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Optimization of Ultrasound-Assisted Extraction of Olive Leaf (var. Halhalı) Extracts

Zeytin Yaprağı (var. Halhalı) Ekstraktlarının Ultrason Destekli Ekstraksiyon ile Optimizasyonu

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ABSTRACT

In this study, the effects of three different independent variables (extraction temperature, extraction time, ultrasonic power) on the total phenolic content, extraction yield and antioxidant capacity (IC_{50}) of olive leaf extracts of Halhalı variety, which is originated from South-eastern Anatolia Region were investigated. As a result of the evaluation made with the Box-Behnken Design Model, the optimum conditions were determined as 62.94°C, 50.67 min, and 64.65% ultrasonic power for the optimization of ultrasonic-assisted extraction. In order to visualize the effect of the extraction method on TPC, extraction yield and IC_{50} value of olive leaf extracts, the results of three extraction methods were compared. The differences among the mean values of the investigated parameters in terms of extraction method were found significantly different (P<0.05). In addition, it can be inferenced that ultrasound assisted extraction accelerated the transition of phenolic substances to solvent in terms of SEM images and investigated other parameters.

Key Words

Olive leaf extract, Halhalı variety, ultrasound assisted extraction, optimization.

ÖZ

Bu çalışmada orijini Güneydoğu Anadolu Bölgesi olan Halhalı zeytin çeşidinin yaprak esktraktlarının toplam fenolik madde, ekstraksiyon verimi ve antioksidan kapasite (IC_{50}) değeri üzerine üç farklı bağımsız değişkenin (ekstraksiyon sıcaklığı, ekstraksiyon süresi ve ultrasonik güç) etkisi araştırılmıştır. Box-Behnken Dizayn Modeli ile yapılan değerlendirmenin bir sonucu olarak ultrason destekli ekstraksiyon için optimum şartlar 62.94°C, 50.67 dakika ve % 64.65 ultrasonik güç olarak belirlenmiştir. Ekstraksiyon metodunun zeytin yaprağı ekstraktlarının toplam fenolik madde, ekstraksiyon verimi ve IC_{50} değeri üzerine etkisini ortaya koymak için üç farklı ekstraksiyon metodunun sonuçları karşılaştırılmıştır. Ekstraksiyon metodu açısından araştırılan parametrelerin ortalama değerleri arasındaki farklılık P<0.05 düzeyinde istatistiksel olarak önemli bulunmuştur. Buna ilaveten, SEM görüntüleri ve araştırılan diğer parametreler açısından ultrason destekli ekstraksiyonun fenolik madde geçişini hızlandırdığı sonucuna varılmıştır.

Anahtar Kelimeler

Zeytin yaprağı ekstraktı, Halhalı çeşidi, ultrason destekli ekstraksiyon, optimizasyon.

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INTRODUCTION

Olive leaves, which represent approximately 10% of the total weight of harvested olive, are generally used for animal feeding. Olive leaves, which are left on to the olive trees after harvesting season, are mostly not used as a specific aim, and they do not provide an economic profit for the producers. They are mostly burned with branches gathered from pruning [1]. They are not only an abundant and cheap source but also rich in phenolic compounds that have bioactive properties such as antioxidant and antimicrobial activity. Therefore, utilization of by-products is important for both producers and consumers. Geographical (soil structure, altitude, location etc.), climatic (light, rainfall, season etc.), genetic (origin, cultivar etc.) and technological (preliminary preparations, solvent type, extraction method and extraction conditions) factors can affect the amount and profile of phenolic compounds in olive leaves [2].

Cleaning and separation of the leaves from foreign matters are the first steps of the preliminary preparations for extraction procedures. Afterwards, leaves are mostly dried by different methods such as air drying, microwave drying, and freeze-drying in order to remove water in leaf cell structure. Thus, leaves become more stable to enzymatic degradation, and it facilitates solvent diffusion into the cells of the leaves much easier than fresh leaves. Since phenolic compounds of olive leaves have different polarities, extraction systems, which consist of two or more solvents, are more preferred. In many studies, it was reported that methanol solvent extraction systems showed the highest extraction yield for extraction of phenolic compounds from the plants including olive leaves [3-9]. Even though methanol is removed after extraction, there can be toxic residues in the final extracts. Therefore, ethanol is the most preferable for extraction of phenolic compounds from olive leaves.

Extraction techniques are classified as traditional and alternative/new extraction techniques. Since much more extraction time and solvent consumption are required in traditional extraction techniques, alternative/new techniques have been developed. Ultrasound-assisted extraction, which is considered one of the alternative/new extraction techniques is a cheap, simple and efficient method especially for extraction of heat-sensitive materials such as phenolic compounds. Principally, the technique involves the transition energy generated from ultrasound waves with frequencies ranging from 20 kHz to 2000 kHz to extraction medium [10]. Mechanical vibrations which are generated by the high-frequency ultrasound waves, evolve to cavitation bubbles and mechanical pressure waves occur in the extraction medium. Since the collapse of the cavitation bubbles destroys the cell wall of the solid material, the solvent can diffuse into the cells and extractive compounds can transit into the solution easily [11, 12].

Table 1. Some researches about ultrasound assisted extraction of olive leaves.

Parameters	System	Referans
Probe position, ultrasound radiation amplitude, percent of ultrasound exposure duty cycle, irradiation time, extract flowrate, extract composition and water bath temperature	Probe	13
Geographical origin of olive leaves and extraction methods (Ultrasound assisted extraction and homogeniser-assisted extraction)	Bath	14
Electric power, emitter surface, extraction temperature	Probe	15
Solid/solvent ratio, extraction time, concentration of ethanol	Bath	16
Solid/solvent ratio, pH, extraction time, extraction temperature	Bath	17
Type and concentration of extraction solvent, extraction temperature, extraction time, ultrasonic power, liquid/solid ratio, extraction pressure	Probe	18
Extraction temperature	Probe	19
Ultrasound extraction mode, sonication extraction time, the liquid–solid ratio	Probe	20
Solvent type and concentration, extraction time, extraction temparature	Bath	21

The studies about ultrasound-assisted extraction of olive leaves [13-21] (Table 1) are generally focussed on the effect of parameters such as ultrasonic power/ amplitude, particle size, solvent concentration, the solvent to solid ratio and, extraction time and temperature. The number of trials, time and energy increases as the number of parameters increases. Furthermore, the interactions between the parameters can occur, and this situation change the results of the extraction procedure. In this context, all of the interaction among the parameters can be examined synchronously and minimized the number of the applications Response Surface Methodology (RSM). RSM is a mathematical and statistical technique in order to optimize the parameters of a system [22]. Ultrasound-assisted extraction can be applied by using an ultrasonic bath and probe systems. Whereas the influence of most parameters on extraction process was investigated using probe systems, there is not a previous optimization study on the effect of ultrasonic power, extraction temperature and extraction time by using bath systems. Therefore, the aim of the study is to optimize the processing parameters of ultrasound-assisted extraction (ultrasonic power, extraction temperature and extraction time) of native olive leaf (Halhalı variety) by using RSM, and examine the effects of single factor experiments and then compare the results of the optimized ultrasound assisted extraction method with some traditional methods.

MATERIALS and METHODS

Materials

Native olive leaves (var. Halhalı) were picked from Derik district of Mardin, Turkey in April 2017. After the twigs of the leaves and other impurities were removed, fresh leaves were washed with tap water and dried at 30±0.5°C in an incubator for 48 hours. Dried leaves were grounded in a coffee grinder (1-2 mm particle size) and then stored at -18°C.

Reagents

Folin-Ciocalteu reagent, sodium carbonate, Gallic acid and DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from Sigma–Aldrich. Ethanol and methanol (>99.5% and >99.8% mass fraction purity) were purchased from Merck. Tea bags were purchased from Silva Textile Company (Sefaköy-Beşyol, İstanbul). All of the solutions were prepared with deionized water (18 mΩ) from a Millipore Milli-Q water purification system.

Extraction experiments

Ultrasound-assisted extraction was performed in an ultrasonic bath (ISOLAB, 40 kHz, tank dimensions (mm): 302 x 239 x 150). Power has been adjusted as a percentage (40%–70%-100%) of full power. A tea bag was filled with 2 g of dried and grounded olive leaves and 40 mL ethanol (60%) were placed together in a Schott glass bottle (250 mL) and then the bottle was placed into the bath that contained approximately eight litres of water for each treatment. After the extraction, the tea bag was removed and, the extract was filtered through a syringe filter (0.45 µm). The filtered extract was stored at -18°C in an amber glass bottle until analysis.

A tea bag was filled with 2 g of dried, grounded olive leaves, and 40 mL ethanol (60%) were placed in a Schott glass bottle (250 mL), and the bottle was left at room temperature (25°C) for 24 hours in the dark for the conventional extraction. After the extraction period, the extracts were filtered through a syringe filter (0.45 µm). The filtered extract was stored at -18°C in an amber glass bottle until analysis.

A tea bag was filled with 2 g of dried and grounded olive leaves, and boiled tap water (~98°C) were placed in a Schott glass bottle (250 mL), and the bottle was left in the dark for 30 minutes for infusion. After the extraction period, the extracts were filtered through a syringe filter (0.45 µm). The filtered extract was stored at -18°C in an amber glass bottle until analysis.

Response surface methodology

In order to optimize the ultrasound-assisted extraction of olive leaves, Box Behnken Design (BBD) was performed to explore the effect of variables on the response parameters by using Design Expert 8.0.7.1 (trial version) software. Three independent process variables and their limits were determined according to literature [15, 19, 23]. The three independent factors studied were the extraction temperature (X_1), extraction time (X_2) and ultrasonic power (X_3). The coded values of the independent factors are shown in Table 2. Furthermore, the responses were selected as total phenolic content (TPC) (Y_1), antioxidant capacity (IC_{50}) (Y_2) and extraction yield (Y_3). As the BBD recommended using five replicates evaluate pure error, 17 experiments were performed as shown in Table 3. Each experiment was carried out in triplicate. In the optimization, the obtained results showed that response variables were fitted to a quadratic polynomial model in Equation (1).

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i \neq j=1}^{3} b_{ii} X_i X_j \qquad (1)$$

In Equation (1), Y is the predicted response, which was proposed by the experimental program according to the individual and interaction effects of the independent variables; b0, bi, bii, bij are the regression coefficients as intercept, linearity, square and interaction respectively; Xi and Xj are the independent variables.

Determination of antioxidant capacity

The antioxidant capacity of olive leaf extract was measured using the DPPH assay according to the method described by [24]. Diluted samples (0.1 mL), standard solutions (0.1 mL) and 3.9 mL of the DPPH (10^{-4} M) working solution were mixed in a glass tube and left in the dark for 40 minutes. The absorbance was measured against control (without sample) at 515 nm using a UV-VIS spectrophotometer. The percentage of DPPH radical scavenging activity (RSA) was calculated using Equation 2.

$$\%RSC(\%Inhibition) = [(Abs_C - Abs_S) / Abs_C]*100 \qquad (2)$$

In Equation 2, Abs_C means the absorbance of the control and Abs_s means the absorbance of the test sample.

The RSA values of the olive leaf extracts were plotted against the concentration (µg/mL) of the extracts. After calculation of concentration required inhibit 50% DPPH radical formation (IC_{50} value) from the log-dose inhibition curve, the results were expressed as IC_{50} values of the extracts and standards.

Determination of total phenolic content (TPC)

The total phenolic content was determined according to the Folin–Ciocalteu method as described by [25]. Briefly, Folin–Ciocalteu's reagent (1500 µL) and 7.5% sodium carbonate solution (1200 µL) were added to the appropriately diluted samples (300 µL). The reaction was kept in dark for 2 h, and the TPC was determined by measuring the absorbance at 765 nm. The results were expressed as gallic acid equivalent per g of the dried leaves (mg GAE/g dry leaf). Calibration curves were prepared using gallic acid solutions (5 mg/L-100 mg/L) with a regression coefficient of 0.9982 for 60% ethanol.

Extraction yield

All of the solvents were removed from the extract in a rotary evaporator, and the residue was weighed. Extraction yield was calculated using Equation 3 [26].

$$Extraction\ yield(\%) = \frac{A}{B} x \frac{C}{D} x \frac{100}{DM} x100 \qquad (3)$$

where A is the amount of residue (g), B is the amount of the olive leaf extract, C is the volume of the extract, D is the amount of olive leaf, DM is the dry matter percentage of the olive leaf.

SEM imaging

After the extraction procedure, the tea bags were removed from the Schott glass bottle, and the olive leaf residue was dried at 30°C by using a laboratory oven. The samples were first sputtered, and then coated with a thin layer of conductive gold at a thickness of 50–100 nm. The shape and surface characteristics of the samples were observed and digitally recorded.

Table 2. Symbols and coded values of independent variables of Box Behnken Design

Independent variables	Symbols	Coded values		
		-1	0	1
Temperature (°C)	X1	30	50	70
Time (min)	X2	20	40	60
Ultrasonic power (%)	X3	40	70	100

Table 3. Experimental mean values of the response variables for ultrasound-assisted extraction by using Box Behnken Design

	Independent variable levels			Mean value of response variables		
Run no	X1 (°C)	X2 (min)	X3 (%)	Y1 (mg GAE/ g dry leaf)	Y2 (%)	Y3 (µg/mL)
1	50	40	70	38.77	25.99	127.44
2	30	20	70	20.57	13.83	142.03
3	70	40	100	48.83	26.66	156.17
4	50	60	40	48.07	25.77	149.77
5	30	40	100	23.13	14.27	155.98
6	50	20	40	32.90	13.38	151.31
7	70	20	70	36.61	20.63	140.02
8	50	60	100	46.89	26.15	152.98
9	50	40	70	39.03	22.27	132.97
10	50	20	100	37.85	18.68	152.18
11	30	40	40	21.66	12.16	157.42
12	70	40	40	51.49	25.97	143.10
13	70	60	70	53.89	27.74	140.00
14	30	60	70	25.29	15.23	146.79
15	50	40	70	43.32	24.19	129.76
16	50	40	70	49.80	23.91	137.32
17	50	40	70	44.65	24.12	128.15

X_1: Extraction temperature, X_2: Extraction time, X_3: Ultrasonic power; Y_1: Total phenolic content, Y_2: Extraction yield, Y_3: Antioxidant capacity (IC_{50}).

Statistical analysis

Two replicate extractions were carried out for each of the samples followed by a minimum of three spectrophotometric measurements from each extract. The statistical software Design Expert (Trial version 7.0) was used to analyse the experimental data for an analysis of variance (ANOVA). In addition, the results of the extraction method were analysed using the ANOVA procedure of SPSS. Tukey's test was used for the determination of the significance between means.

RESULTS and DISCUSSION

Optimisation results of ultrasound-assisted extraction

The obtained results of TPC, extraction yield and IC_{50} values are shown in Table 3, while the results of ANOVA for the determination of the fit of the model are shown in Table 4, indicating the contribution of the variables to the quadratic model. If the model does not fit with the data well, the lack of fit will be significant [27]. Since the lack of fit was not significant (P>0.05), it can be inferred that the response surface results are valid for TPC, extraction yield and IC_{50}.

For TPC (mg GAE/g-dry leaf) and extraction yield, the linear parameter (X_1, X_2) and the quadratic parameter (X_1^2) were significant (P<0.001, P< 0.05 and P < 0.05, respectively), whereas for IC_{50}, the linear parameter (X_1) and the quadratic parameters (X_1^2, X_2^2, X_3^2) were significant (P<0.05, P<0.01, P<0.05 and P<0.001, respectively). However, ultrasonic power (X_3) did not show significant effect (P<0.05) on the response variables. The R^2 of the models for TPC, extraction yield and IC_{50} was calculated as 0.9470, 0.9371 and 0.9510, respectively.

In order to visualize the relationship between the response variables and experimental levels of the independent variables for the ultrasound-assisted extraction, quadratic polynomial model equations of coded factors (Y) was found as follows:

Table 4. ANOVA results according to the effect of model and independent variables on the response variables.

		Total Phenolic Content		Extraction Yield		IC_{50} Value	
Source	DF	SS	P-value	SS	P-value	SS	P-value
Model	9	201.05	0.0011**	49.68	0.0020**	173.68	0.0008***
X1	1	1254.29	< 0.0001***	258.86	0.0001***	65.70	0.0482*
X2	1	266.91	0.0036*	100.68	0.0019*	2.01	0.6885
X3	1	0.83	0.8170	8.99	0.1909	30.87	0.1454
X1 X2	1	39.46	0.1426	8.15	0.2105	5.72	0.5034
X1 X3	1	4.24	0.6052	0.5	0.7419	52.69	0.0696
X2 X3	1	9.39	0.4469	6.04	0.2739	1.37	0.7405
X12	1	211.50	0.0065*	37.53	0.0212*	169.50	0.0064**
X22	1	15.80	0.3307	12.99	0.1253	94.55	0.0241*
X32	1	0.27	0.8959	7.60	0.2247	1037.09	< 0.0001***
Residual	7	14.46		4.29		11.50	
Lack of fit	3	6.18	0.8255	7.69	0.0921	4.81	0.8306
Pure error	4	20.67		1.73		16.52	
Total	16						

***: Significant difference with P<0.001, **: Significant difference with P<0.01, *: Significant difference with P<0.05, DF: Degree of freedom,

SS: Sum of squares

$$Y_1 = +43.11 + 12.52X_1 + 5.78X_2 + 0.32X_3 + 3.14X_1X_2 - 1.03X_1X_3 - 1.53X_2X_3 - 7.09X_1^2 - 1.94X_2^2 + 0.25X_3^2 \quad (4)$$

$$Y_2 = +24.10 + 5.69X_1 + 3.55X_2 + 1.06X_3 + 1.43X_1X_2 - 0.35X_1X_3 - 1.23X_2X_3 - 2.99X_1^2 - 1.76X_2^2 - 1.34X_3^2 \quad (5)$$

$$Y_3 = +131.13 - 2.87X_1 + 0.05X_2 + 1.96X_3 - 1.20X_1X_2 + 3.63X_1X_3 + 0.58X_2X_3 + 6.34X_1^2 + 4.74X_2^2 + 15.69X_3^2 \quad (6)$$

where Y_1 is the predictive TPC value, Y_2 is the predictive extraction yield and Y_3 is the predictive IC_{50} value.

The response surface and contour plots were generated for each of the fitted models as a function of the independent variables while keeping the other variable at the central value (Figures 1-3).

The effects of independent variables on TPC of olive leaf extracts

As shown in Figure 1A, when the extraction time (X_2) was fixed at 59.86 min, it was predicted that maximum total phenolic content could be achieved when the combination of extraction temperature and power were 65.91°C and 83.33%, respectively. The TPC of the extract increased with the increase of extraction temperature from 30°C to 65.91°C. However, the rate of the increase slowed down between the 65.91°C and 70°C. Although it is known that high temperature causes the decrease in the total amount of phenolic compounds due to chemical and thermal degradation [19, 28], the increase in the extraction temperature does not appear to have a negative effect on the TPC of the olive leaf extract. This might be ascribed to the capability of high temperature to increase the solubility of the phenolic compounds by decreasing solvent viscosity and surface tension, thereby increasing the diffusion and extraction rate of these compounds. Similarly, TPC increased with an increase in extraction time from 20 min to 59.86 min as shown in Figure 1B. As mentioned before in section 3.1., ultrasonic power did not have any significant influence on TPC. The interaction of extraction temperature and time are presented in Figure 1C. These results indicate that the effect of extraction temperature is more significant than the extraction time on the TPC.

The effects of independent variables on the extraction yield of olive leaf extracts

The effect of the variables and their interactions on predicted extraction yield can be seen in Figure 2. As shown in Figure 2A, when the extraction time (X_2) was fixed at 51.74 min, it was predicted that maximum extraction yield could be achieved as the combination of extraction temperature and power were 68.6°C and 95.43%, respectively. The extraction yield increased with the increase of extraction temperature from 30°C to 68.6°C. An increase of extraction temperature over 68.6°C resulted in a decrease relatively. As shown in Fi-

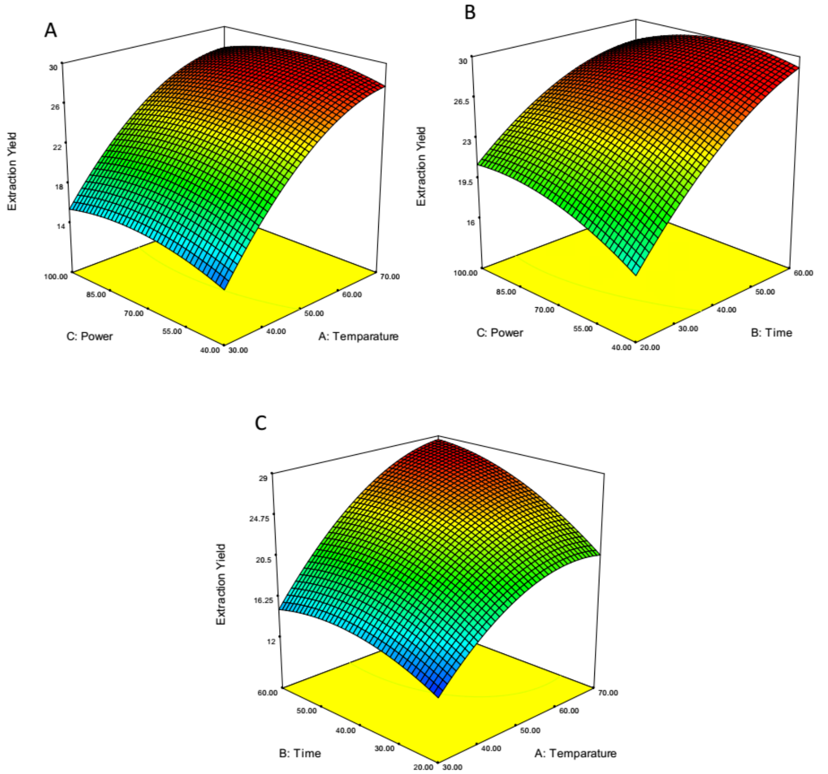

Figure 1. Response surface contour plots for the TPC of olive leaf extracts as a function of (A) power to temperature (Constant time: 59.86 min); (B) power to time (Constant temperature: 65.91°C); (C) time to temperature (Constant power: 83.33%)

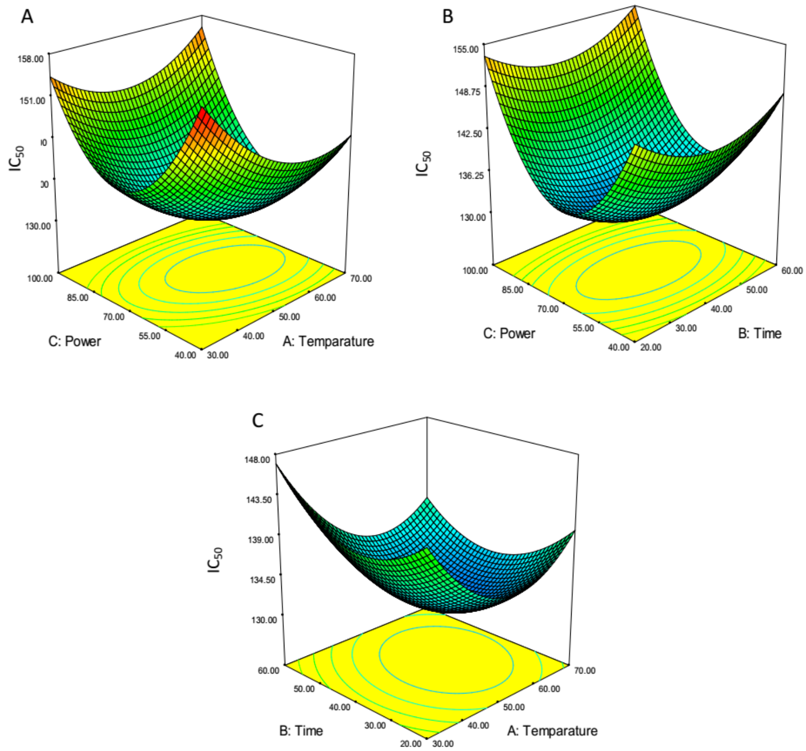

Figure 2. Response surface contour plots for the extraction yield of olive leaf as a function of (A) power to temperature (Constant time: 51.74 min); (B) power to time (Constant temperature: 68.6°C); (C) time to temperature (Constant power: 95.43%)

gure 2B, the extraction yield increased with the increase of extraction time from 20 min to 51.74 min, however, the rate of the increase slowed down between 51.74 min and 60 min. Similar to TPC, ultrasonic power had no significant effect on extraction yield. Figure 2C shows the effects of temperature and time on the extraction yield. As shown in Figure 2C, the extraction temperature had a more significant effect on the extraction yield than the extraction time. Unlike the results obtained in this study, researchers reported that the maximum oleuropein extraction yield was achieved at an extraction temperature of 50°C and the oleuropein extraction yield was slightly decreased above extraction temperature of 50°C till to 60°C [18]. This difference may be mainly due to other experimental conditions such as probe system, ultrasonic power, extraction pressure, ethanol concentration, liquid to solid ratio as well as raw material characteristics such as olive leaf origin, climatic and geographical conditions.

The effects of independent variables on IC_{50} of olive leaf extracts

Figure 3 illustrates the effect of the variables and their interaction on predicted IC_{50} of the olive leaf extracts. The optimized parameter settings for predicted IC_{50} of the olive leaf extracts were determined as 55°C, 39.70 min and 67.28%. As shown in Figure 3A, IC_{50} of the extracts was negatively correlated with extraction temperature when the temperature was lower than 55°C. On the other hand, IC_{50} of the extracts was positively correlated with extraction temperature when the temperature was higher than 55°C. Similarly, IC_{50} decreased with the increase of ultrasonic power from 40% to 67.28%. On the other hand IC_{50} increased with the increase of ultrasonic power from 67.28% to 70%. As shown in Figure 3B, IC_{50} of the extracts decreased with the increase of extraction time from 20 min to 39.70 min, while IC_{50} increased with the increase of extraction time from 39.70 min to 60 min. As shown in Figure 3C, the extraction temperature is the most significant parameter for IC_{50} value.

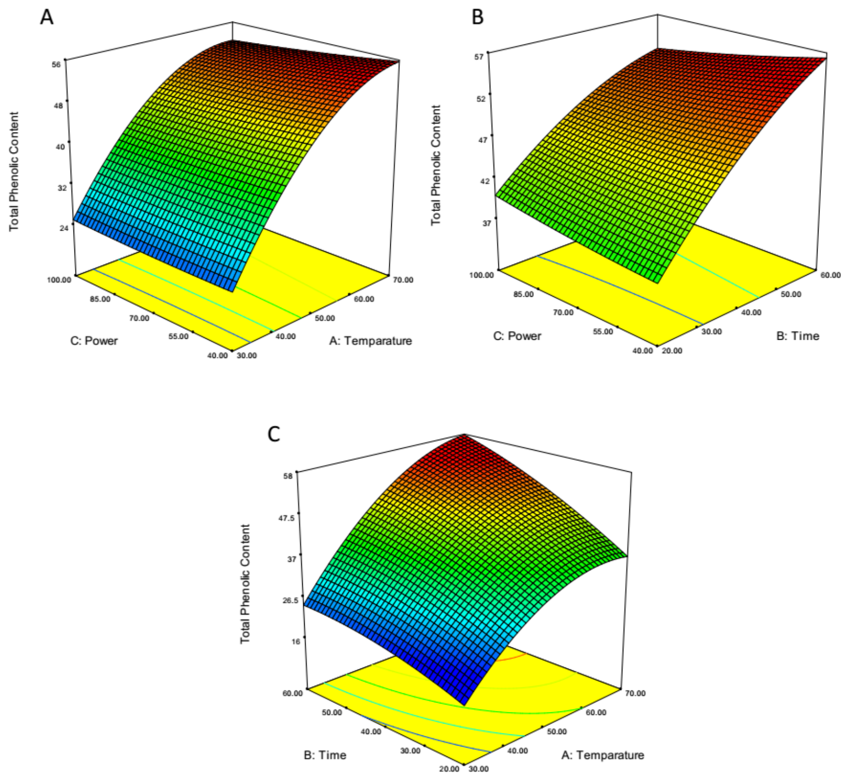

Figure 3. Response surface contour plots for the IC50 of olive leaf extracts as a function of (A) power to temperature (Constant time: 67.28%); (B) power to time (Constant temperature: 55°C); (C) time to temperature (Constant power: 39.70 min)

Optimization of the Model for Ultrasonic Assisted Extraction

Optimum process parameters were determined by simultaneously maximizing TPC and extraction yield, and minimizing IC_{50} value. Because IC_{50} value means the concentration of the required inhibiting 50% DPPH radical formation, the decrease of the IC_{50} value means the increase of the antioxidant capacity. Thus, the graphics obtained by the desirability function are presented in Figure 4 when the independent variables are evaluated together. The maximum desirability (0.919) of the model was obtained at 62.94°C, 50.67 min, and 64.65%. Under these conditions, the maximum TPC, extraction yield and IC_{50} value were found as 52.08 mg GAE/g dry leaf, 28.34% and 132.81 µg/mL, respectively. Similar results were reported by [17]. Researchers found the optimum conditions for ultrasound-assisted extraction as 59.87 min, 59.87°C, 3.52 of pH, 500 mg/19.78 mL solid to solvent ratio and the maximum TPC was found as 56.17 mg-GAE/g-dry matter under these conditions. In this study, experiments were carried out at 62.64°C, 50.67 min and 70% ultrasonic power to investigate the effect of extraction method since the power setting of the ultrasonic bath used in the experiments changed to 40, 70 and 100%.

The effect of the extraction method on the investigated parameters

In order to visualize the effect of the extraction method on TPC, extraction yield and IC_{50} value of olive leaf extracts, the results of three extraction methods were compared. The differences among the mean values of the investigated parameters in terms of extraction method were found significantly different ($P<0.05$). In this context, the lowest TPC (22.26 mg GAE/g dry leaf) and extraction yield (11.28%) and the highest IC_{50} value (235.23 µg/mL) was obtained using the infusion method. Since water is used in the infusion method, results of the TPC, extraction yield and antioxidant capacity were lower than the other extraction methods. As shown in Table 5, the results of investigated parameters obtained

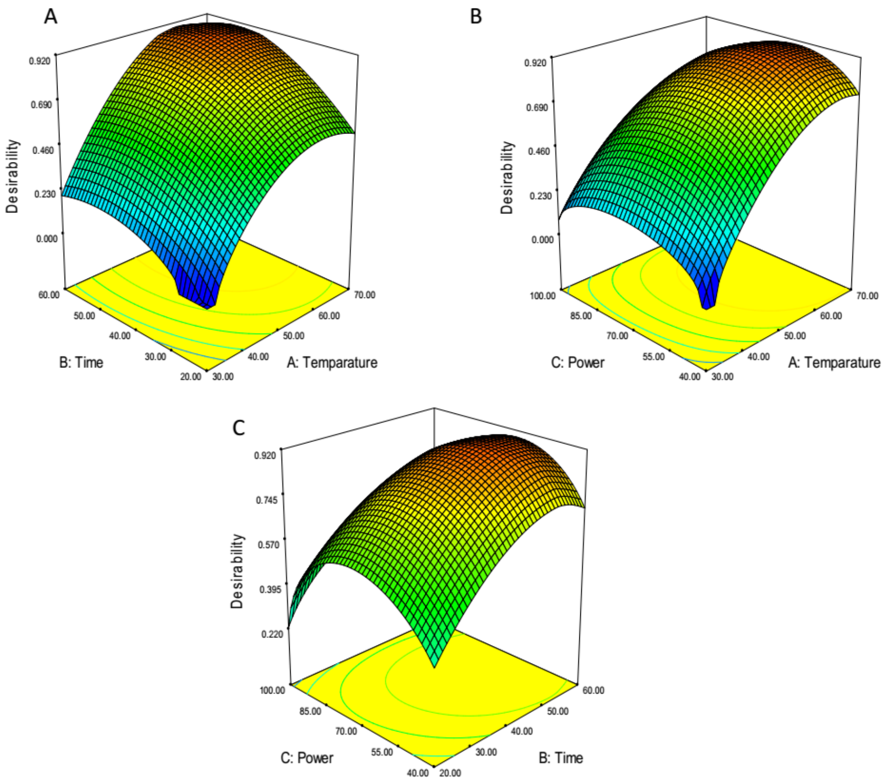

Figure 4. Response surface contour plots for the desirability as a function of (A) time to temperature (Constant power: 64.65%); (B) power to temperature (Constant time: 50.67 min); (C) power to time (Constant temperature: 62.64 °C)

Table 5. The mean values and groups of the parameters investigated in relation to the extraction method.

Extraction method	TPC (mg GAE/g dry leaf)	Extraction yield (%)	IC_{50} value (µg/mL) (µg/mL)
Infusion	22.26 ± 1.53^{a}	11.28 ± 0.07^{a}	235.23 ± 5.14^{c}
Conventional extraction	52.59 ± 0.70^{c}	30.34 ± 0.45^{b}	89.24 ± 1.30^{a}
Ultrasonic assisted extraction	48.93 ±0.69^{b}	31.97 ± 0.10^{c}	92.07 ± 0.60^{b}

by the conventional method, which was applied for 24 hours at room temperature, were very close to the results of ultrasound-assisted extraction method, which was applied under the optimized conditions (50.67 min, 62.64°C, 70% ultrasonic power). It can be inferred that the extraction time decreases with the effect of the ultrasound technique. Similar results were presented by [15]. Researchers reported that ultrasound assisted extraction reduced the extraction time from 24 h to 15 min. It was compared TPC and extract yield of olive leaf extracts obtained with methanol by homogenizer-assisted extraction (28.000 x g for 30 s) and ultrasound-assisted extraction (50 Hz at 25°C) methods [14]. Researchers reported that homogenizer-assisted extraction was superior to ultrasound-assisted extraction in terms of TPC and extraction yield, they interpreted the results as the ultrasonic breakdown of plant matrix by ultrasonic waves might not be as powerful as homogenizer-assisted extraction to create a better performance.

In order to visualize the effect of extraction method on disruption of the cell wall, microstructures of solid phase were screened by scanning electron microscopy (SEM) before and after the extraction procedure. As shown in Figure 5A, the surface of the solid-phase is firm and smooth before extraction. After infusion (30 min at room temperature), there is a slight disintegration in the structure (Fig 5B) and the disintegration increases with the conventional extraction method (24 hours at room temperature) (Fig 5C). After ultrasonic assisted extraction using the optimized results (60.21 ° C, 48.96 min, 70% ultrasonic power), it is seen that the disruption in cellular structure is at the highest level (Fig 5D).

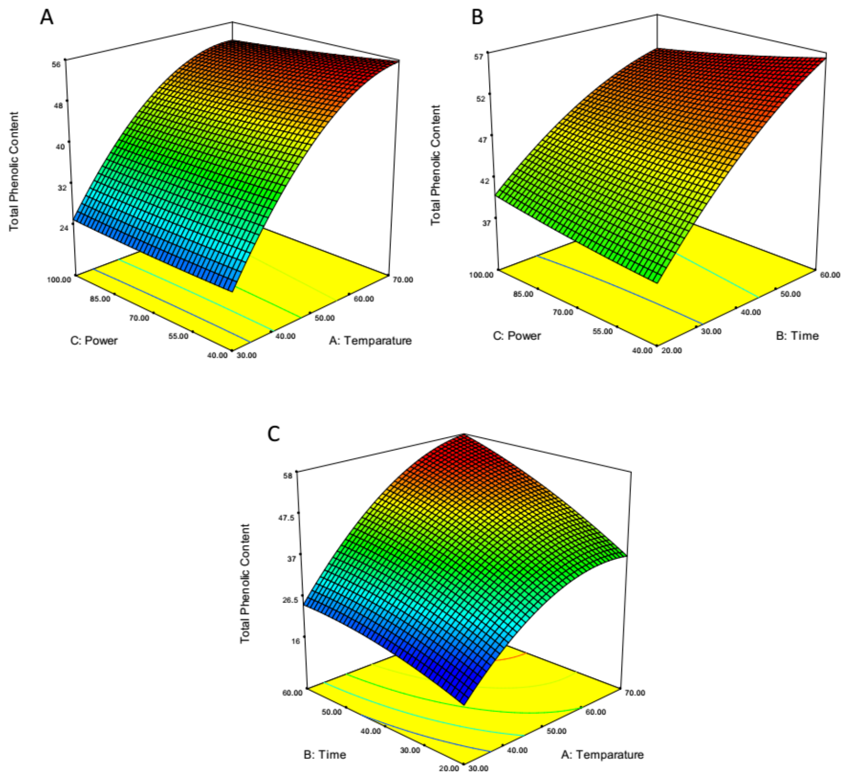

Figure 3. Response surface contour plots for the IC50 of olive leaf extracts as a function of (A) power to temperature (Constant time: 67.28%); (B) power to time (Constant temperature: 55°C); (C) time to temperature (Constant power: 39.70 min)

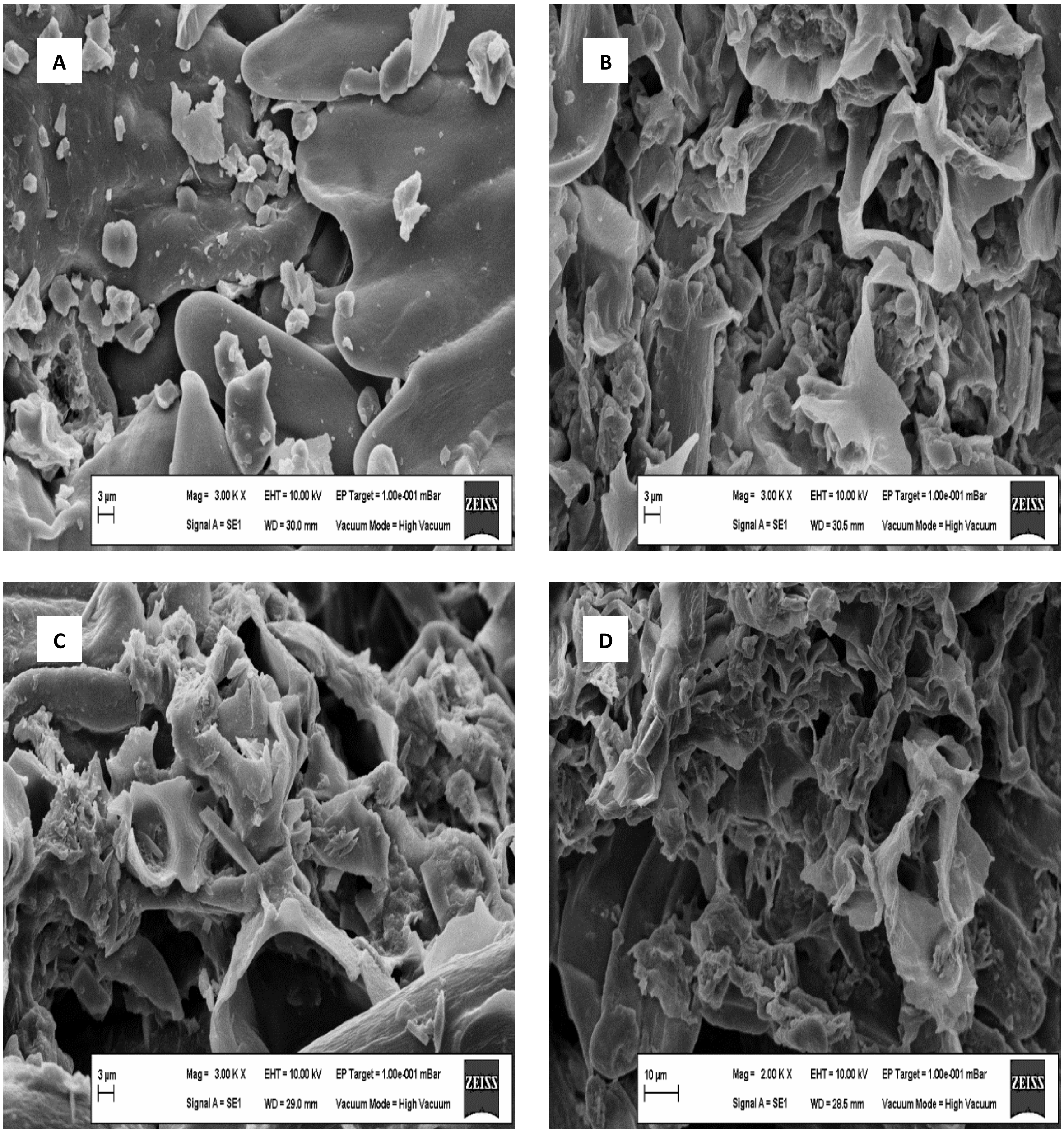

Figure 5. SEM images of olive leaf samples, (A) image of non-treated sample, (B) image of sample after infusion, (C) image of sample after conventional extraction method, (D) image of sample after ultrasonic assisted extraction.

CONCLUSION

The experimental results revealed that the optimum conditions for ultrasound-assisted extraction of olive leaves should be 62.94°C for extraction temperature, 50.67 min for extraction time and 64.65% for ultrasonic power in order to obtain the highest TPC (mg GAE/g dry leaf) and extraction yield (%), and the lowest IC_{50}. The most significant parameter on TPC and extraction yield was linear in terms of extraction temperature whereas the most significant parameter on the IC_{50} value was square in terms of ultrasonic power. The results showing the effect of the extraction method on the investigated parameters indicated that infusion was the least effective method because the viscosity of water decreased the penetration of water to the phenolic compounds. Furthermore, the study showed that ultrasonic-assisted extraction had the advantage of shortening extraction time by increasing the disruption of the cell wall of olive leaf.

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