Citation/Atıf: Özenirler Ç, Zara G. Evaluation of the polyphenol contents and antioxidant activity of propolis extracted with different techniques (Farklı tekniklerle ekstrakte edilen propolisin polifenol içerikleri ve antioksidan aktivitelerinin değerlendirilmesi). U. Arı D. / U. Bee J. 2022, 22(2):176-187. DOI: 10.31467/uluaricilik.1106415

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

EVALUATION OF THE POLYPHENOL CONTENTS AND ANTIOXIDANT ACTIVITY OF PROPOLIS EXTRACTED WITH DIFFERENT TECHNIQUES

Farklı Tekniklerle Ekstrakte Edilen Propolisin Polifenol İçerikleri Ve Antioksidan Aktivitelerinin Değerlendirilmesi

Çiğdem ÖZENİRLER [1*], Golshan ZARE [2]

[1*]Department of Biology, Faculty of Science, Hacettepe University, 06800, Ankara, TÜRKİYE, Corresponding author / Yazışma yazarı: cozenir@hacettepe.edu.tr, ORCID No: 0000-0003-0390-2416

[2]Department of Pharmaceutical Botany, Faculty of Pharmacy, Hacettepe University, 06100, Ankara, TÜRKiYE, ORCID No: 0000-0002-5972-5191

Geliş Tarihi / Received: 20.04.2022 Kabul Tarihi / Accepted: 05.07.2022 DOI: 10.31467/uluaricilik.1106415

ABSTRACT

Propolis is classified as an opotherapeutic medicine due to the botanical origin of the resins. The chemical composition of propolis is greatly influenced by the honeybee species, botanical source and extraction techniques. Within this frame, we compared the same propolis' polyphenol contents and antioxidant activities prepared with different techniques. Four types of extracts were prepared. The first type was prepared classically by ethyl alcohol (*POH)*. The second and third types were extracted by sterile distilled water kept as both sterilised (*PS)* and non-sterilized (*PN)*. The fourth one was prepared with full vacuumed and dried propolis with honey (*PH*). The antioxidant activity of extracts was evaluated with DPPH radical scavenging, ABTS radical cation scavenging, Cupric ion reducing antioxidant capacity. Also total phenolic and flavonoid content of extracts were investigated. POH extract showed significantly high content of total phenol and flavonoids which followed by PN, PS and PH. POH showed approximately two times higher activity on DPPH radical (IC50=4,39µg/mL) compared with quercetin as references. The highest activity on DPPH is shown by POH with 4,39 µg/mL of IC50 value which was followed by aqueous extracts 18,08. The lowest activity was shown by PS with 4,39 µg/mL of IC50 value. The highest scavenging activity against ABTS radical cation was shown by POH (73,37 mg TE/g extract) and the lowest activity was shown by PS (34,21 mg TE/g extract). According to the results, the new aqueous extraction technique is promising with relatively high polyphenol contents and antioxidant activities. Also honey with propolis can be an alternative product, although it has relatively lower values of antioxidant activity.

Keywords: Propolis, Antioxidant, Honey, A new aqueous *extraction*

ÖZ

Propolis, arılardan gelen organik salgıların karmaşık kimyasal bileşimi nedeniyle opoterapotik bir ilaç olarak sınıflandırılır. Propolisin kimyasal bileşimi, bal arısı türü, botanik kaynak ve ekstraksiyon tekniklerinden büyük ölçüde etkilenmektedir. Bu çerçevede toplanan ham propolisten farklı tekniklerle hazırlanmış ekstaraktlar polifenol içerikleri ve antioksidan aktiviteleri bazında karşılaştırılmıştır.Dört tip ekstrakt hazırlanmıştır. Birinci tip klasik olarak etil alkol (POH) ile hazırlanmıştır. İkinci ve üçüncü tipler, hem sterilize edilmiş (PS) hem de sterilize edilmemiş (PN) olarak tutulan steril damıtılmış su ile özütlenmiştir. Dördüncüsü ise tamamen vakumlanmış ve kurutulmuş ballı propolis (PH) ile hazırlanmıştır. Ekstraktların antioksidan aktivitesi, DPPH radikal süpürücü, ABTS radikal katyon

süpürücü, Kuprik iyonu azaltan antioksidan kapasite, toplam fenolik içerik ve toplam flavonoid içerik deneyleri ile değerlendirilmiştir. POH özütü, önemli ölçüde yüksek toplam fenol ve flavonoid içeriği göstermiş ve bunu PN, PS ve PH izlemiştir. POH, referans olarak kuersetin ile karşılaştırıldığında DPPH radikali (IC50=12,24 µg/mL) üzerinde yaklaşık iki kat daha yüksek aktivite göstermiştir. DPPH üzerindeki en düşük aktivite, 56,72 µg/mL IC50 değeri ile PS tarafından gösterilmiştir. En yüksek aktivite POH (271,75 mg GAE/g ekstraktı) tarafından gösterilirken, bunu su ekstraktları takip etmiş ve en düşük değer HP'ye ait olarak tespit edilmiştir. ABTS radikal katyonuna karşı en yüksek süpürme aktivitesi POH (73,37 mg TE/g özü) ve en düşük aktivite PS (34,21 mg TE/g özü) ile gösterilmiştir. Sonuçlara göre, nispeten yüksek polifenol içerikleri ve antioksidan aktiviteleri ile yeni su ekstraksiyon tekniğinin umut vericidir. Ayrıca propolisli balın, nispeten daha düşük değerlere sahip olmasına rağmen alternatif bir ürün olarak tüketilebileceği düşünülmektedir.

Anahtar kelimeler: Propolis, Antioksidan, Bal, Su bazlı yeni bir ekstraksiyon

GENİŞLETİLMİŞ ÖZET

Amaç: Bu çalışmanın amacı, besin takviyesi olarak kullanılmakta olan propolisin su bazlı ekstraktından hazırlanan üç farklı ürünün (balla karıştırılmış propolis, su ile ekstrakte edilmiş ve sonrasında sterilize edilmiş ve edilmemiş propolis), geleneksel yöntemlerden biri olan alkol içerisinde çözme ile üretilmiş propolis ile toplam fenol, flavonoid içeriğini ve antioksidan aktivitesini karşılaştırmaktır.

Gereç-Yöntem: Ham propolis, 2018 ve 2019 yıllarında Türkiye'de Tunceli-Ovacık bölgesinden 9 alt bölgede 12 farklı arılıktan toplanmıştır. Ham proprolisin alkol bazlı ekstrasyonunda %99 saf etil alkol kullanılmıştır (POH). Propolis, tam karanlık koşullarda %10 ham propolis ila %90 çözücü kombinasyonunda 4 hafta boyunca alkolde bekletilmiştir. Su bazlı hazırlanan propolis için T.C. Tarım ve Orman Bakanlığı tarafından 2020 yılında tescil edilen (kayıt no: 007395.20.03.2020) yöntemle pH'ı 4.6 olan steril distile su ile ekstraksiyon gerçekleştirilmiştir. Buradan elden edilen ürün sonrasında steril edilmiş (PS) ve edilmemiş (PN) olarak şişelenmiş, ayrıca aynı yöreden toplanan bal ile karıştırılarak (dördüncü bir ürün olarak-PH) saklanmıştır. Ekstraktların antioksidan aktivitesi, DPPH radikal süpürücü, ABTS radikal katyon süpürücü, Kuprik iyonu azaltan antioksidan kapasite, toplam fenolik içerik ve toplam flavonoid içerik deneyleri ile değerlendirilmiştir.

Bulgular ve Sonuç: Toplam fenol ve flavonoid içerikleri karşılaştırıldığında; alkol ile hazırlanan örnek, su ile hazırlanan örneklerde, steril edilmeyen örnek, steril edilen örnek ve bal ile hazırlanan karışım olarak bir sıralama bulunmuştur. POH, referans olarak kuersetin ile karşılaştırıldığında DPPH radikali (IC50=12,24 µg/mL) üzerinde yaklaşık iki kat daha yüksek aktivite göstermiştir. DPPH üzerindeki en düşük aktivite, 56,72 µg/mL IC50 değeri ile su ile hazırlanan steril örnekte tarafından görülmüştür. En yüksek aktivite POH (271,75 mg GAE/g ekstraktı) tarafından gösterilirken, bunu su ekstraktları takip etmiş ve en düşük değer HP'ye ait olarak tespit edilmiştir. ABTS radikal katyonuna karşı en yüksek süpürme aktivitesi POH (73,37 mg TE/g özü) ve en düşük aktivite PS (34,21 mg TE/g özü) ile gösterilmiştir. Sonuçlara göre, nispeten yüksek polifenol içerikleri ve antioksidan aktiviteleri ile yeni su ekstraksiyon tekniğinin umut vericidir. Ayrıca propolisli balın, nispeten daha düşük değerlere sahip olmasına rağmen alternatif bir ürün olarak tüketilebileceği düşünülmektedir.

INTRODUCTION

Propolis, is the generic name for the resinous substance that is collected by bees from different plant sources -like poplar, birch, pine, alder, willow and palm or sometimes from wounds in several other plants etc. (Çelemli Gençay 2013). Bees then mix these resins with their waxes and β-glucosidase to use in the defence of the bee community by coating and strengthening the inside walls of the hive (Zhang et al. 2011, Simone-Finstrom et al. 2017). It is also used to cover holes and cracks and to repair combs, avoid insect invasions by shortening the entrance and to reduce the microbial growth on the walls of the hive. It prevents wind and water from entering as well as to help maintain hive's inner temperature to an optimal degree (Bhargava et al. 2021).

The chemical composition of propolis is quite complex. Approximately 300 different compounds have been identified lately in propolis samples of diverse origins (Pereira et al. 2015, Salgueiro and

Castro 2016, Lorenzon et al. 2018). Among these compounds, flavonoids are the main active propolis constituents that are responsible for a large part of its biological activities (Huang et al. 2014, Hernandez Zarate et al. 2018). Flavonoids in propolis are classified into flavones, flavonols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans and neoflavonoids (Santos-Buelga et al. 2017). Total flavonoid content has often been used as an index for evaluating the quality of propolis. The flavonoid content of propolis with less than 11% is considered as low quality, whereas 11-17% and the higher percentages are referred to as good quality and high quality, respectively (Gardana et al. 2007). Propolis is composed of resin (flavonoidaglycones, phenolic acids and their esters), waxes (mixture of long-chain non polar compounds), essential oils, pollen and other substances. Minor constituents of propolis are pollen, and other substances such as vitamins, minerals, amino acids and fatty acids (Alvarez-Suárez et al. 2010, Escuredo et al. 2013). Propolis contains a large number of enzymes such as glucose-6-phosphatase, succinic dehydrogenase, adenosine triphosphatase and acid phosphatase (Lotfy 2006, Pasupuleti et al. 2017). Propolis also contains β-glucosidase which hydrolyzes flavonoid glycosides into aglycones (Li et al. 2018, Araghi et al. 2021). Propolis is classified as an opotherapeutic medicine due to its complex chemical composition of organic secretions from bees (Zenebom and Pascuet 2005, Machado et al. 2017). It is difficult to use raw propolis as it is difficult to remove from the human skin. It is hard, brittle with poor solubility and low oral bioavailability (Elbaz et al. 2016, Dallabona et al. 2020). Over the last 30 years, the pharmacological and chemical properties of propolis became the aim of intensive studies (nearly 4000 papers in journals and 2884 patents in which nearly half of them are owned by China, Japan and Russia). Since the end of the 20th century, the paradigm related to the chemistry of propolis has changed drastically. By the 1960s, it was known that propolis is chemically complex, but stable. But lately, it has been understood that the chemical composition of propolis varies considerably, depending on the honeybee species, botanical source and extraction method, which greatly influence its properties. The quality of the propolis also depends on the beekeeper' experience and knowledge, storage conditions and also regarding the transport from the production area, the collecting season, day and time of collecting, the method of collecting and the place of propolis in the hive (EFSA 2010). The effect of the propolis on human health also can vary due to the patient's age, gender, physiology and sometimes lifestyle (Dezmirean et al. 2021).

Local eco-flora has a very strong effect on the chemical composition of propolis (Salatino et al. 2005). To produce propolis, the bees use material from several parts of plants and in different stages of development. Hence, the complexity and the chemical variety of propolis are deeply related to the eco-flora of the geographical region, which the bees commonly visit (Bankova et al. 2014).

This causes classifying propolis into different "types" (eg. in Brazil there are 14 types). The characteristic constituents in temperate region propolis are flavonoids without B-ring substituents, such as chrysin, galangin, pinocembrin, pinobanksin which gives the characteristic colour (Christov et al. 2006, Salatino et al. 2011, Santos-Buelga et al. 2017).

Caffeic acid phenethyl ester (CAPE) is a major constituent of temperate propolis with broad biological activities, including inhibition of nuclear factor κ-B; inhibition of cell proliferation; induction of cell cycle arrest and apoptosis (Huang et al. 2014, Ristivojević et al. 2015).

In tropical region propolis, especially Brazilian green propolis (CAS: 9009-62-5), the dominating chemical components are prenylated phenylpropanoids (e.g., artepillin C) and diterpenes (Midorikawa et al. 2001, Paviani et al. 2010). For propolis produced in the Pacific region, geranyl flavanones are the characteristic compounds which are also found in propolis from the African region (Bankova 2005, Salatino et al. 2011). Anatolian propolis has different chemical profiles in different types. For example, one type is rich in monoterpenes, sesquiterpenes and diterpenes which are generally collected from *Ferula* spp, *Pinaceae* spp and *Cupressaceae* spp by honeybees (Uzel et al. 2005).

There are several different methods (not the solvents) of extraction models that occur for propolis (Bankova et al. 2021). Some of these methods are commercial while some are just for research. Ethanol is the most frequently used solvent, because it possesses more extraction capacity. It removes around 50-60% of the propolis components, while the classical aqueous extraction method removes only around 10% (Park 1998).

The aim of this study is to evaluate the total phenol

and flavonoid content antioxidant activity of ethanol extract of propolis, propolis mixed with honey, aqueous sterilised and non-sterilized extract of propolis and assess the chemical composition of it.

MATERIALS AND METHODS

Preparation of propolis

The raw propolis was collected from Tunceli-Ovacık region in Turkey from 12 different apiaries in 9 sub-localities during 2018 and 2019. In the first step, all of the raw propolis was broken or grated and divided into smaller pieces. Then propolis was washed down with water and the mixture was cooled slowly. During this cooling wax and resin were separated from the mixture with sieves, propolis was moved to a separate area. The cleaned propolis was used for extraction via different solvents.

Extraction techniques

Four types of extracts were prepared. All the extractions were made in registered GMP production laboratories where all the equipment and methods were fully calibrated and validated in 2020 and 2021.

Ethanol extraction: For ethyl alcohol extraction, 99% pure double filtered absolute ethyl alcohol produced by (Botafarm Ltd.) were used. The propolis was extracted in the alcohol for 4 weeks in combination of 10% raw propolis to 90% solvent in full dark conditions.

Aqueous extraction: The propolis was extracted by sterile distilled water with pH of 4.6 with a special method developed by Dr. Aytekin which is registered by the Republic of Turkey Ministry of Agriculture and Forestry in 2020 (Reg. No: 007395.20.03.2020). This method includes raw steps of the following; It was heated at 45-50°C (12 hours), then left to infuse (2 hours), stirred from time to time. Distilled water propolis ratio was used as 10%. The mixture was cooled slowly, the acidity was lowered and kept for 12 hours in dark conditions. It was brought to normal pH and filtered four times. The filtrate was collected after each filtration. It was heated in a separate bowl and filtered again. The aqueous mixture obtained here was combined with the other mixture. This mixture was stirred from time to time and kept in the oven at 45°C for a while. The mixture was drained. Raw filtered. Then the mixture was divided into sterilised (S1) and non-sterilized (NS1) groups. NS1 group was bottled and covered with a lid immediately and S1 is bottled after sterilisation. One bottle is open and sterilised and revored in Class 10000 Clean room the other is covered with airtighed cups. We used the less effective type of sterilisation in closed glass vials and used hot vapour under high pressure which is 121°C.

Honey mixed with propolis: The cleaned propolis was dried and full water was evaporated by an industrial type of Vacuum Freeze Dryer model GZL2 (2012) in 12h F-12hD-6hFD conditions. The full vacuumed and dried propolis were mixed with honey (10% propolis and 90% honey from the same apiaries) and kept at room temperature until analysis. For antioxidant assays 5 gr of honey mixed propolis were macerated with 99% ethanol (100 mL) at room temperature for 24 h and then were filtered through a filter paper. Solvent from the samples was removed using a rotary evaporator.

Antioxidant activity

Determination of total phenolic contents: Total phenolic contents of different propolis and mixture extracts were evaluated by the Folin-Ciocalteu's colorimetric method (Slinkard and Singleton, 1977) using regression equation of calibration curve (Y= 0.0114x + 0.1427, R2: 0.9986) and expressed in gallic acid equivalents and expressed in mg of gallic acid equivalents (GAE) / 1g of extract. Folin-Ciocalteau's reagent was diluted with distilled water (1:10) and then 100 µL of solution was mixed with 20 µL of propolis extract and different concentrations of reference dissolved in ethanol. Finally, 80 µl of sodium carbonate (Na_2CO_3) solution (%7.5) was added to the mixture. The mixture was left at room temperature for 120 minutes in the dark, then absorbance was measured at 765 nm.

Determination of total flavonoid contents: Flavonoid content of propolis extracts were determined by the aluminium chloride colorimetric method (Chang et al., 2002) and were calculated according to the equation (y=0.0055x+0.1098, R2=0.9983) obtained from the calibration curve as quercetin equivalent (mg/g extract). 25 µl of extract and different concentrations of reference dissolved in ethanol were mixed with 75 µl of 95% ethanol, 5 µl of 10% aluminium chloride ($AlCl_3$), 5 µl of 1 M potassium acetate (KCH_3COO) and 140 µl of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. Quercetin was used as reference.

DPPH radical scavenging capacity assay: DPPH radical scavenging capacity of each extract was determined according to Brand-Williams et al. (1995). DPPH radical scavenging capacities of propolis extracts were tested at 12.5, 25, 50 and 100 µg/mL concentrations. The inhibition percentage of extracts on DPPH were calculated. 1mM DPPH reagent (1,1-diphenyl-2-picrylhydrazyl) was solved in ethanol and then 50 µL of this solution was mixed with 150 µL of different concentrations of the extract and Quercetin as reference. The reaction mixture was incubated at room temperature for 30 minutes in the dark, then absorbance was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: Inhibition % = $[(A_{blank}-A_{sample})/A_{blank}]\times100$, where A_{blank} is the absorbance of the blank (containing ethanol instead of sample) and A_{sample} is the absorbance of the extracts or reference. The half-maximal inhibitory concentration (IC_{50}) value for each extract was calculated from the plotted graph of scavenging activity against the concentrations of the sample.

ABTS radical cation scavenging activity assay: ABTS radical cation scavenging activity assay was carried out according Re et al. (1999). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was generated by reacting ABTS stock solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 12-16 hours. $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.02 nm at 734 nm before use. 200 µL of this solution was mixed with 20 µL of the extract and different concentrations of reference dissolved in ethanol. The reaction mixture was incubated for 6 minutes at room temperature in the dark, then absorbance was measured at 734 nm. ABTS radical cation scavenging activities of the propolis extracts were determined in accordance with the equation (y=0,9051x+2,9872, R^2=0.995) of Trolox calibration curve.

Cupric ion reducing antioxidant capacity (CUPRAC) assay: Cupric ion reducing antioxidant capacity assay was carried out according to Apak et al. (2004). 50 µL of copper (II) chloride (CuCl2) solution (1.0x10- 2 M), 50 µL of neocuproine solution (7.5x10-3 M), 50 µL of ammonium acetate (NH4Ac) buffer solution at pH 7.0 (1.0 M) were mixed and then 25 µL of extracts or different concentrations of reference (800 µg/mL to 25 µg/ mL) and 25 µL of distilled water were added to the initial mixture, separately. The absorbance of the final solution was measured at 450 nm after 30 minutes keeping at room temperature in the dark. Cupric ion reducing antioxidant capacities of the propolis extracts were determined according to the equation (y=0.014x+0.0569, R2=0.9998) as gallic acid equivalent (mg/g extract).

All total phenol, flavonoid content and antioxidant assay was carried out in three repeats.

Statistical analysis

Principal component analysis (PCA) was conducted with PAST (Hammer et al. 2001). The four groups were evaluated with their five-character sets.

RESULTS

According to our study, the amount of total phenolics and flavonoid contents in propolis extracts varied from 58,09 ± 2,58 (PH); 286,95 ± 39,1 (POH); 125,61 ± 1,42 (PS); 142,24 ± 16,79 (PN) mg GAE/g extracts and 95,73 ± 9,55; 444,33 ± 20,82; 103,21 ± 21,24; 106, 76± 19,29 mg QE/g respectively (Table 1). These results clearly demonstrated that POH extract showed significantly high content of total phenol and flavonoids which flowed by PN and PS. Lowest amount of total phenol and flavonoid belong to honey-propolis composition extract. As presented in Table 1, the amount of total phenolics and flavonoid contents in propolis extracts varied from 58,09 to 286,95 mg GAE/g extracts and from 95,73 to 444,33 mg QE/g respectively. These results clearly demonstrated that POH extract showed significantly high content of total phenol and flavonoids which flowed by PN and PS. Lowest amount of total phenol and flavonoid belong to honey-propolis composition extract.

In ABTS and DPPH assay honey mixed propolis, after ethanol extract of propolis has highest activity compared with aqueous extract of propolis. This activity can be caused by honey composition. Honey alone displays significant antioxidant activity, similar to many plants (Gheldof et al. 2002). POH extract showed significantly high content of total phenol and flavonoids which followed by PN, PS and PH. POH showed approximately two times much higher activity on DPPH radical (IC50=12.24 µg/mL) compared with quercetin as references. The lowest activity on DPPH is shown by PS with 56,72 µg/mL of IC50 value. The highest activity was shown by POH (271,75 mg GAE/g extract) which was followed

by aqueous extracts and lowest value belonged to HP. The highest scavenging activity against ABTS radical cation was shown by POH (73,37 mg TE/g extract) and the lowest activity was shown by PS (34,21 mg TE/g extract). All propolis extracts showed concentration-dependent inhibitory activity against DPPH radical. IC50 values for DPPH radical scavenging capacity are presented in Table 3.

Table 1. Total phenolic and flavonoid contents of propolis extracts (POH: ethyl alcohol, PS: extracted by sterile distilled water kept as sterilized, PN*:* extracted by sterile distilled water kept as non-sterilized, PH: prepared with full vacuumed and dried propolis with honey *GAE: Gallic acid equivalent, **QE: Quercetin equivalent)

Tablo 1. Propolis ekstraktlarının toplam fenolik ve flavonoid içerikleri (POH: etil alkol, PS: steril olarak saklanan steril damıtılmış su ile ekstrakte edilen, PN: sterilize edilmemiş olarak saklanan steril damıtılmış su ile ekstrakte edilen, PH: tamamen vakumlanmış ve bal ile kurutulmuş propolis ile hazırlanan *GAE: Gallik asit eşdeğeri,**QE: kuersetin eşdeğeri)

Extracts	Total phenolic content (mg GAE*/g extract)	Total flavonoid content (mg QE**/g extract)
PH	58,09 ± 2,58	95,73± 9,55
POH	286,95 ± 39,1	444,33± 20,82
PS	125,61 ± 1,42	103,21± 21,24
PN	142,24 ± 16,79	106,76± 19,29

A lower IC50 value belongs to POH (IC50=4.39 µg/mL) which corresponds to a higher antioxidant activity of the extract. POH showed approximately 2 times higher activity on DPPH radical (IC50=12.24 µg/mL) compared with quercetin, as references. The lowest activity on DPPH is shown by PS with 56,72 µg/mL of IC50 value.

Table 2. The inhibitory effects of propolis extracts on DPPH radical (POH: ethyl alcohol, PS: extracted by sterile distilled water kept as sterilised PN*:* extracted by sterile distilled water kept as non-sterilized, PH: prepared with full vacuumed and dried propolis with honey)

Tablo 2. Propolis ekstraktlarının DPPH radikali üzerindeki inhibitör etkileri (POH: etil alkol, PS: steril olarak saklanan steril damıtılmış su ile ekstrakte edilen, PN: sterilize edilmemiş olarak saklanan steril damıtılmış su ile ekstrakte edilen, PH: tamamen vakumlanmış ve bal ile kurutulmuş propolis ile hazırlanan).

Propolis extracts	IC_{50} value (µg/ml)
PH	18,08
POH	4,39
PS	56,72
PN	47,65
Quercetin	10,83

ABTS radical cation scavenging activities of the propolis extracts were expressed in terms of Trolox equivalent antioxidant capacity (TEAC) in Table 3. A higher TEAC value corresponds to a greater antioxidant activity of the propolis extracts. The highest scavenging activity against ABTS radical cation was shown by PoH (73,37 mg TE/g extract) and the lowest activity was shown by PS (34,21 mg TE/g extract).

Table 3. The inhibitory effects of propolis extracts on ABTS radical cation and cupric ion reducing antioxidant capacity (CUPRAC) (POH: ethyl alcohol, PS: extracted by sterile distilled water kept as sterilised PN*:* extracted by sterile distilled water kept as non-sterilized, PH: prepared with full vacuumed and dried propolis with honey *TEAC: Trolox equivalent antioxidant capacity, **SD: Standard deviation, ***GAE: Gallic acid equivalent)

Tablo 3. Propolis ekstraktlarının ABTS radikal katyonu ve kuprik iyonu antioksidan kapasitesini (CUPRAC) azaltıcı etkisi. (POH: etil alkol, PS: steril olarak saklanan steril damıtılmış su ile ekstrakte edilen, PN: sterilize edilmemiş olarak saklanan steril damıtılmış su ile ekstrakte edilen, PH: tamamen vakumlanmış ve bal ile kurutulmuş propolis ile hazırlanan *TEAC: Trolox eşdeğeri antioksidan kapasitesi, **SD: Standart sapma, ***GAE: Gallik asit eşdeğeri)

Extract	TEAC* (mg TE/g extract) (ABTS)	Percentage of inhibition ± SD** against ABTS radical cation	Antioxidant capacity (mg GAE***a/g extract)
PH	65,13 ± 2,33	61,94 ± 2,11	56,78 ± 2,08
POH	73,37 ± 0,31	69,40 ± 3,4	271,75 ± 5,71
PS	34,35 ± 2,07	33,95 ± 1,87	205,23 ± 5,11
PN	34,62 ± 1,64	34,32 ± 1,49	126,13 ± 7,44

Cupric ion reducing antioxidant capacities of the propolis extracts were given in Table 3. The highest activity was shown by POH (271,75 mg GAE/g extract) which was followed by aqueous extract and lowest value belonged to HP.

PCA reduced the dimensionality of our multivariate data to two principal components and it was visualised with minimal loss of information, by using scatter diagram (Fig. 1).

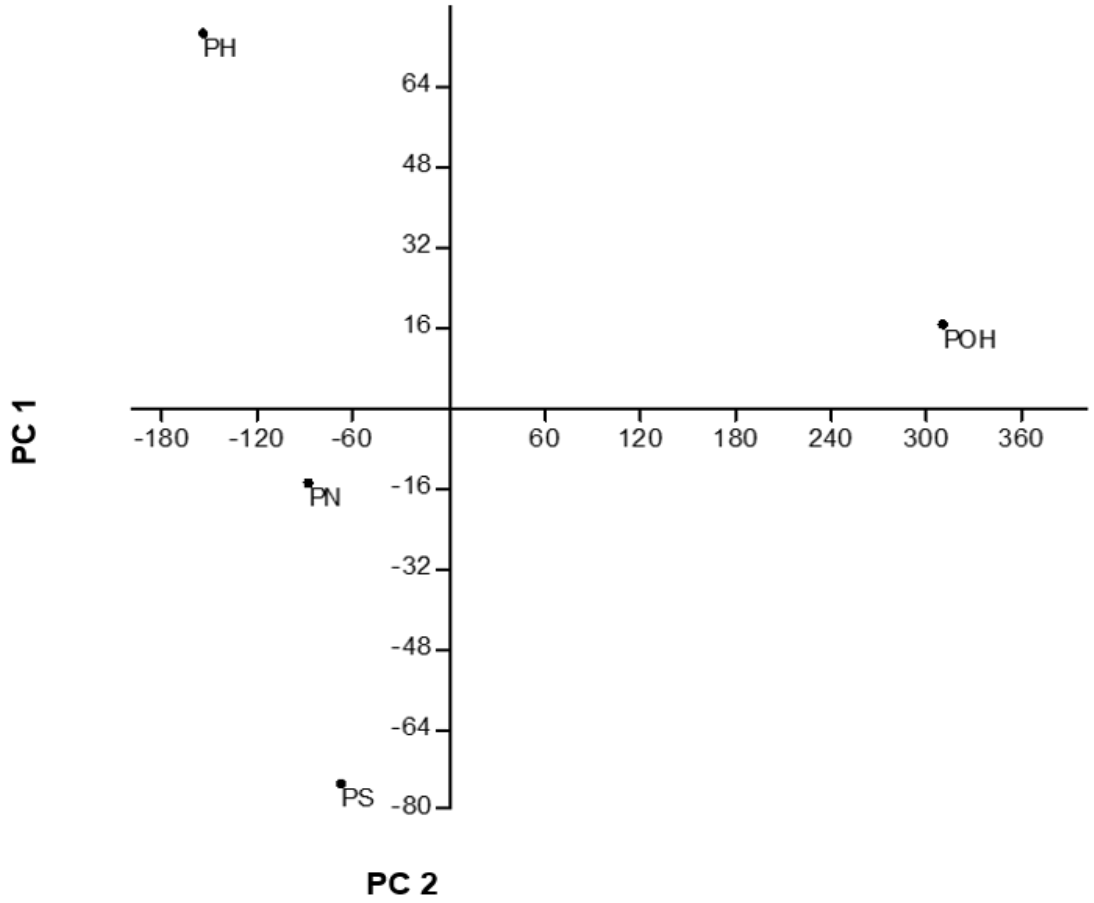

Figure 1. PCA scatter diagram (POH: ethyl alcohol, PS: extracted by sterile distilled water kept as sterilised PN*:* extracted by sterile distilled water kept as non-sterilized, PH: prepared with full vacuumed and dried propolis with honey)

Şekil 1. Temel bileşenler analizi saçılım grafiği (POH: etil alkol, PS: steril olarak saklanan steril damıtılmış su ile ekstrakte edilen, PN: sterilize edilmemiş olarak saklanan steril damıtılmış su ile ekstrakte edilen, PH: tamamen vakumlanmış ve bal ile kurutulmuş propolis ile hazırlanan).

DISCUSSION

Propolis can be classified biologically according to its producer (depending on the bee species) or botanical origin (depending on the plants used by bees). This resinous substance also can be classified chemically. Differentiation among the extraction methods and solvents can give us varied combinations- even if we use the same propolis with exactly the same biological origin.

According to Nalbantsoy et al. (2022), several external factors are present in the production process of propolis. Within this frame, we aim to evaluate different extraction techniques by comparing antioxidant properties of the same propolis with the use of DPPH, ABTS+ and CUPRAC methods. As an opotherapeutic medicine or a human diet supplement, the most important groups we gain from propolis are polyphenolic compounds, especially flavonoids. The antioxidant activity of propolis appears to be largely influenced by both total polyphenol and total flavonoid contents (Sun et al. 2015, Socha et al. 2014, Narimane et al. 2017). Değirmencioğlu et al. reported in their study that with 19 samples from Turkey, total phenolic content found 11.24 -172.98 mg GAE/g and total flavonoid content was 3.88 -58.31 mg QE/g (Değirmencioğlu et al. 2019). Another research carried out with 23 propolis samples from Turkey the total flavonoid content were determined between 21,28- 152,56 mg QE/g and total phenolic content was found between 34,53-259,4 mg GAE/g (Özkök et al. 2021). Güzelmeriç et al. worked with 47 samples produced in Black Sea Region of Turkey and reported that total phenolic content values between 37.25 ± 0.72-592.57 ± 22.39 mg GAE/g; total flavonoid content values between 14.60 ± 0.57-125.58 ± 0.58 mg QE/g (Güzelmeriç et al. 2021). The total phenolics and flavonoid contents in our propolis samples are found relatively higher compared with the other studies held in Turkey (Özkök et al. 2021). All propolis types have very low solubility in water and are soluble in organic solvents, because resins are relatively apolar (Bankova et al. 2021). Beside this fact, maybe because of the botanical origin of our samples, water extractions and dried propolis with honey have higher values than some of the samples in other studies extracted with ethanol. Gençay-Çelemli et al. found that the total phenolic compound of the five samples varies between 27.56±0.05 and 171.93±0.28 mg GAE/g. Also, it was added that total phenolic and flavone-flavonol contents were found highest in the sample that sourced from the taxa belonging to the Brassicaceae family, which is contrary to common belief since the phenolic content of chestnut propolis is higher (Gençay-Çelemli et al. 2019).

According phytochemical research on propolis extract, there is generally a positive correlation between the total phenol and flavonoid content in propolis extraction and their antioxidant activity (Güzelmeriç et al. 2021, Degirmencioglu et al. 2019Gençay et al. 2019,). Phenolic compounds are likely to contribute to the radical scavenging activity of these extracts. According to the results, the new aqueous extraction technique is promising with relatively high polyphenol contents and antioxidant activities. Besides honey with propolis could be an alternative product, although it has relatively lower values. Probably due to the fact that the amount of propolis extract added to honey was not large enough to significantly increase these parameters as it was done before by Osés et al. 2015.

Total phenol and flavonoid contents in non-sterilized aqueous extract of propolis is slightly higher than sterilised aqueous extract. This may result from damage, reduction or alteration of some compounds during the sterilisation process. They show almost the same activity in the ABTS assay whereas in CUPRAC and DPPH assay the antioxidant capacity of sterilised aqueous extract is higher than non-sterilized extract. As we know, the antioxidant capacity of propolis is dependent on its content, but the studies generally aim to compare antioxidant potential of different propolis extract. Although it is a fact that the antioxidant capacity of ethyl alcohol extractions is higher than the others, many commercial ethanol extracted propolis preparations can cause oral mucosal ulceration or gastrointestinal health problems. Moreover, despite the method differences, the results indicated that Tunceli propolis has a relatively high total phenol and flavonoid content compared to other region in Turkey and subsequently possess a high antioxidant potential (Apak et al. 2004; Özkök et al. 2021, Güzelmeriç et al. 2021).

Conclusion

Propolis, as a nutritious product, provides a rich source of nutrients, such as mineral elements, proteins, and antioxidant compounds. The antioxidant capacity of propolis is related to the flavonoid, mineral, and protein contents which derived from botanical origins of the product and extraction solvents. Among the studied samples,

ethyl alcohol extraction of propolis possess highest content of phenol and flavonoid, as well as the highest antioxidant activity. The aqueous extractions also have significant antioxidant capacities. In future studies, there is a need to investigate the eco-floral effect of the antioxidant content of propolis Tunceli-Ovacık region in detail.

Source of Finance: Not applicable.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contribution: Ç.Ö. and G.Z. carried out the experiments, wrote the manuscript.

Ethical issue: Not applicable.

Acknowledgements: We are thankful to the Beekeepers Association of Tunceli during the conduct of the study. The authors thank Dr Emre Çilden for his constructive comments which improved the main text.

REFERENCES

Alvarez-Suárez, J. M., Tulipani, S., Romandini, S., Bertoli, E., & Battino, M. (2010). Contribution of honey in nutrition and human health, a review. Mediterranean Journal of Nutrition and Metabolism, 3(1), 15–23.

Apak R, Güçlü K, Özyürek M, Karademir SE. A novel total antioxidant capacity index for dietary polyphenols, vitamin C and E, using their cupric ion reducing capability in the presence of neocuproine: The CUPRAC method J. Agric. Food Chem. 2004; 52(26): 7970-7981, doi.org/10.1021/jf048741x.

Araghi SH, John A, Googheri, MSS. A molecular dynamics simulations study of the ionic liquid effect on the β-glucosidase active site interactions with a flavonoid glycoside Journal of Molecular Liquids. 2021; 340, 117115, doi.org/10.1016/j.molliq.2021.117115.

Bankova V, Chemical diversity of propolis and the problem of standardization Journal of ethnopharmacology. 2005; 100(1-2): 114-117, doi.org/10.1016/j.jep.2005.05.004.

Bankova V, Popova M, Trusheva B, Propolis volatile compounds: chemical diversity and biological activity: a review Chemistry Central Journal. 2014; 8(1): 1-8, doi.org/10.1186/1752-153X-8-28.

Bankova V, Trusheva B, Popova M, Propolis extraction methods: A review Journal of Apicultural Research. 2021; 60(5): 734-743, doi.org/10.1080/00218839.2021.1901426.

Bhargava P, Mahanta D, Kaul A, Ishida Y, Terao K, Wadhwa R, Kaul SC, Experimental Evidence for Therapeutic Potentials of Propolis Nutrients. 2021; 13(8): 2528, doi.org/10.3390/nu13082528.

Brand-Williams W, Cuvelier ME, Berset , Use of free radical method to evaluate antioxidant activity, LWT-Food Science and Technology,1995; 28(1): 25-30, doi.org/10.1016/S0023-6438(95)80008-5.

Çelemli Gençay Ö, Atakay M., Sorkun K, The correlation between botanical source and the biologically active compounds of propolis, İstanbul Journal of Pharmacy, 2019; 49(2): 81-87, doi.org/10.26650/IstanbulJPharm.2019.435408.

Çelemli Gençay Ö, Chemical properties of propolis collected by stingless bees, In Pot-Honey. Springer, New York, NY, 2013; pp. 525-537.

Chang C, Yang M, Wen H, Chern J, Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis, 2002; 10(3): 178-182.

Christov R, Trusheva B, Popova M, Bankova V, Bertrand M, Chemical composition of propolis from canada, its antiradical activity and plant origin Nat. Prod. Res. 2006; 20: 531–536, doi.org/10.1080/14786410500056918.

Dallabona I D, de Lima GG, Cestaro BI, de Souza Tasso I, Paiva TS., Laureanti EJG, et. al. Development of alginate beads with encapsulated jabuticaba peel and propolis extracts to achieve a new natural colorant antioxidant additive International Journal of Biological Macromolecules, 2020; 163: 1421-1432, doi.org/10.1016/j.ijbiomac.2020.07.256.

Degirmencioglu T, Guzelmeric H, Yuksel E, Kırmızıbekmez, PI, Deniz H, Yesilada E, A new type of Anatolian propolis: Evaluation of its chemical composition, activity profile and botanical origin, Chemistry & biodiversity

2019; 16(12): e1900492, doi.org/10.1002/cbdv.201900492.

Dezmirean DS, Paşca C, Moise AR, Bobiş O, Plant sources responsible for the chemical composition and main bioactive properties of poplar-type propolis Plants, 2021; 10(1): 22, doi.org/10.3390/plants10010022.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) Scientific Opinion on the substantiation of health claims related to propolis (ID 1242, 1245, 1246, 1247, 1248, 3184) and flavonoids in propolis (ID 1244, 1644, 1645, 3526, 3527, 3798, 3799) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. EFSA journal, 2010; 8(10), 1810, doi.org/10.2903/j.efsa.2010.1810.

Elbaz NM, Khalil IA, Abd-Rabou AA, El-Sherbiny IM, Chitosan-based nano-in-microparticle carriers for enhanced oral delivery and anticancer activity of propolis international journal of biological macromolecules. 2016; 92: 254-269, doi.org/10.1016/j.ijbiomac.2016.07.024.

Escuredo O, Míguez M, Fernández-González M, Carmen Seijo M, Nutritional value and antioxidant activity of honeys produced in a European Atlantic area, Food Chemistry, 2013; 138: 851–856. http://dx.doi.org/10.1016/j.foodchem.2012.11.015.

Gardana C, Scaglianti M, Pietta P, Simonetti P, Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry Journal of Pharmaceutical and Biomedical Analysis. 2007; 45(3): 390-399, doi.org/10.1016/j.jpba.2007.06.022

Gheldof N, Wang XH, Engeseth N, Identification and quantification of antioxidant components of honeys from various floral sources J. Agric. Food Chem. 2002; 50(21): 5870–5877, doi.org/10.1021/jf0256135.

Güzelmeriç E, Yuksel P I, Yaman BK, Sipahi H, Celik C, Kırmızıbekmez H, et. al. Comparison of antioxidant and anti-inflammatory activity profiles of various chemically characterized Turkish propolis sub-types: Which propolis type is a promising source for pharmaceutical product development? Journal of Pharmaceutical and Biomedical Analysis, 2021; 203: 114196, doi.org/10.1016/j.jpba.2021.114196.

Hammer Ø, Harper DA, Ryan PD, PAST: Paleontological statistics software package for education and data analysis. Palaeontologia electronica, 2001; 4(1), 9.

Hernandez Zarate MS, Abraham Juarez MDR, Ceron Garcia A, Ozuna Lopez C, Gutierrez Chavez AJ, Segoviano Garfias JDJN, et. al. Flavonoids, phenolic content, and antioxidant activity of propolis from various areas of Guanajuato, Mexico Food Science and Technology, 2018; 38: 210-215, /doi.org/10.1590/fst.29916.

Huang S, Zhang CP, Wang K, Li GQ, Hu FL, Recent advances in the chemical composition of propolis, Molecules, 2014; 19: 19610–19632, doi.org/10.3390/molecules191219610.

Li K, Yao F, Xue Q, Fan, H,Yang, L Li, X., et. al. Inhibitory effects against α-glucosidase and α-amylase of the flavonoids-rich extract from Scutellaria baicalensis shoots and interpretation of structure–activity relationship of its eight flavonoids by a refined assign-score method Chemistry Central Journal. 2018; 12(1): 1-11, doi.org/10.1186/s13065-018-0445-y.

Lorenzon MCA, Castro RN, de Oliveira Pires L, Koshiyama AS, Bento KJB, Biological values of different types of Brazilian propolis Greener Journal of Agricultural Sciences 2018; 8: 90, doi.org/10.15580/GJAS.2018.5.033118054

Lotfy M, Biological activity of bee propolis in health and disease Asian Pac J Cancer Prev. 2006; 7(1): 22-31.

Machado B, Pulcino T N, Silva A L, Tadeu D, Melo RGS, Mendonça IG, Propolis as an alternative in prevention and control of dental cavity Immunity. 2017; 19: 24, doi.org/ ?10.5455/ja.20160726115117.

Midorikawa K, Banskota A H, Tezuka Y, Nagaoka T, Matsushige K, Message D, et. al. Liquid chromatography–mass spectrometry analysis of propolis Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques, 2001; 12(6): 366-373, doi.org/10.1002/pca.605.

Nalbantsoy, A., Sarıkahya, N. B., Özverel, C. S., Barlas, A. B., Kırcı, D., Akgün, İ. H., et. al. Chemical composition and biological activities of Cypriot propolis. Journal of Apicultural Research 2022; 61(2): 233-24, doi.org/10.1080/00218839.2021.1977028.

Narimane S, Demircan E, Salah A, Ozcelik BÖ, Salah R, Correlation between antioxidant activity and phe-nolic acids profile and content of Algerian propolis: influence of solvent Pakistan Journal of Pharmaceutical Sciences. 2017; 30(4):1417–1423.

Osés, SM, Melgosa L, Pascual-Maté A, Fernández-Muiño MA, Sancho MT, Design of a food product composed of honey and propolis Journal of Apicultural Research, 2015; 54(5): 461-467, doi.org/10.1080/00218839.2016.1183934.

Özkök A, Keskin M, Samancı AET, Önder EY, Takma Ç, Determination of antioxidant activity and phenolic compounds for basic standardisation of Turkish propolis Applied Biological Chemistry, 2021; 64(1): 1-10, doi.org/10.1186/s13765-021-00608-3.

Park YK, Ikegaki M, Preparation of water and ethanolic extracts of propolis and evaluation of the preparations Bioscience, biotechnology, and biochemistry. 1998; 62(11): 2230-2232, doi.org/10.1271/bbb.62.2230.

Pasupuleti VR, Sammugam L, Ramesh N, Gan SH, Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits Oxidative medicine and cellular longevity. 2017; doi.org/10.1155/2017/1259510.

Paviani LC, Dariva C, Marcucci MC, Cabral FA, Supercritical carbon dioxide selectivity to fractionate phenolic compounds from the dry ethanolic extract of propolis, Journal of Food Process Engineering. 2010;33(1): 15-27, doi.org/10.1111/j.1745-4530.2008.00256.x.

Pereira DS, Freitas CIA, Freitas MO, Maracaja P, da Silva JBA, da Silva RA, et. al., Histórico e principais usos da própolis apícola Embrapa Amazônia Oriental-Artigo em periódico indexado (ALICE), 2015.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay Free Radical Biology and Medicine 1999; 26(9): 1231-1237, doi.org/10.1016/S0891-5849(98)00315-3.

Ristivojević P, Trifković J, Andrić F, Milojković-Opsenica D, Poplar-type propolis: chemical composition, botanical origin and biological activity, Natural product communications. 2015; 10(11): doi.org/10.1177/1934578X1501001117.

Salatino A, Fernandes-Silva CC, Righi AA, Salatino MLF, Propolis research and the chemistry of plant products Natural product reports. 2011; 28(5): 925-936, doi.org/10.1039/C0NP00072H.

Salatino A, Teixeira ÉW, Negri G, Origin and chemical variation of Brazilian propolis, Evidence-based complementary and alternative medicine 2005; 2(1): 33-38, doi.org/10.1093/ecam/neh060.

Salgueiro FB, Castro RN, Comparação entre a composição química e capacidade antioxidante de diferentes extratos de própolis verde, Química Nova. 2016; 39: 1192-1199, doi.org/10.21577/0100-4042.20160136.

Santos-Buelga C, González-Paramás AM, Phenolic composition of propolis. In Bee Products-Chemical and Biological Properties Springer, Cham.2017, pp. 99-111.

Simone-Finstrom M, Borba RS., Wilson M, Spivak M, Propolis counteracts some threats to honey bee health, Insects. 2017; 8(2): 46, doi.org/10.3390/insects8020046

Slinkard K, Singleton VL, Total phenol analysis: Automation and comparison with manual methods American Journal of Enology and Viticulture. 1977, 28(1): 49-55.

Socha R, Gałkowska D, Bugaj M, Juszczak L, Phenolic composition and antioxidant activity of propolis from various regions of Poland, Natural Product Research 2014; 29(5): pp. 416–422, doi.org/10.1080/14786419.2014.949705.

Sun C, Wu Z, Wang Z, Zhang H, Effect of ethanol/ water solvents on phenolic profiles and antioxidant proper- ties of Beijing propolis extracts Evidence-based Complementary and Alternative Medicine, vol. 2015, Article ID

595393, 9 pages. doi.org/10.1155/2015/595393.

Uzel A, Önçağ Ö, Çoğulu D, Gençay Çelemli Ö, Chemical compositions and antimicrobial activities of four different Anatolian propolis samples Microbiological research. 2005; 160(2): 189-195, doi.org/10.1016/j.micres.2005.01.002.

Zenebom O, Pascuet N, Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Métodos físico-químicos para análise de alimentos 2005; 4.

Zhang CP, Zheng HQ, Hu FL, Extraction, partial characterization, and storage stability of β-glucosidase from propolis Journal of food science. 2011; 76(1): C75-C79, doi.org/10.1111/j.1750-3841.2010.01941.x.