

# Antioxidant Activity and Total Phenolics of Propolis from the Basque Country (Northeastern Spain)

Josep Serra Bonvehí · Arrate Lacalle Gutiérrez

Received: 20 July 2010 / Revised: 24 February 2011 / Accepted: 28 February 2011 / Published online: 23 March 2011  
© AOCS 2011

**Abstract** The purpose of this study was to evaluate the antioxidant activity (AA) of 19 propolis extracts prepared in different solvent (ethanol and propylene glycol). It was observed that all the samples tested had AA, although results varied considerably between extracts, i.e. 420–1,430  $\mu\text{mol}$  Trolox/g (ABTS), 108–291 mg ascorbic acid/g (DPPH), and 1,573–4,669  $\mu\text{mol}$  iron<sup>++</sup> sulfate/g (FRAP). The ethanol may enhance the potency of the AA, and the correlation coefficient between total phenolic content (TPC) (200–340 mg/g propolis extracts) and AA was statistically significant. Total flavonoids ranged from 72 to 161 mg/g propolis extracts. The results indicate that TPC and flavonoids contributed to AA.

**Keywords** Propolis · Total phenolic content · Total flavonoids content · Antioxidant activity · Chemical composition

## Introduction

Propolis (bee glue) is a natural substance collected by honeybees from buds and exudates of trees and plants, which provides an unfavorable environment for micro-organism development, and therefore ensures the asepsis of the beehive [1]. Parameters such as smell, color, constitution, and chemical composition of propolis are due to

different sources from which it is collected. Usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, and waxes, depending on the vegetation at the site of collection [2]. Since there are different original plants in the world, from tropical and sub-tropical to temperate zones, propolis chemical composition and biological activities are extremely diverse [3–5]. Generally, propolis can be classified into two groups: (1) Brazilian-type (*Baccharis*-type) and (2) European-type (poplar-type) mainly of *Populus nigra* L. The Brazilian-type propolis is rich in terpenoids and prenylated derivatives of *p*-coumaric acid [3]. In contrast, propolis from Europe and China contains several kinds of flavonoids and phenolic acid esters [5, 6]. The biological activities of the European-type propolis are mainly due to flavanones, flavones, phenolic acids, and their esters. Owing to the difference in their chemical composition, the biological activity of propolis depends on time, botanical origin, and the area of collection [7]. The honeybee modifies the original composition of plant resins by extracting resinous compounds and mixing them with beeswax and hypopharyngeal gland secretions, especially with the  $\beta$ -glycosidases they secreted during propolis collection [4]. Through this action flavonoid heterosides are hydrolyzed into free aglycones increasing the pharmacological action of the resulting product. Propolis has been proven to possess a wide range of biological activities (antibacterial, antifungal, antiviral, anti-inflammatory, immunostimulating, antitumor, and antioxidant properties), due to different propolis constituents, depending mainly on the floral area from it was collected [6, 8, 9]. The ethanol extracts of propolis (EEP) are effective against several human pathogenic fungi, viruses and Gram-positive bacteria [1]. Recently, inclusion of propolis solutions in the daily diet has gained importance worldwide in view of its

J. S. Bonvehí (✉)  
Research and Development of Nederland Co.,  
P.O. Box 34, 08890 Viladecans, Barcelona, Spain  
e-mail: serrjosep@gmail.com

A. L. Gutiérrez  
Neiker, Basque Institute for Agricultural Research and  
Development, Berreaga kalea 1, 48160 Derio, Bizkaia, Spain

protective role against oxidative damage caused by free radicals (the major cause of diseases such as atherosclerosis, diabetes, and cancer) [10]. Furthermore, flavonoids have been described as the main group of phenolic compounds responsible for the antioxidant activity of the propolis, and its activity could also be associated with their polymerization degree [11]. Moreover, while European-type propolis has been subjected to numerous investigations, there are few articles related to the quality of Spanish propolis [12, 13]. Therefore, the objectives of this study were to determine the physicochemical characterization of collected Basque propolis, and measure the AA of propolis extracts prepared with ethanol or propylene glycol solutions.

## Experimental Procedures

### Propolis Samples

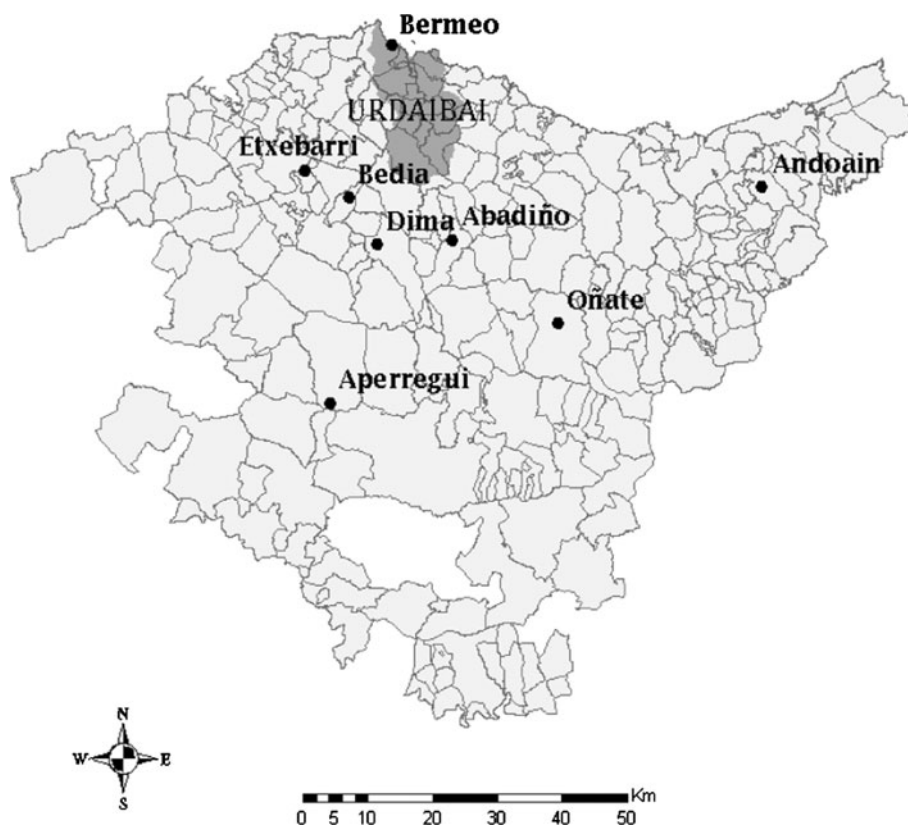
In this study, a representative mixture of 19 propolis obtained from apiaries located in naturally preserved areas of the Basque Country (Bizkaia, Gipuzkoa and Araba) were used (Fig. 1). The raw propolis samples were collected in the same apiary from spring to winter (2005 and 2008) employing the scraping technique described in the protocol of the Basque Apicultural Programme. The

botanical origin that contributed to the propolis was also analyzed: poplars [*Populus* sp. (Aigeros section), ash trees (*Fraxinus* sp.), elms (*Ulmus* sp.), willows (*Salix* sp.), chestnuts (*Castanea sativa*), blackberries (*Rubus ulmifolius*), oaks (*Quercus* sp.), and birches (*Betula* sp.)]. Representative samples were collected (500 g) and sent to the laboratory with the corresponding collection and location data (Table 1). Prior to analysis, macroscopic impurities were removed. Then, 2–3 sub-samples from different parts of each lot were taken for composing the 50-g sample. The samples, previously cooled at  $-20^{\circ}\text{C}$  for 24 h, were milled in an IKA A 10 analytical mill (LabSource, UK). The resulting product was packaged in foils, and 25 g was used as a control sample and 25 g was left to carry out assays in darkness at  $4^{\circ}\text{C}$ . Samples were either homogenized or pulverized if necessary and analyzed in triplicate.

### Reagents and Standards

Folin–Ciocalteu reagent, ethanol, propylene glycol, petroleum ether, methanol, sodium carbonate, potassium persulfate, hydrochloric acid, sulfuric acid, aluminium chloride, potassium acetate, 2,4-dinitrophenylhydrazine, potassium hydroxide, ferric chloride hexahydrate, ferrous sulfate heptahydrate, ascorbic acid, sodium acetate were analytical grade and supplied by Panreac (Barcelona, Spain)

**Fig. 1** Map of Basque Country, with the distribution of sampling areas



**Table 1** Propolis sample collect from different locations throughout the Basque country

Sample no.	Locations	Harvested	Apiary
1	Bermeo	2005	1
2	Urdaibai	2005	2
3	Etxebarri	2005	3
4	Bedia	2005	4
5	Dima	2005	5
6	Abadiño	2005	6
7	Oñate	2005	7
8	Andoain	2005	8
9	Aperregi	2005	9
10	Bermeo	2008	1
11	Urdaibai	2008	2
12	Etxebarri	2008	3
13	Bedia	2008	4
14	Dima	2008	5
15	Dima	2008	5
16	Abadiño	2008	6
17	Oñate	2008	7
18	Andoain	2008	8
19	Aperregi	2008	9

and Merck (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) was obtained from Fluka (Buchs, Switzerland). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), TPTZ (ferric tripyridyltriazine), ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt], polyvinylpyrrolidone, gallic acid, quercetin and naringenin were obtained from Sigma–Aldrich (Steinheim, Germany).

#### Instrumentation

A spectrophotometer Agilent 8453 UV–Vis with a 1-cm quartz absorption cell operated with Agilent ChemStation software for UV–vis spectroscopy (Waldbronn, Germany), an ultrasonic bath model Selecta (Barcelona, Spain), and a high-speed mill Grindomix (Retsch, Germany) were used.

#### Chemical Characteristics

##### *Preparation of Ethanolic and Propylene Glycol Extracts of Propolis (EEP/PEP)*

Ethanol/water (70:30, v/v) and 100% propylene glycol extracts of 19 propolis samples were prepared and used throughout this work. Propolis was frozen at  $-20^{\circ}\text{C}$ , and ground in a mill. The resulting ground powder was used to prepare the propolis extracts.

The finely ground propolis (1 g) was extracted at room temperature with 70% ethanol or 100% propylene glycol (100 mL) for 24 h and then filtered. The filtrate was then made up to 100 mL with the corresponding solvent, and stored in sealed amber bottles at  $4^{\circ}\text{C}$  for 1 week. A second sample of finely ground propolis (1 g) was extracted with 70% ethanol or 100% propylene glycol (25 mL) for 24 h at room temperature and then filtered. The filtrate was then made up to 25 mL with the corresponding solvent, and stored in sealed amber bottles at  $4^{\circ}\text{C}$  for 1 week. These extracts were then used for total phenolic content and total flavonoids content, and antioxidant evaluation in EEP and PEP, respectively.

##### *Water Content*

Water content was determined by gravimetry using 2 g of finely ground propolis in a conventional kiln at  $103\text{--}105^{\circ}\text{C}$  for 2–3 h, until a constant weight was reached [14].

##### *Wax Content*

The wax content was determined in 1.5 g of finely ground propolis utilizing petroleum ether as the solvent ( $40\text{--}60^{\circ}\text{C}$ ) in a Soxhlet extractor for 3 h, and then evaporated to dryness under vacuum at  $60^{\circ}\text{C}$  to a constant weight [14].

##### *Resin and Balsam Content*

Resins and balsams were extracted from 2 g of finely ground propolis in methanol for 45 min with continuous stirring at room temperature. After filtration, the methanol extract was evaporated under vacuum at  $60^{\circ}\text{C}$  to a constant weight [14], and the percentages of resin and balsam in the extracts were calculated as the methanol soluble fraction.

##### *Mineral Salt Content*

The mineral salt of approximately 2 g of finely ground propolis was determined by incineration at  $500\text{--}550^{\circ}\text{C}$  for 8 h. The ash content was calculated as a percentage of the weight of the dry residue [14].

##### *Total Phenolic Substances*

The total phenolic content of propolis extracts was determined using the Folin–Ciocalteu procedure [4]. The EEP or PEP (0.2 mL) were mixed with 3 mL of distilled water, 0.8 mL of the Folin–Ciocalteu reagent and 1.2 mL of 20%  $\text{Na}_2\text{CO}_3$ . The absorbance was measured at 760 nm after

30 min of incubation at room temperature. A blank was prepared by agitating an aliquot of the extract at pH 3.5 with insoluble polyvinylpyrrolidone. Total contents are expressed as mg GAE equiv/g (gallic acid equivalents) using a calibration curve of 0.2, 0.4, 0.6, 0.8 and 1 mM gallic acid ( $y = 232.66x + 2.7333$ ;  $r^2 = 0.999$ ).

#### *Flavone and Flavonol Content*

The aluminium chloride colorimetric method was modified from the procedure reported by Chang et al. [15]. Quercetin (10 mg) was prepared in 80% ethanol and then diluted to concentrations of 10, 25, 50 and 100  $\mu\text{g/mL}$  to make a standard curve. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminium chloride in ethanol solution, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm with a spectrophotometer. The amount of 10% aluminium chloride was replaced by the same amount of distilled water in blank. Similarly, 0.5 mL of EEP or PEP solution reacted with aluminium chloride for determining the flavonoid content (flavones and flavanols). The calibration curve ( $y = 163.08x + 0.4298$ ;  $r^2 = 0.9999$ ) was used to determine total flavonoid content, expressed as mg quercetin per gram propolis.

#### *Flavanone and Dihydroflavonol Content*

The modified method described by Nagy and Grancai [16] was used to determine flavanone and dihydroflavonol content. The solution standard of ( $\pm$ )-naringenin (20 mg) was prepared in methanol and then diluted to 500, 1,000, 2,000, 3,500 and 4,000  $\mu\text{g/mL}$ . One mL of each of the diluted standard solutions was separately reacted with 2 mL of 1% 2,4-dinitrophenylhydrazine reagent (2,4-D) (1 g of 2,4-D in 2 mL of 96% sulfuric acid and then diluting to 100 mL with methanol) and 2 mL of methanol at 50 °C for 50 min. After cooling to room temperature, the solution was mixed with 5 mL of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. Then, 1 mL of the mixture was mixed with 5 mL of methanol and centrifuged at 4,000 rpm for 10 min to remove the precipitate. The supernatant was collected and adjusted to 25 mL with methanol. Later, the absorbance was measured at 495 nm. Similarly, 1 mL of EEP or PEP reacted with 2,4-D reagent for determination of flavanones, using same amount of distilled water as the blank. Total flavanones content were calculated as mg of naringenin per g from a calibration curve ( $y = 2,754.7x$ ;  $r^2 = 0.9975$ ).

#### Antioxidant Activities Measurement

##### *ABTS Scavenging Activity Test*

The Trolox equivalent antioxidant capacity (TEAC) of propolis was determined by the ABTS radical cation decolorization assay developed by Re et al. [17]. Stock solutions of ABTS (7 mM) and potassium persulfate (140 mM) in water were prepared, and the ABTS radical cation was produced by adding potassium persulfate to a final concentration of 2.45 mM. The mixture was left to react overnight (12–16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with ethanol to an absorbance of 0.70 ( $\pm 0.02$ ) at 734 nm. All measurements were performed as follows: 10  $\mu\text{L}$  of EEP or PEP were mixed with 990  $\mu\text{L}$  of ABTS radical solution, and the absorbance measured after exactly 5 min the initial mixing, using ethanol as the blank. The results were obtained, expressed as Trolox equivalent antioxidant capacity (TEAC) ( $\mu\text{mol Trolox/g}$ ), using a calibration curve of 2.5, 5, 10, 15 and 20  $\mu\text{M}$  Trolox in ethanol ( $y = -0.0266x + 0.7093$ ,  $r^2 = 0.9955$ ; % inhibition Trolox  $y = 0.0805x^2 + 21.772x$ ,  $r^2 = 0.999$ ). All measurements were performed in triplicate.

##### *DPPH Scavenging Activity Test*

Antioxidant activity of the propolis extracts was also determined using the DPPH radical scavenging assay described by Brand-Williams et al. [18]. The free radical scavenging capacity using the free DPPH radical was determined by measuring the decrease of absorbance at 517 nm. Briefly, the solution of  $6.5 \times 10^{-5}$  M DPPH in methanol was prepared daily before measurement using a UV–Vis spectrophotometer. Then, 6 mL of DPPH solution were mixed with 3 mL of EEP or PEP. The reaction mixture was vortex-mixed and allowed to stand at 25 °C in the dark for 60 min. Absorbance at 517 nm was measured, at time 0 and 60 min against the methanol blank. The antioxidant content was determined using ascorbic acid as a standard (10, 15, 20, 25, 30, 35 and 40  $\mu\text{g/mL}$ ). The results were expressed as ascorbic acid equivalent antioxidant content (AEAC) (mg AsA/g). The amount of inactivated DPPH\* was proportional to the concentration of added flavonoids. Thus, the calibration procedure based on ascorbic acid as a standard can be used for quantification. One antioxidant  $\mu$ -equivalent is defined as the ability to reduce one micromole of a pro-oxidant ( $y = 0.0001x^2 - 0.0665x + 2.5308$ ;  $r^2 = 0.9994$ ; % inhibition ascorbic acid  $y = 0.0004x^2 - 0.3572x$ ;  $r^2 = 0.9994$ ). All measurements were performed in triplicate.

### Ferric Reducing/Antioxidant Power Assay

The ferric reducing/antioxidant power (FRAP) assay was carried out according to the standard procedure developed by Benzie and Strain [19]. Working FRAP reagent was prepared daily, containing (2.5 mL of a 10 mM TPTZ in 40 mM HCl and 2.5 mL of 20 mM  $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ , and 25 mL of 0.25 M acetate buffer, pH 3.6). An aliquot of 0.4 mL of EEP or PEP were mixed with 3.6 mL of FRAP reagent solution. Absorbance was measured at 593 nm after incubation at 37 °C for 10 min and centrifugation, if necessary, at 10,000 rpm for 15 min. A reagent blank was also prepared as described above 0.4 mL of distilled water was added instead of a test sample. Aqueous solutions of  $\text{FeSO}_4 \times 7\text{H}_2\text{O}$  (200, 400, 600, 800 and 1,000  $\mu\text{M}$ ) were used for the calibration and the results are expressed as  $\mu\text{mol Fe}^{+2}$  sulfate/g propolis ( $y = 534.97x + 6.4824$ ;  $r^2 = 0.9995$ ). All measurements were performed in triplicate.

### Statistical Analysis

Analysis of variance (ANOVA) was applied to determine significant differences among geographical origins of the samples analyzed. ANOVA was performed according to the fixed factor model, considering as sources of variation, locality, year, and composition (total phenolic, total flavonoids, wax, resin and balsam contents, and antioxidant activities) using F distribution and unpaired Student's test at a level of  $P \leq 0.05$ . We also used Spearman to evaluate correlations between concentrations of total phenolic content, total flavonoids content and antioxidant activities. The results were analyzed by means of the software Statistica for Windows 5.5 (StatSoft Inc., Tulsa, OK). For each parameter the mean of three replicates was taken as the variation limit.

## Results and Discussion

### Chemical Composition

Most of the samples had a pleasant odor and were light yellow to dark brown in color. The total resin and balsam content, ranging between 52.5 and 76.2 g/100 g, varied not only with collecting areas but also with collecting season of propolis (2005 and 2008) (Tables 1 and 2). Propolis with high resin and balsam content has a higher content of biologically active components [8]. The percentage of resin and balsam in propolis is an important characteristic because a high percentage of resins and balsams results in a low percentage of wax and insoluble matter. Mineral salts ranged from 0.31 to 0.81 g/100 g, in line with the range commonly accepted by the literature [14].

High water contents (2.35–22.6 g/100 g) were observed and did not agree with the literature values (maximum value of 4 g/100 g). The percentage of moisture in a sample of propolis is affected by the storage and manipulation conditions. The crude propolis cleaning process protocol includes a washing step in which the product is immersed in water. If the subsequent drying step is not done properly the percentage of water in the final product will be affected, exceeding literature values as it was in this particular case. Significant amounts of beeswax were detected (1.8–27.7 g/100 g) probably due to poor beekeeping practices when removing the propolis from the hive. These compounds should not exceed the 20% [4]. Others values of wax of propolis from Brazil showed a range from 4.8 to 19.3 g/100 g, and values close to 30% for samples from China [14].

### Total Phenolic and Flavonoid Compounds

The Folin–Ciocalteu and spectrophotometric procedures are widely used to determine total phenolic and flavonoid contents in propolis samples [3, 4, 9]. These components are the most representative components of the resin and balsam fraction. However, none of the spectrophotometric methods can detect all kinds of flavonoids [15]. Considering the four major groups of flavonoids present in propolis, only flavones and flavonols were found to form a stable complex with aluminium chloride, while flavanones and dihydroflavonols reacted better with 2,4-dinitrophenylhydrazine. Aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, which can be detected at 415 nm. As expected, the absorbance readings of the complexes formed by flavone and flavonol compounds were much higher than those formed by flavanones and isoflavones. On the other hand, 2,4-dinitrophenylhydrazine do not react with flavones, flavonols and isoflavones with the C2–C3 double bond, while the hydrazones of all flavanone standards (i.e., naringin, naringenin and hesperitin), showed maximum absorbance at 495 nm. The contents determined by the two methods were summed to give total flavonoids (Table 2).

The amounts of total phenolic and flavonoid contents varied widely and ranged from 200 to 340 mg GAE/g, and 72–161 mg/g, respectively. The 66.7% of the samples had phenolic content greater than 250 mg GAE/g. In another study, Orantes et al. [12] found that the phenolics content ranging between 129.5 and 364 mg/g in propolis collected in Andalucía (southern Spain), whereas flavonoids content varied between 60.3 and 144.5 mg/g. Investigations of different types of propolis from South America and Brazil had similar (200–300 mg/g) phenolic contents [4]. Lima et al. [20] reported that methanolic extracts of Argentinean

**Table 2** Chemical composition of ethanolic and propylene glycol extracts of Basque propolis (g/100 g)

Sample no.	Total phenolic content	Flavone and flavonol content	Flavanone and dihydroflavonol content	Total flavonoids content	Water content	Ashes	Waxes	Resins and balsams
1	21/23	3.1/2.8	5.5/5.7	8.6/8.5	3.98	0.49	11.9	52.5
2	30/28	5.2/5.7	7.3/6.3	12.5/12.0	12.6	0.45	1.8	71.0
3	27/24	4.5/4.3	5.9/6.2	10.4/10.5	13.5	0.65	6.5	54.7
4	26/24	4.8/4.7	8.9/8.4	13.7/13.1	2.44	0.55	27.7	59.3
5	29/27	6.2/7.8	9.9/8.0	16.1/15.8	5.79	0.59	8.9	65.6
6	30/28	6.1/7.4	10/8.5	16.1/15.9	5.33	0.46	5.4	70.4
7	23/21	3.7/2.6	6.1/6.6	9.8/9.2	22.6	0.52	11.3	57.9
8	28/26	4.9/4.6	8.2/8.3	13.1/12.9	2.62	0.81	9.8	57.2
9	32/28	6.0/5.5	8.2/6.5	14.2/12.0	7.74	0.47	7.9	76.1
10	31/27	5.9/5.2	8.2/6.7	14.1/11.9	5.41	0.34	11.9	76.2
11	28/25	4.4/3.1	7.6/8.2	12.0/11.3	6.41	0.36	17.9	75.6
12	21/20.3	2.6/1.2	6.1/6.0	8.7/7.2	17.5	0.43	12.4	54.5
13	27/26	5.9/2.0	9.6/10.8	15.5/12.8	2.82	0.41	12.0	64.5
14	29/26.5	4.5/1.6	8.0/8.6	12.5/10.2	2.35	0.33	12.7	68.8
15	29/24.7	5.1/2.6	7.0/7.5	12.1/10.1	2.44	0.62	26.2	60.7
16	31/30.3	4.7/2.5	7.3/7.7	12/10.2	3.14	0.47	7.32	67.8
17	33/29.6	5.1/4.7	8.6/7.4	13.7/12.1	7.58	0.31	7.29	73.7
18	21/20	3.9/1.9	7.9/6.7	11.8/8.6	17.4	0.43	5.94	55.9
19	34/30	4.9/3.3	8.6/7.8	13.5/11.1	7.99	0.47	15.1	70.6
X	27.9/25.7	4.82/3.87	7.88/7.47	12.7/11.3	7.88	0.48	11.8	65.2
SD	3.97/3.10	1.00/1.89	1.30/1.23	2.19/2.27	5.87	0.12	6.46	7.96
Range	21–34/20–30.3	2.6–6.2/1.2–7.8	5.5–10/5.7–10.8	8.6–16.1/7.2–15.9	2.35–22.6	0.31–0.81	1.8–27.7	52.5–76.2

Results for different extracts are separated by (/); first value for ethanol extract propolis (EEP); second value for propylene glycol extract propolis (PEP)

SD standard deviation

propolis had great amounts of phenolics (ranging from 257 to 353 mg GAE/g), with a content of flavonoids between 66 and 133 mg/g. New legislation on propolis extracts was published recently in Brazil, and it delineates that the ethanolic propolis preparations must contain at least 0.25% flavonoids and 0.50% phenolic components in relation to the dry weight [21].

Flavonoid contents determined by the aluminium chloride method were generally lower than those determined by the 2,4-dinitrophenylhydrazine procedure. They ranged from 12 to 78 mg/g (flavones/flavonols), while flavanones/dihydroflavonols ranged from 55 to 108 mg/g. The results seem to be opposite to our general knowledge that the major flavonoid components of propolis from the temperate area are flavones and flavonols, indicating the importance of the quantitative determination by 2,4-dinitrophenylhydrazine reaction. Results showed that, among the EEP samples, the lowest level of total flavonoids was around 86 mg/g, similar contents were found in PEP samples. No significant differences were detected between the two extracts ( $P < 0.05$ ).

Flavonoids were predominant in the phenolic fraction, and 83% of the samples showed contents higher than 100 mg/g (Table 2). Our present results agree with those reported by Velazquez et al. [22], who describe the flavonoid composition of Sonoran propolis collected in northwest Mexico (flavones and flavanols: 57.8 mg/g, and flavanones: 150.6 mg/g).

#### Antioxidant Activities

In this study, three different antioxidants assays (ABTS, DPPH and FRAP) were determined and the results obtained are shown in Table 3. Other antioxidant activities were measured in Argentine propolis extracts exhibiting potent antioxidant activity in protecting serum lipids [23].

#### Effects of Propolis Samples on ABTS Radical Cations

The TEAC method is one of the most often used method for the determination of total antioxidant capacity. It is

**Table 3** Antioxidant activities of ethanolic and propylene glycol extracts of Basque propolis

Sample no.	Inhibition ABTS (%)	Inhibition DPPH (%)	TEAC	AEAC	FRAP
1	26.1/23.5	19.1/22.2	780/430	154/129	2,312/2,446
2	41.3/34.0	40.4/26.4	1,260/940	218/177	3,935/2,406
3	28.5/27.2	25.3/24.6	810/610	178/148	2,876/2,496
4	28.1/26.4	23.4/24.2	820/590	167/142	3,113/4,271
5	39.8/30.8	39.2/28.4	1,250/890	200/197	3,771/3,689
6	40.3/31.7	37.6/27.9	1,310/730	206/187	4,343/3,972
7	28.2/27.6	20.8/21.3	820/520	178/152	3,098/3,442
8	29.1/28.0	20.1/25.4	900/460	183/138	3,566/2,309
9	52.3/40.8	38.2/30.1	1,430/810	265/210	4,669/3,485
10	51.2/38.4	38.5/28.2	1,420/820	245/187	4,140/2,493
11	49.3/30.3	37.6/22.6	1,390/720	186/123	3,466/2,939
12	26.8/22.2	19.3/16.8	650/450	148/108	2,353/2,013
13	45.0/26.9	28/20.4	1,140/650	177/166	2,676/2,019
14	41.1/28.6	24.9/23.1	1,030/580	189/150	2,807/2,092
15	27.2/29.4	25.1/27.4	660/600	198/181	2,856/1,961
16	46.7/32.6	40.5/23	1,210/905	239/149	3,387/2,876
17	54.7/38.7	27.4/28.6	1,401/460	254/182	3,409/2,507
18	23.7/23.2	19.5/19.3	560/420	153/118	2,827/1,573
19	38.5/43.6	25.2/28.7	960/600	291/188	3,757/2,151
X	37.8/31.4	29.0/24.7	1,042/641	206/161	3,335/2,692
SD	9.98/6.54	8.21/3.69	291/169	44.2/29.2	657/752
Range	23.7–54.7	19.1–40.5	560–1,430	148–291	2,312–4,669
	22.2–43.6	16.8–30.1	420–940	108–210	1,573–4,271

Results for different extracts are separated by (/); first value for ethanol extract propolis (EEP); second value for propylene glycol extract propolis (PEP)

TEAC:  $\mu\text{mol Trolox/g}$ ; AEAC: mg ascorbic acid/g; FRAP:  $\mu\text{mol iron}^{++}$  sulfate/g

based on the neutralization of a radical cation formed by the single-electron oxidation of a synthetic ABTS chromophore to a strong absorbing ABTS<sup>+</sup> radical (700–750 nm). The ABTS<sup>+</sup> is generated by the oxidation of ABTS with potassium persulfate and reduced in the presence of a hydrogen-donating antioxidant. Most of the samples completed the reaction within 4 min. The results demonstrate that all extracts (i.e., EEP and PEP) of propolis tested were very active. The relation between ABTS radical cation and TFC was also examined, and a positive correlation between them was observed ( $r^2 = 0.486$ ). The radical scavenging activity (RSA) of the EEP varied from 23.7 to 54.7% inhibition of ABTS, which corresponded to 560–1,430  $\mu\text{mol Trolox/g}$  in EEP, and from 22.2 to 43.6% inhibition of ABTS (corresponded to 420–940  $\mu\text{mol Trolox/g}$ ) in PEP (Table 3), with significant differences ( $P \leq 0.05$ ) between extracts. These variations in the results may be due in part to the solvents employed, and on different TFC among the samples analyzed. Andalusian propolis inhibited between 23 and 71.2% of ABTS [12].

#### Effects of Propolis Samples on DPPH Radicals

As shown in Table 3, all propolis extracts had strong DPPH free radical scavenging activity (19.1–40.5% inhibition). These values corresponded to 148–291 mg AsA/g in EEP. The 16.8–30.1% inhibition of DPPH, corresponded to 108–210 mg AsA/g, in PEP, with significant differences ( $P \leq 0.05$ ) between extracts observed. Andalusian propolis had values between 23 and 85.7% of DPPH (mean  $49.12 \pm 16.02\%$ ) in EEP [12]. Similar results were obtained in ethanol propolis extracts collected in various areas of Japan and China [6, 7, 24]. Argentinean propolis of different districts of the province of San Juan had different DPPH values ranging between 46.6 and 89.6% [20]. Additionally, Sonoran propolis showed a strong antioxidant activity (86% at 100  $\mu\text{g/mL}$ ) comparable with those of the reference antioxidants vitamin C (87.4% at 70  $\mu\text{mol/L}$ ) and BHT (66.1% at 140  $\mu\text{mol/L}$ ) [22].

According to the results obtained, antioxidant activity in the ABTS assay was higher than those obtained in the

DPPH assay. The difference between the results of these two radical scavenging assays might be due to the fact that DPPH radical reacts only with lipophilic antioxidants while ABTS radical reacts with both hydrophilic and lipophilic antioxidants [25]. The relationship between DPPH radical scavenging activity and TFC was studied, and positive correlation between them was observed ( $r^2 = 0.797$ ).

The analysis of the relationship between the DPPH activity of Brazilian propolis extracts and their phenolic content showed a medium positive correlation coefficient [3], and non significant Spearman correlations ( $\geq 0.5$ ) between DPPH activity and TFC in Argentinean propolis [20]. In contrast, Andalucian propolis results had an  $r^2 = 0.982$ . Consequently, the constituents of propolis are strongly dependent on the geographical area of collection. It is well established that the DPPH radical scavenging assay determines free antioxidants in products, whereas the Folin–Ciocalteu assay determines both, free and bound phenolics, in order to obtain the content of total phenolic compounds. However, in Uruguayan propolis extracts, the large difference detected between ranges of values of scavenger activity DPPH and microsomal lipid peroxidation, suggest that the complexity of the biologic matrix could modulate the antioxidant effect [26].

#### Effects of Propolis Samples on the FRAP Assay

Both the Folin–Ciocalteu assay and the FRAP assay are electron transfer methods, based on the assumption that the reducing capacity of a sample is a direct measure of its antioxidant capacity by a redox-linked colorimetric reaction [27]. The other assays used in this work are more indirect methods because they measure the inhibition of the free radicals generated in the reaction mixture, and these depends on the reactive species used. FRAP assay showed that Basque extracts of propolis have a significant AA (i.e., 2,312–4,669  $\mu\text{mol Fe}^{2+}$  sulfate/g in EEP; and 1,573–4,271  $\mu\text{mol Fe}^{2+}$  sulfate/g in PEP) (Table 3). The Croatian propolis had a relatively low AA in the FRAP assay (1.6  $\mu\text{mol/L}$  for propolis vs. 2.0  $\mu\text{mol/L}$  for Trolox) [28]. Moreover, literature data showed contrasting results for “in vitro” and “in vivo” oxidant/antioxidant capacity of flavonoid-rich foods [29].

The relationship between FRAP activity and TPC was examined, and a positive correlation between them was observed ( $r^2 = 0.489$ ). However, a consensus on correlation between FRAP and TPC is not always true since some literature data on phenolic contents and their antioxidant activity are contradictory [30].

All the results confirm that Basque propolis extracts possess strong antioxidant activity in the different free radicals studied. The higher antioxidant activities were

obtained with ABTS and FRAP assays using the ethanol extract.

#### Conclusions

In this study, chemical composition and the “in vitro” antioxidant activity of 19 Basque propolis samples were investigated. Flavonoids (flavones and flavanones) are the best candidates to assess the quality of Basque propolis products, due to their different biological properties and predominance in the phenolic fraction. The results reveal that all the extracts tested showed a high free scavenging activity and therefore indicate the possible use of Basque propolis as an important source of natural antioxidants. Furthermore, the solvent employed for the extraction may enhance the potency of the antioxidant activity of propolis extract.

#### References

- Burdock GA (1998) Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem Toxicol* 36:347–363
- Bankova V, Castro SLD, Marcucci M (2000) Propolis: recent advances in chemistry and plant origin. *Apidologie* 31:3–15
- da Silva JF, Sourza MC, Ramalho Matta SR, Ribeiro de Andrade M, Nova Vidal FV (2006) Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. *Food Chem* 99:431–435
- Bonvehi JS, Coll FV (2000) Study on propolis quality from China and Uruguay. *Z Naturforsch* 55c:778–784
- Bankova V, Popova M, Bogdanov S, Sabatini AG (2002) Chemical composition of European propolis: expected and unexpected results. *Z Naturforsch* 57c:530–533
- Ahn MR, Kumazawa S, Usui Y, Nakamura J, Matsuka M, Zhu F, Nakayama T (2007) Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chem* 101:1383–1392
- Kumazawa S, Hamasaka T, Nakayama T (2004) Antioxidant activity of propolis of various geographic origins. *Food Chem* 84:329–339
- Banskota AH, Tezuka Y, Kadota S (2001) Recent progress in pharmacological research of propolis. *Phytother Res* 15:561–571
- Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtziunos I, Karathanos VT (2009) Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chem* 116:452–461
- Kimoto T, Aga M, Hino K, Koya-Miyata S, Yamamoto Y, Micallef MJ, Hanaya T, Arai S, Ikeda M, Kurimoto M (2001) Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. *Anticancer Res* 21:221–228
- Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. *J Nutr Biochem* 13:572–584
- Orantes Bermejo FJ, Torres C, Serra J, Kumazawa S (2007) Capacidad antioxidante y contenido en polifenoles del propóleo en Andalucía. *Vida Apícola* 142:41–46



13. Hegazi AG, Hady FK, Alloh FA (2000) Chemical composition and antimicrobial activity of European propolis. *Z Naturforsch* 55c:70–75
14. Bonvehí JS, Coll FV, Jordà RE (1994) The composition, active components and bacteriostatic activity of propolis in dietetics. *J Am Oil Chem Soc* 71:529–532
15. Chang CC, Yang MH, Wen HM, Cherm JC (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 10:178–182
16. Nagy M, Grancai D (1996) Colorimetric determination of flavanones in propolis. *Farmacia* 51:100–101
17. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26:1231–1237
18. Brand-Williams W, Cuvellier ME, Berset C (1995) Use of free radical method to evaluate antioxidant activity. *Lebensm Wissens Technol* 28:25–30
19. Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. *Anal Biochem* 239:70–76
20. Lima B, Tapia A, Luna I, Fabani MP, Schmeda-Hirschmann G, Podia NS, Wunderlin DA, Feresin GE (2009) Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the Province of San Juan (Argentina). *J Agric Food Chem* 57:2691–2698
21. Brazilian Ministry of Agriculture (2000) Normative instruction #11 Official Dietary of the Union, October, 23rd 2000, Section I, 16–17
22. Velazquez C, Navarro M, Acosta A, Angulo A, Dominguez Z, Robles R, Robles-Zepeda R, Lugo E, Goycolea FM, Velazquez EF, Astiazaran H, Hernandez J (2007) Antibacterial and free-radical scavenging activities of Sonoran propolis. *J Appl Microbiol* 103:1747–1756
23. Isla MJ, Nieva Moreno MI, Sampietro AR, Vattuone MA (2001) Antioxidant activity of Argentine propolis extracts. *J Ethnopharm* 76:165–170
24. Hamasaka T, Kumazawa S, Fujimoto T, Nakayama T (2004) Antioxidant activity and constituents of propolis collected in various areas of Japan. *Food Sci Technol Res* 10:86–92
25. Prior LR, Wu X, Schaih K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:4290–4302
26. Zunini MP, Rojas C, de Paula P, Elingold I, Alvareda Migliaro E, Casanova MB, Iribarne Restucia F, Aguilera Morales S, Dubin M (2010) Phenolic contents and antioxidant activity in central-southern Uruguayan propolis extract. *J Chil Chem Soc* 53:141–146
27. Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53:1841–1856
28. Sobocanec S, Sverko V, Balog T, Saric A, Rusak G, Likic S, Kusic B, Katalinic V, Radic S, Marotti T (2006) Oxidant/antioxidant properties of Croatian native propolis. *J Agric Food Chem* 54:8018–8026
29. Lolito SB, Frei B (2004) Relevance of apple polyphenol as antioxidants in human plasma: contrasting in vitro and in vivo effects. *Free Radic Biol Med* 36:201–211
30. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M (1999) Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem* 47:3954–3962