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# THE EFFECTS OF DIFFERENT EXTRACTION METHODS AND SOLVENTS ON ANTIOXIDANT PROPERTIES OF PROPOLIS

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**Abstract:** Propolis is a complex sticky substance produced by the honey bees (*Apis mellifera*) from the resinous materials they collect from various parts of plant and used by the bees to defend their hives from pathogenic microorganisms. This research aimed to compare the antioxidant properties of propolis extracts produced by using different solvents and extraction methods. The method used in the extraction stage is of great importance as the amount and quality of the bioactive components in the final product are directly affected by the extraction method applied. To obtain propolis extracts, both classical and ultrasonic extraction methods were used with distilled water and 20% propylene glycol-distilled water as solvents. Folin-Ciocalteau method was used to record the total phenolic content of propolis extracts. In this study, to measure the antioxidant activity of extracts, three methods were used that are FRAP, DPPH, and ABTS. Moreover, the aluminum chloride colorimetric method was used for total flavonoid content analysis. According to analysis, brix values recorded between 14.90-27.50 for classic method and 14.40-16.50 for ultrasonic method. The total phenolic content calculated as 721.31-14419.46 mg GAE/L for classic method, and 1212.32-33621.70 mg GAE/L in ultrasonic method. Also, the total amount of flavonoid content was measured as 1137.52-24884.70 mg QE/L extract in ultrasonic method, and 2144.77-74021.42 mg QE/L extract in classic method. DPPH radical scavenging activity of the samples were calculated as 0.46-15.21 $IC_{50}$ µl/ml in classic method, and 1.36-31.86 $IC_{50}$ µl/ml in ultrasonic method, ABTS+ values changed from 0.09-2.71 $IC_{50}$ µl/ml in classic method, and 0.21-4.64 $IC_{50}$ µl/ml in ultrasonic method, and FRAP values measured between 29.22-639.43 µM TE/g in ultrasonic method, and 54.72-1783.02 µM TE/g in classic method. More studies and analysis are needed to investigate the effects of solvents and extraction methods on propolis extracts, as well as on the antioxidant properties of these extracts.

**Keywords:** Propolis, Extraction methods, Flavonoid, Antioxidant activity

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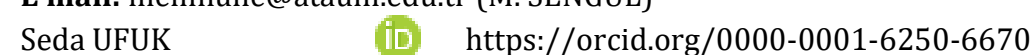
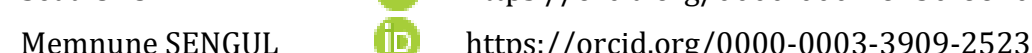
Seda UFUK  https://orcid.org/0000-0001-6250-6670
Memnune SENGUL  https://orcid.org/0000-0003-3909-2523
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## 1. Introduction

Propolis is a natural bee product collected from diverse parts of different plant sources by honey bees (*Apis mellifera L.*) and mixed with materials resulting from bees' metabolism (Freitas et al., 2022; Kegode et al., 2022). Propolis is a gummy, sticky, lipophilic, and balsamic product, comes from two different Greek words 'pro' meaning in defense, and 'polis' meaning the city (Belmehdi et al., 2022; Hossain et al., 2022). Honeybees use propolis to coat openings in the hive and to prevent predators from entering the hives. Also, propolis is used by honey bees to maintain a constant internal temperature in the hive, and to contribute to the creation of an aseptic environment, and generally protect the hive from common microbial infections caused by bacteria, fungi, and yeast (Dogan and Hayoglu, 2012; Tumbarski et al., 2022; Abd Rashid. et al., 2022).

Propolis, one of the most interesting substances produced by honey bees, draws attention in many areas in the field of health. Throughout history, natural products such as propolis have been widely used to alleviate and prevent diseases, also to increase body resistance (Acun and Gul, 2020). Propolis has been used for many years and is used today in various formations such as personal products, handmade medicines, functional food ingredients, food supplements, and over-the-counter products (Freitas et al., 2022; Tumbarski et al., 2022). Along with in vivo and in vitro studies, it has been reported that propolis has many biological activities. Various biological properties of propolis such as antiviral, antioxidant, antiallergic, anti-inflammatory, antibacterial, hepatoprotective, antiparasitic, antiulcerogenic, anticancer, antidiabetic, and other therapeutic effects have been evaluated with various studies (Belmehdi et al., 2022).

The health properties of propolis are attributed to components such as polyphenols, phenolic aldehydes, sesquiterpene-quinones, coumarins, amino acids, steroids, and inorganic compounds (Sagdic et al., 2020). The composition of propolis, which has a characteristic smell and taste, varies according to the conditions of the region where it is collected, the time of collection, and the plant variety from which it is produced. In addition, the

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diversity in beeswax also affects the chemical composition of crude propolis (Dogan and Hayoglu, 2012). Generally, propolis consists of 50% resin and herbal balm, 30% bee wax, 5% pollen, and 10% essential and aromatic oils, and more than 420 components have been identified in its content (Gumus and Kizil, 2022; Escriche and Juan-Borrás, 2018). The main components of propolis include aromatic acids (cinnamic acid, caffeic acid, ferulic acid), aromatic esters (cinnamic and caffeic acid ethyl esters), volatile compounds (geraniol, nerol, farnesol, β-eudesmol), aromatic compounds (vanillin), hydrocarbons (eicosan, trichosan, pentacosan), steroids (cholinasterol, fucosterol, stigmasterol), flavonoids (pinocembrin, chrysin, galangin, apigenin, kaempferol), acids (palmitic acid, melisic acid, serotic acids), minerals (calcium, potassium, magnesium, sodium, zinc, chlorine, iron), vitamins (vitamins A, B1, B2, B3, B5, B6, B7, C and E) and essential oils (Gumus and Kizil, 2022; Ożarowski and Karpiński, 2023; Yildiz and Unal, 2022). Moreover, propolis contains enzymes such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid phosphatase (Dogan and Hayoglu, 2012).

Propolis is commercially available in different formulations such as capsules, mouthwash solutions, cosmetics, powders, shampoos, lotions, lipsticks, nail polishes, beverages, and foods. There are also chewable capsules and tablets such as throat lozenges, chewing gum, and candy (Irigoiti et al., 2021; Dogan and Hayoglu, 2012; Anjum et al., 2019). Propolis cannot be consumed directly as it is taken from the hive. In the first process, the raw wax and foreign materials in propolis must be removed, and then it is made ready for consumption by extraction. The method used in the extraction stage is of great importance because the amount and quality of the bioactive components in the final product are directly affected by the extraction method applied. Today, more efficient extraction methods such as ultrasonic-assisted extraction, microwave-assisted extraction, supercritical extraction, and classical extraction have been developed and used (Sagdic et al., 2020).

Even though many solvents are utilized in the extraction of propolis, merely water, ethanol, olive oil, propylene glycol, and glycerol (glycerin) are included in the legislation. Propylene glycol, which is chemically in the diol class; is miscible with many solvents such as water, chloroform, and acetone (Bakkaloglu and Arici, 2019). Yet, although propolis is appropriate for usage in different areas, there is concern about the solvents used for extraction processes in the food sector. Solvents that do not pose a threat to health should be used in propolis extracts offered for people's consumption. The usage of ethanol as a solvent in the extraction of propolis poses problems, mainly for pregnant women and consumers with halal/haram sensitivity (Bakkaloglu and Arici, 2019). On the other hand, the negative consequences of alcohol (sensory problems in the final product, limited use in cosmetic and pharmaceutical products, inability to use in children and pregnant women) led to the development of non-alcoholic extraction methods (Sagdic et al., 2020). Therefore, to decrease the usage of alcohol as a solvent for extraction of propolis, other types of solvents can be available including distilled water, oils and glycerol. Distilled water, one of the solvents in the legislation, is not preferred because it cannot dissolve the bioactive components in propolis at a sufficient rate (Bakkaloglu and Arici, 2019). For this reason, to increase the extraction efficiency of distilled water during propolis extraction, some tensoactive compounds can be added to the distilled water, such as sodium lauryl sulfate, propylene glycol, polysorbates and polyethylene glycol (Yeo et al., 2015).

Propylene glycol (propane-1, 2-diol) is a colorless, odorless and tasteless substance that is used in foods, drugs and cosmetic products due to its ability to dissolve hydrophobic compounds and water retention, and has a slightly viscous behavior at room conditions, soluble in water, ethanol and acetone. It is a synthetic substance with a density of 1.035 g/mL. Propylene glycol is unstable to sunlight, air, oxidizing agents, acid, base and high temperature and can oxidize (Karadag et al., 2022). Propane-1,2-diol is approved as a food additive (E 1520) in the European Union consistent with Regulation (EC No:1333/2008) on food additives and in the Turkish Food Codex Regulation on Food Additives dated 30/06/2013 (No:28693) (Aggett et al., 2018).

Since the properties of solvents used in propolis extraction are different, different results are found in determining the properties of propolis such as total phenolic substance content, and antioxidant capacity (Galeotti et al., 2018). Therefore, in this study, the effects of the extraction of propolis by different solvents and extraction methods on the amount of soluble solid matter, total phenolic content, antioxidant capacity, the total amount of flavonoid substances of the extracts were examined.

## 2. Materials and Methods

### 2.1. Materials

In this study, crude propolis was produced from different types of plants such as poplar, filbert etc. by *Apis mellifera Caucasica* and this propolis was obtained directly from honey producers in Ardanuç-Artvin in Türkiye. The fresh propolis was stored at refrigerator temperature (4-6°C) until the utilization of analysis.

### 2.2. Propolis Extraction Procedures

For propolis extraction, classic and ultrasonic extraction procedures were utilized, and propolis samples were extracted with distilled water and propylene glycol (20%) solvents (Juodeikaitė et al., 2022; Rodiahwati et al., 2019). For extraction, 25 g crushed propolis samples were macerated in 250 ml distilled water and also 250 ml %20 propylene glycol. The extraction process was prepared according to the propolis/solvent ratio of 1:10 (Keskin, 2018).

In the classic extraction method, 25 g propolis-distilled water (PKS) and 25 g propolis-propylene glycol (20%) (PKPG) samples were agitated in the shaking water bath (JSR, JSSB-30T, Korea) in dark-colored flasks at 40℃ with 90 rpm for 20 hours (Topdas and Sengul, 2021). The extracts were centrifuged at 4500 rpm at 4℃ for 15 minutes and filtered two times with Whatman No:2 and Whatman No:42, respectively. Then, the filtered supernatants were evaporated at 40°C with 150 rpm. After evaporation, the extracts were stored at -20 °C until utilization. Since the evaporation temperature of propylene glycol is high (188°C), and the structural property of propolis will deteriorate after the solvent is evaporated above 50°C, it was not processed in the vacuum evaporator for propylene glycol, however distilled water solution was evaporated and propolis extracts were obtained with the classic extraction method (Arslan et al., 2010).

In the ultrasonic extraction method, 25 g propolis-distilled water (PUS) and 25 g propolis-propylene glycol (20%) (PUPG) samples were agitated in an ultrasonic bath (Bandelin Sonorex Super RK 103 H) at 40 °C, 35 kHz frequency for 20 minutes with four times minutes (Topdas and Sengul, 2021). The extracts were centrifuged at 4500 rpm at 4°C for 15 minutes and filtered two times with Whatman No:2 and Whatman No:42, respectively. Then, the filtered supernatants were evaporated at 40°C with 150 rpm. After evaporation, the extracts were stored at -20 °C until utilization. Also, for ultrasonic extraction method, solution with propylene glycol was not removed by evaporation because it has a high evaporation temperature (Arslan et al., 2010).

**2.3. Experiments**

**2.3.1. Soluble solids content**

An Abbe refractometer device (Carl Zeiss) was used to determine the % dissolved solids of propolis extracts. The values measured using a refractometer were expressed as the propolis extract° Brix value (Cemeroglu, 2013).

**2.3.2. Total flavonoid content**

The aluminum chloride colorimetric method was used for flavonoid analysis. Quercetin (QE) at different concentrations (0.4; 0.3; 0.25; 0.2; 0.16; 0.12, 0.08, and 0.04 mg/L) was used as a standard in the assays. The absorbance of the tubes against distilled water at 415 nm was recorded 40 minutes after the pipetting process was completed. The standard was plotted with the recorded absorbance values versus the concentration. The total flavonoid substance content of propolis extracts was calculated according to the standard graph and the total flavonoid amount was expressed as Quercetin equivalent/L propolis extract (Meda et al., 2005).

**2.3.3. Total phenolic content**

The total phenolic content of propolis extracts was determined using the Folin-Ciocalteau method. A calibration chart using the Gallic acid (GA) standard was prepared using solutions of Gallic acid at different concentrations (10; 25; 50; 75; 100; 150; 200 and 250 mg/L) and results were expressed as mg GAE/L propolis extract in Gallic acid equivalents (Cemeroglu, 2013). The absorbance results were measured at 760 nm after incubation for 30 minutes at room temperature (Asem et al., 2020; Meda et al., 2005)

**2.3.4. Antioxidant capacity**

The antioxidant activity of propolis extracts was determined by three different procedures which are FRAP, ABTS, and DPPH methods.

In the FRAP (Ferric Reducing Antioxidant Power) assay; the Antioxidant activities of the samples were determined by the $Fe^{3+}$ reduction method. The basis of the FRAP assay, known as the iron-reducing capacity, is the reduction of $Fe^{3+}$ ions in the Fe $(TPTZ)^{3+}$ complex to the blue-colored $Fe(TPTZ)^{2+}$ complex in an acidic medium by antioxidant components. For analysis, three different chemicals were used which were acetate buffer (pH 3,6), TPTZ and $FeCl_3.6H_2O$, respectively. For preparation of acetate buffer, 3.1 g Sodium acetate was added in water, then 16 ml %37 acetic acid were added to this and the pH was adjusted 3.6. For TPTZ preparation, 0,156 g TPTZ was added to 50 ml ethanol. Finally, for $FeCl_3.6H_2O$ preparation, 0,5404 g $FeCl_3.6H_2O$ was mixed with water and 2 ml %37 HCl were added, then it was completed 100 ml with distilled water. After completed these three solvents, 8 ml was taken from acetate buffer (pH 3.6), 1 ml was taken from both TPTZ and $FeCl_3.6H_2O$, orderly. With this, FRAP solvent was prepared for the analysis. For analysis, 250 µl was taken and with FRAP, it was completed 2.5 ml. It was waited 4 minutes at room temperature. The absorbance of the formed blue complex was recorded at 593 nm against the pure water reference. Results were calculated in equivalents of Trolox, a standard antioxidant (Kocak et al., 2018; Keskin et al., 2020). The total antioxidant capacity of samples was determined in FRAP units.

In the ABTS method, firstly, to obtain ABTS radical, 2.45 Mm potassium persulfate was mixed with 7 mM ABTS solution and then it was waited for 12-16 hours. This radical was diluted with distilled water to measure 700±25 at 734nm. For analysis, 10-30 µl/ml of the prepared extracts were taken in 250 µl tubes and ABTS radical were added until 250 µl. It was stored for 6 minutes and the absorbance value was measured. The percentage reduction rate was calculated according to the initial and final values. These processes were repeated 3 times (Ozkan et al., 2010; Cemeroglu, 2013).

DPPH assay is the determination of the reducing ability of antioxidants toward DPPH. The ability can be determined by measuring the reduction of its absorbance (Popović et al., 2012). In the DPPH method; 1 mM DPPH was solved in methanol and waited 12-16 hours and this obtained solvents was used in the analysis. In the analysis, 10-30 µl/ml of the prepared extracts were taken in 2000 µl tubes and methanol were added until 2000 µl. Next, 500 µl mixed with the prepared solvents. It was waited 30 minutes at room temperature (Gulcin et al., 2005). In this method, a purple color turns to yellow color after

interaction with the radical and antioxidant agents. The alteration in absorbance owing to colors can be spectrophotometrically displayed at 517 nm (Popović et al., 2012).

**2.4. Statistical Analysis**

Statistical analysis was done with IBM SPSS 20.0 program. Here, 2 different solvents and 2 different extraction methods were used. Analysis of variance was performed on the analysis results. Duncan Multiple Comparison Test, one of the multiple comparison tests, was applied to the different results. Correlation analysis was applied to determine the relationship between analyzes. Moreover, principal component analysis (PCA) was applied to some data to facilitate the identification of similarities and differences between the samples (SIMCA-P +14.1, UMETRICS).

## 3. Results and Discussion

SSC (°Brix), DPPH ($IC_{50}$ µl/ml extract) and ABTS ($IC_{50}$ µl/ml extract), FRAP (µM TE/g extract), Total Phenolic Contents (mg GAE/L extract), and Total Flavonoid Content (mg QE/L extract) of propolis extracts are presented in Table 1.

As a result of the analysis made, it was determined that the SSC value of the propolis extracts with distilled water and propylene glycol varied between 14.40-27.50 °Brix. While it was observed that the °Brix values of the propolis extracts prepared with distilled water (27.50-16.50) were higher than the samples prepared with propylene glycol (14.90-14.40), it was also observed that the extracts prepared by the classical method had higher °Brix values compared to the samples prepared by the ultrasonic method (Table 1). According to study conducted by Keskin and Kolayli (2019), they measured the amount of brix values of twenty various commercial propolis extracts prepared with different types of solvents. The results depicted that the amount of brix values of different commercial propolis extracts were between 0-61 °Brix.

The antioxidant tests of the samples were performed with total phenolic substance content, the total amount of flavonoid substance amount, FRAP, ABTS, and DPPH determination (Table 1). The total phenolic content of the extracts was determined between 721-33.621 mg GAE/L in the current study. This study showed that PKS had a higher SSC value with a greater value of total phenolic contents and PUPG had a lower value of SSC with a lower value of total phenolic contents. Additionally, the extracts with distilled water had again higher soluble solid content value with a greater value of total phenolic contents than the extract with propylene glycol. In addition, the propolis extracted with classical extraction methods had a higher total amount of phenolic substances and soluble solid content value than the propolis extracted with the ultrasonic extraction method. Therefore, the results indicate that the total amount of phenolic substances increases when the solid soluble content values increase consequently (Table 1).

Mujica et al. (2017), worked with propolis dissolved in propylene glycol to analyze the total phenolic compound of the extract. According to results, total phenolic compound (TPC) of the propolis extract prepared with propylene glycol was measured as 22.82 g GAE/L. In addition, Sagdic et al. (2020), worked with fourteen different commercial propolis samples which were prepared with different solvents including ethanol, water and propylene glycol. The results depicted that the total phenolic compounds vary from 2431 mg GAE/L to 127318 mg GAE/L, successively. The result of extract prepared with distilled water is 32490 mg GAE/L, while the result of propolis extract prepared with propylene glycerol is 80467 mg GAE/L. In our study, it was detected that while a higher amount of phenolic substance was detected in water-based samples, a lower amount of phenolic substance was detected in propylene glycol-based samples. This may be due to the solvent used or to the extraction method and conditions.

**Table 1.** SSC (°Brix), DPPH $IC_{50}$ (µl/ml extract) and ABTS $IC_{50}$ (µl/ml extract), FRAP (µM TE/g extract) Total phenolic contents (mg GAE/L extract), and Total Flavonoid Content (mg QE/L extract) of propolis extracts

| Samples | SSC (Brix) | DPPH· ($IC_{50}$ µl/ml extract) | ABTS+· ($IC_{50}$ µl/ml extract) | FRAP (µM TE/g extract) | Total Phenolic Content (mg GAE/L extract) | Total Flavonoid Content (mg QE/L extract) |
|---|---|---|---|---|---|---|
| PKS | 27.50±0.50[a] | 0.46±0.01[c] | 0.09±0.01[d] | 1783.02±74.18[a] | 33620.70±109.41[a] | 74021.42±12128.38[a] |
| PKPG | 14.90±0.10[c] | 15.21±0.08[b] | 2.71±0.02[b] | 54.72±2.88[c] | 1212.32±79.17[c] | 2144.77±105.46[c] |
| PUS | 16.50±0.25[b] | 1.36±0.01[c] | 0.21±0.01[c] | 639.43±63.09[b] | 14419.46±301.27[b] | 24884.70±173.98[b] |
| PUPG | 14.40±0.20[c] | 31.86±1.09[a] | 4.64±0.01[a] | 29.22±1.35[c] | 721.31±7.59[c] | 1137.52±24.78[c] |
| BHA | - | 6.98±0.10 | 4.16±0.14 | - | - | - |
| BHT | - | 19.84±0.56 | 7.87±0.28 | - | - | - |
| Trolox | - | 7.47±0.17 | 3.11±0.04 | - | - | - |
| α-Tocopherol | - | 11.24±0.15 | 12.47±0.12 | - | - | - |
| Severity Level | ** | ** | ** | ** | ** | ** |

PKS= propolis-distilled water, PKPG= propolis-propylene glycol, PUS= propolis-distilled water, PUPG= propolis-propylene glycol. Different letters in each column indicate significant differences of the means at P<0.01.

The amounts of flavonoids, which are a group of phenolic substances, ranged from 1137 to 74021 mg QE/L (Table 1). Flavonoids are the primary components of propolis that function as antioxidants and have antibacterial and anti-inflammatory activities (Gunce et al., 2021; Coneac et al., 2008). In our research the total flavonoid content of propolis extracts was determined at various extraction methods and different types of solvents. Among the extracts, PKS had the highest value of TFC (74021 mg/QE L extract) followed by PUS (24884 mg/QE L), PKPG (2145 mg/QE L), and PUPG (1138 mg/QE L), respectively. These results show that distilled water as an extraction solvent had a higher value of TFC compared to propylene glycol. Moreover, for the extraction method, the classical method increased the value of TFC more than the ultrasonic method (Table 1). As the amount of crude propolis dissolved in the unit volume of solvent increases, the Brix value and accordingly the total amount of polyphenol substance increases (Keskin and Kolayli, 2019). Sagdic et al. (2020) also showed the total flavonoid compounds for fourteen different commercial propolis samples and by means of this study, total flavonoid compounds were found between 104 mg/QE L and 40516 mg/QE L. Moreover, the results were measured as 343 mg/QE L and 20520 mg/QE L for propolis extracts prepared by distilled water and propylene glycol, respectively.

FRAP determination findings of propolis extracts were found to be between 29.22-1783.02 mM Trolox E/g. ABTS determination findings of propolis extracts were measured to be between 0.09-4.64 IC50 µl/ml. Moreover, the DPPH results of propolis extracts were calculated to be between 0.46-31.86 IC50 µl/ml (Table 1). Table 1 illustrates the DPPH $IC_{50}$ (µg/ml) and ABTS $IC_{50}$ (µg/ml) results of standard antioxidants (BHA, BHT, Trolox and α-Tocopherol) below. In the present study, one reducing power assay (FRAP) and two different radical scavenging assays (DPPH and ABTS) were used to calculate the antioxidant potential of propolis extracts prepared with different types of solvents and two types of extraction methods. In the assay of DPPH•, a smaller $IC_{50}$ value shows more antioxidant activity, as a smaller mass of extract is needed to inhibit 50% of the DPPH• (Cottica et al., 2011). The best result was observed in the extract of PKS. According to the results, with the lower $IC_{50}$ values, the distilled water propolis extracts displayed greater antioxidant potential. In addition, since the extracts prepared by the classical method have lower $IC_{50}$ values, which are 0.46-1.36 $IC_{50\mu}$µl/ml extract, compared to the extracts prepared by the ultrasonic method (15.21-31.86 $IC_{50\mu}$µl/ml extract), it was determined that they showed greater antioxidant activity (Table 1). In ABTS assay, a lower $IC_{50}$ result of the extract means greater capturing potential of the ABTS radical, so raised potential of the antioxidant (Asem et al., 2020). In the current study, propolis extracts displayed antioxidant activity in the ABTS scavenging assay and $IC_{50}$ values varied from 0.09 $IC_{50\mu}$µl/ml extract to 31.86 $IC_{50\mu}$µl/ml extract. In the ABTS assay, PKS again showed the highest antioxidant activity with the lowest $IC_{50}$ value. Additionally, PUPG had the smallest antioxidant potential with the highest $IC_{50}$ value. In the FRAP assay, all propolis extracts (PKS, PKPG, PUS, and PUPG) displayed the Trolox Equivalent Antioxidant Capacity (TEAC) of the FRAP method (29.22-1783.02 µM TE/g extract) (Table 1). A higher TE value indicates the strongest reducing potential, therefore shows the greater antioxidant potential of a sample (Asem et al., 2020). According to the results, PKS with a greater TE value shows stronger reducing activity. But, PUPG with lower TE value indicates the lower reducing activity. While greater antioxidant activity (by FRAP and DPPH•) was observed in extract PKS lower antioxidant activity was noted in extract PUPG, both by FRAP and DPPH• (Table 1). Ulloa et al., (2017), used DPPH, ABTS and FRAP methods to demonstrate the antioxidant activity of propolis. Accordingly, DPPH results of propolis extracts were between 0.014 and 0.044 (mmol TE/mL). In addition, ABTS results ranged from 0.079 to 0.149 (mmol TE/mL), while FRAP results ranged from 0.206 to 0.801 ($\mu$mol TE/mL). Antioxidant activity is generally associated with total phenolic content and total flavonoid content in the samples (Zin et al., 2018). There are numerous reasons that could impact the antioxidant activity of the propolis extracts, for example type of bee, propolis collection location, solvents utilized in extraction, plant source, and chemical structures (Syed Salleh et al., 2021). To investigate the antioxidant capacity of propolis extracts, all methods can be compared but this can cause minor differences between the results. Since the results are various in each method, it required to make statistical analysis to calculate the correlation between each method (Asem et al., 2020). According to statistical analysis, a strong correlation was observed between TPC and TFC since flavonoid is classified under the group of phenol compounds.

Pearson's correlation coefficient was utilized to show the antioxidant potential arrays with each other, and also it was used to display correlation with both the total phenolic matter and flavonoid contents of the extracts. In the current study, according to the statistical analysis, SSC, TPC, and TFC displayed correlations on antioxidant potentials of propolis extracts varying from $R^2$=0.642 to $R^2$=1. The correlation between TFC and TPC was significant ($R^2$=0.995). The FRAP assay showed a significant correlation between TPC ($R^2$=0.997) and TFC ($R^2$=0.995). Also, SSC showed a significant correlation with FRAP ($R^2$=0.978), TPC ($R^2$=0.960), and TFC ($R^2$=0.983), respectively. In addition, while ABTS and DPPH assays indicated a significant positive correlation with each other ($R^2$=0.994), FRAP, TPC, TFC, and SSC showed negative correlations shown below in Table 2. There is a positive correlation between SSC and FRAP analyzes in the study. This shows that as the amount of SSC increases, the amount of TPC and TFC increases in the extracts. On the other hand, a positive correlation was found between ABTS and DPPH $IC_{50}$ values (Table 2).

**Table 2.** Correlation analysis among SSC, TPC, TFC and antioxidant activity

| Parameters | SSC | DPPH | ABTS+ | FRAP | TPC | TFC |
|---|---|---|---|---|---|---|
| SSC | 1 | -.642 | -.668 | .978* | .960* | .983* |
| DPPH· | -.642 | 1 | .994** | -.749 | -.783 | -.738 |
| ABTS+· | -.668 | .994** | 1 | -.784 | -.819 | -.772 |
| FRAP | .978* | -.749 | -.784 | 1 | .997** | 1** |
| TPC | .960* | -.783 | -.819 | .997** | 1 | .995** |
| TFC | .983* | -.738 | -.772 | 1** | .995** | 1 |

Additionally, Principal component analysis (PCA) was applied to determine the differences between samples by evaluating some physical properties, antioxidant activities, total phenolic and total flavonoid contents of propolis extracts obtained by using 2 different extraction methods and 2 different solvents. Figures 1a-d show hierarchical clustering, score scatter plot, loading scatter plot and biplot of principal component analysis of propolis extracts. The first two principal components (PC1 = 88.3.3% and PC2 = 11.3%) explained 99.6% of the variance (Figure 1).

As a result of the analysis, propolis extracts could be divided into two main groups (Figure 1a, b). The samples using water as solvent from propolis extracts extracted by classical and ultrasonic methods are on the right side of PCA 1, while the extracts using Propylene Glycol: Water as solvent are on the left side of PCA 1. (Fig. 2a).

While the PKS sample using water as solvent and extracted with the classical method, TPC, TFC, FRAP and SSC analyzes are located close, DPPH and ABTS analyzes are located far away. This indicated that PKS had a higher FRAP capacity and the highest amount of SSC with higher total phenolic substance and total flavonoid substance compared to other samples (Figure 1d). Since it is known that the antioxidant activity increases as the $IC_{50}$ value decreases in ABTS and DPPH methods, it is seen that the PKS sample has the highest antioxidant activity (Figure 1d). When the figures are examined, the PUPG sample extracted by ultrasonic method using Propylene Glycol: water as solvent and TPC, TFC, FRAP and SSC analyzes are located far away, while DPPH and ABTS analyzes are located close. This shows that the PUPG sample has the lowest amount of antioxidant activity, TPC, TFC, SSC (Figure 1).

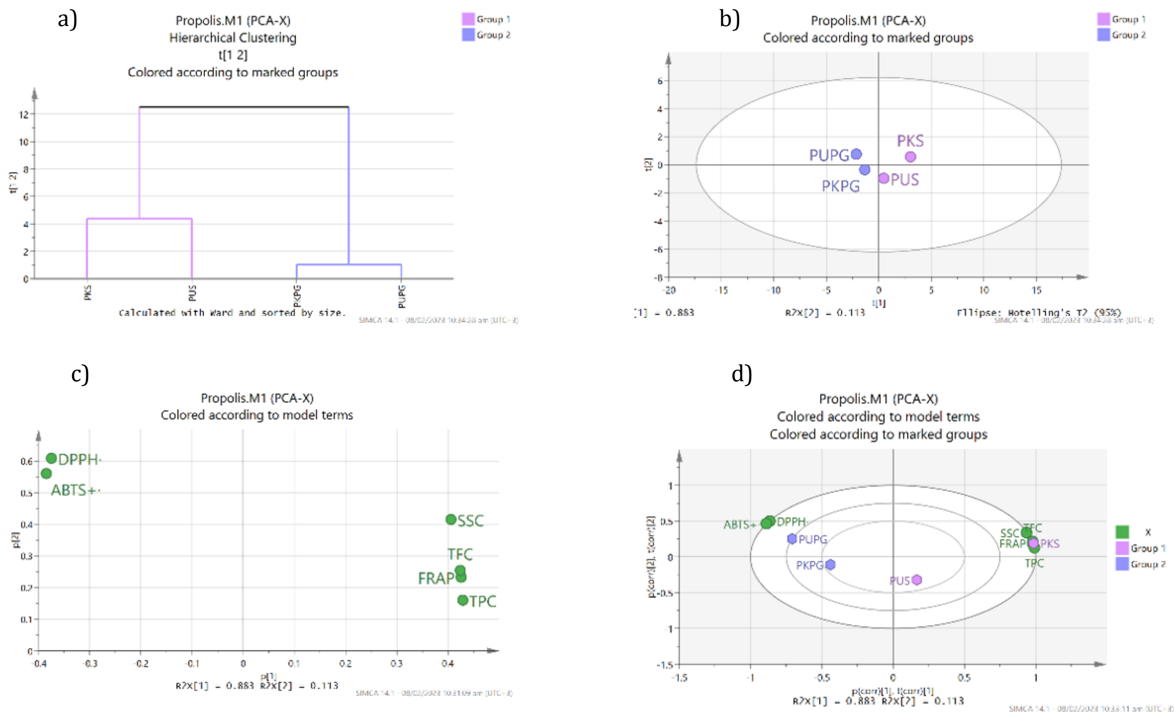


**Figure 1.** Dendrogram (a), score scatter plot (b), loading scatter plot (c), and biplot (d) of the principal component analysis (PCA) (PC1 vs. PC2) for the attributes in propolis extracts.

## 4. Conclusions

The present study showed the effect of various types of solvents and extraction methods on propolis antioxidant activities. All propolis extracts have antioxidant potential. When the results obtained are evaluated, it is clearly seen that there are differences between the amount and variety of bioactive substances of propolis extracts. When

the previous studies were examined, it was seen that the bioactive substance content of the water-based samples obtained by using conventional extraction methods was lower than other samples. On the contrary, in our study, propolis extracted with distilled water and the classical method demonstrated the greatest antioxidant potential compared to other types of propolis extracts. Additionally, propolis extracted with distilled water and the classical method has the highest total amount of phenolic and flavonoid contents. In conclusion, extracts with the highest amount of phenolic and flavonoid contents indicate the strongest antioxidant potential. More studies and analyzes are needed to investigate the effects of solvents and extraction methods on propolis extracts, as well as on the antioxidant properties of these extracts. Since the amount and quality of bioactive components in the final product are directly affected by the extraction method applied, the method used in the extraction stage is of great importance. This study contributes to the literature as different extraction methods are evaluated together.

**Author Contributions**

The percentage of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

| | S.U. | M.S. |
|---|---|---|
| C | 50 | 50 |
| D | 50 | 50 |
| S | 50 | 50 |
| DCP | 50 | 50 |
| DAI | 50 | 50 |
| L | 50 | 50 |
| W | 50 | 50 |
| CR | 50 | 50 |
| SR | 50 | 50 |
| PM | 50 | 50 |
| FA | 50 | 50 |

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

**Conflict of Interest**

The authors declared that there is no conflict of interest.

**Ethical Consideration**

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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