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The use of honey as a green solvent in the extraction of raw propolis

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Abstract

Propolis is a resinous natural mixture taken by scraping beehives. It is used as a food supplement due to its high biological active properties. After extracting crude propolis with various solvents, it is used as propolis extracts. The best propolis extraction is obtained with 70% ethanol, while green solvents are preferred because of some side effects of alcohol. Recently, green solvents have attracted interest in the extraction of propolis. In this study, the solubility of raw propolis in honey was investigated. The results were evaluated as total phenolic content and total antioxidant activity. In the study carried out with honey:water mixtures in different ratios, it was determined that the most ideal ratio was 1:20 (0.333 ± 0.033 mg GAE/mL). As a result, the production of such solvents from beehive products further encourages diversification of bee products and the discovery of new applications using environmentally friendly solutions.

Keywords: Solvent, honey-water, propolis, extract

1. Introduction

Honey is a sweet, viscous food substance produced by bees. Bees produce honey by collecting nectar from flowers and processing it in their digestive system, regurgitating and storing it in honeycombs. The process of creating honey involves enzymatic activity, evaporation, and the addition of enzymes from the bees. Honey has been used as a food, medicine, and sweetener for thousands of years. It has a long history of use in traditional medicine for its antibacterial and wound-healing properties. Honey is also rich in antioxidants and has been associated with a range of health benefits, including reducing inflammation and improving heart health [1,2]. Honey can vary in color and flavor depending on the type of flower nectar collected by the bees. Some common types of honey include clover, orange blossom, and manuka honey. Manuka honey, which is produced in New Zealand and Australia, has gained popularity for its high antibacterial properties and is used in wound care and other medical applications. While honey is generally considered safe for consumption, it should not be given to infants under one year of age due to the risk of botulism. Honey may also cause allergic reactions in some people [3,4].

Propolis is a natural resinous substance collected by honeybees from various plants and trees. Bees use propolis to seal small gaps and cracks in their hives, as well as to protect against infections and other threats. Propolis is a complex mixture of plant resins, beeswax, essential oils, and various organic compounds [5]. Propolis has been used in traditional medicine for centuries, particularly for its antimicrobial and anti-inflammatory properties. Research has also shown that propolis has antioxidant, immunomodulatory, and anticancer effects [6]. There are several types of propolis, which can vary in color and chemical composition depending on the location and plant sources used by the bees. Some of the most common types include Brazilian propolis, European propolis, and Chinese propolis. Propolis can be taken as a supplement or used topically as a natural remedy for various health conditions. Some of the most common uses of propolis include treating sore throat, colds and flu, dental infections, and skin problems. While propolis is generally considered safe, it may cause allergic reactions in some people. It may also interact with certain medications, so it is important to talk to a healthcare provider before using propolis supplements [7].

The escalating utilization of non-renewable substances has prompted researchers to seek alternatives that are renewable and pose lesser risks. Green

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chemistry plays a crucial role in this context. Extensive research has been conducted to explore less toxic or bio-based solvents known as green solvents. Water, solvents derived from biological sources, ionic liquids (ILs), deep eutectic solvents (DESs), green synthetic organic solvents, and supercritical liquids (SCF) stand out as prominent classifications within the realm of green solvents.

Propolis is a complex mixture of plant resins, waxes, essential oils, and other biologically active compounds. The choice of solvent used in propolis extraction can have a significant impact on the chemical composition and bioactivity of the resulting extract. Here are some of the solvents that have been used for propolis extraction along with their advantages and disadvantages:

Ethanol: Ethanol is a commonly used solvent for propolis extraction. It is relatively inexpensive and readily available and has been shown to extract a wide range of bioactive compounds from propolis. However, high concentrations of ethanol can cause degradation of some of the more sensitive components of propolis [8-10].

Methanol: Methanol is another solvent that has been used for propolis extraction. It has similar advantages and disadvantages to ethanol but is generally less effective at extracting certain bioactive compounds [11, 14].

Water: Water is a safe and environmentally friendly solvent for propolis extraction. However, it is generally not as effective as organic solvents at extracting lipophilic compounds and may require the use of additional solvents or extraction techniques to obtain a high-quality propolis extract [12]. Freitas et al. compared the efficiency of honey brandy and mead in propolis extraction with ethanol and water in a study they conducted in 2022 with the hypothesis of environmentally friendly solvent [13]. When we look at the studies on environmentally friendly solvents or green solvents, it is seen that there is an open field for research. It has been observed that there are almost no studies on the effectiveness of the use of honey-water mixture as a solvent in propolis extraction under different extraction conditions.

In this context, it is aimed to determine the ratio of honey-water mixture as green solvent for propolis extraction and the best propolis-solvent ratio with this honey-water mixture. In addition, the antioxidant efficiencies of propolis evaporated after alcoholic extraction in honey, water and honey-water mixture were investigated.

Table 1. The effectiveness of the ethanolic propolis residue in the honey-water mixture

BP1	Honey + 70% EtOH
BP2	Residue + 70% EtOH
BP3	Honey + Residue + 70% EtOH
BP4	Honey-Water Solution
BP5	Residue + Water
BP6	Residue + Honey-Water Solution

2. Material and methods

2.1. Sample extraction

In the study, Anatolian propolis obtained from various beekeepers and flower honey obtained from the Black Sea region were used. Until the time of analysis, propolis was stored at -20 °C and honey was stored under dark and room conditions. Honey and propolis samples used were extracted in methanol and 70% ethanol at a ratio of 1:10 (24 hours, 200 rpm, room temperature), respectively [10]. Antioxidant properties and phenolic components of propolis and honey samples used in the study were determined. Propolis:honey-water extraction efficiency studies were performed on these two samples. This study was planned in three stages.

-The ratio of the honey-water mixture to be used was determined (at a fixed amount of propolis).

-The ideal propolis-solvent ratio was determined in the solvent at the determined honey-water ratio.

-The effectiveness of the ethanolic propolis residue in the honey-water mixture was investigated.

Firstly, honey-water solutions were prepared at 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, 1:20 and 1:40 (propolis-solvent) ratios to determine the ratio of honey-water mixture to be used in propolis extraction (200 rpm, 24 h, rt). Secondly, different amounts of propolis (0.25, 0.5, 0.75, 1, 2, 3 and 4 g in 10 ml solvent) were extracted in the determined honey-water ratio solution. Finally, after the propolis extract extracted with 70% ethanol was evaporated, the residue was dissolved in 2 ml of 70% ethanol. The following experiments were carried out with this residue (Table 1). In the experiments, 1 g of honey, 500 microliters of propolis residue and 10 mL of solvent (70% EtOH, HW and water) were used. The total phenolic (TP) values of the extracts were analyzed, and the results are given as mg GAE/mL extract. Since the main theme of our study was honey-water mixture, firstly, the extraction efficiency of crude propolis was examined. Then, the efficiency of ethanolic propolis residue in the honey-water mixture was tried to be determined.

2.2. Antioxidant capacity

The method described by Slinkard and Singleton (1977) was used to determine the total phenolic content [15]. Initially, 20 µL of the sample was mixed with 680 µL of distilled water. Subsequently, 400 µL of 1:10 diluted Folin-Ciocalteu reagent added to the mixture and

incubated for 4 min. Then, 400 µL of 10% Na_2CO_3 solution was added, and the resulting mixture was allowed to incubate at room temperature for 120 min. Spectrophotometric measurement was performed at 760 nm, and the total amount of phenolic substance present in the sample was determined. The results were expressed as mg gallic acid equivalent (mg GAE/g) using gallic acid as a standard.

To determine the total flavonoid substances, the method outlined by Fukumoto and Mazza (2000) with slight modifications was utilized [16]. First, 2150 µL of methanol was added to 250 µL of the sample, followed by the addition of 50 µL of 10% $Al(NO_3)_3$ and 50 µL of 1 M NH_4CH_3COO solution. After incubation for 40 min, the absorbance of the resulting colored product formed because of the redox reaction between flavonoids and aluminum (III) was measured at a wavelength of 415 nm. The amount of flavonoid substance present was calculated using quercetin as a standard, and the results were expressed as mg quercetin equivalent (mg QUE/g).

2.3. Antioxidant activity

The antioxidant activity was determined using the widely used 2,2-diphenyl-1-picryl hydrazyl (DPPH) test. The DPPH• radical, which has a maximum absorbance at 517 nm, was used as a synthetic radical for this test. The method proposed by Molyneux (2004) was used to assess the scavenging of DPPH radicals with antioxidants present in the sample [17]. A 100 µM methanolic DPPH• solution was employed. In this assay, sample solutions of equal volume were added to the constant DPPH• radical concentration in the medium after being serially diluted. Trolox was utilized as a standard, and the results were expressed as the SC50 value (mg/ml).

The Ferric reducing/antioxidant power (FRAP) assay was used to determine antioxidant activity based on the reduction of Fe(III)-TPTZ-2,4,6-tris(2-pyridyl)-S-triazine complex in the presence of antioxidants [18]. Fresh FRAP reagent was prepared by mixing pH:3.6 acetate buffer, TPTZ , and $FeCl_3$ solution in a ratio of 10:1:1. A volume of 50 µL of the sample was added to 1500 µL of FRAP reagent, and the absorbance was read at 593 nm after 4 min. A standard curve was constructed using different concentrations of $FeSO_4.7H_2O$, and the results were reported as µmol $FeSO_4.7H_2O$/g [10].

2.4. Phenolic component

The HPLC-PDA analysis of the samples for phenolic content specified by Kara et al. involved preparation and subsequent injection into the device [19]. The analysis was performed using a Shimadzu Corporation LC 20AT HPLC system with a mobile phase consisting of a gradient of 70-30% acetonitrile-ultrapure water and 2% acetic acid-ultrapure water. The sample injection volume was 20 µL, the mobile flow rate was 1.0 mL/min, and the column oven temperature was maintained at 30 °C. Analysis of the phenolic content was done using 25 standard substances. The results were expressed in µg of standard phenolic substance per gram of sample.

3. Results and discussion

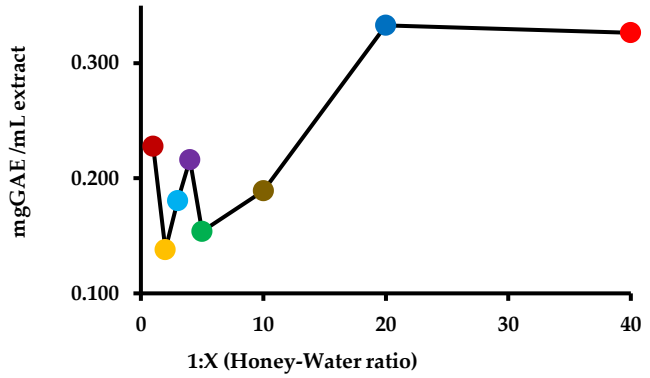
Antioxidant and phenolic analysis results of propolis and honey samples used in the experiment are given in Table 2. According to research, the total phenolic content in propolis varies depending on the source and solvent used. One study by Cottica et al. (2011) found the range to be between 31-299 mg of GAE/g of ethanolic extract [21]. Another study by Stoia et al. (2015) analyzed 10 propolis samples and reported an average of 9.71 ± 0.80 mg GAE/g for extracts [20]. In our study, the TP value of the propolis used was 28.847 ± 0.221 mg GAE/g, highlighting the variability in obtaining a propolis extract rich in polyphenolic components. Research has demonstrated a correlation between the antioxidant capacity of honey and its polyphenol content [22]. Chestnut honey, heather honey, and oak honey possess

Table 2. Antioxidant and phenolic component analysis results of honey and propolis

Analysis		Sample	
		Honey	**Propolis**
TP (mg GAE/ g sample)		0.141 ± 0.013	28.847 ± 0.221
TF (mg QE/ g sample)		0.035 ± 0.002	1.119 ± 0.072
FRAP (µmol $Fe_2SO_4.7H_2O$/g sample)		3.320 ± 0.041	317.347 ± 2.573
DPPH SC_{50} (mg/ml)		20.400 ± 0.085	0.135 ± 0.003
µg phenolic /g sample	Gallic acid	—	47.596
	Protocatechuic acid	—	—
	Chlorogenic acid	—	—
	p-OH benzoic acid	1.726	—
	Epicatechin	—	—
	Caffeic acid	—	231.544
	Syringic acid	—	—
	m-OH benzoic acid	—	—
	Rutin	—	84.681
	Ellagic acid	—	996.589
	p- coumaric acid	2.162	270.568
	Ferulic acid	—	401.705
	Myricetin	—	—
	Resveratrol	—	—
	Daidzein	—	—
	Luteolin	—	24.957
	Quercetin	—	57.726
	t-cinnamic acid	1.587	41.521
	Apigenin	—	93.093
	Hesperetin	1.964	—
	Rhamnetin	—	—
	Chrysin	8.548	775.017
	Pinocembrin	5.672	662.761
	CAPE	—	—
	Curcumin	—	—

Table 3. The ratio of the honey-water mixture for propolis extraction

Ratio	TP (mg GAE/mL extract)
1:1	0.228 ± 0.031
1:2	0.138 ± 0.021
1:3	0.180 ± 0.020
1:4	0.216 ± 0.009
1:5	0.154 ± 0.008
1:10	0.189 ± 0.010
1:20	0.333 ± 0.033
1:40	0.326 ± 0.012



a total polyphenol content of around 1 mg GAE/g. In addition, other types of flower honeys have lower values and as a result, its antioxidant properties are lower. In our study, we determined that the TP value of the flower honey sample used was 0.141 ± 0.013 mg GAE/g.

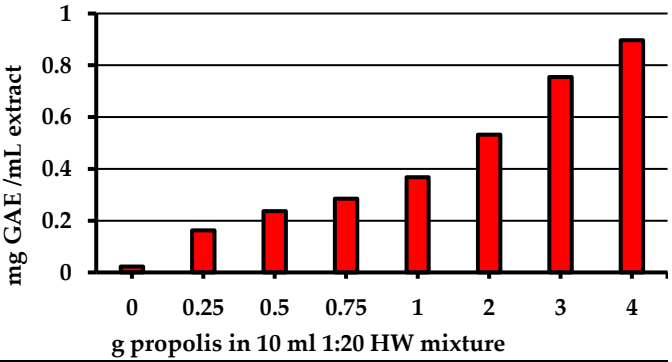
The TP value of the extracts obtained to determine the honey-water mixture to be used in propolis extraction is given in Table 3. The TP value of the extract was calculated by subtracting the antioxidant value of the honey sample. Although there is no clear order in the total TP content obtained in the extracts at the ratios of 1:1 to 1:4, it is seen that the TP value of the extract prepared at the ratio of 1:20 is the highest. It can be thought that the 1:20 ratio of honey-water mixture has the highest ionic and molecular level interactions with the phenolic compounds in propolis. In the following steps, it was decided to use a 1:20 honey-water mixture.

To determine the ideal propolis-solvent ratio, the TP value of the solvent used in the extractions was also calculated. Extraction was performed by adding different amounts of propolis to equal solvent volumes and the TP values of the obtained extract were calculated (Table 4). When we look at the results obtained, it is seen that the TP values per unit volume of the extract increase with the increasing amount of propolis. However, it is also seen that this increase is not directly proportional to the increase in the amount of propolis used in extraction. As a result, the amount of propolis used in the extraction can be increased to obtain higher antioxidant activity, but it should not be ignored that the yield to be obtained from the gram amount of propolis will decrease.

The TP values of the samples after processing were calculated to look at the agonistic or antagonistic activity of ethanolic propolis residue and honey in ethanol and HW mixture (Table 5). When the findings were examined, it was observed that the extract, in which honey and propolis residues were used together (1.313 ± 0.013), was higher than the sum of the TP values of honey and propolis residue prepared separately in ethanol (0.036 ± 0.001 and 1.141 ± 0.006, respectively). Nonetheless, in the extracts prepared using HW mixture and water, it was noted that the TP value of the extract (0.202 ± 0.004), in which the propolis residue was prepared in HW mixture, remained lower compared to the extract (0.279 ± 0.005) where the propolis residue was prepared solely in water. As a result of removing the alcohol from the ethanolic propolis extract and re-dissolving it only in HW mixture, a higher TP value could not be obtained as desired in terms of TP value. In addition, an upward trend was observed in the TP value with the mixing of honey and propolis residue in ethanolic solvent. Based on this information, new and different extraction conditions can be obtained by adding different components or ambient conditions (such as temperature) while preparing the honey-water mixture.

Table 4. The ideal propolis-solvent (1:20 HW mixture) ratio for propolis extraction

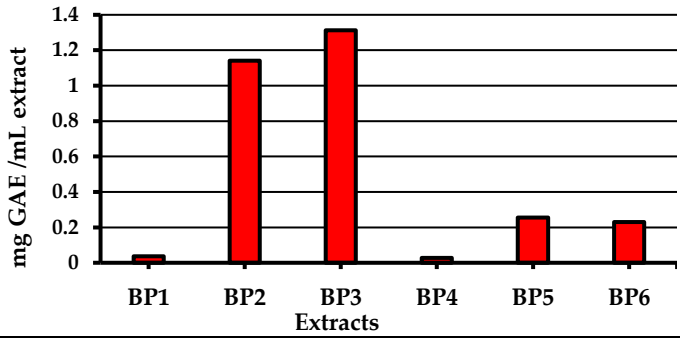
g propolis in 10 ml 1:20 HW mixture	TP (mg GAE/mL extract)
0	0.023 ± 0.005
0,25	0.163 ± 0.006
0,5	0.237 ± 0.006
0,75	0.285 ± 0.013
1	0.368 ± 0.015
2	0.532 ± 0.008
3	0.755 ± 0.019
4	0.897 ± 0.004



*HW: Honey-Water

Table 5. The effectiveness of the ethanolic propolis residue in the honey-water mixture

Extract	TP (mg GAE/mL extract)
BP1	0.036 ± 0.001
BP2	1.141 ± 0.006
BP3	1.313 ± 0.013
BP4	0.028 ± 0.002
BP5	0.256 ± 0.005
BP6	0.230 ± 0.004



In the literature, there are different propolis extraction methods in which maceration, ultrasound assisted, and microwave assisted extraction, Soxhlet, supercritical CO_2 extraction, high pressure methods and different solvents are applied [8]. Although researchers generally agree that the best solvent is ethanol-water mixtures, they are trying to obtain high efficiency propolis extraction with non-alcoholic and green solvents. Researchers studying the effectiveness of honey brandy and mead on propolis extraction state that extracts prepared with these two solvents have a non-negligible antioxidant and antibacterial effect (26.6 ± 2.8% and 6.5 ± 1.0%, respectively)[13]. As an alternative to ethanolic solvents used in propolis extraction, Natural Deep Eutectic Solvents (NADES) has started to come to the fore. In a study conducted to provide equivalent antioxidant content and efficacy, it was stated that the extracts obtained with NADES solvents made from choline chloride-propylene glycol or lactic acid at 50 °C could be equivalent to that with 70% EtOH. Again, in this study, they stated that NADES, aqueous L-Lysine and honey solutions can replace ethanol or water [23]. In another study, the antioxidant properties of aqueous (AqEP), polyethylene glycol-aqueous (Pg-AqEP) and ethanolic (EEP) propolis extracts were compared. As a result of the data they obtained, they stated that phenolic acids and aldehydes constituted 40-42% of all compounds extracted and identified in AqEP and Pg-AqEP and 16% in EEP. As a result of their cell culture study, they reported that they showed similar antioxidant activity, but Pg-AqEP and EEP had better mitochondrial superoxide effect [24]. Researchers, on the other hand, reported that aqueous extracts of propolis have stimulatory activity on cell proliferation in vitro [25]. Considering all these studies it is seen that green solvent studies to replace ethanolic propolis extracts have gained importance in recent years. However, in the extractions made with only water, the lack of efficiency at the level of the ethanolic extract leads to experimenting and using aqueous solvent mixtures. It has been reported that the use of honey, which is also a bee product and its products, has small or large effects on the effectiveness of propolis extracts.

4. Conclusions

The consumption of propolis, which is a valuable natural product with various biological activities among bee products, is increasing day by day. This pushes the researchers to optimize the extraction of this product. Recently, studies have been carried out especially on NADES and water-based extract preparation methods. For this reason, the effect of honey-water mixture as a water-based solvent on the antioxidant efficiency of propolis extract was investigated in our study. When we look at the first step results of our study, a significant increase in antioxidant activity was observed with propolis extraction using 1:20 HW mixture. Higher antioxidant activity can be obtained by improving the extraction conditions. However, the amount of propolis to be extracted in the determined HW mixture is also important. While the high amount of propolis increases the antioxidant activity to a certain extent, there is a decrease in the extraction efficiency after a certain point. Finally, after evaporation of the ethanolic propolis, after the residue was dissolved in HW and EtOH, it was seen that there was no significant difference in antioxidant activity between these two cases. The extract prepared with HW mixture did not provide high antioxidant as expected. Of course, more work is needed to find the best way to extract bioactive compounds from different types of propolis on a green solvent basis.

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