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Ultrasonic-Assisted Extraction of Propolis: Response Surface Optimization and Solvent-Dependent Bioactive Properties

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Abstract

Propolis is a resinous substance produced by honey bees (*Apis mellifera*) and is rich in biologically active compounds. Efficient extraction of these compounds depends largely on solvent selection and the optimization of extraction conditions. In this study, ultrasonic-assisted extraction (UAE) conditions from crude propolis were optimized using two solvents (dimethyl sulfoxide (DMSO) and ethanol) through a Box–Behnken experimental design based on response surface methodology (RSM). Response surface methodology (RSM) was employed to evaluate the effects of solvent concentration (0-100%), extraction temperature (30–50 °C), and extraction time (30-180 min) on extraction yield.

The DMSO-based UAE model showed high statistical significance with an R^2 value of 0.9950 ($F = 154.15$, $p < 0.0001$). The optimal extraction conditions were determined as 98.34% solvent concentration, 42.20°C extraction temperature, and 142.60 min extraction time, resulting in a yield of 54.17%. Similarly, the ethanol-based UAE model was also significant ($F = 113.09$, $p < 0.0001$; $R^2 = 0.9932$), with optimal conditions of 99.19% solvent concentration, 45.26 °C extraction temperature, and 171.5 min extraction time, yielding 55.82%. In both solvent systems, solvent concentration was identified as the most influential parameter affecting extraction yield ($p < 0.0001$). Bioactivity analyses revealed that DMSO extracts contained higher TPC (325.27 ± 0,05 mg GAE/g) and exhibited stronger antioxidant activity (DPPH SC_{50}: 11.90 ± 1.6 µg/mL; ABTS SC_{50}: 147.7 ± 0.4 µg/mL) compared with ethanol extracts. Conversely, ethanol extracts demonstrated stronger antimicrobial activity against *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Bacillus cereus*, and *Staphylococcus aureus*, with consistent MIC values of 25 µg/mL. Overall, the findings indicate that both solvents provide high extraction efficiencies (~54-56%) under optimized UAE conditions. Furthermore, solvent selection plays a decisive role in determining the bioactivity profile. The results show that using DMSO in extraction is suitable for maximizing antioxidant properties, while using ethanol is more suitable for antimicrobial applications.

Keywords: Propolis, Ultrasonic-assisted Extraction, Response Surface Methodology, Antimicrobial Activity, Antioxidant Activity

1. INTRODUCTION

Significant changes have occurred in consumer expectations regarding food products due to improvements in living standards and lifestyle changes. Food consumption is no longer limited to satisfying hunger or meeting basic nutritional needs; rather, individuals increasingly prefer foods that contribute to maintaining a healthy and high-quality life and help prevent diseases (Hasler, 2002; Roberfroid, 2002). In this context, propolis has gained considerable attention as a functional food due to its rich phenolic and flavonoid composition and its well-documented antioxidant, antimicrobial, and immunomodulatory properties (Al-Ghamdi et al., 2017). The term *propolis* originates from the Greek words *pro* (in front of) and *polis* (city), reflecting its protective role within the hive. Propolis is a resinous bee product produced by honeybees (*Apis mellifera* L.) through the

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collection of plant exudates from buds, bark, and leaf surfaces, which are subsequently mixed with beeswax and enzymatic secretions. This biotransformation process results in a complex matrix rich in bioactive compounds (Kumova et al., 2002). Within the hive, bees use propolis to seal cracks and structural openings and to protect the colony against pathogenic microorganisms, thereby acting as an essential biological barrier (Thamnopoulos et al., 2018). Propolis has been used since ancient times in traditional medicine for the treatment of inflammatory and febrile infections (Castaldo and Capasso, 2002). Today, due to its strong antioxidant and antimicrobial properties, its use has expanded significantly, particularly in pharmaceutical research and food formulations. In recent years, studies in food science have highlighted the potential of propolis not only as an antimicrobial agent but also as a natural preservative and edible coating material capable of significantly extending the shelf life of perishable food products (Almuhayawi et al., 2020).

The chemical composition of propolis varies depending on geographical origin, climatic conditions, plant flora, and bee metabolism. Generally, propolis consists of approximately 45-50% plant resins, 25-30% beeswax, 5-10% essential oils, 5% pollen, and about 5% other organic compounds (Marcucci, 1995; Al-Ghamdi et al., 2017). The main compounds responsible for the biological activities of propolis, particularly its antioxidant, antimicrobial, and anti-inflammatory effects, include phenolic acids and their esters, flavonoids, terpenes, β-steroids, aromatic aldehydes, stilbenes, and derivatives of caffeic, cinnamic, and benzoic acids (Bankova et al., 2000; Castro et al., 2007). In addition, propolis contains various vitamins (B1, B2, B6, C, and E), minerals (Ca, Mg, K, Na, Fe, Cu, and Zn), and several enzymes such as dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid phosphatase (Speciale et al., 2006; Bankova et al., 2014).

Due to its complex structure, which includes high amounts of beeswax, resins, and volatile compounds, the extraction technique and solvent selection play a crucial role in efficiently isolating the bioactive components from the propolis matrix (Marcucci, 1995; Bankova et al., 2000). Traditionally, classical extraction methods such as maceration have been widely used for propolis extraction. In this method, propolis is kept in a selected solvent, commonly ethanol, methanol, ethyl acetate, or water, for a certain period to allow the dissolution of bioactive compounds. Although maceration is simple to perform and requires minimal equipment, it has several disadvantages, including long extraction times, high solvent consumption, and the possible degradation of heat- or oxidation-sensitive compounds (Cunha et al., 2004; Trusheva et al., 2007).

To overcome these limitations, modern extraction techniques have been increasingly developed to improve extraction efficiency and reduce processing time. Among these techniques, ultrasonic-assisted extraction (UAE) has attracted considerable attention due to its high extraction efficiency, shorter extraction time, operation at relatively low temperatures, and environmentally friendly characteristics (Chemat et al., 2017; Biçer, 2024). Ultrasonic waves generate cavitation in the liquid medium, which causes the mechanical disruption of plant cell walls, thereby accelerating mass transfer and enhancing the interaction between the solvent and target compounds. As a result, the UAE enables higher extraction yields within shorter processing times compared with conventional extraction methods (Herrera and Luque de Castro, 2005; Biçer, 2024). Furthermore, the ability of the UAE to operate at low temperatures helps preserve heat-sensitive phenolic and flavonoid compounds (Chemat et al., 2017).

The complex matrix and high wax content of propolis also influence the effectiveness of the solvent used during extraction. Previous studies indicate that solvents with different polarities-such as ethanol, methanol, dimethyl sulfoxide (DMSO), propylene glycol, and water-are commonly used for propolis extraction, and solvent polarity plays a critical role in determining both the solubility of bioactive compounds and the overall extraction efficiency (Bankova, 2005; Huang et al., 2014). In addition to solvent type, extraction parameters such as extraction time, solvent concentration, and temperature also significantly affect the chemical composition and functional properties of the resulting extracts (Inui et al., 2014; Chemat et al., 2017).

Therefore, the aim of the present study was to determine the optimal ultrasonic-assisted extraction (UAE) conditions for propolis samples collected from the Sivas region of Turkey using DMSO and ethanol as solvents. In addition, the chemical composition and bioactive properties of the obtained extracts were evaluated.

2. MATERIALS AND METHODS

2.1. Materials

The propolis used in this study was obtained from a beekeeper in the Autumn of 2024 from the Tozanlı Valley region of Sivas, Türkiye (40°20′N, 37°55′Egeographic coordinates). The propolis samples used for analysis were ground in a grinding mill (Emir VortexJet, Turkey) and stored at -18°C until processing and analysis.

The following bacterial cultures, available at the Food Microbiology Laboratory of the Food Engineering Department at Tokat Gaziosmanpaşa University, Tokat, Türkiye, were used as test cultures: *Staphylococcus aureus* ATCC 25923*, Escherichia coli* O157:H7 ATCC 25922*, Salmonella* Typhimurium ATCC 14028*,* and *Bacillus cereus* ATCC 10876. The cultures were activated by incubating them in Brain Heart Infusion Broth at 37°C for 24 hours.

Methanol, ethanol, dimethyl sulfoxide, sodium carbonate, and aluminum chloride were obtained from Merck (Germany). Gallic acid, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical, and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Co. (USA). Folin–Ciocalteu's phenol reagent was purchased from Merck (Germany). Brain Heart Infusion Broth (BHIB; Condalab, 201181, Spain), Brain Heart Infusion Agar (BHIA; Condalab, 1310, Spain), Pepton water (PW, Merck, 1.07224, Germany).

2.2. Optimization of extraction conditions

The extraction conditions were optimized using Response Surface Methodology (RSM). A Box-Behnken experimental design was employed to evaluate the combined effects of the main process variables on extraction efficiency. Experimental design and statistical analyses were performed using Design-Expert® software. Solvent concentration, extraction temperature, and extraction time were determined as independent variables, as they directly affect mass transfer and overall extraction performance; extraction yield was determined as the response.

Ethanol (EtOH) and dimethyl sulfoxide (DMSO) were used as solvents for the extraction of raw propolis, with ultrasonic-assisted extraction (UAE) employed as the extraction method. Propolis samples (1.0 g) were subjected to UAE in an ultrasonic water bath (37 kHz, 150 W, 15.8 W/L) (Elmasonic SH 100, Almanya) with shaking at various ethanol and DMSO concentrations (10 mL; 0-100%), extraction times (30-180 min), and temperatures (30-50 °C). All extractions were performed in duplicate. After extraction, the resulting extracts were filtered through coarse filter paper, and the filtrates were weighed. Extraction yields were then calculated using Equation 1.

$$\text{Yield} = \frac{\text{Extract weight}}{\text{Sample weight}} \text{ x } 100 \qquad \text{(Equation 1)}$$

The selection of the value ranges for the independent variables was based on preliminary experiments and a review of the literature (Oroian et al., 2020). The extraction process was optimized with respect to solvent concentration, extraction time, and temperature. The specific values of the independent variables tested for extraction yield in propolis samples using ultrasonic-assisted extraction (UAE) are presented in Table 1.

Table 1. Independent Variable Values Used in the UAE of Propolis with DMSO and EtOH solvents

Independent Variables	Symbol	-1	0	+1
Solvent concentration (%)	X_1	0	50	100
Time (min)	X_2	30	105	180
Temperature (°C)	X_3	30	40	50

The conditions that provided the highest extraction efficiency were selected as the optimum extraction parameters. The experimental design used in this study is given in Table 2. The effects of process variables specific to the extraction method on the yield were investigated, and the extraction procedure that produced the maximum extract was optimized using the 'desirability' function approach.

2.3. Antioxidant activity

The ABTS radical cation scavenging method

The method described by Knez et al. (2025) was used to evaluate the ABTS radical scavenging activity of the samples. The ABTS•$^{+}$ radical was generated by mixing a 7 mM ABTS solution with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature for 12–16 hours. Prior to analysis, the ABTS solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 50 µL of the propolis solution was mixed with 100 µL of the diluted ABTS solution in a 96-well microplate. The mixture was allowed to react at room temperature for 10 minutes, and the absorbance was then measured at 734 nm. A blank solution without ABTS•$^{+}$ was used as a control, and Trolox was used as the reference antioxidant.

$$\text{ABTS scavenging activity (\%)} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100 \quad \text{(Equation 2)}$$

Results were expressed as IC_{50} values (concentration required to scavenge 50% of ABTS radicals).

Table 2. Experimental Plan for Extracting Propolis by UAE with DMSO and EtOH solvents

Experiment No.	Solvent Concentration (%) (X_1)	Extraction Time (min.) (X_2)	Temperature (°C) (X_3)
1	0	30	105
2	0	40	180
3	0	50	105
4	0	40	30
5	50	40	105
6	50	40	105
7	50	40	105
8	50	30	30
9	50	50	180
10	50	50	30
11	50	30	180
12	50	40	105
13	50	40	105
14	100	40	180
15	100	30	105
16	100	50	105
17	100	40	30

DPPH radical scavenging method

The antioxidant activities of propolis fractions were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The analysis was performed according to the method described by Blois (1958), with slight modifications. 20 µL of test solution was mixed with 180 µL of DPPH solution and placed in a 96-well plate. The plates were left in the dark for 15 min, then the absorbance was read with a reader at 540 nm.

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100 \quad \text{(Equation 3)}$$

Results were expressed as IC_{50} values (concentration required to scavenge 50% of DPPH radicals).

2.4. Total flavonoid content (TFC)

Total flavonoid content was determined using the aluminum chloride ($AlCl_3$) colorimetric method, following the procedure described by Chang et al. (2002). 22.5 µL of propolis samples were taken and 127.5 µL of methanol was added. 150 µL of 2% (w/v) $AlCl_3$ solution was added and vortexed, then incubated at room temperature in the dark for 30 minutes. Absorbance values were measured at a wavelength of 415 nm. Methanol was used as a blank. TFC was calculated using the quercetin standard curve, and the results were expressed as mg quercetin equivalent (QE)/g sample.

2.5. Total phenolic content (TPC)

The total phenolic content of samples was determined using the Folin–Ciocalteu method. The analysis was performed according to the method described by Escriche and Juan-Borrás (2018), applying modifications commonly used in propolis studies. The calibration curve was prepared using absorbance values corresponding to different concentrations of a gallic acid stock solution. For the assay, 10 µL of the propolis samples were mixed with 100 µL of Folin–Ciocalteu reagent and allowed to react at room temperature for five minutes. After this period, 100µL of 7.5% (w/v) sodium carbonate (Na_2CO_3) solution was added to the mixture. Following vortexing, the samples were incubated in the dark at room temperature for 30 minutes. At the end of the incubation, absorbance was measured at 760 nm using a UV–Vis spectrophotometer. TPC of the samples was calculated from the calibration curve, considering the corresponding absorbance, sample dilution, and concentration. The results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.6. Antimicrobial activity

The antimicrobial activity of samples prepared by diluting them at a ratio of 1:10 against *Escherichia coli, Salmonella* Typhimurium*, Staphylococcus aureus,* and *Bacillus cereus* was determined using the 96-well MIC (Minimum Inhibitory Concentration) plate method. Suspensions at a level of 6 log CFU/mL were prepared from the developed 18-24-hour microorganism cultures using 0.1% peptone water. Each culture was tested separately and no culture cocktail was used. Under aseptic conditions, 200 µL of the prepared extracts were added to the first wells. Subsequently, 100 µL of BHI broth medium was added to each well from the second well to the twelfth well for the specified microorganisms. Dilution was then performed from the first well to the eleventh well, and the extract was not transferred to the twelfth well; the last well was used as a positive control. The twelfth well was used as a positive control to observe the growth of test microorganisms, so it did not contain the extract whose antimicrobial effect is being investigated. Subsequently, 100 µL of culture suspensions were added to all wells and incubated at 37°C for 24-48 hours on MIC plates for *Escherichia coli, Salmonella* Typhimurium*, Staphylococcus aureus, and Bacillus cereus*. After incubation, streaks were made from each well of the MIC plates onto BHI agar media and left to incubate, and wells where no growth was observed were identified (Wiegand et al., 2008; Öncul and Karabıyıklı, 2016).

2.7. Statistical analysis

Statistical analyses were performed using SPSS 23.0 (IBM, USA) and Design Expert 13 (Stat-Ease Inc., USA) software packages. The t-test to determine whether there was a difference between the predicted values and the experimental data, and the calculation of Pearson coefficients between samples, were performed using SPSS 23.0 software package; regression analysis, statistical analyses, response surface plots, and optimization processes applied to determine the effect of treatment variables on performance were performed using Design Expert 13.0 software package. Statistical significance levels were expressed with a $p<0.05$ value.

3. RESULTS AND DISCUSSION

3.1. Optimization of propolis extraction

Within the scope of the experimental design, the effects of solvent concentration (X_1), extraction temperature (X_2), and extraction time (X_3) on extraction efficiency (%) were investigated. The RSM design matrix and experimental efficiency results for DMSO-based and EtOH-based ultrasonic-assisted extraction are presented in Table 3.

Table 3 shows that the independent variables significantly affect the extraction yield of DMSO-based ultrasonic-assisted extraction. Experimental yields ranged from 24.60 ± 0.57 % to 54.77 ± 0.05 %. Among the factors examined, solvent concentration appears to be the most dominant parameter. This can be explained by the fact that the solvent increases the solubility of the target compounds and facilitates mass transfer.

In all combinations of temperature and time, the yield increased almost linearly as the DMSO concentration increased from 0% to 100%; this is consistent with the "like dissolves like" principle. In the extraction of complex natural matrices such as propolis, optimizing the solvent polarity is particularly important because it

increases the solubility of phenolic and flavonoid compounds and thus has a significant effect on the overall extraction efficiency (Galanakis, 2012; Picot-Allain et al., 2022).

Increased temperature generally had a positive effect on yield. Increasing the temperature from 30 °C to 50 °C at 0% solvent concentration increased the yield from 25.83% to 31.83%. The increase in molecular kinetic energy and decrease in viscosity at high temperatures may have increased the extraction/mass transfer rate. However, the 54.77% yield obtained at 100% concentration at 50 °C is one of the highest yield points of the system. Similarly, Oroian et al. (2020) stated that high temperatures trigger cell wall rupture, thereby accelerating the release of target components. However, the high yield achieved at 50 °C in our study demonstrates that this temperature is compatible with the thermal stability of the target components.

Table 3. Response surface methodology design and experimental extraction yield (%) for DMSO and EtOH solvents using UAE

Experiment No.	Solvent Concentration (%) (X_1)	Temperature (°C) (X_2)	Time (min.) (X_3)	Yield for DMSO (%)	Yield for EtOH (%)
1	0	50	105	31.83 ± 0.29	33.73 ± 0.12
2	0	30	105	25.83 ± 0.29	30.03 ± 0.05
3	0	40	180	34.00 ± 0.33	34.00 ± 1.22
4	0	40	30	24.60 ± 0.57	29.67 ± 1.43
5	50	30	30	37.33 ± 0.62	39.23 ± 0.69
6	50	50	30	41.30 ± 1.80	43.00 ± 0.00
7	50	40	105	47.20 ± 0.08	46.80 ± 0.73
8	50	40	105	46.53 ± 2.00	45.07 ± 1.80
9	50	50	180	48.33 ± 0.29	48.77 ± 0.05
10	50	30	180	45.67 ± 0.78	45.03 ± 1.43
11	50	40	105	46.03 ± 0.05	46.40 ± 0.41
12	50	40	105	47.07 ± 0.82	47.07 ± 0.33
13	50	40	105	48.07 ± 0.57	54.27 ± 0.61
14	100	40	30	51.03 ± 0.12	52.03 ± 0.05
15	100	30	105	49.13 ± 0.05	48.10 ± 0.88
16	100	40	180	53.67 ± 0.21	54.27 ± 0.61
17	100	50	105	54.77 ± 0.05	55.60 ± 1.39

Increasing the extraction time brought the system closer to equilibrium, thus increasing the yield. At 40 °C and 0% solvent concentration, increasing the extraction time from 30 minutes to 180 minutes resulted in a yield of 34.00%. A similar upward trend was observed at 50% concentration. According to the results, the highest yield (54.77 ± 0.05%) was obtained at 100% solvent concentration and 50 °C, after 105 minutes of extraction. Considering the time factor, the lack of a significant increase in yield after 105 minutes suggests that the system has reached equilibrium. This behavior is consistent with diffusion-controlled extraction kinetics described by Fick's law. The fact that extending the duration to 180 minutes does not provide an additional yield increase supports the saturation state referred to in the literature as "exhausted extraction". Further increasing the duration after the system reaches the equilibrium point may not significantly contribute to mass transfer. Increased temperatures and prolonged cavitation effects can lead to partial degradation of sensitive bioactive compounds (Chemat et al., 2017). In this case, an extraction time of 105 minutes can be considered a balance point in terms of both extraction efficiency and bioactivity preservation. The experimental design for optimization consisted of a total of 17 experiments planned using response surface methodology (RSM), considering three independent variables: solvent concentration (X_1), extraction temperature (X_2), and extraction time (X_3). Five replicates were performed at the center point (50% solvent concentration, 40 °C, and 105 min) to estimate experimental error and evaluate the reproducibility of the process. Regression analysis was applied to reveal the relationship between extraction yield and the independent variables, and a second-order polynomial model was constructed. The quadratic polynomial equation expressed in terms of actual factors is presented in Equation 4.

$$\text{Yield (\%)} = 0.1509 + 0.4566X_1 + 0.3848X_2 + 0.0445X_3 - 0.0015X_1X_2 + 0.00022X_1X_3 + 0.000233X_2X_3 - 0.001624\,{X_1}^2 - 0.00110{X_2}^2 - 0.000148{X_3}^2 \quad \text{(Equation 4)}$$

Analysis of variance (ANOVA) results revealed that the quadratic model created to predict extraction yield was statistically highly significant. The model's values of F=154.15 and p< 0.0001 demonstrate the agreement of the established mathematical structure with the experimental data and indicate that the relationships between the variables are far from random (Table 4). Specifically, the lack of significance in the Lack-of-Fit value (p=0.1855) indicates that the model error is not statistically different from the experimental error. This suggests that the second-order model adequately represents the experimental data. In response surface methodology, a lack of significance in the Lack-of-Fit value is generally considered an indicator that the model is valid and suitable for optimization studies (Myers et al., 2016; Montgomery, 2017).

Solvent concentration, which had the highest F value among the factors (F=1102.34), was determined to be the most dominant parameter affecting yield. When the interactions between variables were evaluated, only the X_1-X_3 (solvent concentration-time) interaction was found to be statistically significant (p=0.0114), while other pairwise interactions were insignificant (p>0.05). These interactions explain how mass transfer behavior changes over time depending on changes in solvent concentration. Statistical significance of all second-order terms (X_1-X_2-X_3) confirms the curvature of the response surface and indicates the existence of a true optimum point within the study area. These results support the use of a second-order polynomial model for optimization. In response surface methodology, significant quadratic terms are generally interpreted as evidence of curvature in the system and the presence of an optimal region within the design space (Bezerra et al., 2008).

Table 4. ANOVA table showing the effect of linear, quadratic, and interaction terms on yield for DMSO-based UAE

Source	df	Sum Of Squares	Mean Square	F-Value	P-Value
Model	9	1343.13	149.24	154.15	< 0.0001
X_1	1	1067.22	1067.22	1102.34	< 0.0001
X_2	1	43.25	43.25	44.67	0.0003
X_3	1	96.61	96.61	99.78	< 0.0001
X_1X_2	1	0.0225	0.0225	0.0232	0.8831
X_1X_3	1	11.22	11.22	11.59	0.0114
X_2X_3	1	0.7225	0.7225	0.7463	0.4163
X_1^2	1	81.79	81.79	84.49	< 0.0001
X_2^2	1	19.60	19.60	20.24	0.0028
X_3^2	1	12.28	12.28	12.68	0.0092
Residual	7	6.78	0.9681		
Lack Of Fit	3	4.51	1.50	2.64	0.1855
Pure Error	4	2.27	0.5680		
Cor Total	16	1349.90			

After determining the statistical significance of the model and the effects of the factors on the response using analysis of variance (ANOVA), goodness-of-fit criteria such as R^2, Adjusted R^2, Predicted R^2, Adequate Precision, PRESS, and coefficient of variation (C.V. %) were evaluated to determine the extent to which the developed model represents the experimental data (Table 5).

Table 5. Statistical parameters obtained for the extraction yield in DMSO-based UAE

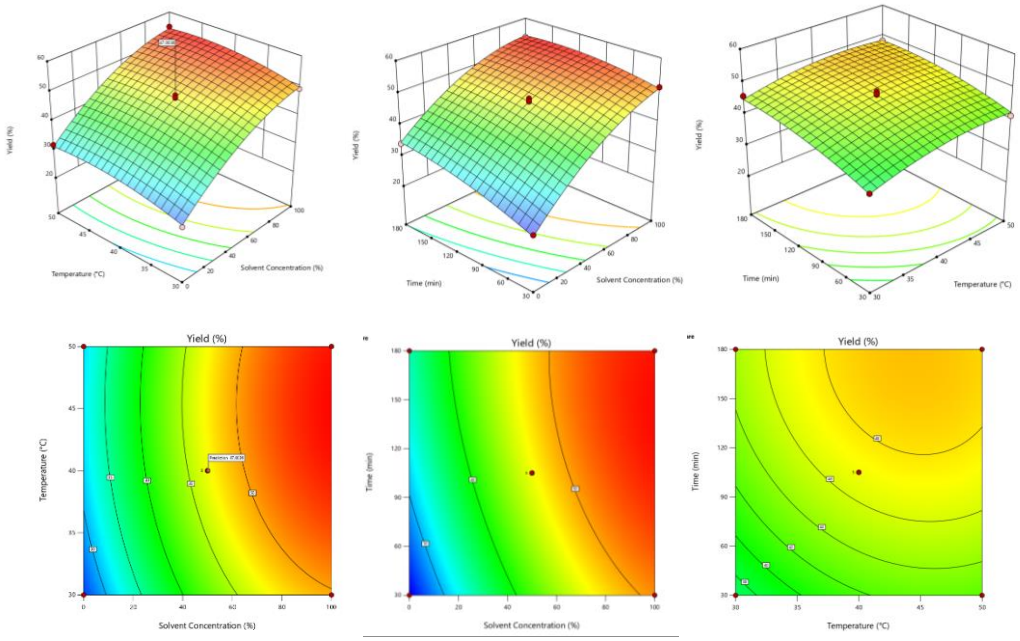
Std. Dev.	0.9839	**R^2**	0.9950
Mean	43.05	**Adjusted R^2**	0.9885
C.V. %	2.29	**Predicted R^2**	0.9440
		Adeq Precision	39.8198

The low standard deviation of the model (0.9838) indicates that the variation between experimental results and model predictions is quite limited. The coefficient of determination (R^2=0.9950) being very close to unity proves that the model has the capacity to explain 99.50% of the total variation. In particular, the difference between the adjusted coefficient of determination (Adj-R^2=0.9885) and the estimated coefficient of determination (Pred-R^2=0.9440) being less than 0.2 confirms the high predictive power of the model, and it is fit with the data. The coefficient of variation is 2.29%, indicating low experimental sensitivity and good

reproducibility of the data. Furthermore, the Adequate Precision was calculated as 39.8198. Since this value is significantly higher than the commonly accepted threshold of 4, the model demonstrates an adequate signal-to-noise ratio and confirms its reliable use for response surface methodology applications.

Figure 1 illustrates the simultaneous effects of solvent concentration (%) and temperature (°C) on extraction yield, respectively, using three-dimensional (3D) response surface and two-dimensional (2D) contour plots.

The steep slope along the solvent axis observed in Figure 1-a is in full agreement with the high F-value (F=1102.34) in the ANOVA results, which is a key finding of the study. The fact that the contour lines exhibit a more parallel and linear structure proves that the interaction between temperature and solvent concentration is statistically insignificant (p=0.8831). This is like the findings of Cujic et al. (2016), which showed that the dissolving power of the solvent is a dominant factor independent of temperature variations.



(a) (b) (c)

Figure 1. Response surface graphs showing the effects of temperature and solvent concentration on the yield obtained from DMSO-based UAE

The steep slope along the solvent axis observed in Figure 1-a is in full agreement with the high F-value (F=1102.34) in the ANOVA results, which is a key finding of the study. The fact that the contour lines exhibit a more parallel and linear structure proves that the interaction between temperature and solvent concentration is statistically insignificant (p=0.8831). This is like the findings of Cujic et al. (2016), which showed that the dissolving power of the solvent is a dominant factor independent of temperature variations.

The ellipse (oval) contour lines presented in Figure 1-b demonstrate a significant synergistic interaction (p=0.0114) between solvent concentration and extraction time. The pronounced curvature observed in the response surface indicates that the system is approaching a saturation point, and the rate of increase in yield gradually stabilizes after a certain period. In response surface methodology, ellipse contour plots are often interpreted as evidence of a strong interaction between variables. Maran et al. (2013) emphasized that such ellipse patterns reflect significant interaction effects, particularly important from the perspective of mass transfer kinetics.

Figure 1-c, which examines the combined effect of temperature and time, exhibits a more planar slope compared to the other graphs. The wider arcs of the contour lines confirm that, although these two factors increase efficiency, their effects on each other are limited and the interaction remains weak (p=0.4163). This linear trend parallels the study by Permukaan (2021), which showed that temperature and time act as independent enhancing factors in the study range.

Figure 2 presents a comparative distribution of the experimentally obtained yield values and those predicted by the second-order model, as shown in the following example. In the graphical analysis, the clustering of data points along and very close to the ideal 45° line of fit confirms the high frequency between the model predictions and the actual data. This supports the regression model's performance in explaining 99.50% of the variability in response.

In studies conducted to obtain high yield from DMSO-based ultrasonic-assisted extraction of raw propolis, the optimum conditions predicted by the program were determined. Using the Design Expert 13 software package, 13 solution points that give the optimum point and are close to each other were determined according to the "desirability" function approach. From these solutions, the 98.34% DMSO concentration, 42.20 °C temperature, and 142.60 min selected by the program were established as the optimum process conditions for extraction (Table 6).

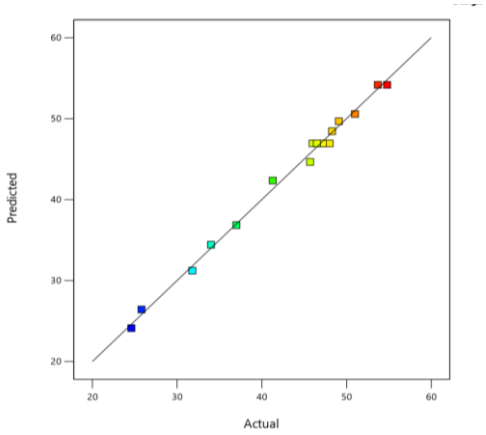


Figure 2. The relationship between experimental data and predicted data in terms of yield

A one-sample t-test was applied to test the agreement between the optimum yield value predicted by the model (54.17%) and the experimentally obtained value (52.58 ± 0.69%). The analysis revealed no statistically significant difference between the experimental data and the model predictions ($p=0.057$; $p>0.05$). This finding confirms that the developed quadratic model has high predictive power and successfully optimizes the extraction process.

Table 6. The relationship between experimental data and predicted data under optimum conditions of DMSO-based UAE

Optimal Conditions		
Solvent Concentration (%)	**Temperature (°C)**	**Time (min)**
98.34	42.20	142.60
Predicted value		
Yield %	54.17	
Obtained Value		
Yield %	52.58 ± 0.69	

The RSM design matrix and experimental efficiency results for ethanol-based ultrasonic-assisted extraction are presented in Table 3. Table 3 clearly shows that solvent concentration has a dominant effect on extraction efficiency. While the efficiency values obtained at 0% solvent concentration remained in the range of 29.67-34.00%, it was determined that the efficiency increased to approximately 39.23-48.77% and 48.10-55.60% at 50% and 100% levels, respectively. Increasing the ethanol concentration increases the mass transfer coefficient by bringing the solvent polarity closer to the target components. In the literature, Cottica et al. (2011) stated that solvent strength is the most critical factor determining the penetration rate into the porous structure of the matrix in propolis extraction, and this is consistent with the linear increase in our study.

The fact that increasing the temperature from 30°C to 50°C relative to the solvent has a positive but less significant effect on the yield indicates that the system has reached thermodynamic equilibrium. This increase observed at higher solvent concentrations can be attributed to the decrease in viscosity and the facilitation of

diffusion with increasing temperature. However, a linear increase in yield was observed as the temperature continued to increase. This can be explained by the possible loss of volatile compounds at high temperatures, as the system reaches thermal equilibrium after a certain point, as stated by Soquetta et al. (2018).

The plateau observed after 105 minutes demonstrates that ultrasonic-assisted extraction (UAE) exhibits typical kinetic behavior. Ultrasound-induced cavitation initially disrupts cell walls and accelerates diffusion by increasing mass transfer. However, as the extraction time increases, the concentration gradient between propolis and solvent decreases, and the yield increase is limited at the point where the system reaches equilibrium.

The highest extraction efficiency (55.60 ± 1.39%) was obtained at 100% solvent concentration, 50°C, and 105 minutes. This result reveals that the combination of high solvent concentration with medium-high temperature and time plays a critical role in maximizing extraction efficiency. The effect of the variables on extraction efficiency was mathematically modeled by constructing a second-order response surface model (RSM). The regression equation, adjusted to actual factor levels, is given in Equation 5.

$$\text{Yield}\ (\%) = -0.0637 + 0.27805X_1 + 1.21725X_2 + 0.07088X_3 + 0.00145X_1X_2 - 0.000133X_1X_3 + (2.52 \times 10^{-19})X_2X_3 - 0.001133{X_1}^2 - 0.013325{X_2}^2 - 0.000161{X_3}^2 \quad \text{(Equation 5)}$$

The results of the ANOVA for the quadratic model describing ethanol-based ultrasonic-assisted extraction yield are summarized in Table 7. The high F-value of the model (113.09) and the probability level below 0.0001 confirm that the regression model is statistically significant. These findings demonstrate that the model adequately explains the observed variability in extraction yield.

Table 7. ANOVA table showing the effect of linear, quadratic, and interaction terms on yield for EtOH based-UAE

Source	df	Sum Of Squares	Mean Square	F-Value	P-Value
Model	9	1004.63	111.63	113.09	< 0.0001
X_1	1	871.53	871.53	882.95	< 0.0001
X_2	1	40.05	40.05	40.58	0.0004
X_3	1	41.40	41.40	41.95	0.0003
X_1X_2	1	2.10	2.10	2.13	0.1878
X_1X_3	1	1.0000	1.0000	1.01	0.3477
X_2X_3	1	0,0000	0.0000	0.0000	1.0000
X_1^2	1	33.78	33.78	34.22	0.0006
X_2^2	1	7.48	7.48	7.57	0.0284
X_3^2	1	3.47	3.47	3.51	0.1030
Residual	7	6.91	0.9871		
Lack of Fit	3	4.40	1.47	2.33	0.2154
Pure Error	4	2.51	0.6280		
Cor Total	16	1011.54			

Examining the linear terms revealed that solvent concentration (X_1), extraction temperature (X_2), and extraction time (X_3) had a statistically significant effect on extraction yield ($p<0.01$). The very high F-value (882.95) exhibited by the solvent concentration proves that this parameter is the main driving force of the system. These findings are similar to those of Trusheva et al. (2007), which showed that solvent power plays a more critical role than temperature and time.

Statistical analysis revealed that the interaction terms between the extraction variables were not significant ($p>0.05$). This suggests that each parameter influenced the yield independently, rather than through a synergistic relationship. Such behavior is commonly observed in extraction systems, where the solvent primarily determines solubility, while variables such as temperature or time mainly influence the extraction rate (Chemat et al., 2017). In this case, changing the level of one factor did not substantially modify the effect of the others.

Analysis of the second-order components showed that the squared effects of both solvent concentration (X_1^2) and temperature (X_2^2) were statistically significant on the response ($p<0.05$). The result supports the existence of a nonlinear relationship between extraction yield and these parameters, and the existence of a specific optimum point. Guan and Yao (2008) stated that the significance of quadratic terms in the extraction of bioactive compounds indicates the formation of a "response variable curvature" (curvature) and that this curvature points to the existence of a true optimum region. In contrast, the second-order effect (X_3^2) of extraction time was not statistically significant ($p>0.05$). This indicates that the effect of time on yield exhibited a linear trend within the selected time interval (30-180 min). In other words, no significant saturation point (plateau) was reached in yield within this period, and it was determined that the yield followed a stable increase or a constant course with time.

In general, the ANOVA results show that solvent concentration, temperature, and time have significant effects on extraction efficiency, especially solvent concentration, which is the main parameter driving the process, and that the quadratic model developed within the scope of response surface methodology is suitable and reliable for the optimization of the ethanol-based ultrasonic-assisted extraction process.

The statistical parameters given in Table 8 demonstrate that the RSM model developed for ethanol-based ultrasonic-assisted extraction is quite robust and reliable. The low standard deviation of the model (Std. Dev.=0.9935) indicates that the deviation between the experimental data and the values predicted by the model is limited, and that the model has high sensitivity. The average extraction yield of 43.85 % shows that the results obtained in this study correspond to a significant yield level. The model's coefficient of determination (R^2=0.9932) indicates that a very high percentage 99.32 % of the variation in the response variable (extraction yield) is explained by the independent variables.

Table 8. Statistical parameters obtained for the extraction yield in Ethanol ultrasonic-assisted extraction

Std. Dev.	0.9935	**R^2**	0.9932
Mean	43.85	**Adjusted R^2**	0.9844
C.V. %	2.27	**Predicted R^2**	0.9266
		Adeq Precision	34.3672

The difference of approximately 0.058 between the adjusted coefficient of determination (Adjusted R^2=0.9844) and the predicted coefficient of determination (Predicted R^2=0.9266) indicates that the model shows a good fit with the experimental data and does not have an overfitting problem (Baş and Boyancı, 2007). The coefficient of variation was 2.27%, which is well below the commonly accepted 10 % threshold. Together with the low standard deviation (0.9935), this suggests that the experiments were conducted with good precision, and the results are reproducible. In response surface studies, low CV values are generally interpreted as evidence that experimental variability is minimal, and the fitted model reflects the data reliably rather than by chance (Baş and Boyacı, 2007). The Adequate Precision value, which reflects the signal-to-noise ratio, was calculated as 34.3672. Since this value is far above the recommended minimum of 4, the model appears to provide a sufficiently strong signal within the design space. A high Adequate Precision value indicates that the model's signal-to-noise ratio is satisfactory and that optimization can be reliably used without being affected by experimental errors (Myers et al., 2016; Montgomery, 2017).

Figure 3 shows the effect of solvent concentration (%), temperature (°C), and extraction time (min) on extraction efficiency. The relationships between the variables were shown using 3D surface plots and corresponding 2D contour maps obtained from response surface methodology.

Figure 3a shows that the strongest effect on extraction yield is solvent concentration. Increasing solvent concentration from 0% to 100% sharply and consistently increases the yield, regardless of temperature. This indicates that solvent concentration is the main factor controlling the extraction process. This dominant trend in the obtained data confirms the effect of solvent polarity on the extraction kinetics of bioactive compounds. In complex matrices such as propolis, extraction efficiency largely depends on the compatibility between the solvent and target compounds. According to the "like dissolves like" principle, solvents with appropriate polarity dissolve phenolic and flavonoid components more effectively (Trusheva et al., 2007; Zhang et al., 2018).

In contrast, the effect of temperature varies depending on the solvent concentration. At low solvent concentrations, increasing the temperature causes only a slight increase in yield; however, at high solvent concentrations, increasing the temperature from 30 °C to 50 °C leads to a more significant increase in yield, indicating the existence of a synergistic interaction between these two parameters. This can be explained by the increase in diffusion rate and the parallel improvement in mass transfer efficiency due to the decrease in solvent viscosity at high temperatures. At the same time, higher temperatures increase molecular mobility within the matrix, allowing the solvent to penetrate the porous structure more effectively (Oroian et al., 2020).

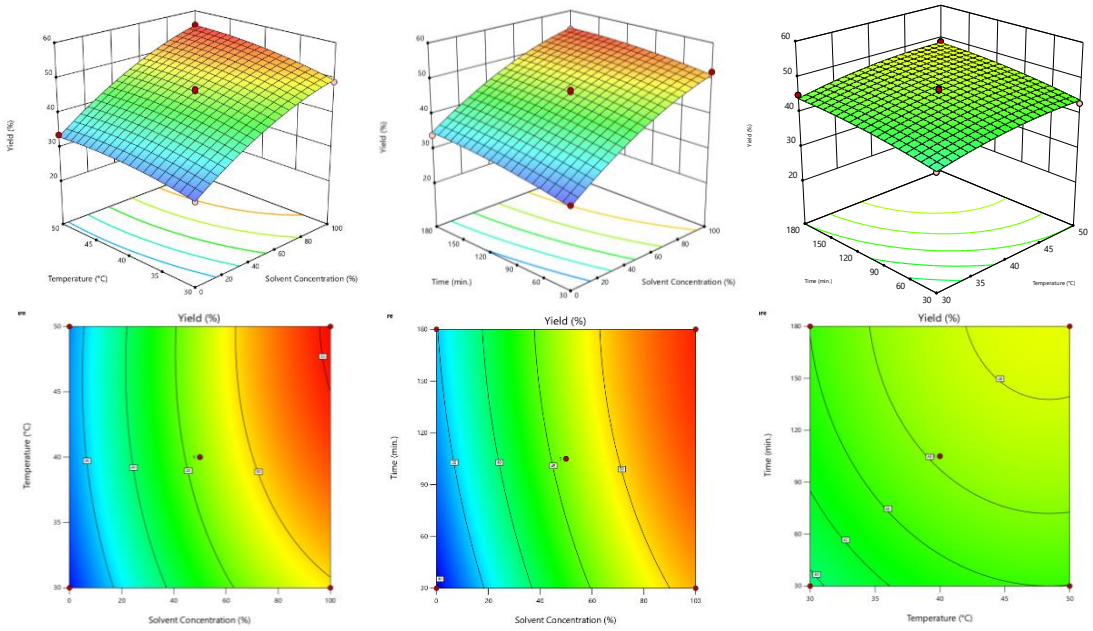


Figure 3. Effects of temperature and solvent concentration on yield in ethanol-based UAE (Response Surface Plots)

Figure 3-b shows that the interaction between solvent concentration (X_1) and extraction time (X_3) significantly increases the yield and that there is a synergistic relationship between these parameters. The distinct elliptical (oval) structure observed in the contour diagram is evidence that the interaction between these two variables is statistically significant ($p<0.05$). It was determined that the yield reaches its maximum in the region where high solvent concentration and increasing time combine (upper right corner of the graph), and the convex curve on the surface indicates that the system has reached saturation after a certain time. In the literature, elliptical contour lines in response surface analyses are defined as a key indicator that the interaction between independent variables is statistically significant and that these variables synergistically maximize the response (Baş and Boyancı, 2007; Myers et al., 2016; Montgomery, 2017).

Figure 3-c presents the combined influence of temperature (X_2) and extraction time (X_3) on extraction yield. Compared with other parameter interactions, the relatively flat response surface and the broader, nearly circular contour lines suggest that the interaction between these two variables is not statistically significant ($p>0.05$). Although both temperature and time positively affect yield, changes in one factor do not substantially modify the effect of the other. These results, consistent with the approach highlighted by Khuri and Cornell (1996), show that low surface slope and wide contour patterns indicate no significant synergistic interaction between extraction temperature and extraction time, and that the variables are independently effective.

Figure 4 shows the relationship between the experimentally obtained yield values and the yield values predicted by the model. The fact that most data points are located on or very close to the 45° reference line reveals that the developed model represents the experimental results with high accuracy. The regular and limited distribution of the points around the reference line indicates that there is no systematic deviation in the model and that the prediction errors are low. These findings confirm that the model is statistically reliable in predicting the yield in the ethanol-based ultrasonic-assisted extraction process.

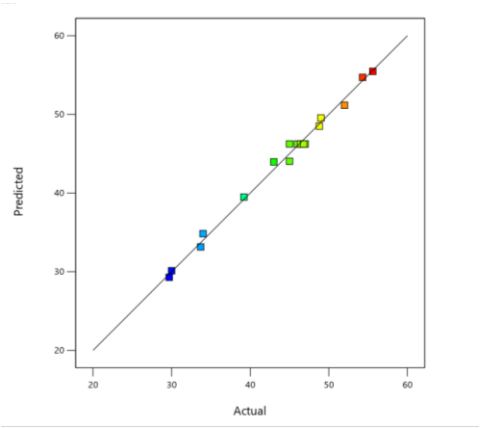


Figure 4. The relationship between experimental data and predicted data in terms of yield

In studies conducted to obtain high yields from ethanol-based ultrasonic-assisted extraction of raw propolis, the optimum conditions predicted by the program were determined. Using the Design Expert 7.0 software package, 13 solution points that give the optimum point and are close to each other were determined according to the "desirability" function approach. From these solutions, the 99.19 % ethanol concentration, 45.26 °C temperature, and 171.49 min selected by the program were established as the optimum process conditions for extraction (Table 9).

Table 9. The relationship between experimental data and predicted data under optimum conditions of EtOH-based UAE

Optimal Conditions		
Solvent Concentration (%)	**Temperature (˚C)**	**Time (min)**
99.19	45.26	171.49
Predicted value		
Yield %	55.82	
Obtained Value		
Yield %	51.94±2.04	

The relationship between the yield value predicted by the model under optimum conditions (55.82 %) and the mean of the experimentally obtained data (51.94±2.04 %) was evaluated using a one-sample t-test. Statistical analysis revealed no significant difference between the experimental data and the model's theoretical prediction ($p=0.0811$; $p>0.05$). This confirms that the developed quadratic model simulates the ethanol-based extraction process with high accuracy and that optimization has been successfully achieved.

3.2. Antioxidant activity

When the bioactive properties of DMSO and ethanol extracts were examined, statistically significant differences were determined between the extracts in terms of all parameters ($p<0.05$).

As seen in Table 10, the TPC was found to be higher in the DMSO extract (327.271 mg GAE/g) compared to the ethanol extract (281.833 mg GAE/g). Similarly, the TFC was also found to be higher in the DMSO extract (15.518 mg RE/g) compared to the ethanol extract (15.167 mg RE/g). This indicates that DMSO is a more effective solvent for extracting phenolic and flavonoid compounds.

When the antioxidant activity results were evaluated, the SC_{50} values for DPPH and ABTS radical scavenging analyses were determined to be higher in the ethanol extract. For the DPPH method, the SC_{50} value was determined to be 1190 µg/mL in the DMSO extract and 1375 µg/mL in the ethanol extract. Similarly, for the ABTS method, the SC_{50} values were determined to be 147.7 and 156.6 µg/mL, respectively. Since a lower SC_{50} value indicates higher antioxidant activity, it is understood that the DMSO extract has a stronger antioxidant capacity for both methods.

When the obtained findings are evaluated together, it is concluded that the DMSO extract is both richer in phenolic and flavonoid compounds and exhibits higher antioxidant activity. This supports a positive relationship between phenolic compound content and antioxidant activity.

Table 10. Comparison of TPC, TFC, and antioxidant activities of DMSO-based and EtOH-based propolis extracts

	Total phenolic mg GAE/g	Total Flavonoid mg RE/g	DPPH Radical Scavenging Activity SC_{50} (µg/mL)	ABTS Radical Scavenging Activity SC_{50} (µg/mL)
DMSO extract	325.271 ± 0.053[a]	15.518 ± 0.007[a]	1190 ± 1.6[b]	147.7 ± 0.4[b]
Ethanol extract	281.833 ± 0.041[b]	15.167 ± 0.007[b]	1375 ± 1.6[a]	156.5 ± 0.6[a]

(n=3, p<0,05)

Figure 5 shows that in both methods, the ABTS radical scavenging activity significantly increases with increasing concentration, which indicates that the antioxidant effect is dose-dependent. It was observed that the ABTS activity of the DMSO extract increased significantly with increasing concentration and showed higher activity compared to the ethanol extract, especially in the medium-high concentration range. The ethanol extract, on the other hand, exhibited lower, but significantly increased, antioxidant activity than the DMSO extract at all concentrations. The plateau tendency of the curves at high concentrations suggests that ABTS radicals were largely scavenged and that the system reached saturation. These results demonstrate that DMSO-based ultrasonic-assisted extraction effectively extracts antioxidant compounds found in propolis and that the resulting extracts possess strong radical scavenging capacity.

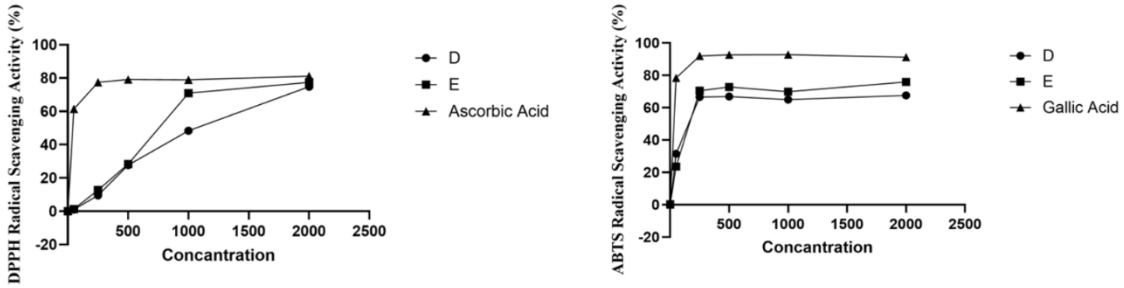


Figure 5. Comparative DPPH and ABTS antioxidant activities of DMSO and EtOH propolis extracts (D: DMSO extract; E: EtOH extract)

3.3. Total phenolic content (TPC) and total flavonoid content (TFC)

The effects of DMSO and EtOH solvents on the recovery of phenolic and flavonoid components in the propolis matrix were investigated, with comparative data for these parameters given in Table 10. As a result of the analyses, the TPC of the propolis extract obtained with DMSO was determined to be 325.271 ± 0.053 mg GAE/g. In addition, the TFC of the DMSO extract was determined to be 15.518 ± 0.007 mg RE/g. Although the flavonoid concentration shows a lower distribution compared to the TPC, it is consistent with the characteristic bioactive composition of propolis. Comparatively, the TPC in the ethanol extract was recorded as 281.833 ± 0.041 mg GAE/g, and the TFC as 15.167 ± 0.007 mg RE/g. Figure 6 shows that the two solvents have a similar effect on the extraction of flavonoids (TFC). However, DMSO is more effective than ethanol at extracting phenolic compounds (TPC). The higher TPC value obtained with DMSO suggests that this solvent is more effective at dissolving phenolic compounds in the propolis matrix, probably because of its polarity. Consequently, the DMSO extract appears to have a richer phenolic profile.

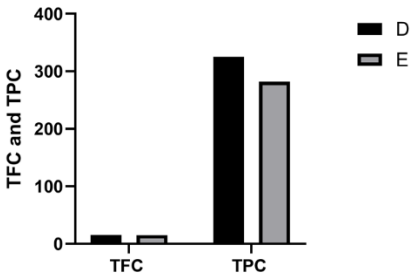


Figure 6. Comparison of TPC and TFC values of DMSO- and EtOH-based propolis extracts

3.4. Antimicrobial activity

The minimum inhibitory concentration (MIC) values of propolis extracts prepared with DMSO and ethanol against selected bacterial strains are shown in Table 11. The findings indicate that antimicrobial activity varies depending on the extraction solvent.

The ethanol extract inhibited all tested bacterial strains at 25 µg/mL, including *E. coli, S.* Typhimurium, *B. cereus, and S. aureus*. In contrast, the DMSO extract required a higher concentration (50 µg/mL) to inhibit the Gram-negative bacteria *E. coli* and *S*. Typhimurium. However, it showed the same inhibitory concentration as the ethanol extract (25 µg/mL) against the Gram-positive bacteria *B. cereus and S. aureus*.

Table 11. MIC of DMSO- and Ethanol-Based Propolis Extracts Against Bacterial Strains

	Minimum inhibitory concentration value (µg/mL)			
	E. coli	*S.* Typhimurium	*B. cereus*	*S. aureus*
DMSO extract	50	50	25	25
Ethanol extract	25	25	25	25

In this study, propolis extract prepared with ethanol exhibited higher antimicrobial activity compared to DMSO extract, despite having lower phenolic and flavonoid content. This indicates that the solvent used is a determining factor in both extract yield and biological activity. Due to the known antimicrobial effect of ethanol at high concentrations, it may not be possible to distinguish how much of the observed activity is due to ethanol in analyses without solvent control. However, the literature states that ethanol alone has a limited effect at low concentrations and that the main activity is based on biological components (McDonnell and Russell, 1999). It is known that ethanol is a more selective solvent in the extraction of more biologically active compounds, such as low molecular weight phenolic acids and flavonoids found in propolis, and that these compounds play a key role in antimicrobial activity (Bankova et al., 2000; Silva et al., 2006). It has also been reported that ethanol can increase the permeability of the bacterial cell membrane, facilitating the entry of phenolic compounds into the cell and thus enhancing the antimicrobial mechanisms of phenolic components, such as membrane damage, protein denaturation, and enzyme inhibition (Cushnie and Lamb, 2011).

In this context, the lower MIC values observed in the ethanol extract cannot be explained solely by the TPC or TFC. The qualitative composition of the compounds, their bioavailability, and possible synergistic interactions between ethanol and phenolic compounds may also play an important role. In other words, antimicrobial activity depends not only on how much phenolic compound is present, but also on which compounds are extracted and how they interact. Previous studies have similarly reported that the antimicrobial activity of propolis extracts varies depending on the solvent used and the resulting compound profile (Sforcin, 2007; Przybyłek and Karpiński, 2019).

4. CONCLUSIONS

In this study, the ultrasonic-assisted extraction (UAE) of propolis was systematically optimised for both dimethyl sulphoxide (DMSO) and ethanol-based systems using response surface methodology. Statistical analyses revealed that solvent concentration was the most significant factor influencing extraction yield in both solvent systems; this highlighted the critical role of solvent polarity and mass transfer mechanisms in the extraction process ($R^2 > 0.99$).

These findings demonstrate that solvent selection plays a decisive role not only in extraction efficiency but also in shaping the functional and biological properties of the resulting extracts. In this context, the results of this study provide a scientific basis for selecting appropriate extraction conditions tailored to the intended functional applications of propolis, such as antioxidant-rich extracts or antimicrobial-focused formulations.

Furthermore, this study contributes to a better understanding of solvent-matrix interactions during propolis extraction and highlights the importance of integrating these into optimisation strategies for both extraction yield and bioactivity parameters. Future studies focusing on scale-up processes and the detailed characterization of individual bioactive compounds could further support the development of propolis-derived functional products for applications in the pharmaceutical, nutraceutical, and food industries.

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AUTHOR CONTRIBUTION

EBB: Conceptualization, Methodology, Validation, Data Curation, Investigation, Formal Analysis, Writing – Original Draft, Writing – Review and Editing. ŞKÇ: Methodology, Validation, Data Curation, Writing – Review and Editing. EY: Methodology, Review and Editing.

DECLARATIONS

Ethics Approval and Consent to Participate

This study did not involve any human participants or animals. Therefore, ethics committee approval was not required.

Conflict of Interest

The authors declare no conflicts of interest. This work is a part of the PhD thesis of the first author.

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