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Comparison of total phenolic contents and antioxidant activities of propolis in different solvents

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ABSTRACT

This study aims to determine which solvent is the best for the solubility of the propolis by using concentrations of total polyphenols and flavonoids, ferric reducing antioxidant power (FRAP) assay, and total antioxidant status (TAS) in extracts of propolis from different provinces of Türkiye prepared with water, ethanol, dimethyl sulfoxide (DMSO), glycerol and acetone. Propolis samples were lyophilized in the same solvents except for that glycerol and acetone. Total concentrations of polyphenols and flavonoids, FRAP, and TAS of both normal and lyophilized extracts were found be consistent when compared with each other. After extraction of propolis and evaluation of the total polyphenol and flavonoid content and antioxidant capacity, we concluded that it is mostly dissolved in DMSO, and after that in ethanol, acetone, glycerol respectively, and the least in water according to our extraction and analysis methods.

Keywords: Propolis, Flavonoids, Polyphenols, Solubility, Different solvents

Introduction

Propolis is a resinous, sticky, natural complex mixture collected by honeybees from various plant sources (Burdock, 1998). It has a characteristic smell and colours changing from yellow, green, red to dark brown (Burdock, 1998; Orsatti et al., 2010). Propolis contains more than 300 kinds of chemical compounds such as polyphenols (flavonoids, phenolic acids, and their esters, phenolic aldehydes, alcohols, and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds (Bankova et al., 2000). In recent years, propolis has gained quite popularity in the food and beverage industries in order to prevent many diseases such as cardiovascular disease, diabetes, and cancer and to protect health (Banskota et al., 2000).

Propolis has a wide range of biological activities such as antioxidant (Nagai et al., 2003; Kumazawa et al., 2004; Mohtar et al., 2020; Peixoto et al., 2021), antifungal, antibacterial, antiviral (Kujumgiev et al., 1999), antiproliferative (Banskota et al., 2002), cytotoxic (Banskota et al., 2000), immunomodulator (Orsolic et al., 2004), antimicrobial (Arslan et al., 2012), anti-inflammatory (Barlak et al., 2015), radioprotective (Benkovic et al., 2008), hepatoprotective (Banskota et al., 2000), preventive and protective effects against DNA damage (Aliyazicioglu et al., 2011).

It has been suggested that the compounds primarily responsible for the biological activities of propolis are phenolic compounds such as flavonoids (Havsteen, 2002). It has been shown that antioxidant activity, which is one of the most important biological activities of flavonoids in propolis, provides protection against lipid peroxidation in the cell membrane, thanks to its ability to scavenge free radicals (Pinchuk and Lichtenberg, 2002).

Since propolis cannot be used in its raw form, it should be purified by extraction with solvents. In this process, inert substances should be removed, and polyphenolic fractions should be protected (Pietta et al., 2002). Propolis extraction methods may affect the activity of propolis, as the use of different solvents may dissolve and extract different compounds in propolis (Sforcin, 2007).

The main purpose of this study is to compare the total phenolic contents and antioxidant activities of normal and lyophilized extracts of propolis, which is collected from different provinces of Türkiye, with the five different solvents determined as water, ethanol, dimethyl sulfoxide (DMSO), glycerol, and acetone.

Materials and Methods

Propolis Origin

Propolis was formed by mixing propolis samples supplied by Fanus Food Company (Trabzon, Türkiye) from different provinces of Türkiye.

Preparation of Water, Ethanol, Dimethyl Sulfoxide (DMSO), Glycerol and Acetone Extracts of Propolis

First, the propolis sample was frozen at -20°C and grated. The grated propolis sample was refrozen at -20°C and was ground in a blender (Arzum AR1002). With our own extraction method, 500 mg of ground propolis were dissolved in 20 mL of pure water, ethanol (Riedel-de Haën), DMSO (Carlo Erba), glycerol (Merck), or acetone (Merck) at 150 rpm and 60°C with the aid of a shaker incubator for 24 hours. After incubation, each extract was centrifuged at 2057 *g* for 10 minutes and filtered through filter paper. Collected supernatants were stored at 4 °C in the dark for further studies. The final concentration of each propolis extract including water extract of propolis (WEP), ethanol extract of propolis (EEP), DMSO extract of propolis (DEP), glycerol extract of propolis (GEP), and acetone extract of propolis (AEP) was adjusted to 25 mg/mL (stock solution). A proportion of 5 mL of the water, ethanol, and DMSO extracts were kept at -80°C for 30 minutes and lyophilized for 6 hours. 5 mL solvent (water, ethanol, or DMSO) was added to those extracts to obtain dissolved lyophilized extracts.

Determination of Total Polyphenol Content

Total polyphenol content was determined spectrophotometrically by modifying the Folin-Ciocalteu colorimetric method and adapting this method to a 96-well microplate reader (Lotito and Frei, 2004). 12.5 µL of diluted (1:50 with deionized water) propolis extracts were mixed by adding 62.5 µL of Folin-Ciocalteu reagent (Sigma) (1:10) and 125 µL of sodium carbonate (Lancaster) (20 %, w/v) into a 96-well microplate. After 30 minutes of incubation at room temperature and in the dark, absorbance was read at 700 nm on the microplate reader (Tunable VERSAmax microplate reader, USA). Gallic acid (Sigma) was used as a standard in drawing the calibration curve. Total polyphenol contents were stated as mg Gallic acid (GA)/g propolis.

Determination of Total Flavonoid Content

Total flavonoid content was determined spectrophotometrically by modifying the aluminium nitrate colorimetric method (Park et al., 1997). 20 µL of diluted (1:20 with deionized water) propolis extracts were mixed by adding 172 µL of 80 % ethanol, 4 µL of 10 % aluminium nitrate (Fluka) and

4 µL of 1 M aqueous potassium acetate (Merck) into a 96-well microplate. After 40 minutes of incubation at room temperature and in the dark, absorbance was read at 415 nm on the microplate reader. Quercetin (Fluka) was used as a standard in drawing the calibration curve. Total flavonoid contents were stated as mg Quercetin (Q)/g propolis.

Determination of Fe^{3+} (Ferric) Reducing Antioxidant Power (FRAP)

The reducing antioxidant power was determined spectrophotometrically according to the method applied by Oyaizu (1986) based on ferric to ferrous ion reduction at low pH (Oyaizu, 1986). To 40 µL of diluted (1:100 with deionized water) propolis extract in 1.5 mL of microtube (Eppendorf) was added 100 µL of 0.2 M sodium phosphate buffer (Merck) (pH 6.6) and 100 µL of 1% potassium ferricyanide (Lancaster) and mixed. The mixture was incubated at 50 °C for 20 minutes and cooled to room temperature. Then, 100 µL of 10% trichloroacetic acid (ABCR) was added to the mixture and centrifuged at 3000 g (Thermo micromax SN: 8035/2) for 10 minutes. 100 µL of the upper phase was taken and transferred to a 96-well plate. The transferred phase was mixed with 100 µL of deionized water and 20 µL of 0.1% $FeCl_3$ (Sigma) in a 96-well plate. It was incubated for 5 minutes at room temperature in the dark and absorbance was read at 700 nm on the microplate reader. Trolox (Fluka) was used as a standard in drawing the calibration curve. Antioxidant potentials of propolis were stated as mg Trolox (Tro)/g propolis.

Determination of Total Antioxidant Status (TAS)

The total antioxidant status was determined according to the colorimetric method applied by Erel (2004). TAS was measured using the TAS kit (Rel Assay Diagnostics, Cat No: RL001) and the results were stated in mmol Trolox (Tro)/100 g propolis.

Results and Discussion

Total Phenolic Contents and Antioxidant Activities of Propolis Extracts

Total polyphenol content, total flavonoid content, ferric reducing antioxidant power (FRAP) and total antioxidant status (TAS) of all normal and lyophilized extracts (DEP, EEP, AEP, GEP, WEP, lyophilized dimethyl sulfoxide extract of propolis (LDEP), lyophilized ethanol extract of propolis (LEEP), and lyophilized water extract of propolis (LWEP)) were determined and the results were stated as mg GA/g propolis, mg Q/g propolis, mg Tro/g propolis and mmol Tro/100 g propolis, respectively. These results were given in Table 1 and were found to be consistent with each other in terms of both the amount of phenolic compounds and antioxidant activity.

As we cannot use propolis in the natural state, it must be refined by extraction using solvents (Pietta et al., 2002). Since different solvents should solve various compositions of propolis in different amounts, the contents of the WEP, EEP, DEP, GEP, and AEP would be different in quality and/or quantity. In most studies, the solvents chosen to dissolve propolis are not used purely, but diluted with water from 15% to 95%, and these diluted extracts have been studied (Schnitzler et al., 2010; Silva et al., 2012; Frozza et al., 2013; Siripatrawan et al. 2013; Wang et al., 2014; Cruz et al. 2021). The reason we used pure solvents was to determine which solvent would achieve the best solubility.

Silva et al. (2012) studied polyphenolic and flavonoid contents of propolis, by preparing hydro-alcoholic, methanol, and water extracts of propolis for every region (Bragança, Coimbra, and Beja). Polyphenol and flavonoid contents of hydro-alcoholic extracts were found to be considerably high as compared to methanol and water extracts. Total phenolic (277.17 ±7.50) and flavonoid (142.32 ±4.52) contents of Bragança propolis were determined to be of quite a high concentration (mg/g), and Coimbra and Beja propolis followed them respectively. Alencar et al. (2007) found that ethanol extract of Brazilian red propolis includes 232 ±22.3 mg/g polyphenol and 43 ±1.0 mg/g flavonoid. In another study, Frozza et al. (2013) found that hydro-alcoholic extract of Brazilian red propolis includes 151.55 ±1.95 mg/g polyphenolic composition as a dry extract. This difference came from the various methods of extraction, and geographical localization as well.

In addition, each researcher works with different solvents, at different absorbance values, at different concentrations, and by modifying the methods, they apply in various ways. Therefore, this affects the amount of polyphenols and flavonoids in propolis extract. For this reason, all these criteria will also affect the antioxidant activity of propolis. For this reason, it seems difficult to make a clear comparison of the differences between the methods in the studies.

The antioxidant activities of propolis samples from different geographical regions (Argentina, Australia, Brazil, Bulgaria, Chile, China (Hebei, Hubei and Zhejiang), Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States and Uzbekistan) were compared by Kumazawa et al. (2004). EEP originated from Argentina, Australia, China, Hungary, and New Zealand had comparatively powerful antioxidant activity and stood in correlation with the total polyphenol and flavonoid contents. But Thailand propolis was found to have the lowest values (Kumazawa et al., 2004). Kumazawa et al. (2004) determined that the polyphenol content

of the ethanolic extract of European and Chinese propolis was ranged from 200 to 300 mg GA/g propolis.

It is suggested that a single constituent of propolis does not have more powerful activity than complete extract and therefore the general biological qualities of propolis emanated from the natural combination of its constituents (Sforcin, 2007). For that reason, instead of isolating the constituents of extracts used in our study and examining their effects separately, we preferred using the whole sample.

In our study, the total polyphenol and flavonoid contents of propolis with DEP were found to be higher than EEP, AEP, GEP, and WEP. Also, DEP was found to have more FRAP capacity and were at a higher level in terms of TAS than EEP AEP, GEP, WEP (Table 1).

Total polyphenol content in the LEEP was found to be higher than as in the LDEP and LWEP. Total flavonoid content, FRAP, and TAS in the LDEP was also found to be higher than as in the LEEP and LWEP (Table 1).

When we used lyophilized propolis, we aimed to separate organic compounds from the resin available and to see the difference between lyophilized and non-lyophilized samples of propolis. But according to the results of our analysis of contents and antioxidant tests, it has been found that there were no great differences between lyophilized and non-lyophilized samples of propolis in terms of content and antioxidant capacity. TAS and FRAP methods to extracts were found to be proportionate to the amounts of polyphenol and flavonoid contents.

The materials in propolis mainly lipophilic compounds. Because it is easy to extract lipophilic compounds by using ethanol, that of EEP is well known and interest greatly (Nakajima et al., 2007). Although using EEP is prevailing, research about WEP has increased in number (Mani et al., 2006).

The WEP has a good antioxidant activity due to its high phenolic compound content. It has been reported that the water extract of propolis has hepatoprotective effect on both chemical and immunological liver injury models, inhibits platelet aggregation, and shows antiviral and anti-inflammatory activity (Nagai et al., 2003; Mani et al., 2006).

Nakajima et al. (2007), in a study they conducted, revealed that water extract of Brazilian green propolis and caffeoylquinic acid derivatives had neuroprotective effects on retinal damage *in vitro* and that these effects were due to their antioxidant properties (Nakajima et al., 2007).

In another study by Nakajima et al. (2009) where they prepared Brazilian WEP and EEP, water extract of royal jelly and ethanol extract of pollen, comparing radical scavenging activity of hydrogen peroxide, superoxide anion and hydroxyl through different antioxidant capacity methods, the antioxidant capacity was found to be in WEP, EEP and ethanol extract of pollen, respectively (Nakajima et al., 2009).

When Laskar et al. (2010) compared various antioxidant determination methods, they suggested that the water extract of Indian propolis is more effective than the ethanolic extract, because of its high polyphenol content, and that it can be used in the prevention of various diseases related to free radicals (Laskar et al. 2010).

DEP is used to some extent in cell culture studies (Azarshinfam et al., 2021; Liao et al., 2021). Studies on the extraction of propolis with glycerol (Thamnopoulos et al., 2018) and acetone (Kheiri et al., 2011) are very few, and antioxidant activity studies have not been conducted in any of them. Therefore, in addition to DEP, AEP and GEP were also included in this study.

Table 1. Total phenolic contents and antioxidant activities of propolis extracts (Arithmetic mean ± SD, n=3)

	DEP	EEP	AEP	GEP	WEP	LDEP	LEEP	LWEP
Total polyphenol content (mg GA/g propolis)	141.2 ±9.99	122.7 ±6.37	100.0 ±8.49	88.0 ±7.75	19.7 ±0.29	136.8 ±4.04	142.0 ±1.41	18.2 ±1.15
Total flavonoid content (mg Q/g propolis)	55.3 ±6.63	47.8 ±8.66	47.3 ±6.43	23.3 ±1.91	1.3 ±0.12	63.5 ±7.07	54.2 ±4.86	2.4 ±1.02
Ferric reducing power (mg Tro/g propolis	273.8 ±11.62	236.9 ±13.92	221.3 ±14.11	141.8 ±18.97	26.2 ±8.57	287.1 ±8.74	232.9 ±19.23	24.0 ±5.55
Total antioxidant status (mmol Tro/100 g propolis)	248.5 ±5.10	233.1 ±1.99	157.5 ±11.06	159.8 ±5.73	15.4 ±5.39	242.9 ±13.48	238.3 ±10.1	26.0 ±1.12

DEP: DMSO extract of propolis; EEP: ethanol extract of propolis; AEP: acetone extract of propolis; GEP: glycerol extract of propolis; WEP: water extract of propolis; LDEP: lyophilized DMSO extract of propolis; LEEP: lyophilized ethanol extract of propolis; LWEP: lyophilized water extract of propolis

Conclusion

As a result; after extraction of propolis using water, ethanol, DMSO, glycerol, and acetone as solvents, evaluated the total polyphenol and flavonoid content and antioxidant capacity, we concluded that it is mostly dissolved in DMSO, and after that in ethanol, acetone, glycerol respectively, and the least in water.

In the light of all this information, propolis can be a natural raw material source for various sectors such as food industry, medicine and cosmetics, thanks to its solubility in various solvents.

Compliance with Ethical Standard

Conflict of interests: The author declares that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: Author declare that this study does not include any experiments with human or animal subjects; therefore, no ethics committee approval is needed.

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