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Research Article

Ultrasonic assisted propolis extraction: characterization by ATR-FTIR and determination of its total antioxidant capacity and radical scavenging ability

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Abstract: In the current study, ultrasonic assisted ethanolic extract of propolis **was** discussed in detail, including their total phenolic content, total antioxidant capacity and radical scavenging capacity. For this purpose, we determined the total antioxidant capacity of propolis extract by CUPRAC and FRAP) assay. At the same time, the free radical scavenging capacity of propolis extracts was investigated via the DPPH˙ and CUPRAC- hydroxyl radical scavenging (HRS) methods. The chemical constituents of propolis extract were characterized by ATR-FTIR. The results revealed that propolis is rich in total phenolic components (189 mg GAE /g extract). According to the CUPRAC assay, the total antioxidant capacity of propolis extract was calculated to be 2.43 ± 0.07 mmol TE/g-propolis extract. FRAP value of propolis extract was determined as 1.11 mmol TE/g-propolis extract. DPPH• scavenging activity of propolis extract was calculated to be 0.71± 0.002 mmol TE / g - extract. On the other hand, according to the CUPRAC method, HRS capacity of propolis extract at different concentrations (5-10 µg/mL) was determined as 68.1% and 77.64 %, respectively. Research findings showed that propolis extract has a strong radical scavenging potential. The FTIR spectra of the functional groups originating from the phenolic compounds in the propolis extract were as expected.

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DPPH,

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1. INTRODUCTION

Propolis is a natural product collected by bees from the cones and barks of trees, buds and shoots of plants. The content of propolis varies depending on the region where it is collected and the season. It has strong antimicrobial (Choi *et al*., 2006), antiviral, anti-inflammatory (Kujumgiev *et al*., 1999), antioxidant (Mohammadzadeh *et al.,* 2007), regenerative effects, and anticancer (Kimoto *et al.,* 2001), containing a mixture of oils, pollen, special resin and waxy substances in its composition (Osés *et al*., 2016). Propolis generally contains various chemical compounds such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids (Kumazawa *et al.,* 2004). Propolis is used in traditional medicine, cosmetics and food industry due to the pharmacological activity of its bioactive components (Banskota *et al*., 2001; Chaillou & Nazareno, 2009; Dezmirean *et al*., 2020).

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Antioxidant components could remove free radicals and prolong shelf life by delaying the lipid peroxidation process, which causes food and pharmaceutical products to deteriorate (Halliwell, 1996). An inquiry of normally happening antioxidant ingredients from plant sources may prompt the advancement of novel medicines, which may diminish the danger of long-term infections brought about by free radicals (Abuja & Albertini, 2001).

The antioxidant activity of propolis deserves attention due to the phenolic components it contains. Since propolis contains a high proportion of phenolic components, it has significant antioxidant activity. Phenolic compounds represent the largest group of propolis components, depending on the amount and type (Oroian *et al*., 2020).

Ultrasound-assisted extractions (UAE) is a new and easy-to-use technique for obtaining bioactive molecules from various sources (Carreira-Casais *et al*., 2021; Jha & Sit, 2021). The intensity of the ultrasonic energy generates more vibrations in the sample components, facilitating the transport of the target molecules from the solid to the liquid solvent medium (Samaram *et al*., 2015). Due to the high yield with short extraction time and the use of a small amount of solvents, the UAE technique is superior to other techniques. In addition, it is an ideal option in the food industry as it can be made quickly, efficiently and at low temperatures (Madhu *et al*., 2019).

In the present study, in addition to identifying the presence or absence of functional groups of phenolic compounds of ultrasound-assisted propolis extracts by ATR-FTIR spectroscopy, the total antioxidant capacity and radical scavenging capacity of propolis extract were determined using various *in vitro* antioxidant methods. For this purpose, we determined the total antioxidant capacity of propolis extract according to "Cupric ion Reducing total Antioxidant Capacity" (CUPRAC) and Ferriccyanide (Fe^{3+}) Reducing Antioxidant Power (FRAP) assay. At the same time, the radical scavenging capacity of propolis extracts was investigated according to the 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) and CUPRAC- hydroxyl radical scavenging (HRS) methods.

2. MATERIAL and METHODS

2.1. Chemicals

Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), catalase from bovine liver (2000-5000 U mg^{-1} solid), and Neocuproine (Nc-$C_{14}H_{12}N_2$), were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethanol (96%) was purchased from ISOLAB Laborgeräte GmbH (Eschau, GERMANY). Ammonium acetate (NH_4Ac), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), sodium salicylate ($C_7H_5NaO_3$), Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), hydrogen peroxide (H_2O_2, 30 wt.%), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and trichloroacetic acid (TCA), were purchased from Merck (Darmstadt, Germany).

2.2. Ultrasound-Assisted Extraction

Propolis samples collected in the region of Isparta, Türkiye (satellite coordinates: 38° 1' 25.5288" North and 30° 52' 22.3032" East) were then dried in the dark until processed. Propolis samples were prepared prior to extraction by then grinding with a coffee grinder (Sinbo SCM 2934-Türkiye). A total amount of 4 g of powdered propolis was soaked in 40 mL of 96% ethanol in a sealed bottle (Cavalaro *et al.,* 2019). The experimental conditions of the extraction procedure were as described previously by Samaram *et al*., (2014). The collected supernatants were filtered from the residue and dried by evaporating the solvents with a rotary evaporator (IKA RV 10 digital, IKA, Germany) at 50 ºC under vacuum.

2.3. Fourier Transform Infrared Spectroscopy (ATR-FTIR) Analysis

The infrared spectra were scanned on an JASCO FT/IR 4700 spectrophotometer (Jasco Co., Tokyo, Japan) at 4 cm^{-1} resolutions in frequency range between 4000 and 400 cm^{-1}.

2.4. *In Vitro* Antioxidant Activity Assays and Total Phenolic Content

It is recommended that the antioxidant activities of foods be compared by more than one method in terms of the mechanisms, selectivity, sensitivity, and applicability of the assays utilized to determine their antioxidant capacity (Apak *et al.,* 2004), For this purpose, we applied DPPH (Bener *et al*, 2022), CUPRAC (Apak et al., 2006), CUPRAC-HRS (Özyürek *et al*., 2008), and FRAP (Berker *et al*., 2007) methods to measure the antioxidant capacity and radical scavenging capacity of propolis extract. In each method, all tests were repeated three times for propolis extract and evaluated with a UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan). A calibration curve was constructed using Trolox and results were expressed as mmol TE /g extract for each method.

Total phenolic content of propolis extract was determined via the the Folin–Ciocalteu method (Li *et al*., 2008). A calibration curve was constructed using gallic acid equivalents (GAE) and results were expressed as mg GAE /g extract.

2.5. Statistical Analysis

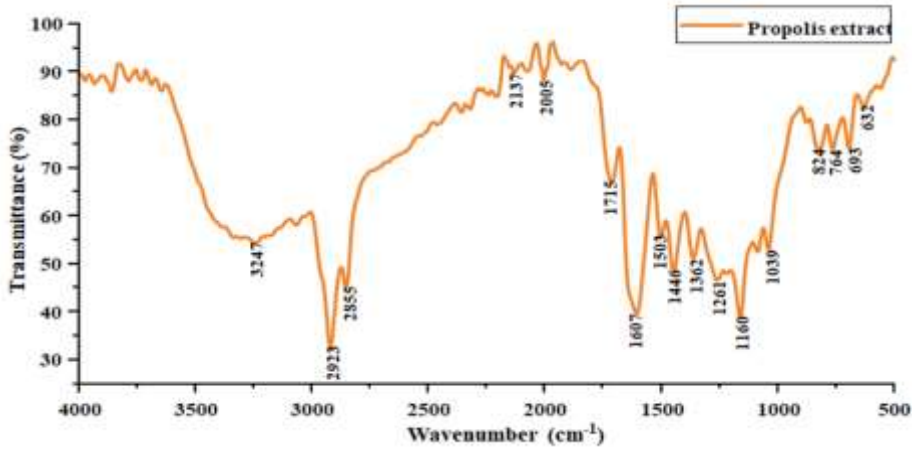
The presented data (mean ± standard deviation) resultedfrom at least three independent experiments and analyzed by SPSS (version 23 for Windows 10 pro, SPSS Inc.). The values were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test, with significance set at $p < 0.05$.

3. RESULTS and DISCUSSION

3.1. Characterization of Propolis Extract by ATR–FTIR

The FT-IR spectra of the propolis extract are presented in Figure 1. It revealed that the FTIR spectrum of the propolis extract had considerable bands at 2923 and 2855 cm^{-1} corresponding to symmetrical and asymmetrical C-H stretching, respectively (Figure 1). Intense patterns located between 1362 and 1039 cm^{-1} illustrate C–O stretching and C–OH bending resulted from alcohols, ethers, esters and carboxylic acids representing functional groups in phenolic compounds which are found in propolis extracts (Soltani *et al.,* 2017). The stretching of the C=O, which originates from the stretching vibration of and C=C from the stretching of the aromatic rings, was 1715 and 1607 cm^{-1}, respectively. In the 3247 cm^{-1} region, a very large broad band was observed, corresponding to the absorption of the OH functional group representing alcohols. The IR spectra of the propolis extract were consistent with previously reported spectra in the literature (da Silva *et al*., 2018). More specifically, the spectra of functional groups originating from the phenolic compounds found in the propolis extract were as expected.

Figure 1. FTIR spectra of propolis extract.



3.2. Total Phenolic Content

As in natural food products, the type and amount of phenolic substances determine the majority of the compounds responsible for antioxidant activity in propolis. Total phenolic content was determined according to the Folin method. According to this analysis; high phenolic content indicates high antioxidant activity. In the studies presented in the literature, it was reported that there was a strong correlation between the folin method and different antioxidant methods (CUPRAC, ABTS/persulfate, FRAP), because all of these methods were electron transfer based assays (Çelik et al., 2008). According to the test used to measure the total amount of phenolic substances, the total amount of all ethanol-soluble phenolic and polyphenolic substances was determined, since the Folin reagent forms a colored complex with all phenolic compounds such as phenolic acids, flavonoids, flavanols, anthocyanins. The total phenolic content of the propolis extract was calculated to be 189.17 ± 3.004 mg/g (GAE/g-extract). However, different results have been reported in the literature. Gulcin *et al*., (2010) reported that the total phenolic content of propolis varied between 31.2 mg/g and 302 mg/g GAE.

Table 1. Antioxidant activity and total phenolic content of propolis extract.

Sample	CUPRAC value (mmol TE/ g-extract)	FRAP value (mmol TE/ g-extract)	DPPH value (mmol TE/ g-extract)	TPC (mg/g-extract)
Propolis extract	2.43 ± 0.07	1.11 ± 0.13*	0.71± 0.002	189 ± 3.004

*Mean ± standard deviation. Abbreviations: CUPRAC, cupric ions (Cu^{2+}) reducing antioxidant capacity, DPPH, 2,2-diphenyl-1-picrylhydrazyl TPC, total phenolic content, FRAP, Ferriccyanide (Fe^{3+}) Reducing Antioxidant Power

3.3. Radical Scavenging Capacity and Total Antioxidant Capacity of Propolis Extract

In our current study, we evaluated total anxidant capacity and the radical scavenging activity of propolis extract by DPPH, CUPRAC, FRAP, and modified CUPRAC – Hydroxyl radical scavenging (HRS), methods. The total antioxidant capacity and free radical scavenging activity of propolis extract according to the applied methods were presented in Table 1 and Figure 2.

Based on the ability of DPPH, a stable free radical, to lighten in the presence of antioxidants, the DPPH test is a direct, practical and reliable method for measuring radical scavenging activity (Hasan *et al.,* 2009). The DPPH is a stable free radical absorbing at 517 nm wavelength. Therefore, it can be said that when the antioxidant donates its electron to DPPH, and this causes the absorption of DPPH radical solution to decrease at 517 nm (Bozkurt *et al.,* 2020). Researchers often express the values of DPPH radical scavenging activity of herbal extracts as % scavenging or IC50. In the current study, the DPPH radical scavenging activity of propolis extract was expressed as mmol trolox equivalents per gram of extract. For this purpose, molar absorption coefficient of TR compound (ε_{TR}: 21600 L mol^{-1} cm^{-1}) was determined in the DPPH method and free radical scavenging activity of propolis extract was calculated to be 0.71 ± 0.002 mmol TE / g - extract (Table 1). In a study reported in the literature, the DPPH radical scavenging activity of propolis extracts in the ultrasonic- assisted extraction in 80% ethanol medium was calculated to be 1.03 mmol TE/g-dry sample (Ulloa *et al*., 2017). The difference in the measured DPPH values could be attributed to the region where the propolis samples were collected, the ethanol concentration used in the extraction, the extraction time and the temperature.

The CUPRAC assay is a method based on the estimation of the total amount of antioxidants as a function of the reduction of copper ions (II). Using bis(neocuproine) copper(II)chloride, a chromogenic redox reagent, the total amount of antioxidants, both hydrophilic and lipophilic, can be easily determined. The CUPRAC method refers to the electron donating power of the antioxidant. Contrary to DDPH, the higher absorbance values recorded at 450 nm depending on the intensity of yellow-orange color formation in the Cuprac method indicate higher antioxidant capacity. According to the CUPRAC assay, the total antioxidant capacity of

propolis extract was calculated to be 2.43 ± 0.07 mmol TE/g-extract (Table 1). However, it was determined that the total antioxidant capacity of propolis samples collected from different geographical regions of Turkey was measured between 0.71 and 8.24 mmol TR/g- propolis extract by the CUPRAC method. Of all these data, it can be deduced that the total antioxidant antioxidant capacity of propolis samples varies according to the geographical region where it was collected and the vegetation.

In the FRAP method, the reducing capacity of propolis extract was accomplished using Fe^{3+} to Fe^{2+} reduction assay. In this analysis, the light color of the FRAP test solution changed to dark colors depending on the concentration of the substance that showed antioxidant activity (Erdoğan, 2022). The presence of reducing agents, which act as antioxidants in the samples, causes the Fe^{3+}/ferricyanide complex to be reduced to the iron form. Thus, Fe^{2+} can be traced by measuring the formation of Prussian blue of pearl at 700 nm (Gülçin *et al*., 2006). The absorbance values of propolis extract and reference antioxidant substances at different concentrations at 700 nm were presented in Table 2. The higher absorption value measured by the FRAP method at 700 nm indicates a higher reduction capacity. The data in Table 2 revealed that BHA had the highest FRAP value at 450 µg/mL concentration, followed by BHT and propolis extract, respectively. However, the absorbance value measured at 700 nm increased depending on the concentration. In addition, the FRAP value of propolis extract was calculated to be 1.11 mmol trolox equivalent / g -propolis extract.

When the in vitro antioxidant methods used to determine the antioxidant capacity of propolis are compared, For the total antioxidant capacity of propolis extract, it was determined that the FRAP value (1.11 mmol TE/g extract) was higher than the DPPH value (0.71 mmol TE/g extract), while it was lower than the CUPRAC value (2.43 mmol TE /g extract).

Table 2. Total reducing power of different concentrations (150–450 µg/mL) of propolis extract, BHA, and BHT

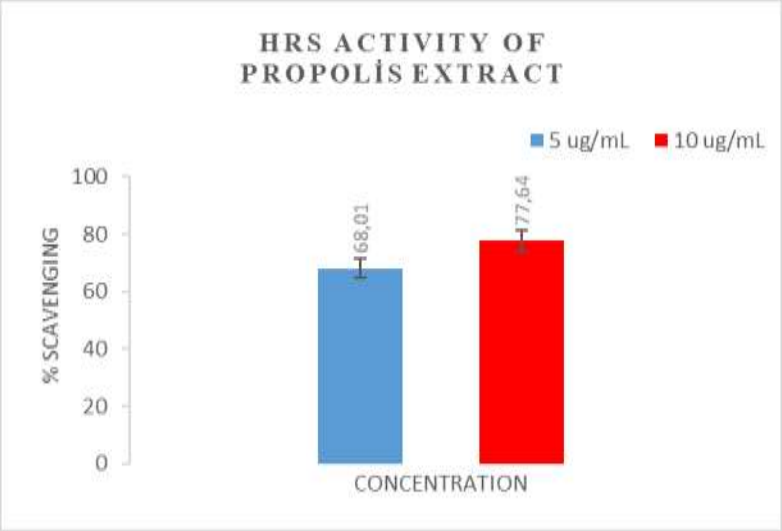
Concentration (µg/mL)	*FRAP value (at 700 nm)*		
	BHA	BHT	Propolis extract
150	0.828 ± 0.041[a]	0.523 ± 0.008[b]	0.237 ± 0.003[c]
300	1.367 ± 0.052[a]	0.840 ± 0.025[b]	0.446 ± 0.012[c]
450	2.704 ± 0.017[a]	0.968 ± 0.071[b]	0.587 ± 0.044[c]

Mean ± standard deviation. Different letters (a, b and c) in each row indicate significantly different ($p < 0.05$).

According to the modified CUPRAC assay, HRS capacity of propolis extract at different concentrations (5-10 µg/mL) was calculated to be 68.1% and 77.64 %, respectively (Figure 2). Research findings showed that propolis extract has strong radical scavenging potential. Free radicals are destructive molecules that break down living cells and cause aging and diseases. Free radicals are molecules with an unpaired electron. Most free radicals in our organism are radicals composed of molecular oxygen. Molecular oxygen tends to form highly reactive oxygen species (ROS) due to its diradical nature. Reactive oxygen species (ROS) are superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), which are formed in small amounts during normal oxygen metabolism (Erdoğan & Erbaş, 2021). ROS is also responsible for damaging crucial biomolecules, including nucleic acids, lipids, proteins and carbohydrates, and might cause DNA damage that can lead to mutations (Ak & Gülçin, 2008). Among ROS, $OH^{\bullet}$ is the most dominant in terms of oxidative activity. $OH^{\bullet}$ is the most toxic radical known, as it can oxidize all biological macromolecules composed of lipids, proteins and nucleic acids at almost diffusion-limited rates (Özyürek *et al*., 2008). In a study previously reported in the literature, it was reported that propolis extract was more effective in delaying the oxidation of olive oil compared to synthetic antioxidants such as BHA and BHT (Erdoğan 2023). The data presented in the present study revealed that propolis extracts

exhibited a great ability to scavenge a toxic radical such as $OH^{\bullet}$, even at very low concentrations.

Figure 2. Hydroxyl radical scavenging (HRS) activity of propolis extract.



4. CONCLUSION

In the current study, propolis extracts were discussed in detail, including their total phenolic content, total antioxidant, and radical scavenging activity. The data showed that propolis is rich in total phenolic content. Using *in vitro* antioxidant methods, propolis was found to possess strong free radical scavenging capacity and antioxidant properties. The characteristic FT-IR spectra of the propolis extracts confirmed the functional groups originated from the phenolic compounds of the propolis extracts. In the light of the data obtained in this study, more detailed studies can be carried out for the purification of individual phenolic compounds of propolis and that will provide some critical insights into the use of the bioactive components of propolis for different applications such as pharmaceutical, cosmetic and food industry. However, due to the undesirable properties of conventional solvents used in propolis extraction, the next step in research efforts should focus on finding green solvents that can provide high extraction yield and effective antioxidant results, such as ethanol.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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