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Chemical and Functional Characterization of Italian Propolis Obtained by Different Harvesting Methods

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ABSTRACT: The composition and antioxidant activity of Italian poplar propolis obtained using three harvesting methods and extracted with different solvents were evaluated. Waxes, balsams, and resins contents were determined. Flavones and flavonols, flavanones and dihydroflavonols, and total phenolics were also analyzed. To characterize the phenolic composition, the presence of 15 compounds was verified through HPLC-MS/MS. The antioxidant activity was evaluated through 1,1-diphenyl-2-picrylhydrazyl radical and reducing power assays. The ability of propolis to inhibit lipid oxidation was monitored by analyzing hydroperoxide and TBARS formation in lipids incorporated into an oil-in-water (O/W) emulsion. Acetone shows the highest extraction capacity. Wedge propolis has the highest concentration of active phenolic compounds (TP = 359.1 ± 16.3 GAEs/g; TFF = 5.83 ± 0.42%; TFD = 7.34 ± 1.8%) and seems to be the most promising for obtaining high-value propolis more suitable to prepare high-quality dietary supplements (TBARS = 0.012 ± 0.009 mmol std/g; RP = 0.77 ± 0.07 TEs/g).

KEYWORDS: *propolis, harvesting method, total phenolics, flavonoid, antioxidant activity*

INTRODUCTION

Propolis, or bee glue, is a product based on resins collected by bees from plant exudates and contains more than 160 constituents.[1] In the beehive, propolis is used by the bees to seal holes, cracks, or openings[2] to guarantee thermal isolation, to defend them from intruders, and to prevent decomposition of animals killed after invading the colony. The smell, color, constitution, and composition of propolis vary according not only to the different botanical sources and geographical origin[3,4] but also to the method of harvest.[5−7] The propolis is characterized by a mean content of 50% balsams and resins, 30% waxes, 10% essential oils, 5% pollen, and 5% various other substances and organic debris. This matrix usually contains a variety of compounds, such as phenolic compounds, flavonoids among them, terpenes, sesquiterpenes, and stilbenes, β-steroids, aromatic aldehydes, and alcohols.[8−10] Flavonoids in particular are thought to be responsible for many of the biological and pharmacological activities, such as anticancer,[11] anti-inflammatory,[12] antimicrobial,[13] and antioxidant.[14] In addition to this, Park et al. have recently demonstrated that the ethanolic extracts of propolis suppress dioxin toxicity.[15] In the past few years, propolis has been widely used in drinks and foods for human nutrition not only to improve health and prevent diseases[16,17] but also as an ingredient in many dietary supplements and nutraceuticals.[18] A nutraceutical is a product isolated or purified from an alimentary or biological material demonstrated to have physiological benefits or provide protection against chronic diseases.[19] The science of nutraceuticals is at the confluence of two major issues in our society: food and health. During the past decade, nutraceuticals have emerged as a major consumer-driven trend, serving the needs of individuals to exercise greater control over health, prevent diseases, delay aging, and enhance well-being and performance. This trend is expected to continue, and scientific information on all aspects of nutraceuticals is very important for the advancement of this emerging sector. Considering the range of propolis properties and the several possible fields of application, there is an increased interest in its activities and composition in the food and nutraceutical industries. Propolis, for instance, should be used alone or in combination with other natural products not only as a dietary supplement but also as a natural antioxidant in food. The present study was conducted to determine the composition and antioxidant activity of 20 propolis samples obtained using one of the following harvesting methods: (1) scraping, which consists of scraping the propolis from the inner surface of the beehive; (2) wooden wedges, which are obtained through scraping the propolis from the thin space created by wooden wedges interposed between the super and the cover of the hive; and (3) plastic nets, in which the beekeeper scrapes the plastic nets interposed between the super and the cover of the hive to induce bees to depose propolis.

The contents of waxes, balsams, and resins were determined by analyzing the raw propolis samples; all other analyses were performed on extracts obtained using ethanol, acetone, or chloroform. It is widely known that the yield of extraction depends on the polarity of the solvent, time of extraction, temperature, and also the composition and physical characteristics of the sample. Under the same extraction time and temperature conditions, the solvent used and the composition of the sample are the two most important factors to be considered. Ethanol was used because it is the solvent for most common commercial products obtained by propolis. Acetone was used because it is known to be one of the best extraction solvents for phenolic compounds.[20] Chloroform was chosen because it can

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better penetrate the waxy matrix and make the active substances more available for the extraction process.

With regard to the composition, the amounts of waxes, balsams, resins, total phenolics, and flavonoid components (total flavones and flavonols, total flavanones, and dihydroflavonols) were determined. In addition to this, to better characterize the phenolic composition of the propolis extracts, the presence of 15 phenolic compounds (apigenin, chrysin, galangin, naringenin, quercetin, kaempferol, pinobanksin, pinocembrin, pinostrobin, caffeic acid, ferulic acid, *p*-coumaric acid, caffeic acid phenylethyl ester, caffeic acid cinnamyl ester, and pinobanksin-3-*O*-acetate) selected among the most significant in poplar propolis[4] was verified through HPLC-MS/MS analysis. The antioxidant activity was studied using three different methods, which assess different aspects of the oxidation process: the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) assay, which is based on both hydrogen atom abstraction (HAT) and electron transfer reaction (SET) mechanisms; the reducing power assay, which is a SET-based method; and the thiobarbituric acid reactive substances (TBARS) and hydroperoxides contents determination in propolis extracts incorporated into an oil-in-water (O/W) emulsion. This last approach was used to correlate with the ability of propolis to inhibit the oxidative deterioration of lipids in food systems. In vitro assays, such as ferric reducing/antioxidant power (FRAP), Trolox equivalent antioxidant activity (TEAC), and oxygen radical absorbant capacity (ORAC), are performed in the absence of lipids. This means that the impact of antioxidant partitioning is not evaluated, thus leading to possible lack of correlation between the results of in vitro assays and antioxidant performance in foods. To accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical, and environmental conditions expected in food products. This is achieved by working in emulsion.[21] The results were compared to (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as antioxidant standard.

MATERIALS AND METHODS

Chemicals and Apparatus. All of the reagents, solvents (analytical grade), and phenolic standard compounds (apigenin, chrysin, galangin, naringenin, quercetin, kaempferol, pinobanksin, pinocembrin, pinostrobin, caffeic acid, ferulic acid, *p*-coumaric acid, caffeic acid phenylethyl ester) were purchased from Fluka (Buchs, Switzerland). Whatman grade no. 41 filter paper was purchased from Whatman International Ltd. (Milano, Italy). The dinitrophenylhydrazine reagent was prepared by dissolving 1 g of 2,4-dinitrophenylhydrazine (2,4-D) in 2 mL of 96% sulfuric acid and then diluting to 100 mL with methanol. The solution used to prepare the O/W emulsions was Tween 80 1% (w/w) in a buffer solution at pH 3.0. The 2-thiobarbituric acid (TBA) solution was prepared by mixing 15 g of trichloroacetic acid (TCA), 0.375 g of TBA, 1.76 mL of 12 N HCl, and 82.9 mL of distilled water. The absorbance was measured with a Varian Cary 50 Bio UV−visible spectrometer (Torino, Italy). The HPLC-MS/MS system consisted of a 6310A Ion Trap LC-MS(n) (Agilent Technologies Inc., Waldbronn, Germany) equipped with an Agilent 1200 series LC with binary pump, an electrospray interface (ESI), and an ion trap mass spectrometer. A Zorbax Eclipse XDB C18 50 mm × 2.1 mm i.d., 1.8 μm, p.s. column was used for the separation.

Samples. Twenty propolis samples were provided by Consiglio per la Ricerca e la Sperimentazione in Agricoltura-Istituto Nazionale di Apicoltura e Bachicoltura (CRA-API) (Bologna, Italy). The propolis samples were all deposited by bees during the spring of 2007 and were collected in the early summer from colonies located near each other in an experimental field in the province of Bologna (Italy) to reduce the influence on the composition due to geographical origin, botanical source, and climatic variability. These 20 samples were obtained by different harvesting methods, in particular, 6 by scraping, 8 by wooden wedges (3−5 mm thick), and 6 by plastic nets (mesh size = 2 mm). All of the analyses were carried out in triplicate.

Wax Extraction and Quantification. The contents of waxes were estimated according to a procedure properly developed by CRA-API. Three grams of frozen propolis was powdered and treated with 120 mL of petroleum ether at 40−60 °C in a Soxhlet extractor for 6 h. The extract was transferred to a previously weighed 150 mL evaporator flask and concentrated under reduced pressure at 50 °C. Then, 120 mL of 70% ethanol was added, heated under reflux until a clear solution was obtained, and then cooled at 0 °C for 1 h to promote wax separation. The mixture was filtered through a previously weighed Whatman grade no. 41 filter paper. The flask and the filter were washed with 70% ethanol, dried at 110 °C for 1 h, and transferred to a desiccator until constant weight. The sum of the residues remaining in the flask and on the filter, expressed as % w/w, represents the waxes.

Balsam Extraction and Quantification. The contents of balsams were estimated according to a procedure properly developed by CRA-API. The 70% ethanolic filtrate obtained during wax extraction (see previous paragraph) was concentrated under reduced pressure at 60 °C. The aqueous residue was transferred to a separating funnel, and 50 mL of dichlorometane was added. After shaking, the organic phase was collected and dried over 30 g of anhydrous Na_2SO_4 and then filtered in a previously weighed 150 mL evaporator flask. The extraction was repeated twice. The solution was evaporated to dryness under reduced pressure at 60 °C, and the flask was transferred to a desiccator until constant weight. The results are expressed as % w/w.

Resin Extraction and Quantification. The contents of resins were estimated according to a procedure properly developed by CRA-API. The residual propolis obtained after the extraction in the Soxhlet equipment was treated with 120 mL of a mixture of chloroform/ethanol 1:1 (v/v) in a Soxhlet extractor for 6 h. The extract was transferred to a preweighed 150 mL evaporator flask and concentrated to dryness under reduced pressure at 70 °C. The flask was dried at 110 °C for 1 h and transferred to a desiccator until constant weight. The results are expressed as % w/w.

Phenolic Compounds and Antioxidant Activity. *Propolis Extraction.* As previously mentioned, three different solvents were used for the extraction: ethanol, acetone, and chloroform. For each extract, 1 g of minced propolis was extracted with 10 mL of solvent under continuous stirring at room temperature for 30 min. The extraction was performed a second time after 24 h of continuous stirring. The ethanolic extract was filtered in a 25 mL volumetric flask and filled to volume with ethanol. The acetone and chloroform extracts were filtered and evaporated under vacuum at approximately 55 °C. Then, each residue was dissolved in ethanol, and the volume was filled to 25 mL.

Total Phenolics Determination. The total phenolics content (TP) was estimated by a properly modified Folin−Ciocalteu method.[22] A volume of 50 μL of extract diluted to 1:50 (v/v) in ethanol was mixed with 2.5 mL of the Folin−Ciocalteu reagent 1:10 (v/v) and 2.0 mL of a hot saturated solution of Na_2CO_3. The absorbance was measured at 760 nm after 5 min of incubation at 50 °C in the dark. Gallic acid was used for the calibration curve (20−800 μg/mL). TP was expressed as milligrams of gallic acid equivalents per gram of propolis (GAEs/g).

Total Flavones and Flavonols Determination. The total flavones and flavonols (TFF) were estimated according to an aluminum chloride method based on the procedure described by Woisky and Salatino.[23] For the calibration curve, four standard solutions of quercetin in 80% ethanol (25, 50, 100, and 200 μg/mL) were prepared. A 0.5 mL portion of standard solutions was separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$ in water (w/v), 0.1 mL of 1 M potassium acetate, and 2.8 mL of 80% ethanol. After incubation at 20 °C for 30 min, the absorbance was measured at 425 nm. The 10% $AlCl_3$ was substituted by the same quantity of distilled water in the blank sample. Similarly, 0.5 mL of each extract diluted to 1:50 (v/v) in 80% ethanol was analyzed as described above. The results are expressed as TFF % w/w.

Total Flavanones and Dihydroflavonols Determination. The total flavanone and dihydroflavonol (TFD) contents were determined using the method described by Nagy and Grancai[24] with minor modifications.

Four standard solutions of pinocembrine in methanol (200, 500, 1000, and 1500 μg/mL) were used for the calibration. One milliliter of each standard solution was separately reacted with 2 mL of the 2,4-D reagent (see Chemicals and Apparatus paragraph for the preparation) and 2 mL of methanol at 50 °C for 50 min. After cooling to room temperature, the reaction mixture was mixed with 5 mL of 10% KOH in 70% methanol (w/v) and incubated at 20 °C for 2 min. Then the mixture was mixed with 5 mL of methanol and centrifuged at 2000 rpm for 5 min. The supernatant was collected and adjusted to 25 mL. The absorbance of the supernatant was measured at 495 nm. A blank solution for the calibration with 1 mL of methanol instead of the pinocembrine standard solutions was used in an analogous procedure. The extracts were analyzed as described above, but in this case a blank solution with 2 mL of methanol instead of the 2,4-D reagent was used. The TFD content is expressed as TFD % w/w.

Free Radical Scavenging Activity. The scavenging activity of DPPH• was assayed according to the method of Brand-Williams et al.[25] Different extract aliquots of extract were dissolved in methanol to reach the final volume of 2.7 mL, and subsequently, 0.3 mL of methanolic DPPH• solution (0.1 mmol) was added. The mixtures were shaken and incubated at 37 °C in the dark. The absorbance was read at 517 nm every 5 min until a stable absorbance was obtained, using the absorbance of DPPH• as reference (A_{blank}). The loss of color was calculated as follows: fade percentage = ($A_{blank} - A_{propolis\ extract}$/$A_{blank} \times 100$). Ethanolic solutions with different Trolox concentrations (0.2−1.6 mmol/L) were analyzed, as described above. The DPPH• scavenging activity, expressed as the Trolox concentration required to provide 50% inhibition (IC_{50}) (mmol of Trolox equiv/g of propolis) (TEs/g), was extrapolated from the dose−response curve. Additionally, for each extract, the DPPH• bleaching kinetic rate was also evaluated using 0.25 mL of the extract diluted to 1:50 (v/v). The reduction of the DPPH• at 37 °C was measured by monitoring the decrease in the absorption at 517 nm, and the kinetic constant k was calculated. The same Trolox standard solutions previously prepared were analyzed, and the k of each standard solution was calculated. The antioxidant activity, expressed as millimoles of Trolox equivalents per gram of propolis (TEs/g), was extrapolated from the dose−response curve.

Reducing Power Determination. The reducing power (RP) was determined according to the method described by Oyaizu.[26] Different volumes (0.1−1.0 mL) of each extract were diluted to 1:50 (v/v) in ethanol and were mixed with a phosphate buffer solution at pH 6.6 (3.4−2.5 mL) and 2.5 mL of 10 mg/mL potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 2.5 mL of 100 mg/mL TCA was added, the mixture was centrifuged at 4000 rpm for 5 min, and then 5 mL was mixed with 1 mL of 1.0 mg/mL $FeCl_3$. The absorbance was measured at 700 nm. The slope calculated from the dose−response curve indicated the RP of the extract. Ethanolic solutions with different Trolox concentrations (0.1−1.0 mmol/L) were prepared and analyzed, as reported above. The slope of the dose−response curve obtained by analyzing each Trolox solution was plotted against the Trolox concentration, and the equation was used to obtain the RP, expressed as millimoles of Trolox equivalents per gram of propolis (TEs/g).

Preparation of the O/W Emulsions and Lipid Oxidation Measurements. An appropriate O/W emulsion system suitable to evaluate the antioxidant activity of the propolis extracts was developed. Soybean oil was chosen as the lipid source for its high unsaturated fatty acid and low free fatty acid contents. The O/W emulsions were prepared by homogenizing the soybean oil at a final concentration of 10% w/w and the aqueous emulsifier solution at pH 3.0 (1% w/w of Tween 80) at the final concentration of 90% w/w (10 min at 13500 rpm). The lipid hydroperoxides (LH) and the TBARS contents of the emulsions, prepared by adding 1 mL of each extract into the oil phase, were assayed after 96 h of storage at 20 °C under magnetic stirring. The LH were measured according to the method described by Shantha and Decker[27] with minor modifications. An aliquot of 20 μL of each sample was mixed with 2.975 mL of a 2:1 (v/v) methanol/butanol solution, 25 μL of 0.2 M HCl, 15 μL of a NH_4SCN/water solution (3.94 M), and 15 μL of an iron(II) chloride/water solution (72 mM). The solution was then vortexed, and the absorbance at 510 nm was measured after 20 min. A calibration curve was constructed with standards containing 0.2−40 μg of iron(III) chloride, and the results extrapolated from the dose−response curve were expressed as micrograms of Fe^{3+} per gram of propolis. The TBARS were determined according to the method described by McDonald and Hultin.[28] For each sample, 100 μL of each sample was mixed with 1.9 mL of distilled water and 4 mL of TBA solution. The mixture was vortexed and heated in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 1000 rpm for 15 min, and the absorbance was measured at 532 nm. A calibration curve was constructed with standards containing 0.005−1 mmol of 1,1,3,3-tetraethoxypropane (TEP) std/mL, and the results were expressed as millimoles of std per gram of propolis.

HPLC-MS/MS Analysis. The phenolic composition of the propolis extracts was determined through HPLC-MS/MS analysis. Propolis extracts (40 mg/mL) were filtered through a 0.22 μm filter and diluted 1:100 with methanol, and 5 μL was injected in the HPLC system. Standard solutions were prepared by dissolving the corresponding analytical standard in methanol and used to prepare calibration curves. All standards were filtered through a 0.22 μm filter, and 1 μL was injected in the HPLC system. The eluents were (A) 0.1% formic acid in a water/acetonitrile 98:2 solution and (B) 0.1% formic acid in a acetonitrile/water 98:2 solution. Separations were performed at 30 °C at a flow rate of 0.2 mL/min with the following gradient: 30% B for 1 min, 30−60% B in 6 min, 60−80% B in 3 min, 80% B for 4 min, 80−30% B in 2 min. The capillary voltage was set to −3500 V, and the desolvating temperature was 350 °C. Nitrogen was used as a drying (flow rate = 8 L/min) and nebulizing gas (pressure = 25 psi). The mass spectrometer operated in negative full-scan mode, which has been demonstrated to have higher sensitivity toward the metabolite of interest,[29,30] in the scan range 100−700 Da. The softwares used for the analyses and quantification were 6300 Series Trap control version 6.2 and Data Analysis for 6300 Series LC/MS Ion Trap control version 4.0 (Agilent Technologies Inc.). The identification of the 15 phenolic compounds through HPLC-MS/MS analysis was performed by comparing the experimental retention time (RT) and the ESI-MS and ESI-MS/MS fragmentation patterns with those of pure standard compounds[10] and corroborated by literature data.[31,32] The quantification of phenolic compounds was performed using standard calibration curves when possible. Caffeic acid cinnamyl ester and pinobanksin-3-*O*-acetate were quantified only by using internal standard (syringic acid) for the lack of pure compounds; therefore, the related results are to be considered only tentative. The full mass limits of detection (LOD) and quantitation (LOQ) for the phenolic compounds analyzed were estimated as the signal-to-noise ratio = 3 and 10, respectively, and calculated between 0.15 and 0.6 μg/mL for the LOD and between 0.5 and 2 μg/mL for the LOQ.

Statistical Analysis. The analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to evaluate the statistical significance of the measured differences between the propolis extracts. To evaluate the most important variables that discriminate between the propolis extracts, a post hoc test was performed using the Tukey "Honest Significant Difference test" (HSD). For all of these tests, the P level was set at 0.05. All of the analyses were performed using Statistica 6 for Windows (StatSoft Italia, Vigonza, Italy).

RESULTS AND DISCUSSION

In Table 1, the contents of waxes, balsams, and resins for all of the samples are reported. As evident, the major wax mean content is obtained for the scraping samples, in particular, for sample S1. The wedge samples show the lowest mean content, sample W4 having the lowest amount at all, whereas the plastic net samples have an intermediate content. Considering the balsams, the highest mean amount is obtained for the plastic net samples, whereas the wedges show the lowest mean content; however, wedge sample W7 contains the major balsam amount, whereas the scraping samples have an intermediate balsam content. For

Table 1. Wax, Balsam, and Resin Contents of Propolis Samples

sample	waxes (% w/w)	balsams (% w/w)	resins (% w/w)
scraping			
S1	41.0	6.95	39.1
S2	23.8	5.87	58.0
S3	20.1	5.38	58.5
S4	27.1	3.69	58.7
S5	24.6	5.77	63.1
S6	22.1	3.84	61.2
mean ± SD	26.4 ± 7.5	5.25 ± 1.3	56.4 ± 8.7
wedges			
W1	33.5	5.17	48.3
W2	19.6	2.31	60.7
W3	12.8	6.37	59.9
W4	7.40	4.61	71.4
W5	26.7	3.19	63.5
W6	15.5	5.48	72.7
W7	16.9	8.45	66.7
W8	17.5	1.78	71.1
mean ± SD	18.7 ± 8.1	4.67 ± 2.2	64.3 ± 8.1
plastic nets			
N1	27.1	6.20	54.9
N2	27.8	5.83	52.9
N3	22.7	6.02	62.6
N4	13.8	7.09	67.8
N5	16.3	5.28	64.6
N6	23.4	5.98	57.6
mean ± SD	21.9 ± 5.7	6.07 ± 0.6	60.1 ± 5.8
overall mean ± SD	22.0 ± 7.6	5.26 ± 1.6	60.7 ± 8.0
P (ANOVA)	0.179	0.300	0.194
P (MANOVA)		0.483	

the resins, the wedge samples, particularly sample W6, which shows the highest content, have the highest mean concentration, followed by the plastic nets and the scraping samples. None of the differences is statistically significant ($P < 0.05$). These results confirm the only literature data available at the present time, in which the wax, balsam, and resin contents of Italian propolis samples collected in the years 1999, 2000, and 2002 are reported. In particular, the results of 1999, the year in which all three harvesting methods were used, are very interesting. These results are in complete agreement with our data; the wedge propolis indeed shows the lowest mean content of waxes (7.53%) and the highest mean amount of resins (79.1%), whereas the highest mean amount of balsams is obtained for the plastic net samples (12.7%).[7]

In Figures 1 and 2, the phenolic compound contents and the antioxidant activities, divided according to the extraction solvent, are reported. From these data, it is evident that the acetone possesses the highest extraction capacity toward the phenolic substances considered; however, the extraction capacity of ethanol is still confirmed. Indeed, the differences between acetone and ethanol are not statistically significant, as demonstrated by the post hoc test results, except for the TFD of the wedges. The extraction capacity of chloroform is limited and less than the ethanol and acetone capacities. This result is confirmed by the post hoc test, which shows that the differences are statistically significant. Considering the antioxidant activity, even though the high extraction capacity is from acetone, the acetone extracts do not show the greatest antioxidant activity. The best results were

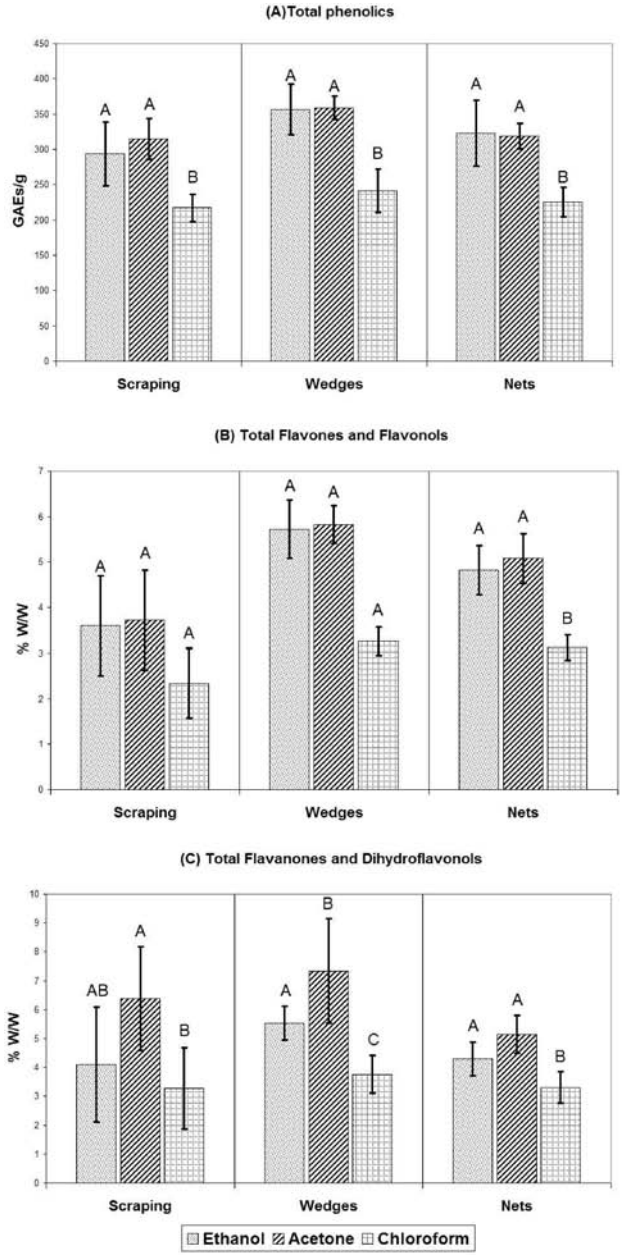

Figure 1. Phenolic compounds of the propolis extracts. Results of the HSD test: for each type of propolis, the same letter in the same box indicates no significant differences ($P < 0.05$).

obtained from the ethanol extracts, except for the DPPH IC_{50}; in this case, the chloroform extracts show the highest antioxidant activity and the most statistically significant differences.

To verify the influence of the harvesting method, the results of the ethanol, acetone, and chloroform extracts are reported in Tables 2–4. Considering the ethanol extracts (Table 2), statistically significant differences for TP, TFF, DPPH *k*, and RP are found; the post hoc test, in particular, shows that wedges and plastic nets gave similar results in terms of TP and TFF, which are significantly higher than scraping propolis. With regard to DPPH *k*, the scraping and wedges show a significant difference from plastic nets. Wedge and plastic net propolis gave the best results on phenolics ethanol extraction and also in terms of in vitro antioxidant activity evaluated as DPPH *k* and RP. Concerning the phenolic compound contents, our results are in

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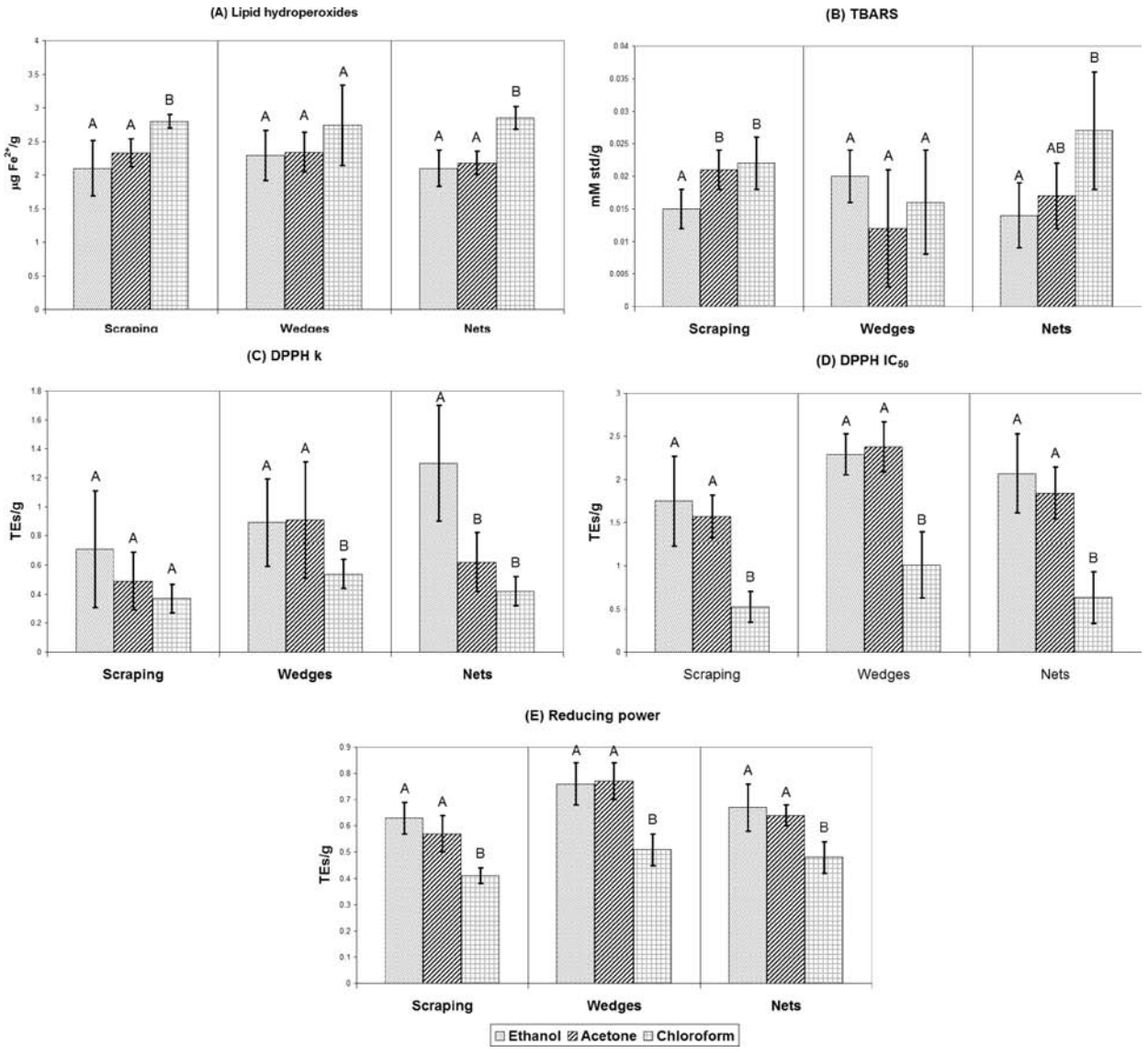

Figure 2. Antioxidant activity of the propolis extracts. Results of the HSD test: for each type of propolis, the same letter in the same box indicates no significant differences ($P < 0.05$).

agreement with the literature data, although expressed in different ways.[33−37] With regard to the acetone extracts (Table 3), statistically significant differences not only for TP, TFF, DPPH *k* and RP but also for TBARS and DPPH IC_{50} are found. The post hoc test shows no significant differences between the wedge and plastic net propolis in terms of phenolic compounds contents, whereas scraping propolis samples have significantly lower amounts, which is the same as observed for ethanol extracts. Additionally, the wedge and plastic nets propolis acetone extracts showed the highest antioxidant activity in three of the five analyses performed, so that, also in this case, the in vitro antioxidant activity is in accordance with the phenolics composition. With regard to the chloroform extracts (Table 4), statistically significant differences only for TFF, DPPH IC_{50}, and RP are found, and the post hoc test confirms the significant differences also observed in the ethanol and acetone extracts. In this case, the wedge and the plastic net propolis show the highest contents of phenolic compounds and the greatest antioxidant activity, except for the DPPH IC_{50}, for which the highest values are obtained for the scraping propolis.

The results of phenolic compound quantification are reported in Table 5. As shown, the high extraction capacity toward the phenolic substances considered of ethanol and acetone is still confirmed, because all of the compounds analyzed, except for caffeic acid, are more abundant in these two propolis extracts. With regard to the harvesting method, 10 of the 15 phenolic compounds considered gave statistically significant differences. The post hoc test, in particular, shows that wedges and plastic nets have the higher contents of chrysin, galangin, kaempferol, pinostrobin, caffeic acid phenylethyl ester, and caffeic acid cinnamyl ester, whereas quercetin, naringenin, and ferulic acid are more concentrated in wedge and scraping propolis extracts.

The literature available, although extensive, makes it difficult to compare our compositional and functional results with similar data because they are often expressed in different ways, and the propolis extracts are prepared using different procedures. To our knowledge, no literature data on acetone and chloroform propolis extracts are available; therefore, it was not possible to do a comparison with similar results. Considering the results reported in Table 1 and those related to the composition of the ethanol propolis extracts shown in Figure 1 and, despite the different

Table 2. Phenolic Compounds Contents and Antioxidant Activity of Ethanol Propolis Extracts[a]

sample	TP (GAEs/g)	TFF (% w/w)	TFD (% w/w)	LH (μg Fe^{3+}/g)	TBARS (mmol std/g)	DPPH k (TEs/g)	DPPH IC_{50} (TEs/g)	RP (TEs/g)
scraping								
S1	267.3	3.83	2.59	2.42	0.018	0.78	2.10	0.71
S2	378.8	5.34	5.94	2.37	0.021	0.79	2.61	0.69
S3	250.8	3.62	2.15	1.64	0.013	1.4	1.58	0.64
S4	292.8	2.08	2.95	1.92	0.014	0.38	1.42	0.58
S5	276.0	2.85	3.79	1.65	0.012	0.50	1.61	0.57
S6	296.8	3.89	7.17	2.59	0.014	0.38	1.15	0.59
mean ± SD	293.8 ± 45.0(A)	3.60 ± 1.1(A)	4.10 ± 2.0(A)	2.10 ± 0.41(A)	0.015 ± 0.003(A)	0.71 ± 0.4(A)	1.75 ± 0.52(A)	0.63 ± 0.06(A)
wedges								
W1	381.4	6.08	5.54	2.88	0.016	0.93	2.37	0.86
W2	355.6	6.50	4.83	2.05	0.013	0.87	2.18	0.84
W3	387.7	6.11	4.93	2.29	0.021	0.93	2.48	0.66
W4	409.2	6.49	5.74	2.25	0.020	1.4	1.91	0.68
W5	296.6	5.04	6.76	2.77	0.025	0.46	2.12	0.64
W6	332.4	5.31	5.39	2.08	0.026	0.85	2.68	0.78
W7	347.2	5.05	5.60	2.22	0.017	0.83	2.20	0.81
W8	339.5	5.17	5.44	1.75	0.020	0.82	2.35	0.77
mean ± SD	356.2 ± 35.7(B)	5.72 ± 0.64(B)	5.53 ± 0.59(A)	2.29 ± 0.37(A)	0.020 ± 0.004(A)	0.89 ± 0.3(AB)	2.29 ± 0.24(A)	0.76 ± 0.08(B)
plastic nets								
N1	305.5	5.29	3.99	2.28	0.017	1.2	1.62	0.52
N2	302.3	4.92	3.61	2.32	0.017	0.97	2.31	0.67
N3	400.6	5.46	4.05	1.89	0.005	1.9	2.53	0.73
N4	261.6	3.98	5.30	1.82	0.011	1.5	1.38	0.62
N5	332.0	4.75	4.45	2.44	0.017	0.60	2.38	0.76
N6	335.9	4.51	4.40	1.88	0.019	1.4	2.18	0.73
mean ± SD	323.0 ± 46.4(AB)	4.82 ± 0.54(B)	4.30 ± 0.58(A)	2.10 ± 0.27(A)	0.014 ± 0.005(A)	1.3 ± 0.4(B)	2.07 ± 0.46(A)	0.67 ± 0.09(AB)
overall mean ± SD	327.5 ± 47.8	4.81 ± 1.2	4.73 ± 1.3	2.17 ± 0.35	0.017 ± 0.005	0.94 ± 0.4	2.06 ± 0.45	0.69 ± 0.09
P (ANOVA)[b]	**0.041**	**<0.001**	0.077	0.546	0.074	**0.040**	0.076	**0.026**
P (MANOVA)[b]					0.052			

[a]Results of the HSD test: the same letter in the same column indicates no significant differences ($P < 0.05$). [b]The statistically significant values are given in bold.

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Table 3. Phenolic Compounds Contents and Antioxidant Activity of Acetone Propolis Extracts[a]

sample	TP (GAEs/g)	TFF (% w/w)	TFD (% w/w)	LH (μg Fe^{3+}/g)	TBARS (mmol std/g)	DPPH k (TEs/g)	DPPH IC_{50} (TEs/g)	RP (TEs/g)
scraping								
S1	280.6	3.40	6.48	2.40	0.019	0.36	1.63	0.56
S2	320.1	5.32	4.42	2.70	0.022	0.71	1.97	0.66
S3	293.3	3.63	3.87	2.34	0.020	0.43	1.22	0.54
S4	301.9	1.99	7.93	2.23	0.024	0.66	1.46	0.53
S5	337.3	3.99	7.40	2.17	0.025	0.45	1.68	0.66
S6	357.9	3.97	8.18	2.13	0.017	0.31	1.45	0.50
mean ± SD	315.2 ± 28.9(A)	3.72 ± 1.1(A)	6.38 ± 1.8(A)	2.33 ± 0.21(A)	0.021 ± 0.003(A)	0.49 ± 0.2(A)	1.57 ± 0.25(A)	0.57 ± 0.07(A)
wedges								
W1	391.7	5.78	8.29	2.42	0.0040	1.6	2.53	0.83
W2	342.3	5.73	6.75	2.28	0.022	0.34	2.05	0.74
W3	359.2	6.11	4.72	2.75	0.022	0.80	2.00	0.64
W4	350.5	6.60	4.44	2.78	0.023	1.1	2.55	0.76
W5	373.8	6.02	8.54	2.16	0.0042	0.60	2.26	0.71
W6	351.3	5.55	8.98	1.95	0.0074	0.86	2.55	0.83
W7	357.1	5.65	9.03	2.28	0.0053	1.1	2.84	0.82
W8	346.5	5.17	7.95	2.10	0.0068	0.88	2.26	0.81
mean ± SD	359.1 ± 16.3(B)	5.83 ± 0.42(B)	7.34 ± 1.8(A)	2.34 ± 0.30(A)	0.012 ± 0.009(B)	0.91 ± 0.4(B)	2.38 ± 0.29(B)	0.77 ± 0.07(B)
plastic nets								
N1	335.3	5.36	6.38	2.16	0.019	0.48	1.89	0.63
N2	290.1	5.33	4.92	2.45	0.018	0.94	2.31	0.65
N3	339.3	5.83	4.64	2.07	0.0070	0.73	1.63	0.70
N4	319.8	4.85	5.22	2.30	0.022	0.50	2.00	0.63
N5	310.5	4.92	5.00	2.08	0.017	0.55	1.45	0.58
N6	316.4	4.21	4.66	2.00	0.018	0.50	1.73	0.66
mean ± SD	318.6 ± 17.8(A)	5.08 ± 0.55(B)	5.14 ± 0.65(A)	2.18 ± 0.17(A)	0.017 ± 0.005(AB)	0.62 ± 0.2(AB)	1.84 ± 0.30(A)	0.64 ± 0.04(A)
overall mean ± SD	333.7 ± 29.2	4.97 ± 1.1	6.39 ± 1.8	2.29 ± 0.24	0.016 ± 0.007	0.69 ± 0.3	1.97 ± 0.44	0.67 ± 0.1
P (ANOVA)[b]	**0.002**	**<0.001**	0.062	0.425	**0.047**	**0.031**	**<0.001**	**<0.001**
P (MANOVA)[b]					**<0.001**			

[a]Results of the HSD test: the same letter in the same column indicates no significant differences ($P < 0.05$). [b]The statistically significant values are given in bold.

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Table 4. Phenolic Compounds Contents and Antioxidant Activity of Chloroform Propolis Extracts[a]

sample	TP (GAEs/g)	TFF (% w/w)	TFD (% w/w)	LH (μg Fe^{3+}/g)	TBARS (mmol std/g)	DPPH k (TEs/g)	DPPH IC_{50} (TEs/g)	RP (TEs/g)
scraping								
S1	211.4	2.17	2.12	2.63	0.025	0.57	0.372	0.43
S2	235.5	3.58	3.29	2.84	0.025	0.53	0.431	0.46
S3	208.2	2.30	2.18	2.81	0.023	0.28	0.780	0.38
S4	198.6	1.16	2.46	2.85	0.020	0.24	0.512	0.39
S5	202.9	2.30	3.68	2.74	0.015	0.26	0.714	0.40
S6	247.2	2.51	5.93	2.91	0.027	0.35	0.352	0.38
mean ± SD	217.3 ± 19.5(A)	2.34 ± 0.77(A)	3.28 ± 1.4(A)	2.80 ± 0.10(A)	0.022 ± 0.004(A)	0.37 ± 0.1(A)	0.527 ± 0.18(A)	0.41 ± 0.03(A)
wedges								
W1	311.6	3.80	3.59	1.63	0.010	0.53	1.65	0.61
W2	221.1	3.19	4.20	2.53	0.013	0.33	0.872	0.47
W3	221.6	3.48	3.27	3.03	0.031	0.58	0.441	0.48
W4	235.7	3.35	3.07	2.69	0.028	0.76	0.630	0.49
W5	235.2	3.28	5.05	3.24	0.011	0.35	1.28	0.43
W6	215.6	2.76	3.22	3.61	0.011	0.53	1.01	0.54
W7	249.6	3.08	3.64	2.86	0.011	0.63	1.06	0.54
W8	240.5	3.11	4.03	2.35	0.014	0.58	1.13	0.56
mean ± SD	241.4 ± 30.6(A)	3.26 ± 0.31(B)	3.76 ± 0.65(A)	2.74 ± 0.60(A)	0.016 ± 0.008(A)	0.54 ± 0.1(A)	1.01 ± 0.38(B)	0.51 ± 0.06(B)
plastic nets								
N1	228.8	2.88	3.53	2.75	0.028	0.47	0.320	0.43
N2	219.8	3.31	2.55	2.88	0.026	0.60	0.352	0.46
N3	197.6	3.44	3.62	2.93	0.012	0.33	0.961	0.44
N4	257.3	3.36	3.71	2.76	0.028	0.46	0.423	0.53
N5	237.2	2.93	2.67	3.11	0.042	0.30	0.781	0.58
N6	211.2	2.80	3.76	2.63	0.026	0.36	0.964	0.42
mean ± SD	225.3 ± 20.9(A)	3.12 ± 0.28(B)	3.31 ± 0.55(A)	2.85 ± 0.17(A)	0.027 ± 0.009(A)	0.42 ± 0.1(A)	0.633 ± 0.30(AB)	0.48 ± 0.06(AB)
overall mean ± SD	229.3 ± 25.9	2.94 ± 0.62	3.48 ± 0.92	2.79 ± 0.38	0.021 ± 0.009	0.45 ± 0.2	0.752 ± 0.36	0.47 ± 0.07
P (ANOVA)[b]	0.212	**0.007**	0.560	0.895	0.058	0.086	**0.022**	**0.006**
P (MANOVA)[b]					0.053			

[a]Results of the HSD test: the same letter in the same column indicates no significant differences ($P < 0.05$). [b]The statistically significant values are given in bold.

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Table 5. Phenolic Composition Obtained through HPLC-MS/MS Analysis of the Propolis Extracts[a]

	apigenin			chrysin			galangin			kaempferol		
	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform
scraping	5.37 ± 2.12 (A)	5.90 ± 1.05(A)	5.05 ± 0.60(A)	5.06 ± 3.29(A)	5.73 ± 2.43(A)	5.67 ± 1.74(A)	11.98 ± 6.08(A)	13.55 ± 4.67(A)	12.64 ± 3.39(A)	4.56 ± 2.28(A)	5.14 ± 1.78(A)	4.64 ± 1.22(A)
wedges	5.81 ± 0.69 (A)	5.97 ± 1.01 (A)	3.62 ± 1.05 (A)	8.11 ± 1.41(B)	8.23 ± 1.19(B)	4.70 ± 1.46(A)	18.41 ± 2.16(B)	18.52 ± 1.79 (B)	10.63 ± 3.02 (A)	7.02 ± 0.84(B)	7.10 ± 0.64(B)	3.95 ± 1.11(A)
plastic nets	4.39 ± 1.01(A)	5.83 ± 0.64 (A)	4.00 ± 1.33 (A)	5.52 ± 1.78(A)	7.72 ± 1.36(AB)	5.06 ± 1.77(A)	13.11 ± 3.47 (AB)	17.72 ± 2.48 (AB)	11.57 ± 3.98 (A)	4.99 ± 1.31 (AB)	6.81 ± 0.98 (AB)	4.34 ± 1.45(A)
P (ANOVA)[b]	0.174	0.960	0.059	**0.039**	**0.039**	0.564	**0.018**	**0.022**	0.566	**0.016**	**0.017**	0.596
P (ANOVA)[b,c]		**<0.001**		**0.005**				**<0.001**			**<0.001**	
	quercetin			naringenin			pinocembrin			pinostrobin		
	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform
scraping	8.02 ± 2.92 (AB)	9.03 ± 1.46(A)	7.35 ± 1.15(A)	8.67 ± 2.93(AB)	9.70 ± 1.28(A)	9.16 ± 1.64(A)	19.22 ± 9.75(A)	21.45 ± 8.80(A)	21.46 ± 8.63(A)	1.97 ± 0.94(A)	2.33 ± 0.81(A)	2.32 ± 0.99(A)
wedges	10.28 ± 1.00 (A)	10.33 ± 1.54 (A)	5.81 ± 1.76 (A)	10.73 ± 0.91(A)	11.00 ± 1.64(A)	7.27 ± 0.98(A)	24.37 ± 1.95(A)	24.89 ± 3.51 (A)	16.08 ± 4.03 (A)	3.65 ± 0.51(B)	3.60 ± 0.15(B)	2.39 ± 0.72(A)
plastic nets	7.24 ± 1.59 (B)	9.77 ± 1.29 (A)	6.14 ± 2.27 (A)	7.89 ± 1.79(B)	10.65 ± 1.24(A)	7.68 ± 2.64(A)	17.72 ± 3.87(A)	23.67 ± 3.44 (A)	18.31 ± 7.07 (A)	2.46 ± 0.80(A)	3.29 ± 0.66(B)	2.37 ± 0.95(A)
P (ANOVA)[b]	**0.022**	0.277	0.286	**0.037**	0.259	0.341	0.106	0.534	0.341	**0.002**	**0.002**	0.988
P (ANOVA)[b,c]		**<0.001**		**<0.001**				**0.038**			**0.029**	
	pinobanksin			*p*-coumaric acid			ferulic acid			caffeic acid		
	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform
scraping	6.62 ± 1.65(A)	7.28 ± 1.82(A)	6.29 ± 1.57(A)	8.60 ± 3.60(A)	10.08 ± 1.67(A)	8.53 ± 1.18(A)	1.78 ± 0.56(AB)	2.03 ± 0.31(A)	1.82 ± 0.31(A)	5.39 ± 3.54(A)	7.10 ± 1.90(A)	7.27 ± 1.35(A)
wedges	7.31 ± 1.83 (A)	7.45 ± 1.86 (A)	4.53 ± 1.17 (A)	10.77 ± 1.37(A)	11.01 ± 1.87(A)	6.51 ± 2.02(A)	2.13 ± 0.15(A)	2.17 ± 0.33(A)	1.39 ± 0.39(A)	7.09 ± 0.79(A)	7.56 ± 1.28(A)	5.53 ± 2.26(A)
plastic nets	5.95 ± 1.49 (A)	7.39 ± 1.85 (A)	4.47 ± 1.12 (A)	8.31 ± 2.13(A)	10.98 ± 0.98(A)	7.26 ± 2.60(A)	1.56 ± 0.33(B)	2.09 ± 0.31(A)	1.43 ± 0.53(A)	5.26 ± 2.00(A)	7.85 ± 1.72(A)	6.14 ± 2.76(A)
P (ANOVA)[b]	0.283	0.990	0.172	0.141	0.515	0.211	**0.031**	0.725	0.153	0.258	0.724	0.365
P (ANOVA)[b,c]		**0.040**		**<0.001**				**<0.001**			0.057	

	caffeic acid phenylethyl ester			caffeic acid cinnamyl ester[d]			pinobanksin-3-*O*-acetate[d]		
	ethanol	acetone	chloroform	ethanol	acetone	chloroform	ethanol	acetone	chloroform
scraping	2.36 ± 1.13(A)	2.83 ± 0.99(A)	2.81 ± 1.13(A)	2.17 ± 1.02(A)	2.60 ± 0.91(A)	2.63 ± 1.13(A)	12.31 ± 5.90(A)	15.39 ± 5.38(A)	15.70 ± 6.75(A)
wedges	4.27 ± 0.55(B)	4.18 ± 0.54(B)	2.77 ± 0.83(A)	3.91 ± 0.55(B)	4.03 ± 0.20(B)	2.59 ± 0.78(A)	19.54 ± 2.74(A)	23.31 ± 0.92(B)	15.98 ± 4.79(A)
plastic nets	3.01 ± 1.05(A)	3.89 ± 0.42(B)	2.80 ± 1.12(A)	2.82 ± 0.93(A)	3.64 ± 0.73(B)	2.60 ± 1.04(A)	14.73 ± 4.71(A)	16.97 ± 3.39(A)	15.43 ± 6.17(A)
P (ANOVA)[b]	**0.003**	**0.003**	0.976	**0.002**	**0.002**	0.976	0.114	**0.018**	0.995
P (ANOVA)[b,c]		**0.031**			**0.034**			**0.046**	

[a]Each result refers to the average amount in mg/g of the propolis extracts analyzed. Results of the HSD test: the same letter in the same column indicates no significant differences ($P < 0.05$). [b]The statistically significant values are given in bold. [c]The ANOVA and the HSD test results for solvents show statistically significant differences between chloroform extracts and the other ones. No significant differences were ever observed between ethanol and acetone extracts. [d]The quantification is to be considered only tentative.

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ways to express the content of the balsams, it is possible to affirm that the propolis analyzed in this study can be identified as poplar propolis, as characterized by Popova et al. in 2007.[36] This is confirmed also by Nagy et al.[38] and Greenaway et al.,[39] who demonstrated that, in temperate zones, the bud exudates of the *Populus* species are the main source of propolis. Indeed, this is the main propolis type present in Italy and in particular in the limited geographical area from which the samples of this study were collected. In addition, it is important to recall that types of propolis other than those of poplar origin have different chemical compositions.[40] With regard to the antioxidant activity, the difficulty arises from the different methods of calculations and the use of different standard reference compounds. According to our data, it is difficult to identify exactly the propolis with the greatest antioxidant activity because the results vary according to the procedures. There is a wide variety of methods to assess antioxidant capacity, each having advantages and disadvantages; besides, no single assay accurately reflects all of the radical sources or all antioxidants in a mixed or complex system; therefore, it is fundamental to use different methods, which assess different aspects of the oxidation process, that can comprehensively determine antioxidant capacity. However, it would be desirable to achieve an agreement on standardized methods by which the antioxidant capacity can be measured accurately and quantitatively. This allows for guidance for appropriate application of assays, meaningful comparisons of results, and a means to control variation within or between the samples. This work also aims to do this. Considering both the bioactive substance contents and the antioxidant activities reported, the wedge propolis, which is richer in resins and balsams, appears to have the most potential to yield high-value functional products. Our results confirmed that the extracts of propolis from different solvents have different chemical compositions, and the composition and the antioxidant activity of propolis depend on the harvesting method employed. The wax, balsam, and resin contents are in agreement with the literature, in which the propolis obtained by wooden wedges shows the lowest wax content and the highest resin content.[7] These results can be explained by the ability of the bees to produce and deposit pure propolis on the smooth and narrow surfaces of the thin space created by the wedges, whereas they deposit propolis mixed with the highest amount of wax on the largest and irregular surfaces of the beehive. Therefore, the propolis composition depends on the location in the hive where it is used and on the dimension of the holes to be filled, as also demonstrated by Alì et al.[41]

The most efficient solvents for the phenolic compound extraction and the antioxidant activity evaluation are acetone and ethanol, whereas chloroform is the least effective solvent, even though it can better penetrate the waxy matrix of the propolis.

The health-promoting properties of propolis mainly depend on the phenolic compounds, which are considered to be the biologically active components in this matrix. The quality of propolis can be related to the high resin and balsam contents because the bioactive substances are concentrated in these fractions.[42,43] Therefore, the wedge method seems to be the most appropriate technique to obtain the best quality of the propolis, as reported in the literature.[7] This research could provide much useful information for the food and nutraceutical industries to choose suitable conditions for extracting desirable phenolic components from propolis and could serve as a basis for other researchers to investigate propolis antioxidants in future research. In addition, because it is difficult to compare our results with similar literature data, it is necessary to standardize the methods by which the results are reported.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ANOVA, analysis of variance; 2,4-D, 2,4-dinitrophenylhydrazine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAEs/g, gallic acid equivalents/g; HSD, Tukey Honest Significant Difference test; LH, hydroperoxides; MANOVA, multivariate analysis of variance; RP, reducing power; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; TCA, trichloroacetic acid; TEP, 1,1,3,3-tetraethoxypropane; TEs/g, Trolox equivalents/g of propolis; TFD, total flavanone and dihydroflavonol; TFF, total flavones and flavonols; TP, total phenolics; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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