Nox1 or Nox2 isoforms (Figure 5). Therefore, propolis could increase the bioavailability of $^{\bullet}NO$ at the endothelium by increasing the expression of the enzyme that produces $^{\bullet}NO$ and at the same time by inhibiting the enzyme that produces superoxide, thus avoiding the formation of peroxynitrite, the strong oxidant formed by the diffusion-controlled reaction between $^{\bullet}NO$ and superoxide.[30]

■ DISCUSSION

An imbalance generated between the production of reactive oxygen and nitrogen species (ROS and RNS) and the endogenous antioxidant mechanisms, in favor of the former, is called oxidative stress, and it is associated with the pathogenesis of several cardiovascular and neurodegenerative diseases as well as cancer and aging.[31]

The search for potential antioxidants able to ameliorate this oxidative stress has received increasing attention, especially in the fields of medicine, nutrition, health, and biology as well as agrochemistry. Epidemiological studies indicate that the consumption of fruits and vegetables reduces the risk of cardiovascular problems and cancer, and this protective effect is associated with the presence of antioxidant phenolic compounds from dietary plant sources.[32−34] The synergism that can take place between the different components of a plant extract is greatly sought, and it is an extra point when compared with supplementation with isolated antioxidant compounds or synthetic antioxidants.

There is a need for a reliable, simple, and fast method to determine the antioxidant value of a natural product. Various methods have been developed to assess the antioxidant capacity of pure compounds and their mixtures. The in vitro methodologies mostly used can be grouped into noncompetitive and competitive methods.[18] An example of the first approach is the DPPH assay, in which the reaction of the antioxidant compound with a stable free radical such as DPPH is studied. It is common to report the antiradical efficiency as percent of remaining DPPH after a certain time and antioxidant concentration, which makes comparisons difficult under different assay conditions. Another disadvantage of DPPH is its very low solubility in water, so that hydrophilic antioxidants should be evaluated using acetone/water (9:4) mixtures. In contrast, ORAC is a competition method; the antioxidant compound must compete with the fluorescent probe for the peroxyl radicals (similar to those radicals produced in biological systems). The ORAC method takes into account both percent inhibition and inhibition time of peroxyl reduction by the antioxidant and, importantly, ORAC values are expressed as Trolox equivalents per weight or volume of sample, giving an easy way of comparison between different antioxidant products. This index is being widely used for foods and beverages. In fact, there is a useful list of ORAC values of fruit and vegetable extracts on the U.S. Department of Agriculture Website, http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/ORAC/ORAC_R2.pdf. The ORAC values determined for Uruguayan propolis were extremely high (8000 μmol TE/g propolis), 600 times higher than ORAC values for honey[35] and 200 times higher than ORAC values reported for red wine.[22]

A good linear correlation was found between the ORAC values and the total polyphenol and flavonoid content. Flavonoids were found as major constituents of Uruguayan propolis,

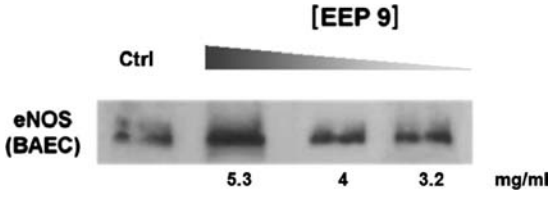

Figure 4. Induction of eNOS expression. Bovine aortic endothelial cells (BAEC) were treated either with ethanol (Ctrl) or with increasing concentrations of EEP9 for 16 h and analyzed by Western blot for the expression of eNOS with specific anti-eNOS antibody. The same amount of cellular homogenate was loaded to each line (10 μg of protein). As compared to control, propolis caused a marked increase in protein expression in a concentration-dependent manner.

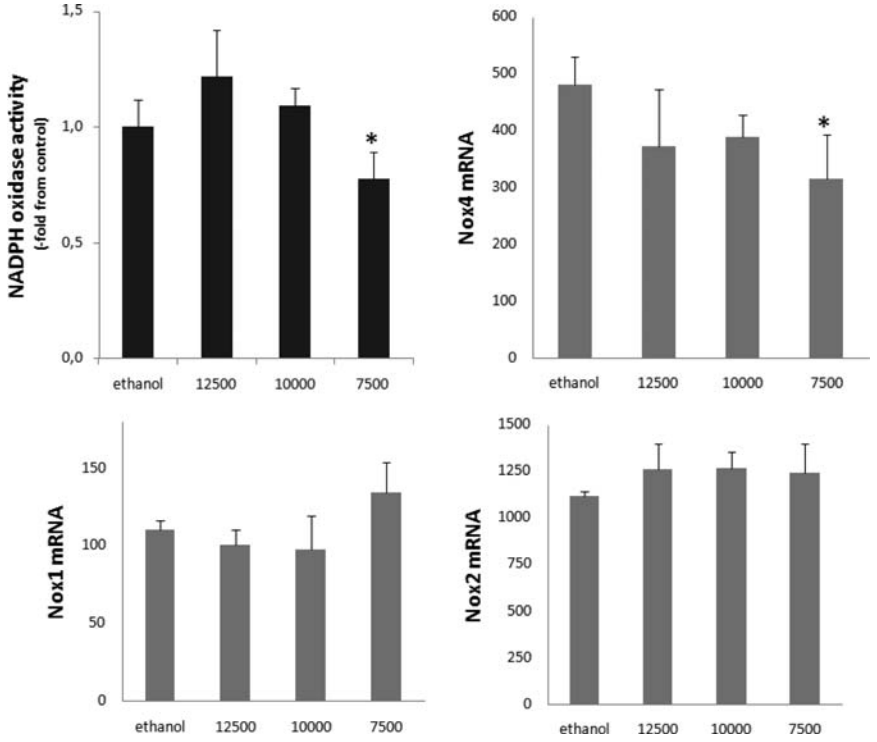

Figure 5. NADPH oxidase activity and expression in the presence of EEP9. Rabbit aortic endothelial cells (RAEC) were treated with ethanol (control) or increasing concentrations of EEP9 for 16 h (1:12.500, 1:10.000, or 1:7.500, which correspond to 3.2, 4.0, or 5.3 mg total polyphenols/mL). (A) Nox activity measured in membrane-enriched homogenates (15 μg of protein) stimulated with NADPH (250 μM) in the presence of AmplexRed reagent (0.25 mM) and HRP (10 units/mL), for 30 min at 37 °C. (B−D) Endothelial Nox isoforms (copy number/150 ng RNA) were measured by real-time PCR and normalized by GADPH. Values are the mean ± SD (n = 3−6). ∗, P < 0.05 versus ethanol.

in contrast with propolis from north of Brazil (group 6) that mainly contained fatty acid esters and no flavonoids.[7]

The ORAC value is an index of radical scavenging but does not show the capacity for the inhibition of oxidation of lipids or proteins where other radicals with different reactivities and compartmentalization are involved. In this work we also assayed the ability of Uruguayan propolis to inhibit lipoprotein LDL oxidation and nitration of tyrosine residues. Oxidation of LDL is considered to be an early event in the development of atherosclerosis.[36] Flavonoids have been found to protect against LDL oxidation, and this activity is related to their chemical structure.[37] Moreover, consumption of red wine polyphenols has been reported to decrease the susceptibility of LDL to oxidation in vivo.[38] Therefore, the evaluation of the particular mixture of polyphenols present in propolis to inhibit LDL oxidation in vitro can provide useful preliminary evidence of its potential biological antioxidant effect. We also studied the effect of propolis on tyrosine nitration. Nitration of tyrosine residues in proteins has been observed to be associated with different pathologies related to an excessive production of nitric oxide.[39] Peroxynitrite is a relevant nitroxidative agent in vivo, and 3-nitrotyrosine is considered to be a biomarker of its formation in biological systems (although other sources of nitrogen dioxide could also be responsible for this post-translational modification).[39] Nitration of proteins can affect protein functionality, not only by loss of activity but also by gain of function, affecting key cellular processes.[39] Catechin polyphenols (at 10 μM) were reported to inhibit peroxynitrite-mediated tyrosine nitration.[24] We here observed that Uruguayan propolis high in polyphenol content was an effective inhibitor of LDL oxidation as well as an inhibitor of tyrosine nitration. A low concentration of propolis extract was sufficient to inhibit LDL oxidation, whereas a higher concentration was needed to prevent tyrosine nitration, indicating the preference of this polyphenol mixture to protect lipid membranes and lipoproteins against oxidative damage, probably due to their hydrophobic nature.

Finally, we investigated the effect of propolis on endothelial cells, focusing on the production of nitric oxide and superoxide. Nitric oxide is a key vasoprotective biomolecule produced enzymatically in the endothelium by eNOS.[40] eNOS is mainly regulated by post-translational modifications but can also be influenced on the transcriptional level.[41] We observed a clear increase in the expression of eNOS after long incubation with Uruguayan propolis (high in polyphenols), a result that was reported before for red wine polyphenols[42] and isolated flavonoids such as (−) epicathechin.[43] Interestingly, the same propolis extracts were able to inhibit NADPH oxidase activity, in association with lower levels of Nox4 mRNA. In fact, Nox4 activity is known to closely correlate with its mRNA expression levels.[44] The effect of propolis on Nox4 levels is in line with the known relative importance of this isoform regarding baseline ROS generation, as opposed to greater importance of Nox1 and Nox2 in agonist-stimulated ROS production.[45] Thus, our results show that propolis could potentially increase the bioavailability of vasoactive $^{\bullet}$NO in the endothelium, not only by increasing the expression of eNOS but also by diminishing the production of superoxide anion, thus avoiding the consumption of $^{\bullet}$NO toward peroxynitrite formation.

In summary, we evaluated the quality of Uruguayan propolis as a natural antioxidant using in vitro as well as cellular assays. Our results show that the mixture of polyphenols in Uruguayan propolis displays an extraordinary radical scavenging activity with ORAC values orders of magnitude higher than those of other natural products (honey, berries) in addition to inhibiting LDL lipoperoxidation and protein nitration. At the cellular level, we observed that Uruguayan propolis was effective at increasing eNOS expression and inhibiting Nox activity, which together point to an increase in $^{\bullet}$NO bioavailability that may antagonize the development of endothelial dysfunction.

■ ASSOCIATED CONTENT

Supporting Information. HPLC chromatograms of all propolis samples and PCA for high and low ORAC groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

EEP, ethanolic extracts of propolis; LDL, low-density lipoprotein; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; RNS, reactive nitrogen species; $^{\bullet}$NO, nitric oxide; eNOS, endothelial nitric oxide synthase; Nox, NADPH oxidase; PCA, principal component analysis.

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