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# Assessment of Propolis Treated by Different Extraction Methods

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**HIGHLIGHTS**

- The effects of different extraction methods on bioactive compounds of propolis.
- The highest antioxidant activity was obtained by ultrasound application.
- The highest phenols and esters were obtained by microwave-assisted method.

**Abstract:** Propolis is a valuable bee product with rich phenolic compound content. Extraction procedures play an important role for the final composition of propolis and determine its properties. The aim of this study was to determine the effect of different extraction techniques (maceration, ultrasound and microwave applications) and different solvents (ethanol and water) on some phenolic groups and antioxidant properties of propolis. The extraction of propolis was done by different solvents (70% ethanol and water) with ratio of 1/20 (w/v) propolis/solvent. After applications of these treatments chemical analyses as total phenolic compounds, total flavanol, tartaric ester content and antioxidant activities were performed. The highest antioxidant activity were determined in samples subjected to ultrasound application, whereas using of microwave-assisted extraction method lead to higher content of total phenolic compounds, total flavanol content and tartaric esters concentration. The results demonstrated the importance of used extraction techniques on propolis composition and consequently on its possible medical properties. These results demonstrated that the extraction of bioactive compounds in propolis could be optimized and properly used for healthy properties.

**Keywords:** propolis; bioactive groups; extraction; maceration; microwave; ultrasound.

## INTRODUCTION

Propolis is a resinous material collected by honey bees (*Apis mellifera L*.) from various plant sources. Propolis comes to the fore as a result of mixing the substances from different parts of the plants with salivary secretions of bee [1,2]. Propolis collected from the hive is crude propolis and can't be used directly.

Propolis constitutes mainly from resin, wax, essential oils, pollen and organic compounds including phenolic compounds. It is widely used as natural product for treatment of many diseases due to its antimicrobial, antifungal, antiviral properties for a long time [3,4].

Extractions with a suitable solvents or different applications must be done for the release of valuable compounds especially phenolic compounds found in propolis. Generally, as the best solvent for extraction is

used ethanol. Other solvents such as ethyl ether, water, methanol, chloroform, dichloromethane and acetone could be also used to extract the compounds present in propolis [3,4]. Additionally, there are some studies using combinations of different solvents such as polyethylene glycol and water mixture [5,6].

Except chemical compounds recently new ways for extraction have been examined. Maceration and Soxhlet extraction are among the traditional methods used during extraction of some compound found in propolis. Since the application of traditional extraction methods possess some properties such as long extraction time, high extraction cost, large amount of solvent evaporation, need of high temperature application and instability of some compounds (aromatic compounds) as more promising new methods have been proposed [7,8].

These applications include supercritical fluid extraction, ultrasound-assisted extraction, high-pressure-assisted extraction and microwave-assisted extraction applications. Most of these extraction methods have promising advantages such as shortening the long extraction time, lowering the production cost, preventing excessive evaporation of solvents and allowing the stability of compounds affected by temperatures [7,9,10]. In this ways important key compounds of propolis (phenolic compounds) become stable during processing and allowed the positive effects on human health (antioxidants, antimicrobial and antiviral properties) [6].

Maceration is the oldest method used for extraction of active compounds from plant materials.by using different immersing liquids [7].

Ultrasonically-assisted extractions are briefly performed on the base of mechanical effects of the acoustic cavitation [11]. The microwave-assisted extraction method is briefly performed by heating the solvent with the effect of heat and adhering the extractable material to the solvent. Some researchers determined that this method shortens the extraction time thanks to the rapid heating of the solvent [12]. Additionally, the microwave assisted extraction significantly shortened the extraction time comparing with ultrasound-assisted extraction and maceration. However, ultrasonic application is performed with less heat than microwave assisted extraction since it provides a large amount of energy in the form of the final heat status [11]. Trusheva and coauthors have reported that ultrasound and microwave-assisted extractions significantly shorten the extraction time compared with traditional extraction methods providing a less amount of energy cost and allowed the use of less solvent in the extractions. In the same study, they obtained successful results related to extraction of propolis with ultrasound-assisted extraction for 30 minutes [7]. In other studies, the effects of these techniques were evaluated [13,14], leading to different results. So, the evaluation of different extraction methods at different conditions and used solvents are required in order to maximize the bioactive compounds of propolis. The evaluation of these methods were at different conditions and their effects on propolis are required.

So, the main objective of this study was to determine the effects of ultrasound-assisted extraction, microwave-assisted extraction and maceration extraction method applied at different conditions on some bioactive compounds of propolis.

## MATERIAL AND METHODS

### Materials

The propolis samples were obtained from the local company found in Sivas, Turkey with geographical coordinates of the north latitude: 39° 26.5734′ and the east latitude: 36° 94.1971′. Samples were frozen, finely ground in a laboratory mill and passed through a 35 mesh sieve prior to beginning of exactions and stored at -20°C prior all experiments.

All procedures were performed at Ege University Food Engineering Department Brach of Biotechnology. Chemicals used during analyses were suppled from Merck (Germany), Fulka (Germany), Tekkim (Turkey), Sigma-Aldrich (USA) and Carlo Erba (France).

### Extraction procedures

As material for extraction procedures were used 1 gram of propolis. The propolis / solvent ratio chosen as 1/20 (w/v). As solvents were used ethanol / water (70:30, v/v) and water (100%). After centrifugation at 4,000 x g for 5 minutes the supernatants were prepared and stored at +4°C for analyses. The extraction procedures were carried out by using different methods given below in order to evaluate better extraction of bioactive compounds found in propolis. Obtained samples were used for extraction procedures.

*Ultrasound-assisted extraction*

Ultrasound-assisted extraction was carried out using a 300 W ultrasonic bath. The system was adapted for food treatment with preliminary experiments. Previously prepared samples were exposed to ultrasound procedure at 25°C for 5, 10, 15, 20, 25 and 30 minutes.

*Microwave-assisted extraction*

Microwave-assisted extraction was performed using a multimodal household microwave oven (Arçelik MD-570) at 700W. Samples placed in a 100 mL flask were exposed to microwave irradiation (irradiation cycle as 10 s “power on”, followed by 10 s “power off” for 10, 15, 20, 25 and 30 seconds [7].

*Maceration*

Maceration extractions were performed in a 100 mL flask. The corresponding amount of solvents were added to the sample and left in the dark for 24h, 48h and 72 h at room temperature.

All extraction procedures were performed in triplicate.

**Analysis**

*The total phenolic content*

The total phenolic content was determined by the Folin-Ciocalteu method of Singleton and Rossi with some modifications [15]. Diluted samples (0.02 µL) were mixed with 100 µL of the Folin-Ciocalteu reagent and 1.58 mL water. The mixture was held at room temperature in dark place for 5 minutes before adding 300 µL of 20% sodium carbonate (w/v). Then, mixture was held at room temperature in dark place for 2 h before measuring the absorbance at 765 nm with spectrophotometer (Thermo Scıentıfıc USA Genesys 10s UV-VS). The results were expressed as gallic acid equivalents (GAE) using a calibration curve (y=(x-0,0291)⁄0,0007)) against gallic acid with R2 value as 0.9976.

*The antioxidant activity*

The antioxidant activity was determined by the 2,2, -diphenyl-2-picryl-hydrazyl (DPPH) method of Blois with some modifications [16]. Diluted samples (100 µL) were mixed with 100 mL of the 0.1 mM DPPH reagent. The mixture was held at room temperature in dark place for 30 minutes before measuring the absorbance at 517 nm with spectrophotometer (Thermo Scıentıfıc USA Genesys 10s UV-VS). The antioxidant activity (AC) using the following formula (1):

$$\boldsymbol{AC}(\%) = \frac{\boldsymbol{A(control)} - \boldsymbol{A(sample)}}{\boldsymbol{A(control)}} * \mathbf{100} \quad (1)$$

*The total flavanol content*

After preliminary treatment (dilution of propolis samples using 9 mL 10% ethanol) to each 0.25 mL propolis samples were added 0.25 mL 1% HCl and 4.55 mL 2% HCl and mixed for 15 minutes. The absorbance values were determined by spectrophotometer (Thermo Scıentıfıc USA Genesys 10s UV-VS) at 360 nm. The results were expressed as caffeic acid equivalents using a calibration curve $(y = (x - 0{,}0076/0{,}0038))$ against caffeic acid with R2 value as 0.9968 [17]

*The tartaric acid ester content*

After preliminary treatment (dilution of propolis samples with 9 ml of 10% ethanol) to each 0.25 ml portions of samples were added 0.25 ml 1% HCl and 4.55 ml 2% HCl and mixed for 15 min. The absorbance values were determined by spectrophotometrically (Thermo Scıentıfıc USA Genesys 10s UV-VS) at 320 nm for the tartaric ester. The results were expressed as quercetin equivalents using a calibration curve $(y = (x - 0{,}006/0{,}024))$ against quersetin with R2 value as 0.9943 [17].

All analyses were performed in triplicate.

### Statistical evaluation

Significant differences between average values were obtained at the 95% level. By using a Post-Hoc test, the least significant differences (LSD) test was performed. Using multivariate exploratory techniques, principal component analysis (PCA) was performed.

## RESULTS AND DISCUSSION

This study has been carried out to present the effects of different extraction methods on some bioactive compounds of propolis. The main results are presented in Table 1.

**Table 1.** Used methods with specification of parameters and values of results.

| Code | Used Method | Time | Solvent | Total Phenols (mg/L GAE) | Tartaric Acid Esters (mg/L quercetin) | Total Flavonols (mg/L caffeic acid) | Antioxidant Activity (%) |
|---|---|---|---|---|---|---|---|
| maE1g | maceration | 1 day | Ethanol | 856 | 332 | 89 | 65 |
| maS1g | maceration | 1 day | Water | 124 | 91 | 27 | 52 |
| maE2g | maceration | 2 day | Ethanol | 723 | 428 | 117 | 84 |
| maS2g | maceration | 2 day | Water | 181 | 144 | 54 | 36 |
| maE3g | maceration | 3 day | Ethanol | 1.216 | 360 | 96 | 90 |
| maS3g | maceration | 3 day | Water | 187 | 150 | 55 | 20 |
| miE10s | microwave | 10 seconds | Ethanol | 1.218 | 356 | 98 | 79 |
| miS10s | microwave | 10 seconds | Water | 96 | 135 | 55 | 84 |
| miE15s | microwave | 15 seconds | Ethanol | 1.330 | 422 | 128 | 91 |
| miS15g | microwave | 15 seconds | Water | 117 | 85 | 30 | 34 |
| miE20s | microwave | 20 seconds | Ethanol | 1.341 | 314 | 99 | 37 |
| miS20s | microwave | 20 seconds | Water | 123 | 75 | 25 | 19 |
| miE25s | microwave | 25 seconds | Ethanol | 1.706 | 394 | 110 | 55 |
| miS25s | microwave | 25 seconds | Water | 151 | 140 | 56 | 48 |
| miE30s | microwave | 30 seconds | Ethanol | 1.536 | 413 | 114 | 37 |
| miS30s | microwave | 30 seconds | Water | 136 | 133 | 53 | 27 |
| uE5d | Ultrasound | 5 minutes | Ethanol | 864 | 256 | 70 | 30 |
| uS5d | Ultrasound | 5 minutes | Water | 130 | 79 | 24 | 64 |
| uE10d | Ultrasound | 10 minutes | Ethanol | 1.227 | 346 | 106 | 46 |
| uE15d | Ultrasound | 10 minutes | Water | 110 | 71 | 23 | 37 |
| uE15d | Ultrasound | 15 minutes | Ethanol | 896 | 272 | 87 | 64 |
| uS15d | Ultrasound | 15 minutes | Water | 153 | 75 | 24 | 22 |
| uE20d | Ultrasound | 20 minutes | Ethanol | 921 | 255 | 70 | 90 |
| uS20d | Ultrasound | 20 minutes | Water | 174 | 135 | 51 | 22 |
| uE25d | Ultrasound | 25 minutes | Ethanol | 1.098 | 285 | 77 | 85 |
| uS25d | Ultrasound | 25 minutes | Water | 157 | 139 | 54 | 64 |
| uE30d | Ultrason | 30 minutes | Ethanol | 1.191 | 373 | 112 | 87 |
| uS30d | Ultrason | 30 minutes | Water | 166 | 82 | 25 | 67 |

The results of propolis extraction with different solvents by the maceration method are shown in Figure 1.

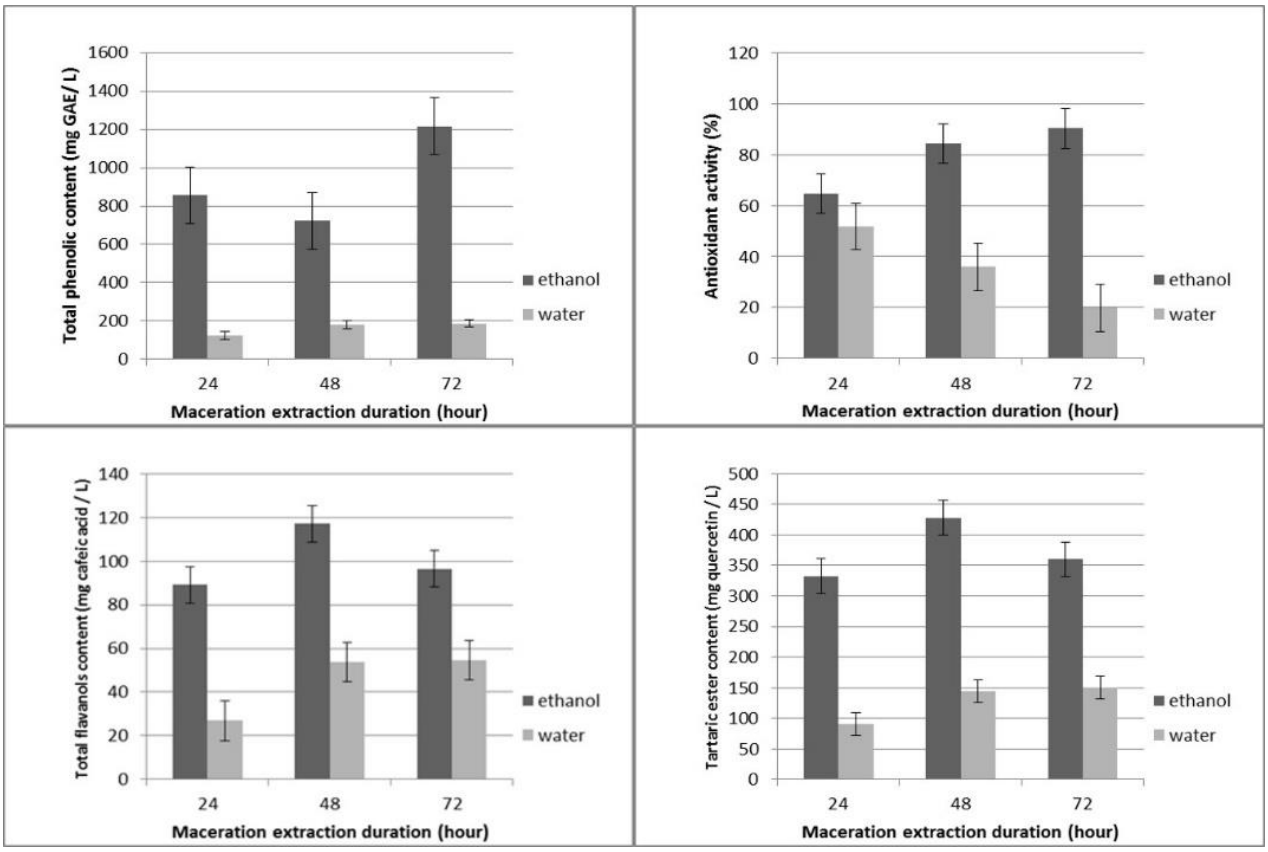


**Figure 1.** The effects of extraction on total phenolic content, antioxidant activity, total flavanols content, tartaric ester content of propolis extract during maceration at 24, 48, 72 hour using solvent of 70% ethanol and water with 1:20 sample / solvent ratio.

The highest amount of phenolic content value was found as 1215 mg gallic acid / L, in samples macerated with ethanol for 72 hours. The lowest amount of phenolic content value was found in the sample macerated with water for 24 hours as 124.142 mg gallic acid / L. The highest antioxidant activity value was found as 90.23% in sample macerated with ethanol for 72 hours while the lowest value was found 19.87% in sample macerated with water for 72 hours. Total highest flavanols and tartaric ester values were found to be 116.94 mg caffeic acid / L and 427.5 mg quercetin / L, respectively, in the sample macerated with ethanol for the 48 hours. The lowest flavanols and tartaric ester values were found to be 26.94 mg caffeic acid / L and 90.83 mg quercetin / L, respectively, in the sample macerated with water for 24 hours.

The total phenolic content, antioxidant activity, total flavanol and tartaric ester concentrations were higher in the samples using ethanol as the solvent.

Total phenolic content, total flavanol content and tartaric ester content increased during maceration application with water, whereas antioxidant activity decreased after 24 hours of maceration. The main explanation of this is that some phenolic compounds related to high antioxidant activity are extracted within 24 hours.

The total phenolic content and antioxidant activity of samples macerated with ethanol reached the maximum value as the extraction duration increased. However, the total flavanol and tartaric ester values reached a maximum value by 48 hours of maceration.

The highest amount of phenolic content value was found as 1705.571 mg gallic acid / L, when macerated with ethanol for 72 hours and subjected to microwave treatment for 25 seconds. This value was followed by 72 hours of macerated sample and sample treated for 30 minutes by ultrasound application using ethanol as solvent, with values as 1215.517 mg / L and 1191.285 mg / L, respectively.

Microwave-assisted extraction allows the acceleration of energy transfer, facilitating the solvation of compounds and additionally promoting the disruption of weak hydrogen bonds found in phenolic compounds. Since the reversible nature of hydrogen bonds they have great potential for separation of bound molecules

and also to accomplish selective and strong complex formation. The results of microwave energy depend upon many factors, corresponding to the character of each solvent and solid matrix, the kind of target compound to be extracted and especially the sample and solvent dielectric constants [18].

Many examples within the scope of phytochemical analysis recommended that microwave-assisted extraction has some extensive deserves, resembling shorter extraction time, higher extraction yield and less solvent consumption compared to traditional extraction methods [19]. Beside that as disadvantage of the microwave-assisted extraction is related to its low selectivity because it is heavily addicted to the solvent nature and the extraction temperature [20].

The results of propolis extraction with different solvents by the microwave-assisted extraction method are shown in Figure 2. The highest total flavanols and tartaric ester values were determined as 91,128.26 mg caffeic acid / L and 422.08 mg quercetin / L, respectively in 15 second microwave treated samples where ethanol was used as solvent. When water was used as solvent, the highest total phenolic compound, total flavanols, tartaric ester values were determined as 151.28 mg gallic acid / L, 55.63 mg caffeic acid / L and 140.41, respectively in 25 second microwave treated samples.

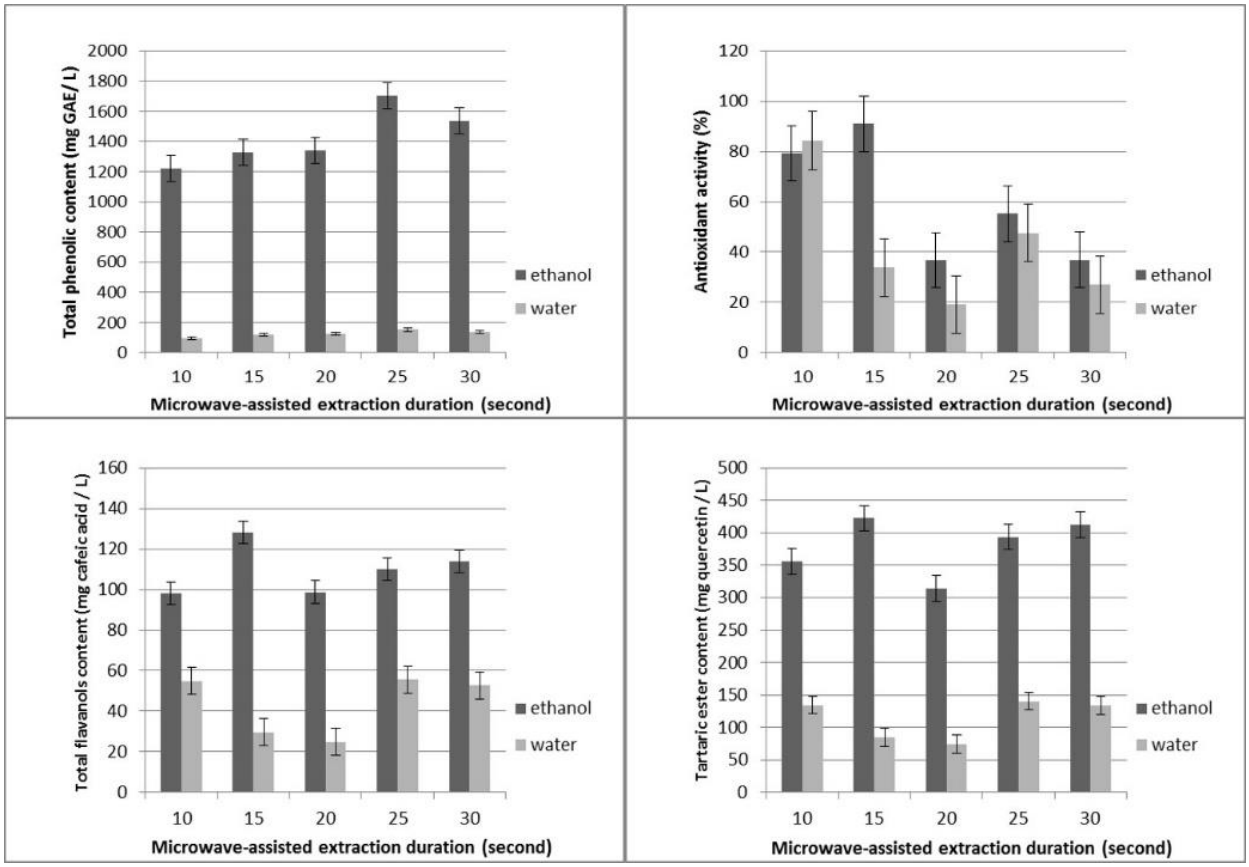


**Figure 2.** The effects of extraction on total phenolic content, antioxidant activity, total flavanols content, tartaric ester content of propolis extract during microwave-assisted extraction at 700 W using solvent of 70% ethanol and water with 1:20 sample /solvent ratio.

Ultrasound-assisted extraction is used in the extraction of bioactive compounds from many different materials by supporting the effects of acoustic cavitation. The propagation of ultrasonic waves provides a larger solvent penetration into the sample matrix, increasing the contact between samples and therefore the solvent (or reagent) caused the increases in mass transfer rate. This system permits performing synchronic extractions, utilization of low quantities of solvent, reduction of operating times and increases in yield and quality of extract. Moreover, ultrasound-assisted extraction is additionally cheap, fast, and versatile compared to traditional techniques, since could be used many solvents of various polarities. However, ultrasound-assisted extraction has some drawbacks, as well as difficulties in combination with alternative instruments and automation [18]. Even these obstacles, ultrasound-assisted extraction possess clean methodology that avoids utilization of a large amount of solvents and voluminous extraction vessels like Soxhlet and long standing procedures as maceration. The reduced environmental impact of ultrasound-assisted extraction is clearly advantageous in terms of energy and time [21].

The results of propolis extraction by the ultrasound-assisted extraction method are shown in Figure 3. The highest antioxidant activity and total phenolic compound values were determined as 90.06% and 1227 mg gallic acid / L, respectively in the samples treated by ultrasound application for 10 minutes and 20 minutes, using ethanol as solvent. When water was used as solvent, the highest antioxidant activity and total phenolic compound values were determined as 66.90% and 174.14 mg gallic acid / L, respectively in the samples treated by ultrasound application for 20 minutes and 30 minutes. The total flavanols and tartaric ester values reached maximum values as 112.47 mg caffeic acid / L and 373.33 mg quercetin / L, respectively, by using ethanol as a solvent in application of 30 minutes ultrasound power. The total flavanols and tartaric ester values were found to be 53.52 mg caffeic acid / L and 139.16 mg quercetin / L, respectively, when water was used as solvent during 30 minutes ultrasound application.

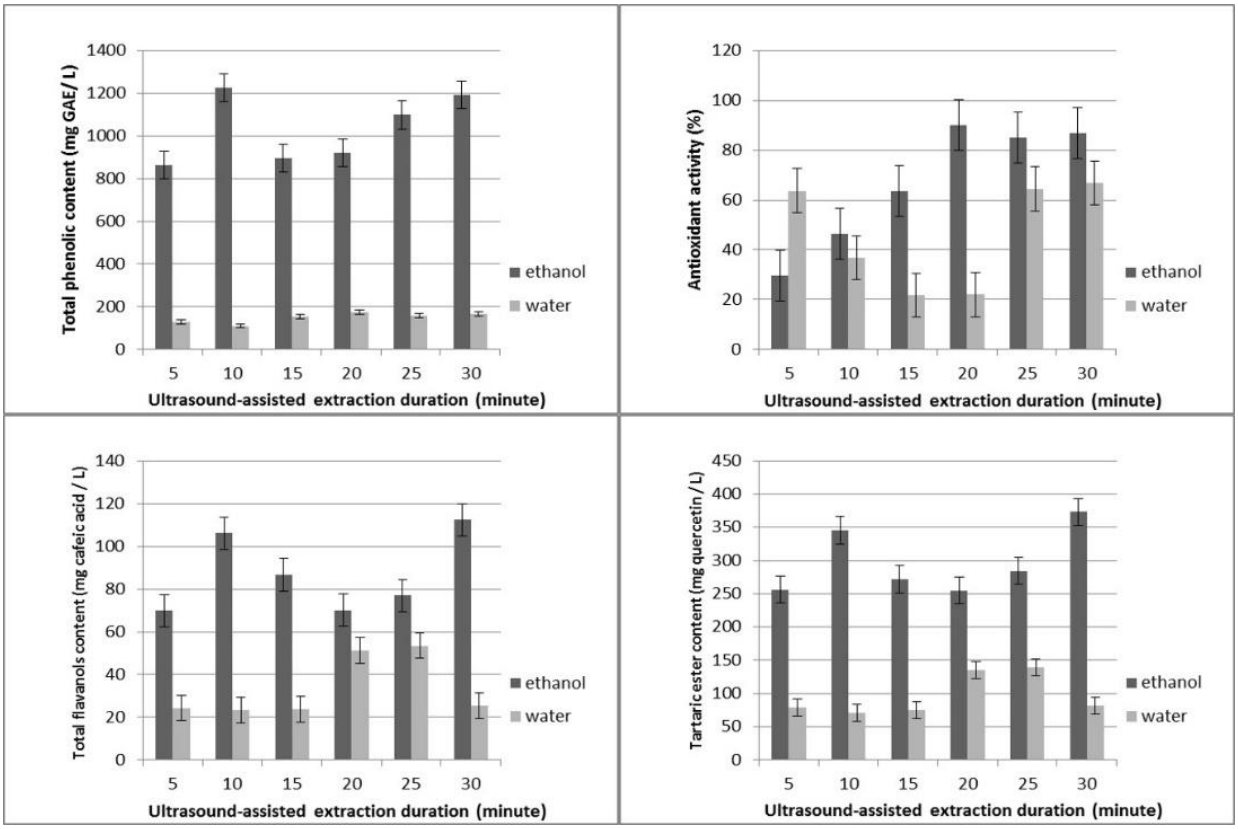


**Figure 3.** The effects of extraction on total phenolic content, antioxidant activity, total flavanols content, tartaric ester content of propolis extract during ultrasound-assisted extraction at 300 W using solvent of 70% ethanol and water 1:20 with sample / solvent ratio.

Maceration is an associate extractive technique conducted at room temperature. It consists of immersing a plant in a liquid (water, oil, alcohol, etc.) within an airtight container, for a variable time based on the material and liquid used. It is still practiced as an easy and economic extraction technique. The application of this method depends on several parameters like sample origin, duration and used immersing liquid [22]. Even some disadvantages (long time) it can be effective methodology for extraction of phenolic compounds [23].

The highest amount of total flavanols value was found as 128.263 mg caffeic acid / L, in samples macerated with ethanol and applied to microwave assisted application done for 25 seconds. The highest amount of tartaric ester value and antioxidant activity were found as 422.083 mg quercetin / L; 91.038%, respectively in samples macerated with ethanol and subjected to microwave treatment for 15 seconds.

Using multivariate exploratory techniques, principal component analysis (PCA) was performed. Principal component analysis permits the visualization of the original arrangement of propolis samples in an n-dimensional space by identifying the directions in which most of the information is retained. The eigenvalue number determined by PCA (principal component analysis) was found to be more than 60%, demonstrating the accuracy of performed analyses. As the distribution of analyzed parameters (Figure 4) in samples produced by different extraction solvent (Figure 5) and different extraction procedures (Figure 6) have the

same x- and y-values (79.26 x 17.52) these figures were evaluated together to determine the relationship among parameters and samples.

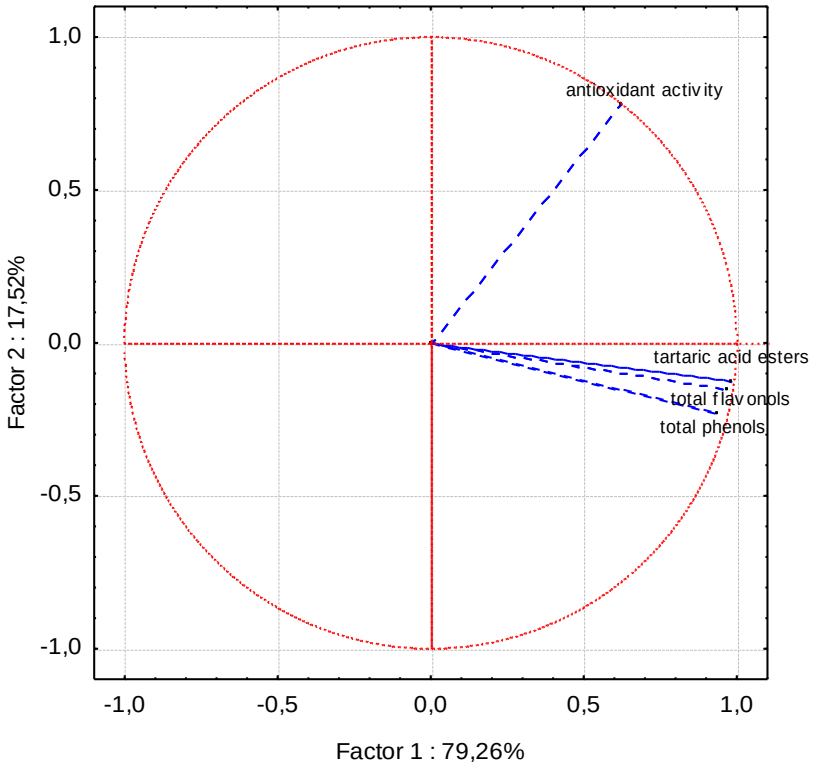


**Figure 4.** Distribution of analyses parameters

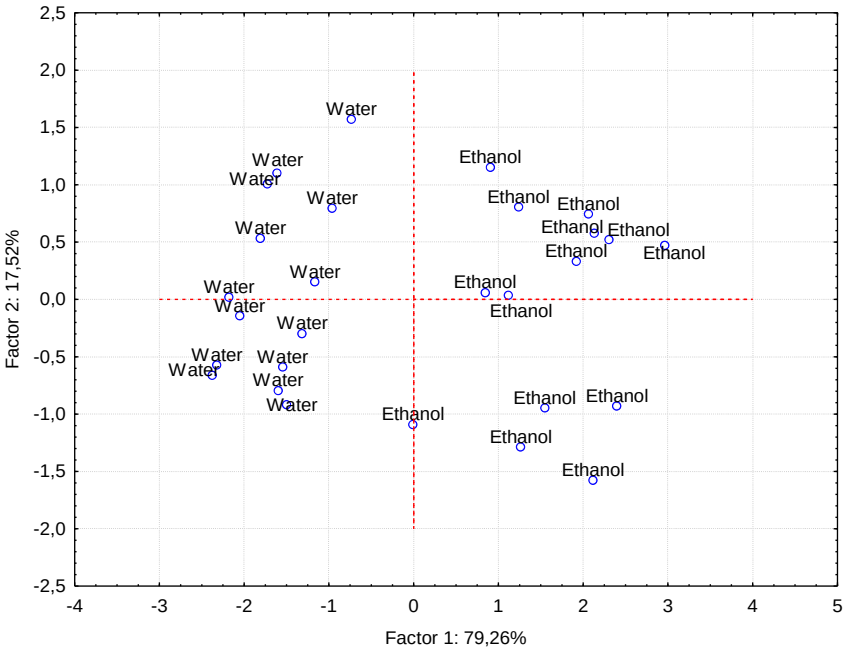


**Figure 5.** Distribution of propolis samples treated by different solvent.

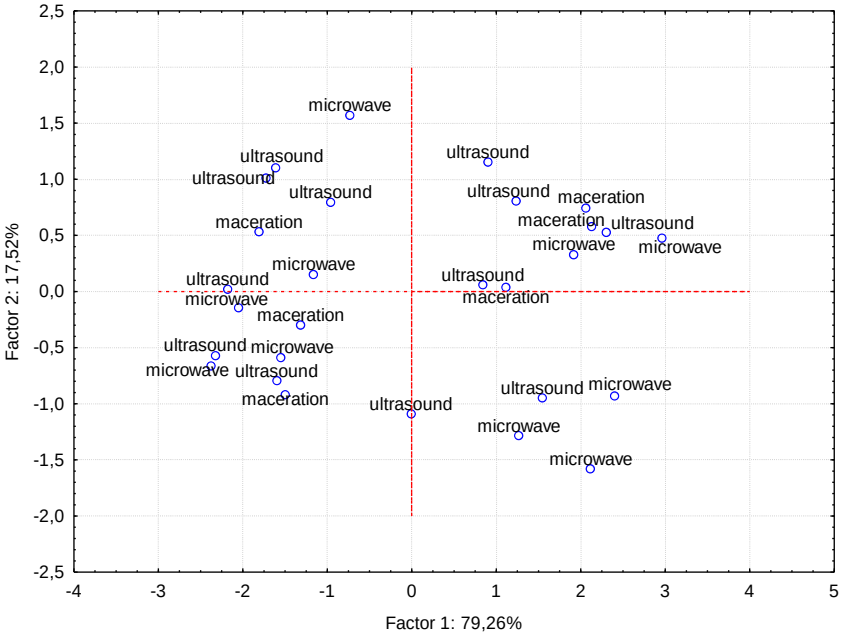


**Figure 6.** Distribution of propolis samples treated by different extraction methods.

Evaluations of bioactive compounds were done considering all targeted parameters. The least significant differences (LSD) were determined considering different extraction procedures and solvents ($p<0,05$). The most prominent differences were related to conditions of using ethanol and water used as solvents ($p<0,05$).The strong effects of ethanol and less importance of water as a solvent was demonstrated in other studies. Trusheva and coauthors determined that the use of water instead of ethanol during propolis extraction reduced the amount of bioactive compounds by 10-fold [7]. Margeretha and coauthors had determined that solvent mixtures containing 40%, 60%, 90% ethanol caused significant differences in total phenolic compound and total flavonoid amounts [24]. In this study, the use of ethanol (70%) as solvent caused significant differences in bioactive compounds content of propolis. This fact demonstrated that the effectiveness of extraction procedures are strongly affected by used solvents.

Hamzah and Leo had reported that the total phenolic compound content increases with prolonged duration of microwave-assisted extraction (100W / 70% ethanol [8]. In our study, by increasing the duration of microwave-assisted application (increased to 25 seconds), the total amount of phenolic compound has been increased in case of using solvent 70% ethanol and water as solvents.

Trusheva and coauthors had reported that the total amount of phenolic compound decreased by increasing duration time using 70% ethanol at 1/10 (w/v) propolis/solvent ratio and microwave-assisted extraction (800 W) [7]. In this study, it was reported that the total amount of phenolic compound and antioxidant activity decreased after microwave-assisted extraction applied at 700 W for 25 seconds using both solvents. However, total flavanols and tartaric ester amounts increased after microwave applications for 30 seconds in samples using 70% ethanol as solvent.

Oroian and coauthors, 2019 demonstrated that that the ultrasonication process is better than the microwave or maceration process for the extraction of total phenols, flavanone and dihydroflavonol content. Comparison among studies is unappropriated since used conditions (time, power) are different [25].

Sun and coauthors reported that the ethanol extract of propolis contained more flavonoids than the water extract of propolis [26]. It was demonstrated that the difference between ethanol and water extract of propolis had an effect on antioxidant activity, also [27]. In a study done by Zin and coauthors, the best results were proposed to be obtained by 5 days maceration considering total phenols of propolis. Additionally it was reported that the extraction by sonication for 30 minutes with 70% of ethanol lead to higher flavonoid content and antioxidant activity [13]. In our study related to this method we demonstrated that 30 minutes caused higher flavonoid content but for highest antioxidant activity 20 minutes was enough.

The composition of samples is the most important factor in the selection of extraction methods. The extraction method, time of application, solvents and temperature ranges should be selected specifically according to desired composition related to product quality.

The results of principal component analysis demonstrated close relation among antioxidant activity and ultrasound application extraction. Since the distribution of analyzed parameters (Figure 4) and used

extraction methods (Figure 6) have the same x- and y-values (79.26 x 17.52) these figures were fitted overlap and evaluated together to determine the relationship among parameters and used methods. As was seen from Figure 4 the antioxidant activity is located in the upper left side of coordinate. Just in the same place of coordinate in Figure 6 is the location of ultrasound application. Additionally there were determined positive relation among total phenolic content and microwave-assisted extraction method. Similar results were obtained for total flavanols values, its changes could be simulate by microwave-assisted extraction.

## CONCLUSION

This study has been carried out to present the effects of different extraction methods and solvents on bioactive compounds of propolis. Comparing the ultrasound, microwave and maceration applications, the most prominent result was related to requirement of using of different extraction methods for specific groups. The highest antioxidant activity was obtained by ultrasound application, whereas microwave-assisted extraction method lead to higher content of total phenolic content, total flavanol content and tartaric esters.

These results demonstrated that the extraction methods of propolis could be further optimized and properly used for healthy properties related to antioxidant activities.

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