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Impact and Optimization of the Conditions of Extraction of Phenolic Compounds and Antioxidant Activity of Olive Leaves (*Moroccan picholine*) Using Response Surface Methodology

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Abstract: The *Moroccan picholine* tree's leaves contain phenolic compounds that benefit human health. However, the amount and type of these compounds can vary based on factors such as the extraction method and conditions. This study aimed to improve phenolic compounds' extraction while minimising harmful chemicals' use. It has been found that using ethanol as a solvent with ultrasonic extraction is the most effective and environmentally friendly technique. Several parameters, such as the extraction time, solid/solvent ratio, and ethanol concentration as independent variables, were evaluated using a surface response method (RSM) based on the Box–Behnken design (BBD) to optimize the extraction conditions. The experimental data were fitted to a second-order polynomial equation using multiple regression analysis and also examined using the appropriate statistical methods. In optimal conditions, the ultrasonic time, the ratio (solvent/solid) and the concentration (ethanol/water), the content of total polyphenols (TPC), total flavonoids (TFC), and antioxidant activity (by DPPH, ABTS, FRAP) were, respectively, 74.45 ± 1.22 mg EAG/g DM, 17.08 ± 1.85 mg EC/g DM, 83.45 ± 0.89% 82.85 ± 1.52%, and 85.01 ± 2.35%. The identification of phenolic compounds by chromatography coupled with mass spectrum (HPLC-MS) under optimal conditions with two successive extractions showed the presence of hydroxytyrosol, catechin, caffeic acid, vanillin, naringin, oleuropein, quercetin, and kaempferol at high concentrations.

Keywords: olive leaves; extraction; optimization; ultrasound; polyphenols; flavonoids; antioxidant

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1. Introduction

The food and pharmaceutical industries are interested in agricultural wastes due to their high content of phenolic bioactive compounds, carbohydrates, oils, and other biochemical molecules [1].

The olive tree is commonly found in the Mediterranean region and is widely spread throughout Morocco, covering 65% of the national tree area. The regions of Fez-Meknes and Marrakech-Safi have the highest concentration of olive-growing areas, covering 54% of the total area and meeting 19% of the demand for edible oils. The olive transformation by-products, including the skin, pulp, pits, and leaves, have caught the attention of the food and pharmaceutical industries due to the presence of phenolic compounds.

Studies have shown that phenolic compounds found in olive tree leaves have beneficial properties, such as antioxidants [2], anticancer, antimicrobial [3], and hypolipidemic activities [4]. However, the amount of phenolic compounds present can vary based on

climate, moisture, plant age and variety [5], and extraction methods [6]. Traditional extraction methods, such as maceration and Soxhlet extraction, are slow and yield low amounts of bioactive products [1]. The ultrasonic method, a newer extraction technique, has been developed to efficiently extract organic bioactive compounds from plants [7]. This step is critical in the production of bioactive.

Ultrasonic-assisted extraction (UAE) is considered the greenest extraction process compared to microwave-assisted extraction (MAE), meeting the requirements of the green extraction method [8] as it reduces the temperature, time, and solvent usage [9–11]. This method has been widely used to extract valuable bioactive compounds from various plant materials. One of the food and pharmaceutical industries' dilemmas is improving extraction efficiency while reducing costs, which can be achieved by optimizing extraction conditions [12]. In addition, this technique is usually performed to study some independent factors, requiring more experiments, leading to increased cost and time [13].

Response surface methodology (RSM) is a powerful statistical tool that optimizes complex processes. It has gained popularity for its effectiveness in extracting methods, identifying optimal variable combinations, and simplifying experiment interpretation. This tool has been widely used in various fields [14]. The objective of this study was to examine the effect of certain independent factors of extraction (time, solid/solvent ratio, ethanol (%)) of bioactive compounds (TPC, TFC) and antioxidant activity (DPPH, ABTS, FRAP) with the ultrasonic-assisted extraction (UAE) method using response surface methodology (RSM).

2. Materials and Methods

2.1. Preparation of the Powder

Olive leaves of the Moroccan picholine variety were harvested in Marrakech, Morocco. The leaves were rinsed with water and dried in a ventilated oven (OVEN 19L DRYING AND STERILIZATION DIGITHEAT J.P.SELECTA) with a thickness of 1 cm at 80 °C (according to previous studies [15]) for 5 h (stable weight), then ground using a propeller mill (Mill Grinder For Spices And Professional Coffee, 1 kg). The leaf powder obtained was sieved (digital vibrating laboratory analysis sieve/GKM Siebtechnik GmbH) into four fractions (>125 µm, (125 µm; 50 µm), (50 µm, 25 µm), and <25 µm). The particle size was set at 25–50 µm. The leaf powder was stored at 4 °C in plastic bags.

2.2. Chemicals

The reagents used were pure ethanol, methanol (HPLC grade), Folin–Ciocalteu's, Sodium carbonate (Na_2CO_3), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), aluminum trichloride ($AlCl_3$), potassium persulfate ($K_2S_2O_8$), tripyridyltriazine complex (TPTZ), sodium acetate buffer (C_2H_3 NaO_2 $3H_2O$ and $C_2H_4O_2$), and hydrochloric acid (HCl).

2.3. Experimental Design and Statistical Analysis

The Box–Behnken design was used to determine the best combination of extraction variables for organic bioactive compounds based on the results of the preliminary single-factor test. Different variables such as extraction time and temperature, sample/solvent ratio, solvent percentage, and pH influence the determination of the content of phytochemicals [16]. Extraction time (min, X1), sample/solvent ratio (g/mL, X2), and ethanol concentration (*v/v*, X3) were chosen as independent variables, and their coded and uncoded (real) levels of independent variables are shown in Table 1.

Table 1. Coded and actual values for Box–Behnken design (BBD).

Code Symbols	Independent Variables	Level		
		−1	**0**	**+1**
X1	Time (min)	30	45	60
X2	Ratio (mL/g)	5	12.5	20
X3	Ethanol (%)	20	60	100

The variation of the response values (Y), with respect to the three variables was fitted into a response surface model and presented in the form of the second-order polynomial equation, is as follows:

$$Yi = \beta 0 + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXi^2 + \sum_{j=1} \sum_{<i=2}^{k} \beta ijXiXj + \varepsilon,$$

where Yi are the experiment responses; β0 represents the theoretical mean value of the response; βi, βj are the coefficients of the linear terms; βii, are the coefficients of the quadratic terms; βij are the coefficients of the interaction terms, and ε the error term.

2.4. Ultrasound-Assisted Extraction of Bioactive Compounds

Extraction with organic solvents has economic and environmental drawbacks. The "green chemistry" concept encourages developing and using less hazardous processes and materials without reducing efficiency [17]. Consequently, solvent extraction of bioactive compounds must be optimized for maximum response using fewer organic solvents. Thus, water was chosen to be studied in combination with ethanol. Ethanol was chosen instead of methanol as the extraction solvent due to the high toxicity of methanol in the human body [18]. Ethanol has the highest affinity for phenolic compounds; therefore, it the first choice for extracting phenolic compounds from fruit and vegetable wastes [19]. We mixed 1 g of olive leaf powder with ethanol/water. The extraction process was performed using a typical ultrasonic apparatus (Heating cleaning bath "Ultrasound HD"-Model 3000866), and the extract was filtered to collect the supernatant. A UV-T80 spectrophotometer was used to analyse the polyphenols, flavonoids, and total antioxidant activity in the samples.

2.5. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The determination of total polyphenols by the method using the Folin–Ciocalteu reagent is described by Singleton et al. [20]. A total of 0.25 mL of leaf extract was mixed with 0.25 mL of Folin–Ciocalteu and 2 mL of distilled water; the mixture was vortexed. After 3 min, 0.25 mL of sodium carbonate (20%) was added; the mixture was stirred and then incubated for 30 min in the dark at room temperature. The absorbance was measured at 750 nm using a UV/VIS spectrophotometer "T80-PG Instruments. The calibration curve for gallic acid was performed, and the results are expressed as mg gallic acid per g dry matter (mg GAE/g DW).

Flavonoid content was determined based on the formation of a flavonoid–aluminum complex that absorbs at 430 nm. The flavonoid assay was performed according to the protocol described by Djeridane et al. (2006) [21]. A total of 1.5 mL of the olive leaf extract was added with 1.5 mL of aluminum trichloride (AlCl3: 2%). After 30 min incubation at room temperature, the absorbance of the reaction mixture was read at 430 nm using a UV/VIS spectrophotometer (T80-PG Instruments). The flavonoid content in the extracts was calculated by reference to a calibration curve established with catechin. Results are expressed as mg catechin equivalent per 1 g dry matter (mg EC/g DW).

2.6. In Vitro Antioxidant Activity

2.6.1. DPPH Radical Reduction Test

For the anti-radical activity of the different extracts of the leaves dried at different temperatures, we used the method based on DPPH (1,1-diphenyl-2-picrylhydrazyl) as a

relatively stable radical, according to the protocol described by Abdel Hameed et al. [22]. Briefly, 1 mL of leaf extract was added to 1 mL of DPPH solution (prepared by solubilizing 4 mg of DPPH in 100 mL of ethanol). The mixtures were incubated in the dark for 30 min at room temperature. The decolorization compared to the negative control containing only DPPH solution measured at 517 nm using a UV/visible spectrophotometer type T80. The radical-scavenging activity of DPPH was calculated as follows: %(AA) = ((A517 control − A517 sample)/A517 control) × 100. A517 control is the absorbance of DPPH solution (without sample extract), and A517 sample is the absorbance of the sample with DPPH solution.

2.6.2. ABTS Radical Test

The ABTS•+ radical cation decolorization test also evaluated the anti-radical activity according to the method used by Aadesariya et al. [23]. The ABTS+- radical was generated by the reaction of 7 mM ABTS+ and 2.45 mM potassium persulfate. An equal mixture volume was incubated in the dark for 12–16 h. The ABTS+-solution was diluted with methanol to an absorbance of 0.700 ± 0.02 at 734 nm before use. Then, 1 mL of ABTS+-solution was mixed with 10 µL of leaf extract. The mixture was incubated for 30 min at 30 °C, and the absorbance was measured at 734 nm. The radical scavenging activity was expressed as the percentage of free radical inhibition by the sample and was calculated by the following formula:

$$\text{ABTS scavenged (\%)} = [(\text{A734 control} - \text{A734 sample})/\text{A517 control}] \times 100.$$

A734 control is the absorbance of the control reaction, and A 734 test is the absorbance in the presence of the sample extracts.

2.6.3. Ferric Reducing Antioxidant Power (FRAP) Test

The FRAP (ferric reducing antioxidant power) method is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This method evaluates the declining power of compounds at low pH [24]. The ferrous tripyridyltriazine (TPTZ) complex has an intense blue color measured by a spectrophotometer at 593 nm. The FRAP assay was performed according to the protocol of [25]. The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer (3.1 g $C_2H_3NaO_2$ $3H_2O$ and 16 mL $C_2H_4O_2$), pH: 3.6; 10 mM solution of TPTZ in 40 mM HCl; and 20 mM $FeCl_3$ at a ratio of 10:01:01 ($v/v/v$). One hundred microliters of each extract were added to 3 mL of FRAP reagent and 300 µL of H_2O. After incubation at 37 °C for 30 min; the absorbance was measured at 593 nm against the blank [26].

2.7. Model Verification

The extraction conditions were numerically optimized for maximum TPC and TFC content with high antioxidant activities based on regression analysis and 3D surface curves of independent variables. Responses were determined according to the recommended extraction conditions.

2.8. Qualitative and Quantitative Analysis by HPLC-MS

Identification and quantification of phenolic compounds by HPLC-MS were performed according to the method used by Puigventos et al. (2015) [27]. The injection volume of each sample was 10 µL with separation between solvent A (0.1% aqueous formic acid solution) and solvent B (methanol) as follows: 0–3 min, linear gradient from 5 to 25% B; 3–6 min, at 25% B; 6–9 min, from 25 to 37% B; 9–13 min, at 37% B; 13–18 min, from 37 to 54% B; 18–22 min, at 54% B; 22–26 min, from 54 to 95% B; 26–29 min, at 95% B; 29–29. 15 min, back to initial conditions at 5% B; and 29.15 to 36 min, at 5% B. The mobile phase flow rate was 1 mL/min. Ion transfer tube temperature was set at 350° and the full scan MS acquisition mode to *m/z* 50–1000. The polyphenolic compounds were obtained at 31 min.

2.9. Statistical Analysis

Analysis of variance (ANOVA) and multiple regression analysis were performed to fit the mathematical model using Design Expert 13 software. Significant terms ($p < 0.05$) in the model for each response were found by analysis of variance, and significance was judged by the F statistic calculated from the data. The experimental data were evaluated with various descriptive statistical analyses such as *p*-value, F-value, sum of squares (SS), degrees of freedom (DF), coefficient variation (CV), the mean sum of squares (MSS), coefficient of determination (R2), and adjusted coefficient determination (Radj2); this is to obtain the statistical significance of the developed quadratic mathematical model.

3. Results and Discussion

3.1. Evaluation and Optimization of Extraction Conditions

This study determined the relationship between response functions and process variables using a three-factor based on the Box–Behnken (BBD) design. The goal was to optimize the extraction conditions for bioactive compounds such as total polyphenols (TPC), total flavonoids (TFC), their corresponding antioxidant activities. Similar scientific studies were used as a reference. [28].

The outcomes of the conducted responses are reported in Table 2. The total polyphenol (TPC) content ranged from 48.69 to 72.98 mg EAG/g DM. The total flavonoid (TFC) content ranged from 10.45 to 16.36 mg EC/g DM. The entire content of TPC and TFC was obtained for trials 13, 14, and 15 under the experimental conditions of X1 = 45 min; X2 = 12.5 mL/g; and X3 = 60%. Regarding DPPH radical scavenging capacity, ABTS and FRAP ranged from 70.95% to 85.69%, 74.67 to 85.41%, and 71.92 to 86.95%, respectively. The highest antioxidant activity was obtained for tests 2 and 13 under X1 = 60 min; X2 = 5 mL/g; X3 = 60% and X1 = 45 min; X2 = 5 mL/g; and X3 = 60%, respectively. Based on these data, the extraction process was optimized to achieve the maximum desirable response.

Table 2. The experimental run from Box–Behnken design (BBD).

N°Exp	Time (min; X_1)	Ratio (mL/g; X_2)	Concentration (%; X_3)	TPC (mg EAG/g DM; Y1)		TFC (mg EC/g DM; Y2)		DPPH (%; Y3)		ABTS (%; Y4)		FRAP (%; Y5)	
				Reel	Predicts	Reel	Predicts	Reel	Predicts	Reel	Predicts	Reel	Predicts
1	30(−1)	5(−1)	60(0)	59.23	58.65	11.12	11.14	76.12	74.97	76.34	76.89	73.65	72.21
2	60(1)	5(−1)	60(0)	66.36	63.65	13.96	14.05	85.69	84.59	85.41	84.05	76.55	79.15
3	30(−1)	20(1)	60(0)	52.98	55.69	11.85	11.77	76.25	77.35	79.69	81.05	82.65	80.05
4	60(1)	20(1)	60(0)	53.31	53.90	11.96	11.95	75.36	76.51	76.23	75.68	73.76	75.20
5	30(−1)	12.5(0)	20(−1)	56.96	56.02	11.08	10.88	77.36	76.46	75.66	74.58	72.36	74.92
6	60(1)	12.5(0)	20(−1)	55.25	56.43	12.15	11.88	78.85	81.84	79.84	81.22	76.02	74.53
7	30(−1)	12.5(0)	100(1)	48.69	47.51	10.69	10.96	70.95	80.99	83.46	82.08	74.12	75.61
8	60(1)	12.5(0)	100(1)	49.36	50.31	12.85	13.05	78.36	79.26	77.24	77.77	80.65	78.09
9	45(0)	5(−1)	20(−1)	60.26	61.79	10.96	11.15	75.28	77.34	76.08	76.06	75.14	74.03
10	45(0)	20(1)	20(−1)	56.45	54.69	10.45	10.74	76.52	76.33	74.67	73.84	71.92	71.96
11	45(0)	5(−1)	100(1)	51.96	50.31	12.39	12.10	77.39	79.58	76.87	77.70	72.18	72.14
12	45(0)	20(1)	100(1)	49.65	48.12	11.23	11.04	74.96	72.90	75.69	75.71	76.98	78.10
13	45(0)	12.5(0)	60(0)	72.98	72.41	15.99	16.34	82.25	81.65	82.96	82.65	86.95	86.28
14	45(0)	12.5(0)	60(0)	72.26	72.41	16.98	16.34	81.12	81.65	83.23	82.65	86.45	86.28
15	45(0)	12.5(0)	60(0)	71.99	72.41	16.06	16.34	81.59	76.99	81.75	82.65	85.45	86.28

Pearson's test showed a strong positive correlation, r = 0.8- 0.85, between TPC and TFC and between TFC and FRAP. This implies that these answers evolve proportionally. The positive average correlations r = 0.5–0.75 appear between the other answers.

3.2. Fitting the Model and Analysis of Variance

The extraction process was optimised by applying the second-order polynomial model fit. The results are presented in Table 3. The model shows a high significance level and a good fit, with the experimental data of TPC and TFC contents showing less variation around the mean (R2 values 0.969 and 0.991), respectively.

Table 3. Experimental design of the surface response and statistical table of results.

Source	Sum of Squares	Estimation of Coefficients	Degree of Freedom	Medium Square	Value F	Value *p*	Remarks
			TPC				
Model	990.43		9	11005	17.53	0.0028	significant
Intercept, X_0		72.41 *					
Linear							
X_1	5.15	0.8025	1	5.15	0.8209	0.4065	
X_2	80.77	−3.18 *	1	80.77	12.87	0.0157	
X_3	107.02	−3.66 *	1	107.02	17.05	0.0091	
Interaction							
X_1 X_2	11.56	−1.70	1	11.56	1.84	0.2328	
X_1 X_3	1.42	0.5950	1	1.42	0.2256	0.6548	
X_2 X_3	0.5625	0.3750	1	0.5625	0.0896	0.7767	
Quadratic							
X^2_1	249.94	−8.23 *	1	249.94	39.83	0.0015	
X^2_2	142.51	−6.21 *	1	142.51	22.71	0.0050	
X^2_3	498.34	−11.62 *	1	498.34	79.40	0.0003	
Residual	31.38		5	6.28			
Lack of fit	30.86		3	10.29	39.27	0.0249	significant
Error	0.5238		2	0.2619			
Total	1021.81		14				
Accuracy Adequacy	12.17						
CV%	4.28						
R2	0.969						
R2Ajust	0.91						
Average	58.51						
			TFC				
Model	61.99		9	6.89	31.55	0.0007	significant
Intercept, X_0		16.34 *					
Linear							
X_1	4.77	0.7725 *	1	4.77	21.87	0.0055	
X_2	1.08	−0.3675	1	1.08	4.95	0.0767	
X_3	0.7938	0.3150	1	0.7938	3.64	0.1148	
Interaction							
X_1 X_2	1.86	−0.6825 *	1	1.86	8.54	0.0330	
X_1 X_3	0.2970	0.2725	1	0.2970	1.36	0.2960	
X_2 X_3	0.1056	−0.1625	1	0.1056	0.4838	0.5177	
Quadratic							
X^2_1	12.54	−1.84 *	1	12.54	57.44	0.0006	
X^2_2	19.16	−2.28 *	1	19.16	87.76	0.0002	
X^2_3	29.11	−2.81 *	1	29.11	133.35	<0.0001	
Residual	1.09		5	0.2183			
Lack of fit	0.4811		3	0.1604	0.5253	0.7074	Not significant
Error	0.6105		2	0.3052			
Total	63.08		14				
Accuracy Adequacy	14.69						
CV%	3.69						
R2	0.98						
R2Ajust	0.95						
Average	12.65						
			DPPH				
Model	165.56		9	18.40	5.19	0.0423	significant
Intercept, X_0		81.65 *					
Linear							
X_1	38.63	2.20 *	1	38.63	10.89	0.0215	
X_2	16.22	−1.42	1	16.22	4.57	0.0855	
X_3	5.04	−0.7937	1	5.04	1.42	0.2867	
Interaction							
X_1 X_2	27.35	−2.61 *	1	27.35	7.71	0.0390	
X_1 X_3	8.76	1.48	1	8.76	2.47	0.1768	
X_2 X_3	3.37	−0.9175	1	3.37	0.9493	0.3747	
Quadratic							
X^2_1	8.06	−1.48	1	8.06	2.27	0.1920	
X^2_2	12.24	−1.82	1	12.24	3.45	0.1224	

Table 3. *Cont.*

Source	Sum of Squares	Estimation of Coefficients	Degree of Freedom	Medium Square	Value F	Value *p*	Remarks
X^2_3	53.19	−3.80 *	1	53.19	14.99	0.0117	
Residual	17.74		5	3.55			
Lack of fit	17.09		3	5.70	17.68	0.0540	Not significant
Error	0.6445		2	0.3222			
Total	183.30		14				
Accuracy Adequacy	8.2473						
CV%	2.42						
R2	0.9032						
R2Ajust	0.73						
Average	77.87						
			ABTS				
Model	163.76		9	18.20	8.05	0.0168	significant
Intercept, X_0		82.65 *					
Linear							
X_1	1.59	0.4462	1	1.59	0.7045	0.4395	
X_2	8.86	−1.05	1	8.86	3.92	0.1046	
X_3	6.14	0.8762	1	6.14	2.72	0.1602	
Interaction							
X_1 X_2	39.25	−3.13 *	1	39.25	17.36	0.0088	
X_1 X_3	27.04	−2.60 *	1	27.04	11.96	0.0181	
X_2 X_3	0.0132	0.0575	1	0.0132	0.0058	0.9420	
Quadratic							
X^2_1	0.0000	−0.0033	1	0.0000	0.0000	0.9968	
X^2_2	38.42	−3.23 *	1	38.42	16.99	0.0092	
X^2_3	47.68	−3.59 *	1	47.68	21.08	0.0059	
Residual	11.31		5	2.26			
Lack of fit	10.06		3	3.35	5.40	0.1602	Not significant
Error	1.24		2	0.6212			
Total	175.07		14				
Accuracy Adequacy	8.31						
CV%	1.90						
R2	0.94						
R2Ajust	0.82						
Average	79.01						
			FRAP				
Model	364.85		9	40.54	5.21	0.0418	significant
Intercept, X_0		86.28 *					
Linear							
X_1	2.20	0.5250	1	2.20	0.2836	0.6171	
X_2	7.59	0.9737	1	7.59	0.9757	0.3686	
X_3	9.01	1.06	1	9.01	1.16	0.3309	
Interaction		0.5250					
X_1 X_2	34.75	−2.95	1	34.75	4.47	0.0881	
X_1 X_3	2.06	0.7175	1	2.06	0.2649	0.6287	
X_2 X_3	16.08	2.00	1	16.08	2.07	0.2099	
Quadratic							
X^2_1	98.21	−3.95 *	1	57.58	7.41	0.0417	
X^2_2	275.63	−5.68 *	1	119.19	15.33	0.0112	
X^2_3	101.96	−6.55 *	1	158.25	20.36	0.0063	
Residual	57.58		5	7.77			
Lack of fit	119.19		3	12.57	21.55	0.0447	significant
Error	158.25		2	0.5833			
Total	38.87		14				
Accuracy Adequacy	6.29						
CV%	3.59						
R2	0.90						
R2Ajust	0.73						
Average	77.79						

* Significant ($p < 0.05$).

The antioxidant activity (DPPH, ABTS, and FRAP) shows that the model is significant, and the polynomial equation fit using the coefficient of determination (R2) 0.90, 0.93, and 0.90, respectively. The regression coefficients for the dependent variables were obtained by multiple linear regressions, as shown in Table 3.

- The linear effect of extraction time (X_1) was significant for TFC and DPPH;
- The solvent/solid ratio (X_2) was significant for TPC;
- The concentration (X_3) was significant for the TPC;
- The quadratic effect of solvent concentration (X_3) and ratio (X_2) was significant for all responses except DPPH;
- The X_1 X_2 interaction effect also significantly impacted TFC, DPPH, and ABTS;
- The X_1 X_3 interaction was significant for ABTS.

The ANOVA result for each variable response indicates that at least one of the model parameters can explain the experimental variation of the response variables (Table 3).

The corresponding variables would be more significant if the F-value becomes larger and the *p*-value becomes smaller [29]. The *p*-value < 0.05 showed that the model terms were significant. In terms of coefficients of variation (CV), the models recorded a CV for CPT, CFT, DPPH, ABTS, and FRAP of 4.24%, 3.69%, 2.42%, 1.9%, and 3.59%, respectively.

Generally, the acceptable coefficient of variation (CV) value should be less than 20%. The diagnostic diagram as the predicted versus actual values (Figure 1) evaluates the relationship and model satisfaction between the experimental and predicted values obtained from the developed models. From Figure 1, it is observed that the data points are located near the straight line, which means a high correlation between experimental and predicted data obtained for TFC and a medium correlation between experimental and predicted data of TPC and antioxidant activity (DPPH, ABTS, and FRAP) from the models.

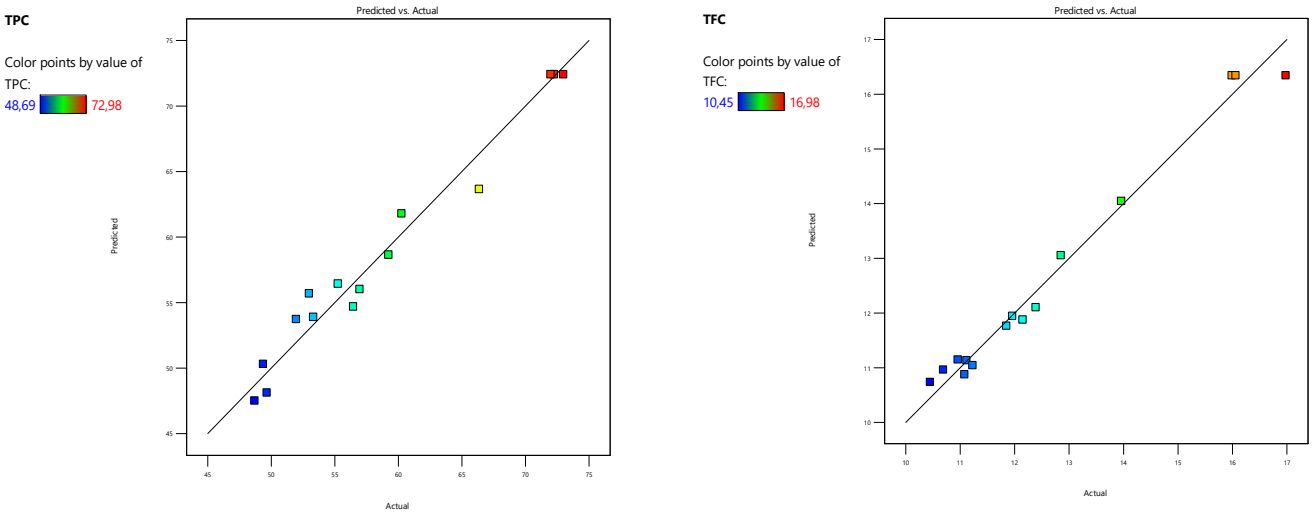

Figure 1. *Cont.*

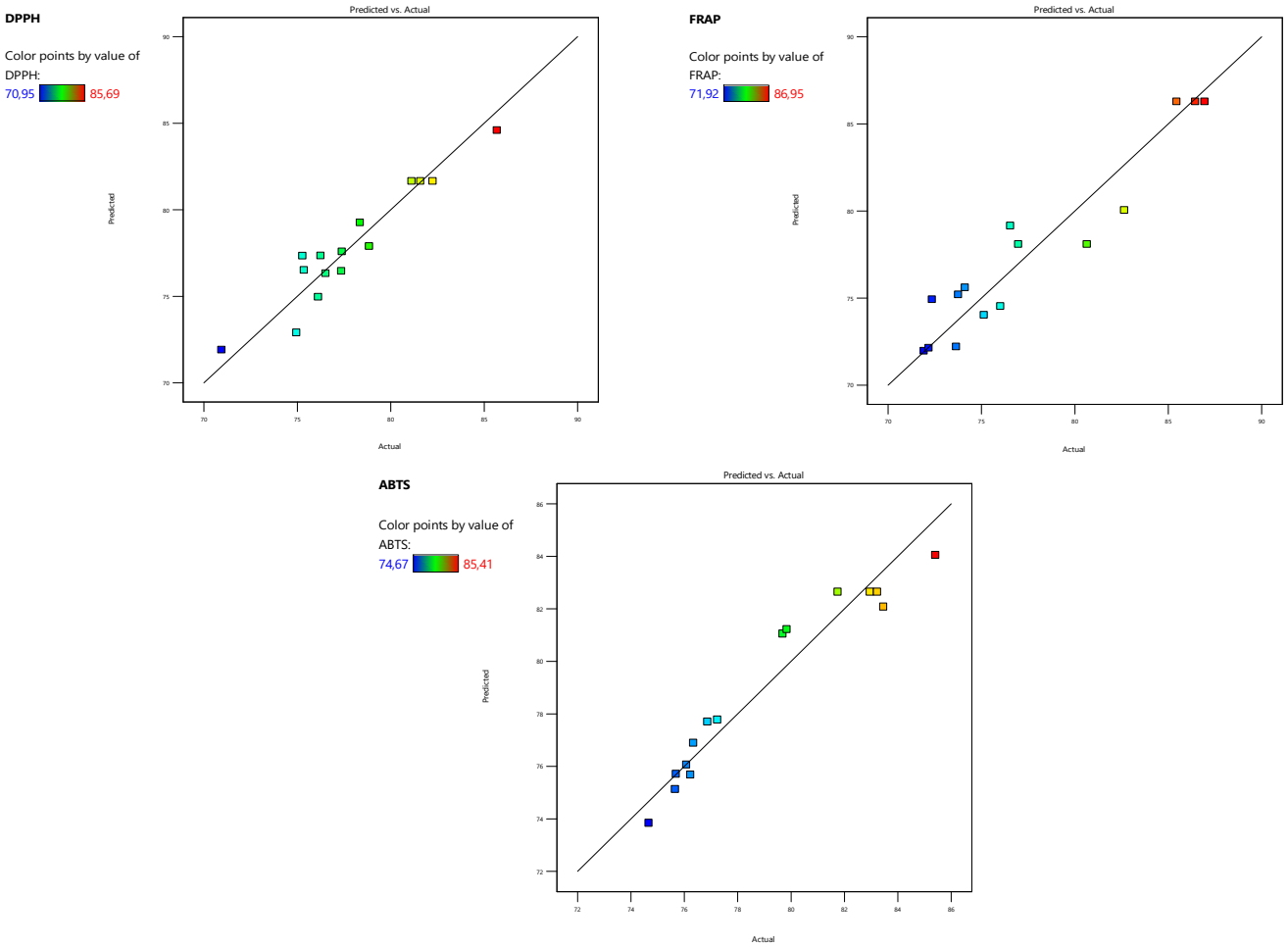

Figure 1. Diagnosis between experimental and predicted values for TPC, TFC, DPPH, ABTS, and FRAP.

3.3. Development of Second Order Polynomial Models

A statistical analysis was conducted using a second-order polynomial equation with interaction terms to model the connection between three process variables and the efficiency of ultrasonic extraction. This model can be utilized to anticipate the efficiency of ultrasonic extraction for varying combinations of process variables.

Five models were developed from this study to have the ultrasound extraction efficiency of TPC, TFC, DPPH, ABTS, and FRAP from olive leaves. The second-order equations of the responses in terms of coded factors are given below:

$$Y_{TPC} = 72.41 - 3.18X_2 - 3.66X_3 - 8.23X^2{}_{11} - 6.21X^2{}_2 - 11.62X^2{}_{33}$$

$$Y_{TFC} = 16.34 + 0.77X_1 - 0.68X_1X_2 - 1.84X^2{}_{11} - 2.28X^2{}_{22} - 2.81X^2{}_{33}$$

$$Y_{DPPH} = 81.65 + 2.2X_1 - 2.61X_1X_2 - 3.8X^2{}_3$$

$$Y_{ABTS} = 82.65 - 3.13X_1X_2 - 2.60X_1X_3 - 3.23X^2{}_2 - 3.59X^2{}_{33}$$

$$Y_{FRAP} = 86.28 - 3.95X^2{}_1 - 5.68X^2{}_2 - 6.55X_3{}^2.$$

3.4. Effect of Process Variables

This study analyzed the impact of process variables on the extraction of bioactive compounds (TPC, TFC, and free radical antioxidants) from olive leaves using a two-level Box–Behnken design with three factors: extraction time, solid/solvent ratio, and ethanol concentration. The results were presented using a three-dimensional response surface,

demonstrating the relationship between the independent and dependent variables. By holding two factors constant and varying the third, the response surface curves display the main effects and interactions of the independent variables on/with the dependent variables [1]. These graphs provide insight into how the different variables affect the extraction process.

3.4.1. Effect of Extraction Time

Based on the results obtained, a longer contact time between the sample and solvent leads to a higher transfer rate of bioactive compounds, ultimately resulting in better extraction efficiency. Figure 2 illustrates that the extraction time of 30–45 min was particularly impactful for flavonoids and DPPH. The linear effect of extraction time was significant ($p < 0.05$) for these two variables but not for the other independent variables. This effect is likely due to the extended time the plant matrix is exposed to the solvent, improving the solubility of the leaves' constituents. Essentially, the duration of the solvent exposure facilitates the migration of chemical compounds into the solution [30].

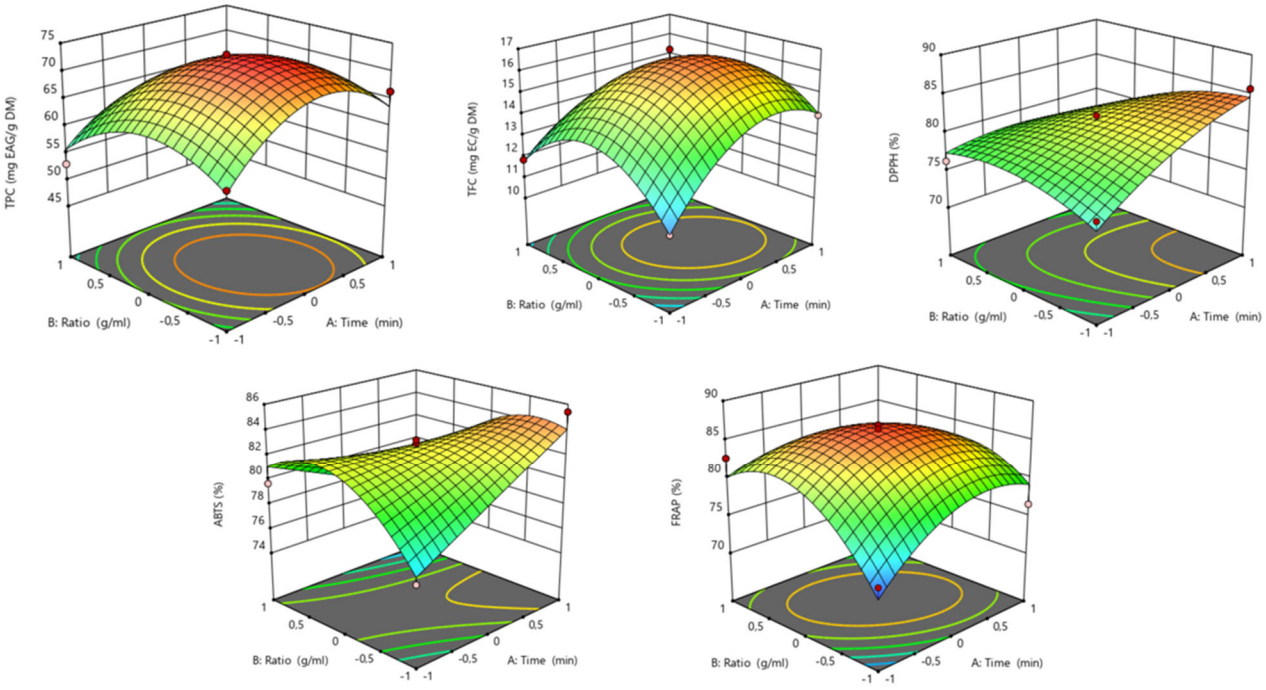

Figure 2. Response surface plot showing the variation of responses as a function of extraction time.

Studies have shown that the longer the extraction time, the higher the content of polyphenols and flavonoids. However, it is essential to note that excessively long extraction times can lead to the degradation of the bioactive compounds, as indicated in [31]. On the other hand, some studies found that extraction time was not a significant factor in the ultrasound-assisted extraction of phenolic compounds [32,33]. In other studies, such as those on Genipap berry pulp, blueberries, and carob pulp, the extraction time for bioactive compounds using ultrasonic-assisted extraction (UAE) was around 49, 50, and 57 min, respectively [34–36].

3.4.2. Effect of Solid–Liquid Ratio

The amount of solvent used in organic bioactive compounds and antioxidant extraction is a crucial factor. When evaluating its impact on the extraction of phenolic compounds, the results, presented in Figure 3, indicate that increasing the solid-liquid ratio up to 12.5 mL/g resulted in an increase in polyphenol and flavonoid content and antioxidant activity. This indicates that the volume of solvent used plays a significant role in

achieving good infusion and easy release of bioactive compounds into the surrounding environment [37]. However, according to Zakaria Fazila (2021) [38], a high solvent-to-solid ratio (between 10–30 mL/g) can lead to a decrease in phenolic compound content. The most effective ratio for maximum phenolic compound extraction was found to be 10 mL/g.

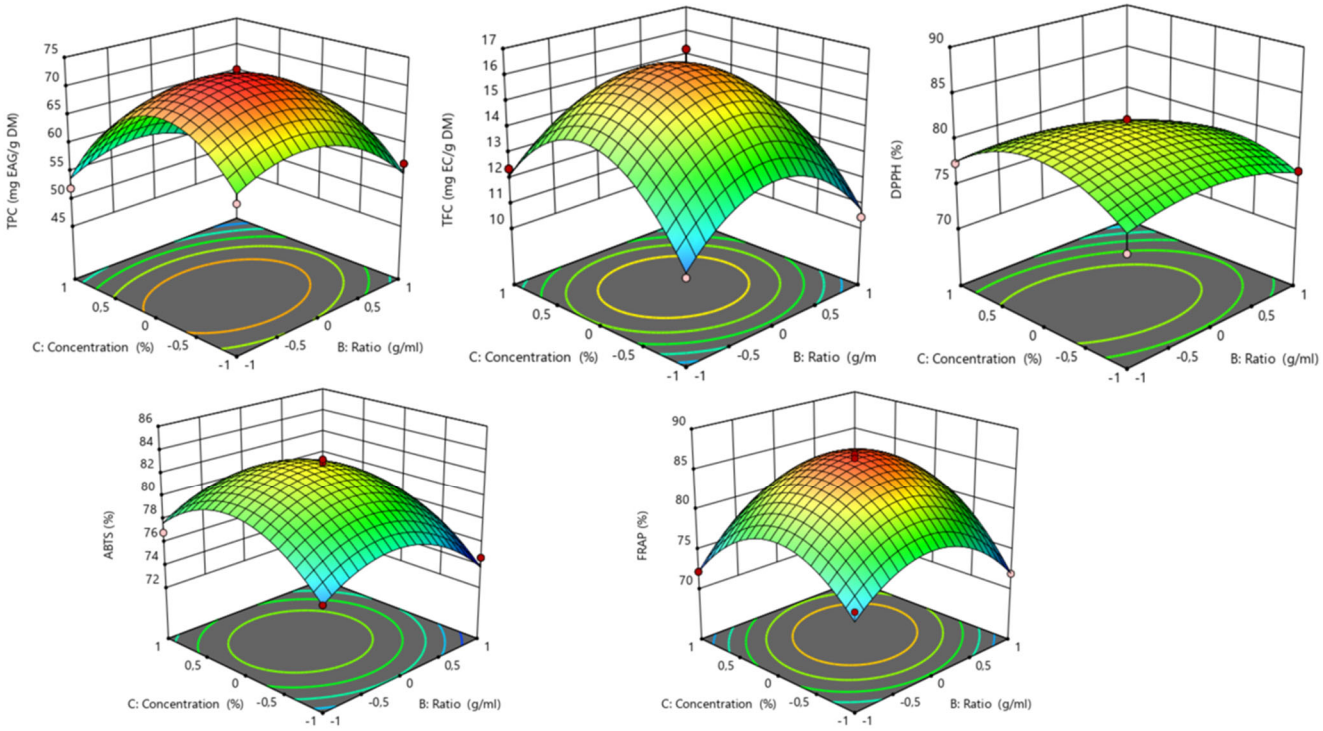

Figure 3. Response surface plot showing the variation of responses as a function of solid/solvent ratio.

This research found that once the solid–liquid ratio surpasses 12.5 mL/g, the solution becomes oversaturated with solute. This can lead to a reduction in the rate of mass transfer and a hindrance in the penetration of organic bioactive compounds into the solution, ultimately resulting in a decrease in the yield of the extraction process.

3.4.3. Effect of Solvent Concentration

The solubility of organic bioactive compounds can be enhanced by varying the concentration of the solvent [39]. Olive leaves were extracted using different ethanol concentrations at no more than 50 °C for 60 min under ultrasonic conditions. The results, shown in Figure 4, indicate that the samples containing 60% ethanol had the highest TPC, TFC, DPPH, ABTS, and FRAP values. This is because the solubility of polyphenol compounds increases with increasing ethanol concentrations. This may explain why the presence of water in ethanol improves the swelling of the plant material, while ethanol disrupts the binding between the solute and the plant. Other studies [40,41] have found that the recovery of organic bioactive compounds increased and peaked at an ethanol concentration of around 70% before slightly decreasing. Studies have shown that increasing the ethanol concentration can enhance the yield of phenolic compounds up to an average concentration of 40% ethanol [42]; however, Caldas et al. [43] observed that the highest phenolic compound content was achieved at an average concentration of 60% ethanol, possibly due to the different polarities of organic bioactive compounds in grape skin. For *Triticum aestivum*, the maximum yield of phenolic compounds was obtained using an ethanol concentration of 56% [44]. Similarly, studies on various plants such as *Jaboticaba* bark, blueberry, and apple pulp have found that the concentration of ethanol required to extsract the maximum amount of phenolic compounds ranges from 40–80% (*V*/*V*) [35–46].

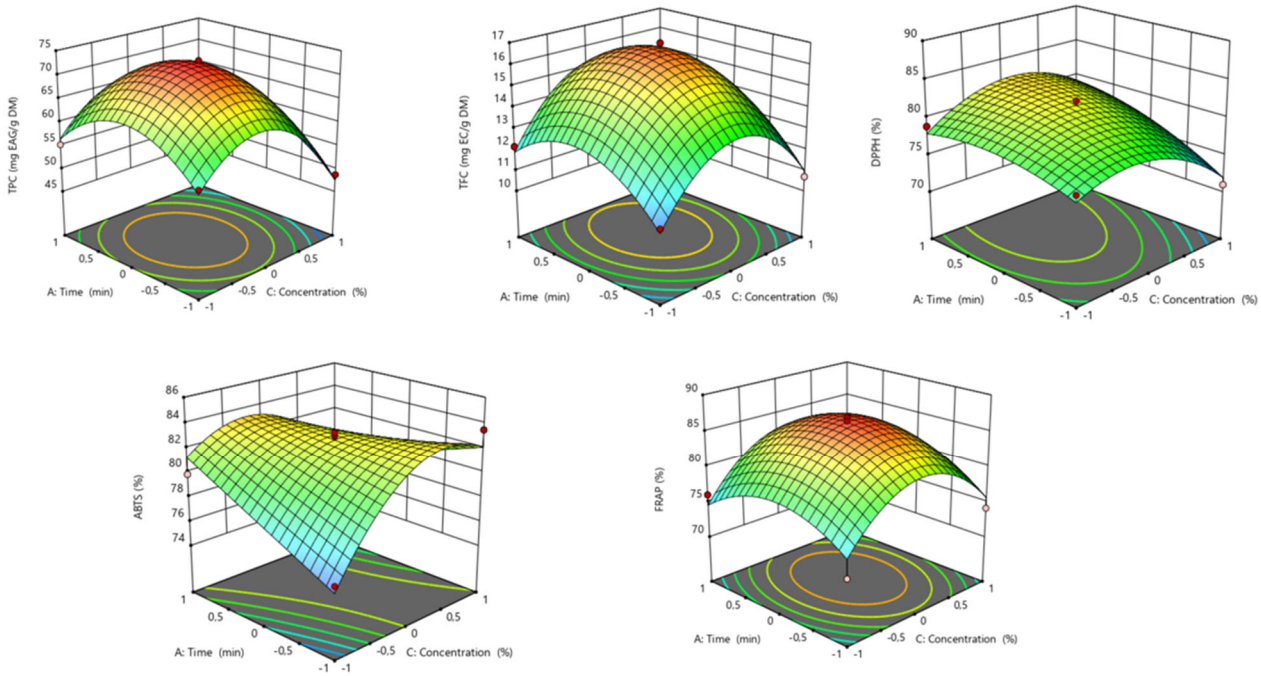

Figure 4. Response surface plot showing the variation of responses with ethanol concentration.

3.5. Determination and Validation of Optimal Conditions

This study aimed to determine the best experimental conditions for extracting phenolic compounds that benefit human health, such as antioxidant activity. This involves determining the ideal sonication time, solid/liquid ratio, and ethanol concentration for maximum extraction yield. The software Design Expert Version 13 was used to find the composite optimum for achieving the highest bioactive compounds extraction yield and the most significant antioxidant activity.

The optimization process involved assigning the response's desirability values between 0 and 1. Figure 5 displays a desirability value of 0.8735 and the predicted optimal values. Experiments were conducted under optimal triplicate conditions to compare the experimental and predicted values of the responses. The mean values are presented in Table 4. The optimal conditions for the experiments were 53.52 min, a solid/liquid ratio of 9.83 mL/g, and an ethanol concentration of 59.7% (*V*/*V*). The experimental values for TPC and TFC bioactive compounds were 74.45 ± 1.22 mg EAG/g DM and 17.08 ± 1.85 mg EC/g DM, respectively, while the antioxidant activity DPPH, ABTS, and FRAP were 83.45 ± 0.89%, 82.85 ± 1.52%, and 87.01 ± 2.35%, respectively. These experimental results were found to be similar to the predicted model for TPC (72.40 mg EAG/g DM), TFC (16.42 mg EC/g DM), DPPH (82.58%), ABTS (83.06%), and FRAP (85.79%). Therefore, there is a synergy between the results found and the Box–Behnken design.

Table 4. Optimal conditions and predicted and actual response values of olive leaf extract.

The Optimal Conditions			**The Answers**									
X1	X2	X3	TPC		TFC		DPPH		ABTS		FRAP	
(min)	(mL/g)	(%)	(mg GAE/g DM)		(mg EC/g DM)		(%)		(%)		(%)	
53.5	9.83	59.7	Reel	Predict	Reel	Predict	Reel	Predict	Reel	Predict	Reel	Predict
Objective			Maximum		Maximum		Maximum		Maximum		Maximum	
Optimized values			74.45 ± 1.22	72.40	17.08 ± 1.85	16.42	83.45 ± 0.89	82.58	82.85 ± 1.52	83.06	87.01 ± 2.35	85.79

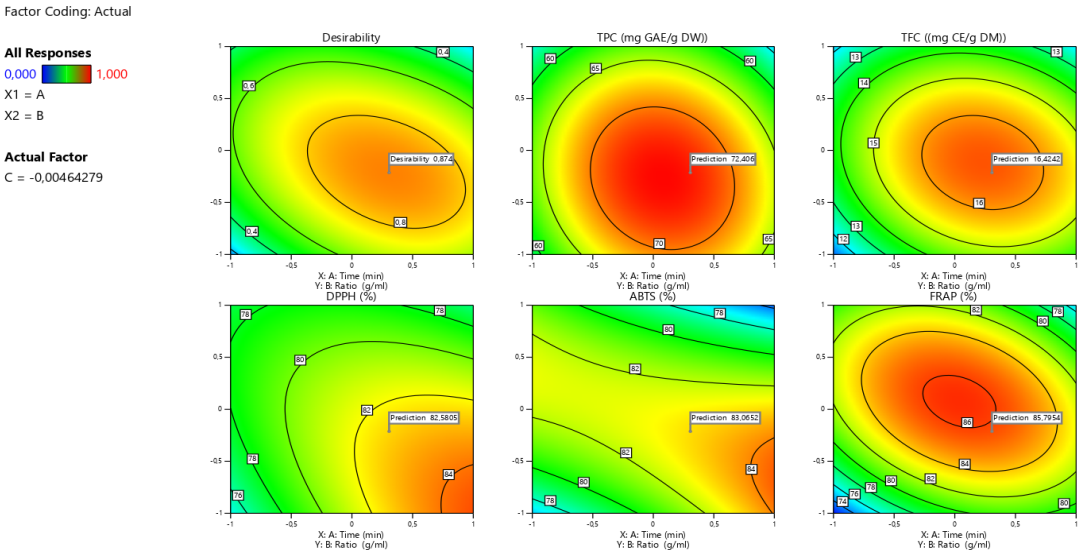

Figure 5. The predicted optimal values and desirability of olive leaf optimization.

3.6. HPLC-MS Analysis

Figure 6 displays the phenolic compounds identified in olive leaf extracts through ultrasound in the first and second extractions. The peaks on the graph correspond to these compounds. The results indicate that the first extraction in optimal conditions (Figure 6a) resulted in a high release of phenolic compounds. In contrast, the second extraction (Figure 6b) had fewer bioactive compounds. This suggests that the ultrasonic extraction method is effective. Eight phenolic compounds were identified: hydroxytyrosol, catechin, caffeic acid, vanillin, naringin, oleuropein, quercetin, and kaempferol. As shown in Table 5, oleuropein was the most abundant compound, with a concentration of 114.10 mg/g DM, followed by hydroxytyrosol, caffeic acid, and kaempferol. The optimized ultrasound-assisted extraction method was likely responsible for the high amount of phenolic compounds in the first extraction.

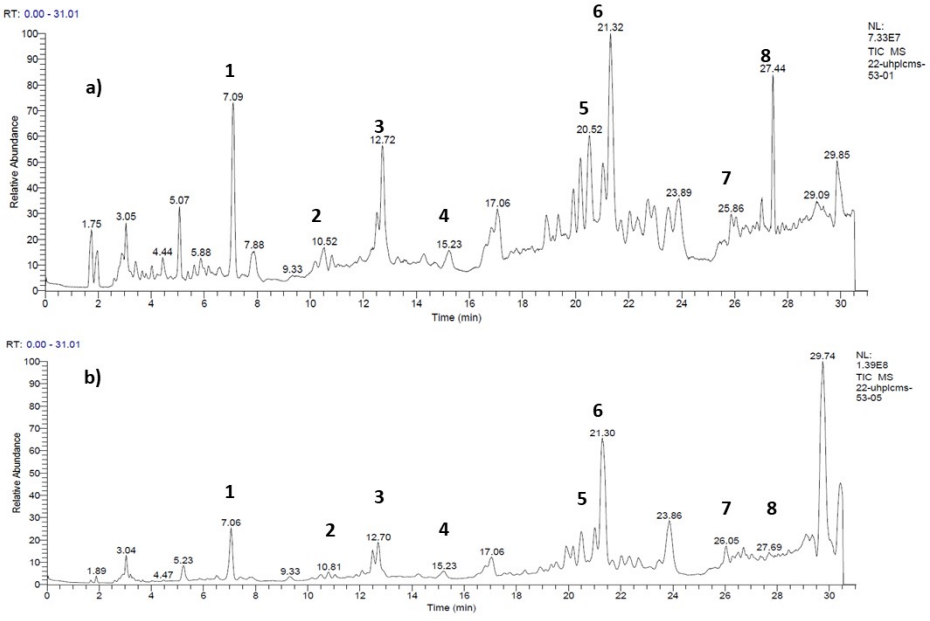

Figure 6. HPLC chromatograms of polyphenols of olive leaf extracts from the First UEA under the optimal conditions (**a**) and the Second UEA under the same optimal conditions (**b**).

Table 5. Concentration of phenolic compounds identified in olive leaf extracts under the optimal conditions of the first and second ultrasound extraction (mg/g DM).

N° PIC	T_R (min)	Phenolic Compounds	Chemical Formulas	Concentration of the First Extraction (mg/g)	Concentration of Second Extraction (mg/g)
1	7.09	Hydroxytyrosol	$C_8H_{10}O_3$	45.40 ± 1.2	10.02 ± 2.12
2	10.52	Catechin	$C_{15}H_{14}O_6$	12.90 ± 1.35	2.21 ± 1.36
3	12.72	Caffeic acid	$C_9H_8O_4$	79.50 ± 1.25	15.32 ± 3.36
4	15.23	Vanillin	$C_8H_8O_3$	12.70 ± 1.36	0.98 ± 2.36
5	20.52	Naringin	$C_9H_8O_3$	45.40 ± 2.45	32.23 ± 1.25
6	21.30	Oleuropein	$C_{25}H_{32}O_{13}$	114.10 ± 3.42	40.23 ± 2.78
7	26.48	Quercetin	$C_{15}H_{10}O_7$	23.00 ± 2.38	12.21 ± 1.45
8	27.44	Kaempferol	$C_{15}H_{10}O_6$	29.00 ± 1.96	9.36 ± 1.69

3.7. Scanning Electron Microscopy

After being dried and ground, the leaf powder was examined under a scanning electron microscope. There was a visible difference between the untreated sample and the one treated with EWM and UAE (as shown in Figure 7). The untreated sample was densely compacted, while the treated sample showed structural changes. UAE caused more damage and formation of cracks than maceration, possibly due to the cavitation effects of the ultrasound [47]. During extraction, high ultrasound intensities can enhance solvent penetration and destroy cell membranes [48], releasing more bioactive compounds from the sample matrix.

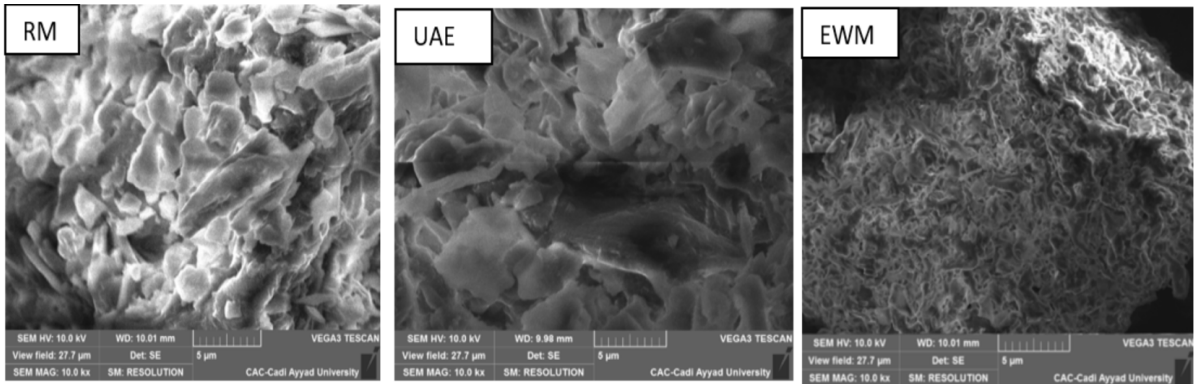

Figure 7. SEM images of olive leaf powder before extraction (RM), leaf powder after ultrasound-assisted extraction (UAE) under optimal conditions, and leaf powder treated by ethanol water maceration (EWM).

4. Conclusions

The Box–Behnken design (BBD) method, along with the surface response design approach (RSM), was used to study the impact of ultrasonic-assisted extraction (UAE) process parameters on the content of polyphenolic compounds in *Moroccan picholine* olive leaves. The results showed that this eco-friendly technique is beneficial in optimizing the conditions for extracting phenolic compounds (TPC, TFC). To achieve a high yield, it is recommended to use an extraction time of 53.5 min, a solvent/solid ratio of 9.95 mL/g, and an ethanol concentration of 59.7%. The content of TPC and TFC are 74.45 ± 1.22 mg EAG/g DM and 17.08 ± 1.85 mg EC/g DM, respectively, while the antioxidant activity of DPPH, ABTS, and FRAP are 83.45 ± 0.89%, 82.85 ± 1.52%, and 87.01 ± 2.35% respectively. The presence of certain phenolic bioactive compounds in high concentrations, specifically oleuropein and hydroxytyrosol, was confirmed through analysis by HPLC-MS.

To better understand the extraction and optimization phenomena, it would be beneficial to study additional parameters such as sonication temperature, frequency, and solvent nature.

Author Contributions: E.M.E.A.: doctoral student, practical work, and main leader of the operative manipulations; A.M.: doctoral assistant of operations and preparation of raw material; N.E. (FSSM/UCA), A.J. (FSSM/UCA) and M.M. (Emeritus FSSM/UCA): professors who initiated the follow-up and planning of the thesis work; M.O. and N.H.: assistants doctors of analysis; K.B., doctor, pharmacist, and CEO of the company, Iris Cosmétologie (IRCOS) Laboratoires, our industrial partner: availability of raw materials, logistics and production machines. All authors have read and agreed to the published version of the manuscript.

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