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A comparative study of solvent effect on propolis extraction by ultrasound-assisted extraction

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Abstract

Propolis is a natural bee product obtained from beehives as raw propolis. Propolis extracts obtained from raw propolis with different polarities solvents are used as food supplement agent. The composition of propolis extracts depends on the raw propolis species, extraction methods and extraction solvent. In this study, it is expressed how the phenolic composition of propolis extracts varies depending on the solvent polarity used. The ultrasonic-assisted maceration technique was used to extract an Anatolian raw propolis sample with five different polarity solvents, namely water, methanol, ethanol, isopropanol, and n-butanol using sequential and gradual extractions. The extraction capacity was evaluated by total phenolic substance content (TPC), total flavonoid substance content (TFC), individual phenolic compounds, and antioxidant capacity. The phenolic compositions were analyzed by High Performance Liquid Chromatography (HPLC-PDA) according to the twenty-five phenolic standards. As a result, propolis directly extracted with water, methanol (98%), ethanol (98%), isopropanol (98%), n-butanol (98%) and 70% ethanol, TPC value of 147.98 mg GAE/g, TFC value of 47.18 mg QUE/g, FRAP value of 1144.33 μM $FeSO_4.7H_2O$/g and DPPH analysis results of 0.03 SC50 (mg/mL). It was determined that 70% ethanolic extract contained the highest phenolic compounds and had the highest antioxidant capacity compared to propolis extracted with solvents gradually.

Keywords: Propolis, extraction, solvent, polyphenols, antioxidant

1. Introduction

Propolis is a natural substance produced by honeybees (*Apis mellifera*) from resin-like materials from plant sources collected in the hive or in special propolis traps. Honeybees use propolis as an isolation, antifungal and antimicrobial agent [1,2]. After being removed from the hives, raw propolis is extracted in different solvents and using various extraction techniques. It is consumed in liquid or capsule form in complementary medicine. Raw propolis is a mixture of wax (5-30%), balsam (20-50%), and volatile compounds (1%), the majority of its bioactive components being in the balsamic portion [3,4]. The balsamic fraction obtained by extraction with ethanol is rich in polyphenols. The types and amounts of polyphenols in propolis samples vary according to the flora of the region where it is produced and the needs of the bee population. However, caffeic acid and its esters and flavonoids such as chrysin and pinocembrin are abundant in all propolis specimens [4–6]. Propolis extracts exhibit a wide range of antioxidant, [5–8] antimicrobial, [6–8], antiviral, [9–11] antidiabetic [12]and antitumoral [13] activities and is used in traditional and complementary medicine as supplementary food [6,14].

One crucial factor in preparing propolis extracts from raw propolis involves the selection of the extraction technique, solvent type, and raw propolis ratio. These elements significantly affect the resulting extract's composition and properties. Various extraction techniques, such as maceration, ultrasonic extraction, Soxhlet extraction, and microwave and supercritical extraction, are used for the preparation of commercial propolis extracts [6,14–16]. Different polarity solvents such as water, ethanol, polypropylene glycol (PPG), and green solvents are used in the extraction [3,17]. However, ethanol is regarded as the optimal solvent for extraction [18,19]. Most propolis extracts consumed as a food supplement are produced using dietary ethanol, such as alcohols made from wheat, sugar beet, or sugar cane [6,15,19]. A number of studies have shown that ultrasound-assisted maceration is the most practical and economical technique in the extraction of raw propolis[14,18–20].

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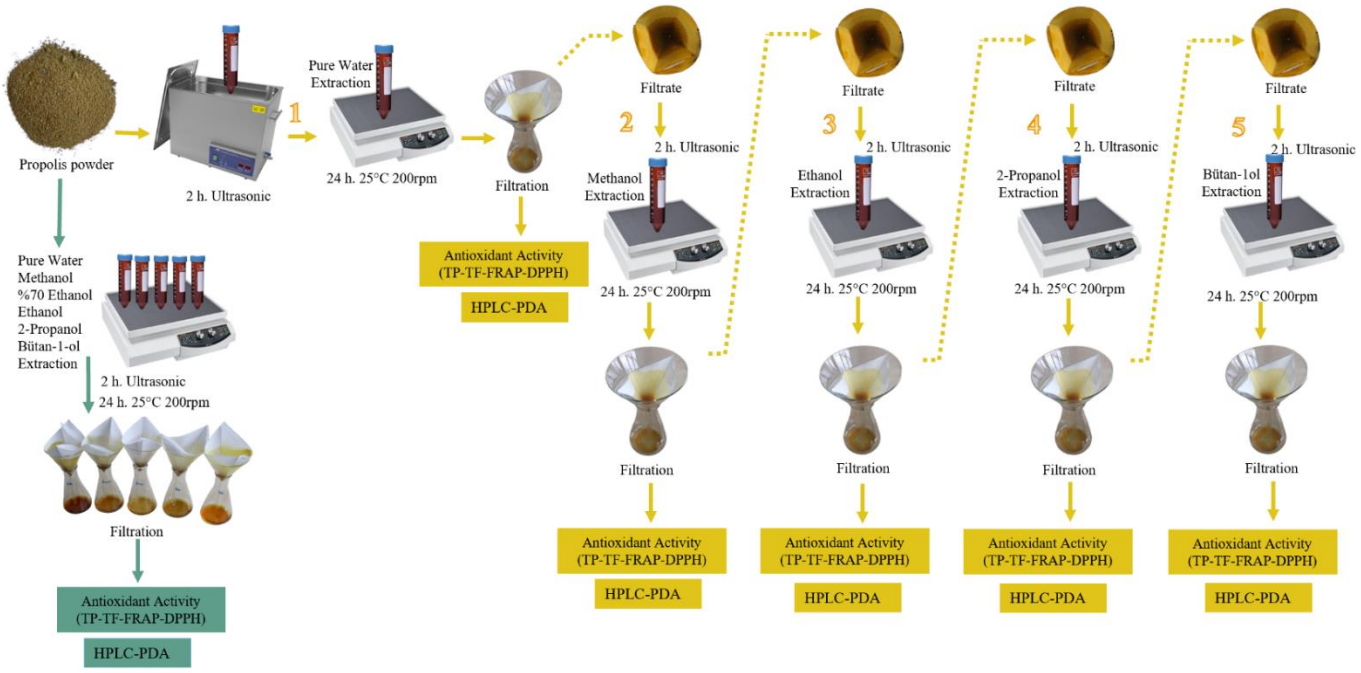

Figure 1. Schematic of the extraction procedure of propolis sample

However, due to the known disadvantages of ethanol, the search for ecological and green solvents for propolis continues. In addition to organic solvents, a variety of deep eutectic solvents, green solvents and alkaline or acidic extractions are also available [21–24].

In addition, which solvent would be more appropriate for the polyphenols found in raw propolis is still a matter of debate. The aim of this study is to investigate which solvent or solvents would be more useful for elucidating the composition of propolis. This research therefore evaluated the effects of solvent polarities on the ultrasonically-assisted propolis extractions.

2. Materials and methods

2.1. Chemicals

Methanol (Merck, 106009), ethanol (Symras, 3050500), isopropanol (Merck,1.00272), and n-butanol (Sigma Aldrich,34867), diethyl ether (Sigma Aldrich, 24004), ethyl acetate (Sigma Aldrich,27227), acetic acid (Sigma Aldrich, 27225), acetic acid (Sigma Aldrich, 27225), acetonitrile (Sigma Aldrich, 34851) were used as analytical purity. The phenolic standards of quercetin (Sigma Aldrich, Q4951), gallic acid (Sigma Aldrich, G7384), caffeic acid (Sigma Aldrich, C0625), protocatechuic acid (Sigma Aldrich, 03930590), p-OH benzoic acid (Sigma Aldrich, 240141), syringic acid (Sigma Aldrich, S6881), epicatechin (Sigma Aldrich, E1753), p-coumaric acid (Sigma Aldrich, C9008), ferulic acid (Sigma Aldrich, 128708), rutin (Sigma Aldrich, R5143), myricetin (Sigma Aldrich, M6760), resveratrol (Sigma Aldrich, R5010), daidzein (Sigma Aldrich, D7802), luteolin (Sigma Aldrich, L9283), trans-cinnamic acid (Sigma Aldrich, C80857), hesperetin (Sigma Aldrich, W431300), chrysin (Sigma Aldrich, C80105), pinocembrin (Sigma Aldrich, P5239), caffeic acid phenethyl ester (CAPE) (Sigma Aldrich, C8221), chlorogenic acid (Sigma Aldrich, C3878), m-OH-benzoic acid (Sigma Aldrich, 36333), ellagic acid (Sigma Aldrich, E2250), apigenin (Sigma Aldrich, 10798), rhamnetin (Sigma Aldrich, 17799), and curcumin (Alfa Aesar, B21573) were used.

2.2. Extraction procedure

Raw propolis samples obtained from 10 different regions of Anatolia of Turkiye, were mixed and turned into a single propolis sample. The raw propolis mixture was frozen in a deep freeze, pulverized with the help of an electric grinder, and returned to the deep freeze. The extraction process was carried out in two parts. In the first part, propolis was extracted separately in different solvents (pure water, methanol ethanol, isopropanol, and n-butanol), while in the second part, the sample was extracted gradually with these solvents. For each extraction, 30 mL of solvent was added to 3 g of powdered propolis. The mixture was placed in a falcon tube and then extracted in an ultrasonic bath (Everest Ultrasonic CleanEX N-1011, Turkiye) for 2 hours with 99 amplitudes. The mixture was next extracted by maceration in a shaker (Heidolph MR 3001, Germany) for 24 hours at room temperature. Following the extraction, the mixtures were filtered through filter paper (Sartorius Stedim Grade 391) and used for analysis (Fig. 1).

2.3. Total phenolic content (TPC)

The TPC of propolis extract was measured for each solvent using the classic Folin-Ciocalteu's method [25]. For this purpose, 20 µL of extract and 400 µL of 0.2 N Folin-Ciocalteu's reagent were mixed in a test tube, to which was added 680 µL of distilled water. After 3 min of incubation, 400 mL of 10% sodium carbonate was added, and the mixture was incubated for 2 h at room temperature. Following incubation, the absorbance was read on a spectrophotometer (Thermo Scientific Evolution TM 201, UV-VIS Spectrophotometer, USA) at 760 nm. Different concentrations of gallic acid (0.5, 0.25, 0.125, 0.125, 0.0625, 0.03125, and 0.015625 mg/mL) were used in the preparation of the standard graph. This was produced with the absorbance values corresponding to the concentration, and the amount of phenolic substance as gallic acid equivalent was determined using the drawn graph.

2.4. Total flavonoid content (TFC)

The TFC of each propolis extract was measured following spectrophotometric assay [26]. Briefly, to 250 µL of each extract were added 2.15 mL absolute methanol (Merck, 106009), 50 µL 10% aluminum nitrate (Sigma Aldrich, 237973), and 50 µL 1 M ammonium acetate. The mixture was then incubated for 40 min at room temperature. After incubation, absorbance was measured at 415 nm. Different concentrations of quercetin (0.5; 0.25; 0.125; 0.125; 0.0625; 0.03125, and 0.015625 mg/mL) were used to prepare the standard graph. This was drawn with the absorbance values at 415 nm against the concentration, and the amount of quercetin equivalent flavonoid substance was determined according to the graph.

2.5. Determination of Iron (III) reducing antioxidant power-(FRAP)

The ferric reducing antioxidant power assay (FRAP) was used to measure total antioxidant capacity [27]. Freshly prepared FRAP reagent was prepared by mixing 300 mM pH 3.6 sodium acetate buffer, 10 mM TPTZ, and 20 mM $FeCl_3$ (Carlo Erba, 451695) solutions (10:1:1). Briefly, 1.5 mL of FRAP reagent and 0.05 mL of sample were placed in a test tube. After incubation at 37º C for 4 min, the absorbance was read at 595 nm. Different concentrations (31.25, 62.5, 125, 125, 250, 500, and 1000 µM) of $FeSO_4.7H_2O$ (Merck, 103965) were used to prepare the standard graph. The results were expressed as $FeSO_4.7H_2O$ equivalent antioxidant power.

2.6. DPPH˙ Radical scavenging activity

It was detected by the decrease in the maximum absorbance of the purple-violet colored commercial DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical at 517 nm in the presence of antioxidant substance. Equal amounts of DPPH solution and sample solutions were mixed and left at room temperature for 50 minutes and absorbances were measured. SC50 values were calculated by plotting the concentrations corresponding to the absorbances found[28]

2.7. Phenolic component analysis by HPLC-PDA

Before measuring the phenolic component in the propolis extracts in different solvents using HPLC-PDA analysis, phenolic component enrichment was performed by liquid-liquid extraction. The solvents of propolis extracts in different solvents were removed in a rotary evaporator (IKA®-Werke RV 05 Basic) at 40°C. Then, 10 ml of pH:2 distilled water was added and extracted, first with diethyl ether and then with ethyl acetate and combined. After all the solvents had been removed, the residue remaining in the flasks was dissolved with 2 ml of absolute methanol, passed through a 0.45 µm filter (Isolab 094.01.003), and then transferred to an instrument for phenolic analysis.

In the phenolic composition analysis method, all validations were completed against 25 phenolic standards using an RP-HPLC system (Shimadzu Corporation LC 20AT, Japan) coupled with a photodiode-array (PDA) detector. The sample was injected into the HPLC system with a reverse phase C18 column (250 mm x 4.6 mm, 5 mm; Fortis). Acetonitrile, water, and acetic acid were used for the mobile phase by applying a programmed gradient. The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile: water (70:30). Samples and standard injection volume was set to 20 µL, column temperature to 30 °C and flow rate to 1.0 mL/min[19]. Standard calibration curves of phenolic compounds were constructed with chromatograms recorded at 250, 280, 320, or 360 nm as their maximum absorbance, and the results were expressed in µg/g.

2.8. Statistics

The Kruskal Wallis non-parametric test was applied to investigate the solvent differences in propolis samples. Since a non-parametric test was used, the results are expressed as mean ± standard deviation, mean rank, and median values. The Kruskal-Wallis test comparing these groups revealed a statistically significant difference between the median values ($p<0.05$). Dunn's post-hoc test was applied to determine which group or groups caused the difference between the solvent groups. IBM SPSS version 25 software was used for all statistical analyses.

Table 1. Total phenolics and antioxidant capacities of direct propolis extractions

	TPC mg GAE/g			TFC mg QUE/g			FRAP μM $FeSO_4.7H_2O$/g			DPPH SC_{50}(mg/mL)		
	Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median
Water	3.43 ± 0.24	2.00	3.20^{a}	0.18 ± 0.02	2.00	0.18^{a}	35.58 ± 0.10	2.00	35.55^{a}	1.03 ± 0.02	17.00	1.02^{b}
Methanol (98%)	147.50 ± 5.07	14.33	148.50^{bd}	33.07 ± 0.90	14.00	33^{bcd}	801.53 ± 8.67	8.00	799.58^{ac}	0.04 ± 0.01	5.83	0.04^{ac}
Ethanol (98%)	146.33 ± 1.53	12.67	146^{bc}	30.14 ± 1.49	11.00	30.68^{bcd}	1020.67 ± 3.06	14.00	1020^{bc}	0.04 ± 0.01	5.83	0.04^{ac}
Isopropanol (98%)	130.69 ± 3.05	8.00	130.08^{acd}	25.21 ± 1.60	7.17	24.60^{ad}	981.67 ± 5.51	11.00	982^{bc}	0.08 ± 0.01	13.83	0.08^{bc}
n-Butanol (98%)	96.33 ± 2.00	5.00	97.80^{ac}	24.01 ± 0.98	5.83	24 ^{ac}	465.67 ± 6.03	5.00	465^{ac}	0.06 ± 0.01	10.83	0.06^{ab}
Ethanol (70%)	147.98 ± 2.42	15.00	148.60^{bd}	47.18 ± 1.48	17.00	47.43^{b}	1144.33 ± 7.09	17.00	1143^{b}	0.03 ± 0.01	3.67	0.03^{a}
p-value		<.01			<.01			<.01			<.01	

The different letters of a, b, c and d indicated a significant difference between the solvent groups ($p < 0.05$)

3. Result and discussion

Ultrasonic extraction of the raw propolis sample was carried out using five species' polarity solvents. The same extraction method was applied in two different ways, as direct and consecutive extractions. The extraction efficiency or capacity was evaluated according to the total phenolic contents and antioxidant capacities. The results of the first extraction values are shown in Table 1. Comparison of the total phenolic contents (TPC) of the five different solvents from polar to apolar solvent, revealed that ethanol and methanol were the phenolic substances with the highest contents, followed by isopropanol, n-butanol, and water. Among the five solvents, water is the most polar solvent, followed by methanol, ethanol, isopropanol, and n-butanol. The results showed that ethanol and methanol exhibited similar extraction capacities to the sample. Although propolis is an overly complex natural product, polyphenols represent the majority of biomolecules that can be extracted with different solvents [3,24]. Although the polyphenols in propolis have different polarities, water is not a good solvent for propolis in general, as confirmed by the results of the present study. While the amount of TPC in water was 3.43 mg GAE/g, the equivalent value in ethanol (70%) was 147.98 mg GAE/g. The TPC values of the methanolic and ethanolic solvents were remarkably close to one another. Methanol exhibits higher polarity than ethanol and was identified as a good solvent for raw propolis as ethanol. However, since methanol is a toxic solvent, it is not used for supplemental propolis extraction. A comparison of the TPC values of the two solvents with lower polarity, isopropanol and n-butanol, revealed that isopropanol had a higher TPC. However, both solvents contained smaller amounts of TPC than ethanol and methanol [29]. No significant difference was observed between the two ethanolic solvents' TPC values (Table 1). Indeed, in a previous propolis extraction study using varying percentages (from 10% to 90%) of ethanolic solutions, ethanolic solvent between 65% and 70% was identified as most effective [18].

Total flavonoid contents (TFC) were also measured in addition to TPC in this study. The amounts of TFC in the five different solvents ranged from 0.18 to 47.18 mg QUE/g. The lowest amount was found in water, and the highest in the ethanolic (70%) extract. No significant difference was determined between the 98% methanolic and ethanolic extracts. Although there were no significant differences in TPC values between these ethanolic solvents, significant differences were observed in TFC values. Analysis revealed that 70% ethanolic solvent was more successful in the extraction of flavonoids. In this study, the antioxidant capacity of the propolis extracts was determined by means of two different tests, FRAP and DPPH radical scavenging

Table 2. Total phenolics and antioxidant capacities of consecutive propolis extractions

		TPC mg GAE/g			TFC mg QUE/g			FRAP μM $FeSO_4.7H_2O$/g			DPPH SC_{50}(mg/mL)		
		Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median	Mean ± Std. Dev.	Mean Rank	Median
Water	**1.step**	3.38 ± 0.16	8.00	3.35^{a}	0.19 ± 0.02	2.00	0.18 ^{ac}	36.68 ± 0.22	8.00	36.50^{a}	1.10 ± 0.09	8.00	1.06 ^{b}
Methanol (98%)	**2.step**	130.30 ± 1.63	14.00	130 ^{ad}	41.30 ± 1.18	14.00	41.6 ^{acd}	1016.67 ± 5.74	1016	799.58^{acd}	0.04 ± 0.001	2.00	0.04 ^{bc}
Ethanol (98%)	**3.step**	15.00 ± 0.82	11.00	15 ^{ade}	3.40 ± 0.03	11.00	3.42 ^{a}	120.00 ± 1.63	11.00	120^{ac}	0.41 ± 0.02	5.00	0.42^{ab}
Isopropanol (98%)	**4.step**	2.00 ± 0.16	5.00	2 ^{bd}	1.63 ± 0.06	8.00	1.62^{bc}	19.77 ± 1.11	5.00	20 ^{bc}	5.20 ± 0.10	11.00	5.2 ^{ac}
n-Butanol (98%)	**5.step**	0.40 ± 0.02	2.00	0.4 ^{bce}	0.87 ± 0.06	5.00	0.84 ^{bd}	2.70 ± 0.22	2.00	2.6 ^{bcd}	51.23 ± 2.70	14.00	50.20 ^{a}
p-value			<.01			<.01			<.01			<.01	

The different letters of a.b.c and d are indicated a significant difference between the solvent groups ($p < 0.05$)

activity. The ferric reducing antioxidant power (FRAP) ranged from 35.58 to 1144.33 μM $FeSO_4.7H_2O$/g. The highest FRAP value was found in 70% ethanolic extracts and the lowest in water. When similar concentrations of methanol and ethanolic propolis extracts were compared, 98% ethanol exhibited a greater antioxidant capacity. The greater antioxidant capacity of the ethanolic extracts may be attributable to their higher flavonoid content. The lowest DPPH radical scavenging activity (SC_{50}) value was in the ethanolic extracts, similarly to the FRAP values, and the highest was observed in water and isopropanol.

Since one of the aims of the study was to classify the polyphenols in propolis according to their polarity, propolis extraction was performed by gradual extraction. The data obtained by sequential extraction are given in Table 2. The powdered raw propolis sample was first extracted with distilled water, followed sequentially by methanol, ethanol, isopropanol, and butanol. This low TPC value indicates that a very small part of the total phenolic substance in the raw propolis was dissolved in water. After aqueous extraction, the remaining propolis pulp was extracted with 98% methanol using a similar extraction technique. The TPC value obtained in the methanolic extract was 130.30 mg GAE/g, most of the phenolic substances being extracted from the raw propolis. The TPC values of the ethanolic extraction was found very lower (15 mg GAE/g) since the extraction was second, first methanolic extraction mostly phenolic were extracted. The TPC values in isopropanol and butanol were very low, since most of the phenolic components in propolis (approximately 95%) were extracted with methanol and ethanol. In the sequential extraction, the majority of flavonoid substances were obtained from metabolic extraction, although a small amount was extracted in ethanol. The highest antioxidant values were observed in methanolic extract, similarly to the TPC and TFC values in sequential extraction.

The phenolic components of the extracts obtained in both extractions were analyzed using HPLC-PDA. The measurement results based on twenty-five phenolic

Table 3. Phenolic composition of direct and sequential propolis extracts

	Phenolic Standards (μg/g)	Water	98% Methanol	98% Ethanol	98% isopropanol	98% n-butanol	70% Ethanol	Water (1)	Methanol (2)	Ethanol (3)	2-Propanol (4)	Butan-1-ol (5)
Phenolic acids	***Hydroxybenzoic acids***											
	***p*-OH Benzoic acid**	47	48	—	36	—	74	45	46	6	2	1
	***m*-OH Benzoic acid**	—	—	—	—	—	—	—	—	—	—	—
	Protocatechuic acid	63	—	—	—	—	—	48	—	—	—	—
	Gallic acid	13	31	—	—	—	—	12	—	—	—	—
	Chlorogenic acid	—	—	35	—	—	99	—	—	—	—	—
	Syringic acid	—	—	—	—	—	27	—	—	—	—	—
	Ellagic acid	153	—	—	—	—	—	230	—	—	36	—
	Hydroxycinnamic acids											
	***t*-cinnamic acid**	7	373	289	205	195	331	40	384	47	7	2
	Ferulic acid	251	1544	1344	1214	756	2121	344	1255	152	18	4
	***p*-Coumaric acid**	170	1192	1054	856	574	1561	335	893	114	13	3
	Caffeic acid	753	1335	1121	1023	636	1719	912	1025	131	17	3
	CAPE	—	2150	1785	1530	1112	2237	—	2207	255	—	—
Flavonoids	***Flavonol***											
	Rhamnetin	—	390	—	—	—	—	—	360	—	—	—
	Quercetin	—	301	256	144	164	451	—	272	72	3	—
	Rutin	—	—	—	—	—	—	—	—	—	—	—
	Myricetin	—	—	—	—	—	—	—	—	—	—	—
	Flavan-3-ols											
	Epicatechin	—	—	—	—	—	—	—	—	—	—	—
	Flavones											
	Chrysin	15	5534	5046	4878	3106	7851	21	5928	744	74	16
	Daidzein	—	—	—	—	—	—	—	—	—	—	—
	Apigenin	—	609	509	555	298	741	—	443	59	7	2
	Luteolin	—	—	—	—	—	19	—	—	—	—	—
	Flavanones											
	Pinocembrin	16	5466	5072	5162	3116	8013	25	5645	705	76	15
	Hesperetin	87	2496	2240	2095	1318	441	101	2655	322	27	5
	Other polyphenols											
	Curcumin	—	—	—	—	—	—	—	—	—	—	—
	Resveratrol	—	—	—	—	—	—	—	—	—	—	—

(—): not detected

compounds are given in Table 3. In the first extraction technique, the majority of the phenolic acids (hydroxybenzoic and hydroxycinnamic acids) were detected in water extract, although a small number of flavonoids were also detected. The most abundant phenolic acid in the aqueous extract was caffeic acid. A previous study using honey as a green solvent also reported that aqueous propolis extract is rich in caffeic acid [24]. This is one of the derivatives of hydroxycinnamic acids, and the hydroxyl groups it contains bestow a polar character on it. However, since this polarity is lower than in water, it is more prone to dissolve in ethanolic and methanolic solvents. Derivatives of hydroxycinnamic acids from polyphenol subclasses emerged as leachable with all the solvents used. In addition, the derivatives of hydroxycinnamic acids, one of the subclasses of polyphenols, emerged as the molecules with the highest extractable quality in the five solvents. Chrysin, one of the flavone derivatives, was soluble in all organic solvents except for water, but was extracted at the highest level with 70% ethanol. Chrysin, one of the major components of propolis, is an important component of complementary medicine due to its high biological activity [30].

The methanolic and ethanolic propolis extracts in this study exhibited similar phenolic compositions. However, ethanol should be used in consumable propolis extracts due to the toxic effect of methanol, although methanol can be used as a suitable solvent for analytical studies. In the present study, two different concentrations of ethanol were used, and it may be concluded that 70% ethanol is more suitable for propolis extraction. Phenolic compounds derived from hydroxybenzoic acids were detected at low levels in the propolis sample, while water and 70% ethanolic were the most suitable for these compounds. The isopropanol and n-butanoic extracts were found to be rich in flavonoids.

CAPE, the most important compound in propolis, was detected at the highest level in methanol and 70% ethanol. CAPE is a polyphenol compound that has attracted considerable attention in recent years due to its high antioxidant properties, as well as significant anti-inflammatory, neuroprotective, and antitumoral activities [30–33]. The first aqueous extraction was performed using the sequential extraction technique. The profiles of phenolic compounds obtained in both aqueous extractions were very similar, although there were small differences between the amounts of phenolic compounds. Similarly to the first extraction, the methanol and ethanolic extracts were found to be rich in both phenolic acid and flavonoids in the second extraction. A large amount of CAPE was extracted with methanol. The phenolic component profiles of isopropanol and butanoic extracts were similar to one another but contained very small amounts of phenolic compounds.

4. Conclusion

Ethanolic propolis extracts exhibited high phenolic acid and flavonoid contents and were also the most antioxidant-rich extracts. Solvents with higher polarities also contained larger amounts of phenolic acids, while lower polarity solvents exhibited larger quantities of flavonoids. The solvent with the highest phenolic components and antioxidant capacity was 70% ethanol.

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Declaration of competing interest

The authors declare no competing interest.

Author contributions

Sevgi Kolaylı: Designed the hypotheses, statistical analysis, evaluated the results, and drafted the manuscript. Ceren Birinci: Formol analyses and HPLC studies.

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