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CHEMICAL SCIENCES

Desirability Function Approach for the Optimization of Hydroalcoholic Solvent Extraction Conditions for Antioxidant Compounds from Olive Leaves

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Absract: The goal of this work was to obtain a rich olive leaf extract with high antioxidant activity due to its content of oleuropein (OLE), hydroxytyrosol (HT) and other phenolics, which have a synergistic effect on total antioxidant activity (TAA). The extraction parameters in the solvent extraction were investigated using response surface methodology (RSM) to produce the best results of HT and TAA in olive leaf extracts. A Box Wilson-CCD design was applied, the multi-objective optimization (MOO) was computed with Pareto solutions, and the desirability function (DF) was employed to define the best process variables. The optimized conditions (solvent concentration, temperature, time, solvent:solid ratio) were as follows: 63.30 %, 36 °C, 62 min, 11.80 mL/g for MeOH:H_2O extracts and 43.80 %, 52 °C, 58 min, 9.40 mL/g for EtOH:H_2O extracts. Under these conditions, the highest results of HT were reached to 0.809±0.110 mg/g dw and 0.175±0.004 mg/g dw for MeOH:H_2O and EtOH:H_2O extracts, respectively. Similarly, the best results for TAA were obtained at higher concentrations in MeOH:H_2O extracts (451±2.32 mM Trolox) than in EtOH:H_2O extracts (297±0.817 mM Trolox). Overall, the synergistic effect of OLE, HT, and flavonoids could make the olive leaf extract a potential cheap source for high value-added products.

Key words: Total antioxidant activity (TAA), Hydroxytyrosol (HT), Desirability function, optimization, method validation.

INTRODUCTION

Olea europaea L. is an evergreen tree that is commonly considered to have strong antioxidant activity through its fruits, oil, and leaves (Jemai et al. 2009). Olive leaf (OLL) is a significant byproduct of both olive tree agriculture and the olive-processing industry that accumulates in vast quantities, producing economic and environmental problems (Herrero et al. 2011, Abaza et al. 2015, Clodoveo et al. 2022). Researchers are interested in the leaves because they are rich source of natural bioactive compounds with the potential to promote health. As a result, OLL have a great deal of potential for use in the food, medicine, and cosmetic industries (Quirantes-Piné et al. 2013, Rahmanian et al. 2015, Clodoveo et al. 2022).

Secoiridoids (oleuropein-OLE), simple phenolic compounds (hydroxytyrosol-HT and tyrosol), flavonoids (apigenin-7-O-glucoside, luteolin-7-O-glucoside, quercetin-7-O-rutinoside), and phenolic acids (verbascoside and caffeic acid) are the most active phenolics in OLL (Rahmanian et al. 2015, Talhaoui et al. 2015). These bioactive compounds have anti-cancer, anti-obesity, antioxidant, antiviral, anti-inflammatory, anti-diabetes, anti-microbial, anti-atherogenic properties, as

well as cardioprotective, gastroprotective, and hepatoprotective properties (Lama-Muñoz et al. 2019, Taamalli et al. 2012, Hassen et al. 2015, Markovic et al. 2019).

OLE and HT are the most frequent phenolic compounds found in olive leaves. When the phenolic content of olive leaves was investigated, it was discovered that the major phenolic compound was OLE, followed by HT. OLE and HT content in olive leaf extract was determined to be 24.54% and 1.46% with antioxidant capabilities of 0.88±0.09 and 1.57 ± 0.12 mM (ABTS), respectively (Benavente-Garcia et al. 2000). HT attracts particular attention with its high antioxidant activity. It originates from the degradation of OLE and has higher antioxidant activity than OLE (Paradiso et al. 2016, Yao et al. 2019). The health advantages of HT have also been thoroughly investigated. It has been demonstrated to be an effective peroxyl radical scavenger. In numerous human systems, it crosses cell membranes and counteracts the reactive oxygen species' harmful effects (D'Angelo et al. 2005). Moreover, it is known to have inhibiting proliferation, antimicrobial properties, anti-atherogenic capacity, cardioprotective, antidiabetic, lipid regulating, anti-obesity effects, retino-protective activity, and skin-related effects (D'Angelo et al. 2005, Granados-Principal et al. 2010, Haloui et al. 2011, Wani et al. 2018, Markovic et al. 2019, Gallardo-Fernández et al. 2022). In recent years, the antiviral, anti-inflammatory, and antithrombotic characteristics of HT have been investigated against COVID-19 disease (Takeda et al. 2021, Crudele et al. 2022, Abdelgawad et al. 2022), and it has been suggested that because of its various qualities, it might be a useful natural resource for the treatment of COVID-19 infection (Abdelgawad et al. 2022).

While the amount of HT in olive leaves is lower than in other olive processing wastes, it adds significantly to the total antioxidant efficacy of the leaves (Taamalli et al. 2012, Dias et al. 2019). It was discovered that HT in leaves has a higher antioxidant capacity than OLE and other phenolics (Benavente-Garcia et al. 2000, Erbay & Icier 2010). Additionally, Benavente-Garcia et al. (2000) found that olive leaf extract had a higher potential for antioxidant protection than either pure hydroxytyrosol or the vitamins C and E. In general, it has previously been shown that the flavonoids included in olive leaves may considerably add to the extracts' antioxidant capacity. As a result, the importance and effect of the flavonoids present should not be underestimated (Goulas et al. 2010, Herrero et al. 2011). This is mostly due to the synergy of all phenolics found in olive leaves, including flavonoids, oleuropeosides, and substituted phenols (Benavente-Garcia et al. 2000).

HT is a remarkable bioactive compound that has numerous health benefits (Wani et al. 2018). Although the antioxidant potential of HT increases its value, extracting pure HT from natural sources is difficult. It is an easily oxidized chemical that is difficult and expensive to synthesize (Erbay & Icier 2010). As a result, it has been attempted to extract it from natural sources, such as olive leaves. HT can also be produced from OLE, but it takes additional procedures (Bouaziz & Sayadi 2005, Erbay & Icier 2010, Rahmanian et al. 2015). Because the properties of a bioactive individual component may differ in the presence of other compounds present in the extracts due to synergy between flavonoids, oleuropeosids, and phenols, olive leaf extract may be more beneficial than individual components (Benavente-Garcia et al. 2000, Şahin et al. 2015). The use of phenolic-rich olive leaf extracts as an alternative functional source to expensive purified biomolecules like oleuropein has the benefit of being a low-cost technique of processing since it avoids the requirement for the purification stage

while being considerably effective. It has been stated that the most potent olive leaf products available on pharmacy shelves are made from leaf extracts rich in organic biophenols, which interact naturally to optimize the health advantages of the plant (Şahin & Bilgin 2018, Medina et al. 2019, Clodoveo et al. 2022). As a result, we tried to emphasize the significance of the extraction conditions of olive leaf extract, including phenolics with synergistic activities such as HT, OLE, and other active polyphenols in olive leaf. By effectively extracting phenolic substances from olive leaf, a low-cost raw material, high-value products can be obtained (Rahmanian et al. 2015). However, various factors, including olive cultivar, geographical origin, extraction circumstances, and solvent type, have a important effect on the phenolic content of olive leaves (Şahin & Bilgin 2018, Dias et al. 2019).

Extraction is a crucial step in the detection and quantification of these valuable phenolics in olive leaves. Although novel methods such as microwave-assisted extraction and superheated liquid extraction have been investigated (Rahmanian et al. 2015), in this study, an effective, easier and more economical extraction method that does not require special equipment was proposed in order to find the ideal conditions for the extraction of antioxidant phenolics from olive leaves. The solvent extraction (SE) technique, a traditional method, has been used extensively for the recovery of biotherapeutic substances (Rahmanian et al. 2015). Aqueous solvents of MeOH and EtOH have been employed for the SE of polyphenols from OLLs (Abaza et al. 2015, Tsakona et al. 2012). Due to their diverse solubilities and polarity, OLE, HT, flavonoids, and phenolic acids in olive leaves have been reported to be extracted using various solvents (Mohamed & Khan 2013, Herrero et al. 2011, Lee & Lee 2010, Quirantes-Piné et al. 2013). In this investigation, the extracts were made using aqueous MeOH and EtOH since the choice of solvent affects the concentration of phenolic components and, consequently, the antioxidant activity of olive leaf extracts. Studies on the effective recovery of phenols by identifying the ideal extraction conditions should be increased in considering the effective factors.

In order to get the best results for HT and TAA of MeOH aqueous and EtOH aqueous olive leaf extracts, the parameters of solvent concentration, solvent solid ratio, temperature, and time were examined. RSM, a multivariate statistical technique, was used to gather information on the relationship between the variables (Habibi et al. 2018, Zuorro et al. 2019, Zuorro 2020). The real problem in a process often has more than one quality feature. The desirability function (DF) approach is the most commonly employed approach for simultaneous quality feature improvement. The major challenge of in-process optimization is determining the optimal operating conditions that best represent a process's multi-objective features. This is a MOO issue in RSM and an important field of research in experimental design (DOE) (Chen et al. 2012, Karande et al. 2013, Algan Cavuldak et al. 2019). Harrington (1965) developed the DF approach, which has been widely employed in industry to deal with MOO problems.

The goals of this work are as follows: a) To create phenolic-rich antioxidant extracts from olive leaves utilizing hydroalcoholic EtOH and MeOH as solvents, and to analyze the phenolic content of the OLL extracts using advanced characterisation methods. b) The goal of a MOO issue handled by the Pareto-optimal front and Harrington's DF was to maximize HT and TAA at the same time. c) Then, these compounds -oleuropein as secoiridoids, tyrosol, and hydroxytyrosol as substituted phenols, luteolin, apigenin-7-glucoside, and luteolin-7-glucoside

as flavones- were determined and quantified by HPLC. d) A simple and reliable method for separating and measuring six bioactive components was designed and validated. The ICH approach was used to build and validate in-house validation procedures.

MATERIALS AND METHODS

Material

Olea europaea L. was collected from an olive grove in Ayvalık-Turkey region at locations 39°16′40.55K and 26°42′47.77D. The sampling location was about 270 meters above sea level. Sampling was done in October 2019. Initial steps such as transportation, drying, reduction in size, and storage before analysis were done according to Vural et al. (2020).

Standards and chemicals

Analytical standards (Gallic acid (GA), 99%, Sigma); (hydroxytyrosol (HT), 98%, Sigma); (tyrosol (Ty), 99.5%, Fluka); (Luteolin-7-glucoside (L7G), 98%, Fluka); (oleuropein (OLE), 98.6%, Extrasynthese); (apigenin-7-glucoside (A7G), 97%, Fluka); (luteolin (Lut), 97%, Fluka) were HPLC grade. Chemicals (MeOH, 99.9%; EtOH, 99.5%; sodium carbonate (Na_2CO_3), 99.5%; Trolox, 97%; potassium persulfate, 99%; and acetic acid, 90.8-100.5%) were purchased from Sigma (USA). Deionized water was purified (18.2MΩ) with Destup (Ankara, Turkey). The TOC content of the ultrapure water was 3.27 µg/L.

Solvent extraction of bioactive compounds

Extraction was performed according to Vural et al. (2020) with different concentrations of MeOH/H_2O and EtOH/H_2O solvent systems using 1 g OLL sample (Table I). Hydroalcoholic solvent extraction treatment was performed with a glass reactor in a water bath, with a cooling system at the base and around the glass reactor jacket. Cooling the reactor jacket using a coolant (ethylene glycol-water) allowed for temperature control of the reaction.

A Box Wilson-CCD/small factorial DOE

A Box Wilson-CCD/small factorial DOE was made to determine the optimum extraction conditions and 22 experimental points were determined. To investigate the relationships between the constituents and observed outcomes and to improve the operating circumstances, a small/central composite design (CCD) with four elements, sixteen randomized trials, and six duplicates of the central point was utilized (Table I). In addition, the run sequence was randomized to reduce the influence of uncontrollable factors. Table IV(a,b) shows the coded and real components of the experimental design.

TAA (mM Trolox) and the amount of HT (mg/g dw) were determined as the dependent

Table I. Experimental values and coded levels of independent variables used in CCD/small factorial design in MeOH/H_2O and EtOH/H_2O solvent extraction systems.

Coded levels						Coded levels					
Independent variables	**-α**	**-1**	**0**	**+1**	**+α**	**Independent variables**	**- α**	**-1**	**0**	**+1**	**+α**
X_1: MeOH conc (% v/v)	12	25	45	65	79	X'_1: EtOH conc (% v/v)	12	25	45	65	79
X_2: Temperature (°C)	20	30	45	60	70	X'_2: Temperature (°C)	20	30	45	60	70
X_3: Time (min)	8	25	50	75	92	X'_3: Time (min)	8	25	50	75	92
X_4:Solvent:solid ratio (mL/g)	2	5	9.5	14	17	X'_4: Solvent:solid ratio (mL/g)	2	5	9.5	14	17

(α=±1.68; Non-center points:16; Center points:6).

variables (Table I). The ranges for the independent variables (the different ratios of hydroalcoholic solvent mixtures, temperature, time and solvent:solid ratio) were found as 12-79% (v/v), 20-70°C, 8-92 min, 2-17 (mL/g leaf), taking into account the values found in the literature and early research (Vural et al. 2020). Stat-Ease Design-Expert 10.0 (Minneapolis, USA) was utilized for chemometric techniques in this work.

Desirability function (DF) approach

Finding an input variable set for which all output variables (responses) fall to the desired values or are as near to them as feasible is the goal of the DF optimization strategy. The DF is used to convert each of the *m* predicted responses $\hat{y}_1, \hat{y}_2, \ldots, \hat{y}_m$ from the *m* potentially various models to an individual DF *di*, where $0 \le di \le 1$, for a given set of factor values (Harrington 1965).

The DFs are defined by the answers to be optimized, which are divided into three categories: (a) the nominal-the-best (NTB) type response, (b) the larger-the-better (LTB) type response, and (c) the smaller-the-better (STB) type response (Karande et al. 2013, Harrington 1965, Wu 2004).

The DF from Eq. (1) is written as follows:
for the LTB-type response,

$$d_i = \begin{cases} 0 & for\ \hat{y}_i \le y_i^{min}, \\ \left(\dfrac{\hat{y}_i - y_i^{min}}{y_i^{max} - y_i^{min}}\right)^r & for\ y_i^{min} < \hat{y}_i < y_i^{max}, \\ 1 & for\ \hat{y}_i \ge y_i^{max}, \end{cases} \quad (1)$$

for the STB-type response,

$$d_i = \begin{cases} 1 & for\ \hat{y}_i \le y_i^{min}, \\ \left(\dfrac{y_i^{max} - \hat{y}_i}{y_i^{max} - y_i^{min}}\right)^r & for\ y_i^{min} < \hat{y}_i < y_i^{max}, \\ 0 & for\ \hat{y}_i \ge y_i^{max}, \end{cases} \quad (2)$$

for the NTB-type response,

$$d_i = \begin{cases} \left(\dfrac{\hat{y}_i - y_i^{min}}{y_i^{max} - y_i^{min}}\right)^{r1} & for\ y_i^{min} \le \hat{y}_i \le T_i, \\ \left(\dfrac{y_i^{max} - \hat{y}_i}{y_i^{max} - T_i}\right)^{r2} & for\ \ T_i < \hat{y}_i < y_i^{max}, \\ 0 & for\ \hat{y}_i < y_i^{min}\ or\ \hat{y}_i > y_i^{max}, \end{cases} \quad (3)$$

where y_i^{min} and y_i^{max} are the lower and the upper bound on the i^{th} response, respectively, $r1 > 0$ and $r2 > 0$ are the two shape parameters, and T_i is the target value of the i^{th} response which is a NTB-type one.

Different values of *r*, *r*1, and *r*2 will result in different desirability shapes, and small values of *r*1 and *r*2 should be chosen. Both shape parameters in such DFs can be set to different values, and the range type conversion is frequently selected since it is easy. The DF can be given as follows:

$$d_i = \begin{cases} 1 & for\ y_i^{min} < \hat{y}_i < y_i^{max} \\ 0 & for\ \hat{y}_i \le y_i^{min}\ and\ \hat{y}_i \ge y_i^{max} \end{cases} \quad (4)$$

All individual DFs are then integrated into a general DF after choosing a workable individual DF for each predicted response (Harrington 1965) using the geometric mean as in Eq (5):

$$DF = (d_1 d_2 \cdots d_m)^{1/m} \quad (5)$$

Total antioxidant activity (TAA) assay

TAA was determined for the extracts using the 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) technique reported by Re et al. (1999), with minor modifications. The method was preferred due to being used for aqueous and lipophilic systems. 2.45 mM potassium persulfate was mixed with 7 mM ABTS solution to make the ABTS+ radical solution. After that, the mixture was maintained at room temperature in the dark for 12 to 24 hours. It was diluted with phosphate buffer (PBS) before use to attain an absorbance of 0.700±0.02 at 734 nm. 50 µL of each sample was mixed into 950 µL of ABTS+ solution. At 0 and 6 minutes of reaction time, the absorbance was measured. Each sample was examined three times. Standard Trolox solutions (0-700 M) were utilized to generate a calibration curve. The results are given in mM Trolox equivalent g^{-1} of dw.

Analysis of bioactive compounds

The Shimadzu HPLC apparatus and LC Solution software were employed. In the analysis, a PDA detector was used in the wavelength range 190-550 nm, and the quantitative measurements were made at 280 nm wavelength. HPLC analyses of HT, Ty, L7G, OLE, A7G and Lut in OLL extracts were performed. It was used by altering the gradient technique utilized by Vural et al. (2020). Two solvents (A: H_2O +%1 Acetic Acid, B: MeOH) were used in the gradient procedure. The column oven's temperature was programmed at 30°C. The flow rate was 1.0 mL/min, and the following elution procedure was used: 0-10 minutes from 85 to 60% A; 10-15 minutes from 60 to 30% A; and 15-30 minutes from 30 to 85% eluent A. The elute was analyzed for bioactive chemicals at 280 nm using an Inertsil ODS-4 (250x4mm; 5m) column and a 25 µL injection volume.

Analytical method validation

Stock solutions of the standards (HT, Ty, L7G, OLE, A7G and Lut) were freshly prepared (200 mg L^{-1}) by dissolving 6 authentic compounds in MeOH:H_2O (80:20) except for OLE. OLE stock solution was prepared within the range of 2000 mg L^{-1}. The concentration range of the standard solutions was chosen based on the expected analyte levels in the samples (Table II). The calibration curve was constructed by graphing the standard concentration (mg mL^{-1}) vs the peak area for each standard. Standard solutions were then filtered using a 0.22 µm syringe membrane filter, injected in triplicate in quantities of 25 µL, and evaluated under chromatographic conditions. For each identified analyte, the limits of detection (LOD) and quantification (LOQ) were computed.

Six standards were identified and quantified by comparing retention times and peak areas to those of the standard and by co-injection with the sample (spike test), respectively. The standard solutions were applied to the samples in three different quantities, and recovery was evaluated in triplicate. Accuracy was evaluated with the mean percentage recoveries method. For comparison, an unspiked sample was concurrently prepared and analyzed. Repeatability was assessed through the relative standard deviation (RSD) values. Precision was evaluated by the performance of intraday (repeatability) by three replicated injections of the same solution, same analyst within the same day

Table II. Assay validation parameters of the proposed HPLC method for determination of bioactive compounds.

Parameter	HT	Ty	L7G	OLE	A7G	Lut
Accuracy[a] (mean recovery % ±SD)	97.32±0.51	98.85±1.02	97.98±0.88	95.85±1.45	98.33±0.79	98.43±0.92
Precision						
Repeatability[b]	±1.01	±0.70	±0.55	±1.70	±0.70	±0.35
Intermediate precision[c]	±2.00	±2.25	±1.99	±4.25	±2.25	±1.80
Linearity						
Slope	191262	258476	3.10^7	65974	295601	634194
Intercept	+123121	+114873	-87408	$+1.10^6$	196496	300382
Correlation cofficient (r)	0.9979	0.9968	0.9993	0.9952	0.9983	0.9921
Range (mg L^{-1})	0.5-100	0.01-20	0.01-20	2.5-1750	0.25-50	0.010-20
LOD[d] (mg L^{-1})	0.012	0.005	0.008	$0.4.10^{-3}$	0.011	$0.6.10^{-3}$
LOQ[d] (mg L^{-1})	0.042	0.015	0.026	$1.7.10^{-3}$	0.037	$2.0.10^{-3}$

[a]The accuracy average of (*n* = 3). Analytical results are the average of triplicates (mean ± sd).
[b]The intraday (*n* = 3), an average of three concentrations (0.5, 5 and 10 mg L^{-1}) Ty, L7G, Lut and (5, 10 and 25 mg L^{-1}) HT, A7G and (50, 500 and 1000 mg L^{-1}) OLE, for compounds repeated three times within the day.
[c]The inter-day (*n* = 3), an average of three concentrations (0.5, 5 and 10 mg L^{-1}) Ty, L7G, Lut and (5, 10 and 25 mg L^{-1}) HT, A7G and (50, 500 and 1000 mg L^{-1}) OLE, for compounds repeated three times in 3 days.
[d]Determined via calculations, LOD = 3.3 (SD of the response/slope), LOQ = 10 (SD of the response/slope).

and inter-day (reproducibility) was determined by analyzing the same solution on three different days (three injections a day). Satisfactory results were obtained for the used method. The validation was performed according to International Conference on Harmonization (ICH) guidelines (ICH 2005), and the calculated validation parameters were shown in Table II.

RESULTS AND DISCUSSION

Fitting the model

In this study, the DOE was employed in which the independent variables were chosen as solvent concentration (SolC), temperature (Temp), time (T), solvent:solid ratio (SSR) (X_1, X_2, X_3, X_4, X'_1, X'_2, X'_3, X'_4) for two different hydroalcoholic solvent systems and the response variables were HT and TAA (Y_1, Y_2, Y'_1, Y'_2). MeOH and EtOH mixtures with water were used to optimize the extraction terms to have the maximum yield of HT and TAA of bioactives in the OLL. After the determination of optimum extraction points, the prominent bioactives in OLL -Ty, L7G, OLE, A7G and Lut- were measured.

Table III(a) demonstrates the ANOVA results for HT and TAA values acquired by MeOH:H_2O extraction. Both models were highly significant (*p <0.0001)* and had high F values of 107.22 and 16.26 for HT and TAA, respectively. The R^2 of the predicted models were 0.9877 and 0.7928, respectively. The models for (MeOH:H_2O) SE were adequate to describe the experimental results and were described by the following equations:

$$Y_{1\,HT} = +0.17+0.34X_1+0.054X_2+0.046X_3+0.029X_4+0.061X_1X_4-0.23X_2X_3+0.084X_2X_4+0.24X_3X_4+0.14X_1^2 \quad (6)$$

$$Y_{2\,TAA} = +345.81+73.48X_1-0.57X_2+38.99X_3+31.28X_2^2 \quad (7)$$

ANOVA results for HT and TAA amounts obtained by (EtOH:H_2O) SE can be seen in Table III(b) as well. According to the ANOVA results of the models for the HT and TAA, the model performance was good, with correlation coefficients of 0.9939 and 0.9998, respectively. The models were demonstrated as significant (*p<0.0001*) and high F-values of 88.26 and 1729.93, respectively. The models for (EtOH:H_2O) SE were illustrated by the subsequent equations:

$$\mathbf{Y'_{1\,HT}} = +0.13 - 0.01X'_1 + 0.06X'_2 + 0.018X'_3 - 0.03X'_4 - 0.06X'_1X'_2 - 0.017X'_1X'_3 + 0.063X'_1X'_4 + 0.021X'_2X'_3 + 0.031X'_3X'_4 - 0.033X'^2_1 + 7.45\,10^{-3}X'^2_2 - 0.037X'^2_3 - 0.014X'^2_4 \quad (8)$$

Table IIIa. ANOVA results and second order polynomial equation for HT and TAA for MeOH/H_2O solvent system (Backward Elimination Regression).

	Y_1:HT				**Y_2: TAA**			
	Hierarchial Terms added after Backward Elimination Regression: $X_4, X_1^2, X_2^2, X_3^2, X_5^2, X_3X_5, X_2X_4, X_4X_5, X_1X_3, X_3X_4, X_2X_5, X_1X_4$				**Hierarchial Terms added after Backward Elimination Regression: $X_4, X_1^2, X_2^2, X_3^2, X_5^2, X_3X_5, X_2X_3, X_1X_3, X_2X_4, X_1X_4, X_4X_5, X_3X_4, X_2X_5$**			
	SSE	**df**	**F value**	***p value***	**SSE**	**df**	**F value**	***p value***
Model	2.41	9	107.22	**< 0.0001**	$1.099\ 10^5$	4	16.26	**< 0.0001**
X_1 (MeOH con.)	0.65	1	262.11	**< 0.0001**	74409.20	1	44.04	**< 0.0001**
X_2 (Temperature)	0.016	1	6.49	**0.0256**	4.34	1	$2.566\ 10^{-3}$	0.9602
X_3 (Time)	0.029	1	11.49	**0.0054**	20745.38	1	12.28	**0.0027**
X_4 (solvent:solid ratio)	0.012	1	4.62	0.0527				
X_1X_4	0.012	1	4.87	**0.0476**				
X_2X_3	0.42	1	168.59	**< 0.0001**				
X_2X_4	0.024	1	9.51	**0.0095**				
X_3X_4	0.45	1	182.08	**< 0.0001**				
X_1^2	0.31	1	125.39	**< 0.0001**				
X_2^2					14759.24	1	8.74	**0.0089**
Residual	0.030	12			28721.66	17		
Lack of Fit	0.022	7	1.96	0.2386	22948.33	12		0.3013
Pure Error	$8.009.10^{-3}$	5			5773.33	5		
Cor Total	2.44	21			$1.386\ 10^5$	21		
R^2	0.9877				0.7928			
R^2_{Adj}	0.9785				0.7441			
R^2_{Pred}	0.9467				0.5957			
PRESS	0.13				56047.29			
Significant Model Terms	$X_1, X_2, X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, X_1^2$				X_1, X_3, X_2^2			
Second order polynomial equation by CCD-Optimal Responce Surface-after Backward Elimination	$\mathbf{Y_{1\,HT}} = +0.17 + 0.34X_1 + 0.054X_2 + 0.046X_3 + 0.029X_4 + 0.061X_1X_4 - 0.23X_2X_3 + 0.084X_2X_4 + 0.24X_3X_4 + 0.14X_1^2$				$\mathbf{Y_{2\,TAA}} = +345.81 + 73.48X_1 - 0.57X_2 + 38.99X_3 + 31.28X_2^2$			

Table IIIb. ANOVA results and second order polynomial equation for HT and TAA for EtOH/H_2O solvent system (Backward Elimination Regression).

	Y'_1:HT				Y'_2: TAA			
	Hierarchial Terms added after Backward Elimination Regression: $X'_2X'_4$				Hierarchial Terms added after Backward Elimination Regression: ----			
	SSE	**df**	**F value**	***p* value**	**SSE**	**df**	**F value**	***p* value**
Model	0.090	13	88.26	**< 0.0001**	78865.70	14	1729.93	**< 0.0001**
X'_1 (EtOH con.)	1.45 10^{-3}	1	18.43	**0.0036**	13122.00	1	4029.65	**< 0.0001**
X'_2 (Temperature)	0.020	1	254.37	**< 0.0001**	6962.00	1	2137.97	**< 0.0001**
X'_3 (Time)	4.59 10^{-3}	1	58.41	**0.0001**	1398.93	1	429.60	**< 0.0001**
X'_4 (solvent:solid ratio)	5.00 10^{-3}	1	63.59	**< 0.0001**	1984.50	1	609.42	**< 0.0001**
$X'_1X'_2$	0.012	1	155.17	**< 0.0001**	524.66	1	161.12	**< 0.0001**
$X'_1X'_3$	2.18 10^{-3}	1	27.70	**0.0012**	288.00	1	88.44	**< 0.0001**
$X'_1X'_4$	0.013	1	168.15	**< 0.0001**	1536.80	1	471.94	**< 0.0001**
$X'_2X'_3$	3.70 10^{-3}	1	47.03	**0.0002**	264.50	1	81.23	**<0.0001**
$X'_2X'_4$					554.34	1	170.23	**< 0.0001**
$X'_3X'_4$	7.69 10^{-3}	1	97.78	**< 0.0001**	1860.50	1	571.34	**< 0.0001**
X'^2_1	0.016	1	208.15	**< 0.0001**	10495.98	1	3223.22	**< 0.0001**
X'^2_2	8.06 10^{-4}	1	10.25	**0.0150**	4213.67	1	1293.98	**< 0.0001**
X'^2_3	0.020	1	256.76	**< 0.0001**	9110.50	1	2797.75	**< 0.0001**
X'^2_4	2.91 10^{-3}	1	37.02	**0.0005**	1672.56	1	513.63	**< 0.0001**
Residual	5.50 10^{-4}	8			19.54	7		
Lack of Fit	4.30 10^{-4}	3	4.78	0.0824	14.74	2	6.14	0.0604
Pure Error	1.20 10^{-4}	5			4.80	5		
Cor Total	0.091	21			78885.24	21		
R^2	0.9939				0.9998			
R^2_{Adj}	0.9827				0.9992			
R^2_{Pred}	0.7540				0.9839			
PRESS	0.022				1272.51			
Significant Model Terms	**X'_1,X'_2, X'_3, X'_4, $X'_1X'_2$, $X'_1X'_3$, $X'_1X'_4$, $X'_2X'_3$, $X'_3X'_4$, X'^2_1, X'^2_2, X'^2_3, X'^2_4**				**X'_1,X'_2, X'_3, X'_4, $X'_1X'_2$, $X'_1X'_3$, $X'_1X'_4$, $X'_2X'_3$, $X'_2X'_4$, $X'_3X'_4$, X'^2_1, X'^2_2, X'^2_3, X'^2_4**			
Second order polynomial equation by CCD-Optimal Responce Surface-after Backward Elimination	$Y'_{1\,HT}$= +0.13- 0.01X'_1+ 0.06X'_2 +0.018X'_3 -0.03X'_4 -0.06$X'_1X'_2$ – 0.017$X'_1X'_3$+ 0.063$X'_1X'_4$ + 0.021$X'_2X'_3$ +0.031$X'_3X'_4$ – 0.033X'^2_1 + 7.45 $10^{-3}X'^2_2$ -0.037X'^2_3 - 0.014X'^2_4				$Y'_{2\,TAA}$=+285.55 +47.65X'_1+ 35.40X'_2+ 10.13X'_3 -18.90X'_4 -12.65$X'_1X'_2$ +6.00$X'_1X'_3$+ 21.65$X'_1X'_4$ +5.75$X'_2X'_3$ -12.85$X'_2X'_4$ + 15.25$X'_3X'_4$ – 26.05X'^2_1 – 17.03X'^2_2 -24.737X'^2_3 -10.73X'^2_4			

$$Y'_{2\ TAA} = +285.55 + 47.65X'_1 + 35.40X'_2 + 10.13X'_3 - 18.90X'_4 - 12.65X'_1X'_2 + 6.00X'_1X'_3 + 21.65X'_1X'_4 + 5.75X'_2X'_3 - 12.85X_2X'_4 + 15.25X'_3X'_4 - 26.05X'^2_1 - 17.03X'^2_2 - 24.737X'^2_3 - 10.73X'^2_4 \quad (9)$$

Analysis of response surfaces

The extraction variables; SolC, Temp, T, and SSR were used to find optimum extraction conditions in SE. Generally, the linear terms of MeOH concentration (X_1) showed the largest effect ($p < 0.0001$) for HT and TAA in the MeOH solvent system. For HT, MeOH concentration (X_1) was followed by time (X_3) and Temp (X_2), whereas it was followed by time (X_3) for TAA. For the EtOH solvent system, the linear terms of all the variables (X'_1, X'_2, X'_3, X'_4) had the greatest influence on TAA, however, extraction Temp (X'_2) and SSR (X'_4) showed the largest effects ($p<0.0001$), followed by time (X'_3) ($p=0.0001$), and EtOH concentration (X'_1) ($p=0.0036$) for HT. Furthermore, 3D response surface plots of HT and TAA, including interaction effects of independent variables, are presented in Figs. 1 and 2 for the MeOH solvent system, and Figs. 3 and 4 for the EtOH solvent system, respectively.

Solvent (MeOH:H_2O and EtOH:H_2O) concentration (SolC)

SE is a traditional procedure that has been used for many years. The solvent type is one of the most critical factors impacting the performance of traditional SE of phenolics from olive by-products (Nakilcioğlu-Taş & Ötleş 2019). All compounds cannot be removed by a single solvent because of their various solubilities and polarities (Mohamed & Khan 2013, Sifaoui et al. 2016). Plants contain a variety of phenolic chemicals with varying chemical characteristics and polarity. Choosing the solvent is very

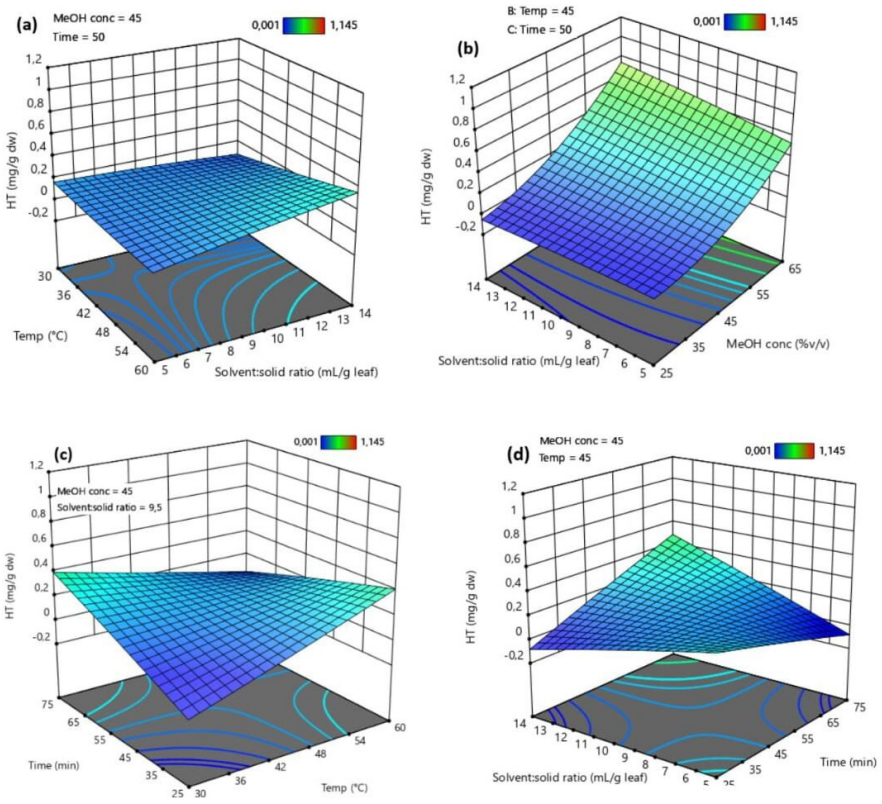

Figure 1. Response surface plots of HT of olive leaves extracts in MeOH/H_2O hydroalcoholic solvent extraction as affected by (a) temperature and solvent:solid ratio (b) MeOH conc. and solvent:solid ratio (c) temperature and time (d) time and solvent:solid ratio.

important, as it affects the amount and type of phenols extracted (Nakilcioğlu-Taş & Ötleş 2019, Wissam et al. 2016, Chan et al. 2009). Additionally, the difference between the type and composition of bioactive compounds affects the TAA of the plant. The solubility and extraction efficiency of bioactive polyphenols may be affected by differences in solvent polarity (Nakilcioğlu-Taş & Ötleş 2019). This might be owing to the polarity of the phytochemicals in OLLs, which allows them to be extracted using solvents of varying polarities (Wissam et al. 2016).

Depending on the target analytes and sample matrix, several ratios of MeOH:H_2O and EtOH:H_2O were used for phenolic compound extraction (Pérez-Serradilla et al. 2007, Habibi et al. 2018). Alcohols have intermediate polarity rather than more or less polar solvents (Wissam et al. 2016, Galanakis et al. 2013). In this study, a range of 25–65% MeOH:H_2O mixtures was used. It was shown that the change in hydroalcoholic composition influenced both HT and TAA significantly (*p<0.0001*) (Table IIIa). Fig. 1(b) shows the effect of MeOH:H_2O concentration

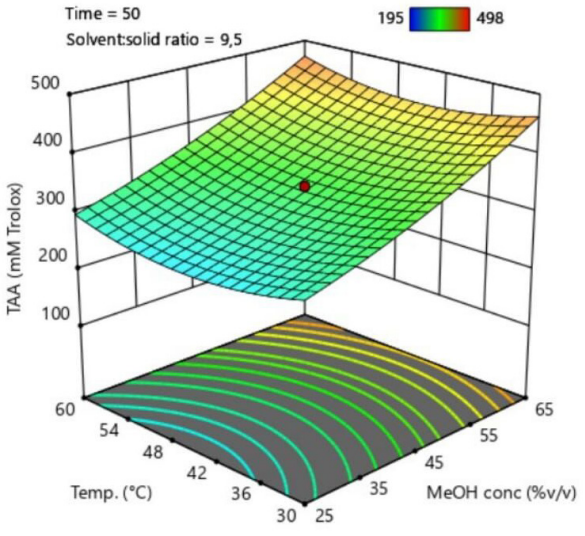

Figure 2. Response surface plots of TAA of olive leaves extracts in MeOH/H_2O hydroalcoholic solvent extraction as affected by MeOH conc. and temperature.

with SSR on HT, which is shown (*p=0.0476*) in Table III(a) also. However there was no significant relationship between Temp and MeOH:H_2O concentration for HT and TAA in the ANOVA results which are shown in Fig. 2(a). The quadratic term of MeOH:H_2O concentration (X_1^2) was significant for HT (*p<0.0001*).

Table III(b) shows the ANOVA results of optimization conditions of EtOH:H_2O (25-65%) solvent system for HT and TAA. Similar to MeOH results, HT was affected by changes in EtOH:H_2O concentration (*p=0.0036*) and TAA (*p<0.0001*) (Table IIIb). For both HT and TAA, the binary relationship of EtOH:H_2O concentration with Temp (*p<0.0001*, *p<0.0001*), with T (*p=0.0012*, *p<0.0001*) and with SSR (*p<0.0001*, *p<0.0001*) were significant which can be seen in Figs. 3(a), (b), (c) and Figs. 4(a), (b), (c) respectively. Quadratic term of EtOH:H_2O concentration (X'^2_1) was significant for both HT and TAA (*p<0.0001*).

It was determined that the hydroalcoholic solvent had a positive linear effect on both responses of HT and TAA. Sifaoui et al. (2016) showed that MeOH was an acceptable solvent for extracting bioactive compounds from OLLs with highest extraction yield whereas EtOH provided lower concentrations of phenolics. Similarly, Nakilcioğlu-Taş & Ötleş (2019) specified that obtaining high yields of TAA and polyphenols including HT was easier in MeOH, implying that these compounds were more polar than EtOH. Contrary to these findings, using EtOH:H_2O mixtures as an inexpensive and non-toxic extraction solvent, the optimal ratio for the simultaneous SE of OLE and HT in olive pomace was determined to be 60% EtOH:40% H_2O (Habibi et al. 2018). In a similar study, compared to MEOH, EtOH was chosen as the ideal solvent for the SE from OLLs to acquire extracts with high TPC and TAA due to being a food-grade solvent and being classified as generally recognized as safe (GRAS) (Wissam et al. 2016). In most investigations, a

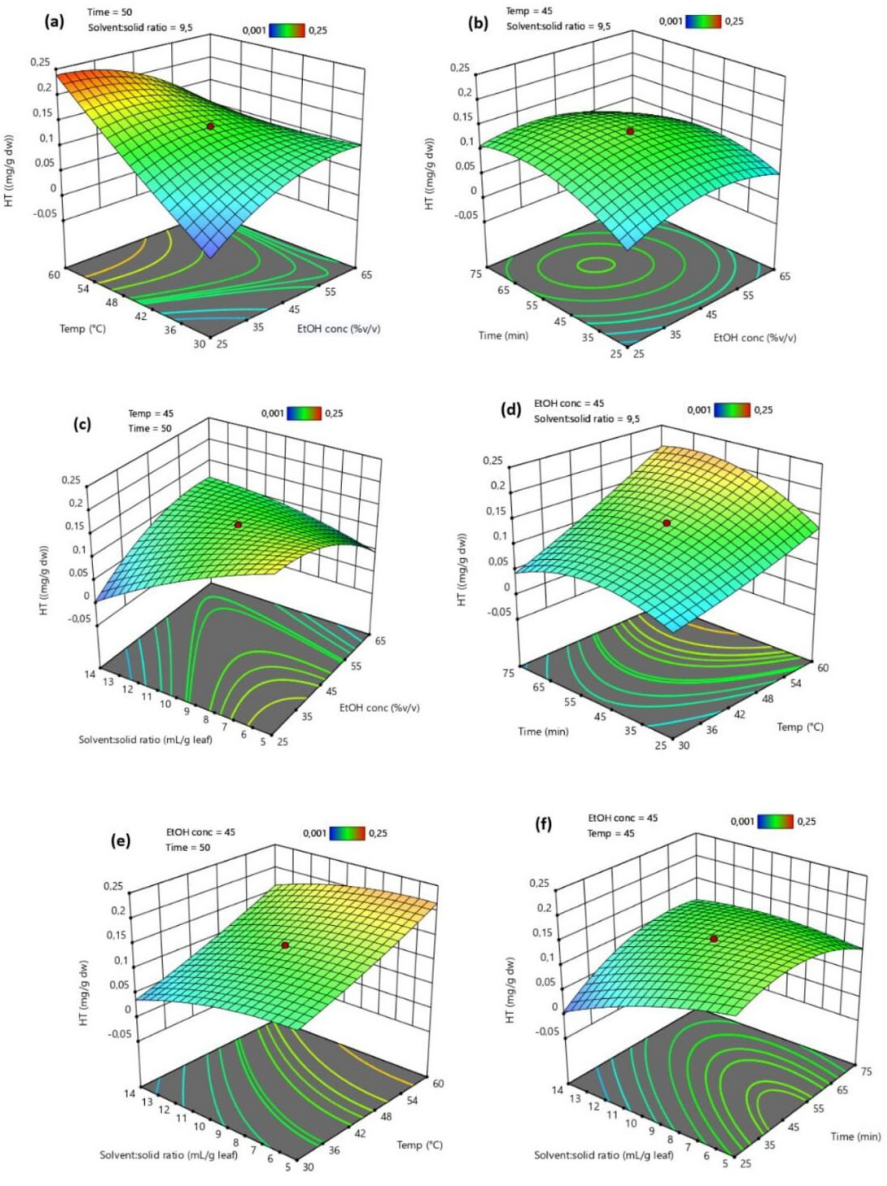

Figure 3. Response surface plots of HT of olive leaves extracts in EtOH/H_2O hydroalcoholic solvent extraction as affected by (a) EtOH conc. and temperature (b) EtOH conc. and time (c) EtOH conc. and solvent:solid ratio (d) temperature and time (e) temperature and solvent:solid ratio (f) time and solvent:solid ratio.

40% EtOH:60% H_2O solution was determined to get a high phenolic yield (Wissam et al. 2016, Şahin et al. 2015, Thoo et al. 2010) in which they observed a drop as EtOH concentration in the solvent increased. Studies on the SE of bioactive compounds derived from OLLs have indicated an EtOH:H_2O ratio of 40-80% v/v (Mylonaki et al. 2008, Japón-Luján et al. 2006). Stamatopoulos et al. (2014) demonstrated the effect of EtOH:H_2O on TPC, with the highest value reached at a concentration of 70% EtOH:H_2O. SolC showed also a significant influence ($p<0.05$) on TAA (Irakli et al. 2018) similar to our results. The greatest TAA values were attained at concentrations of 50% and 70% concentration, however, the yield of HT was maximal at the lowest organic solvent concentration and declined dramatically as the solvent concentration grew, which may explain why HT has a more polar structure. In several studies on HT and TAA, the maximal HT and TAA were obtained by H_2O, 80% MeOH, and 44% EtOH using ultrasound-assisted extraction

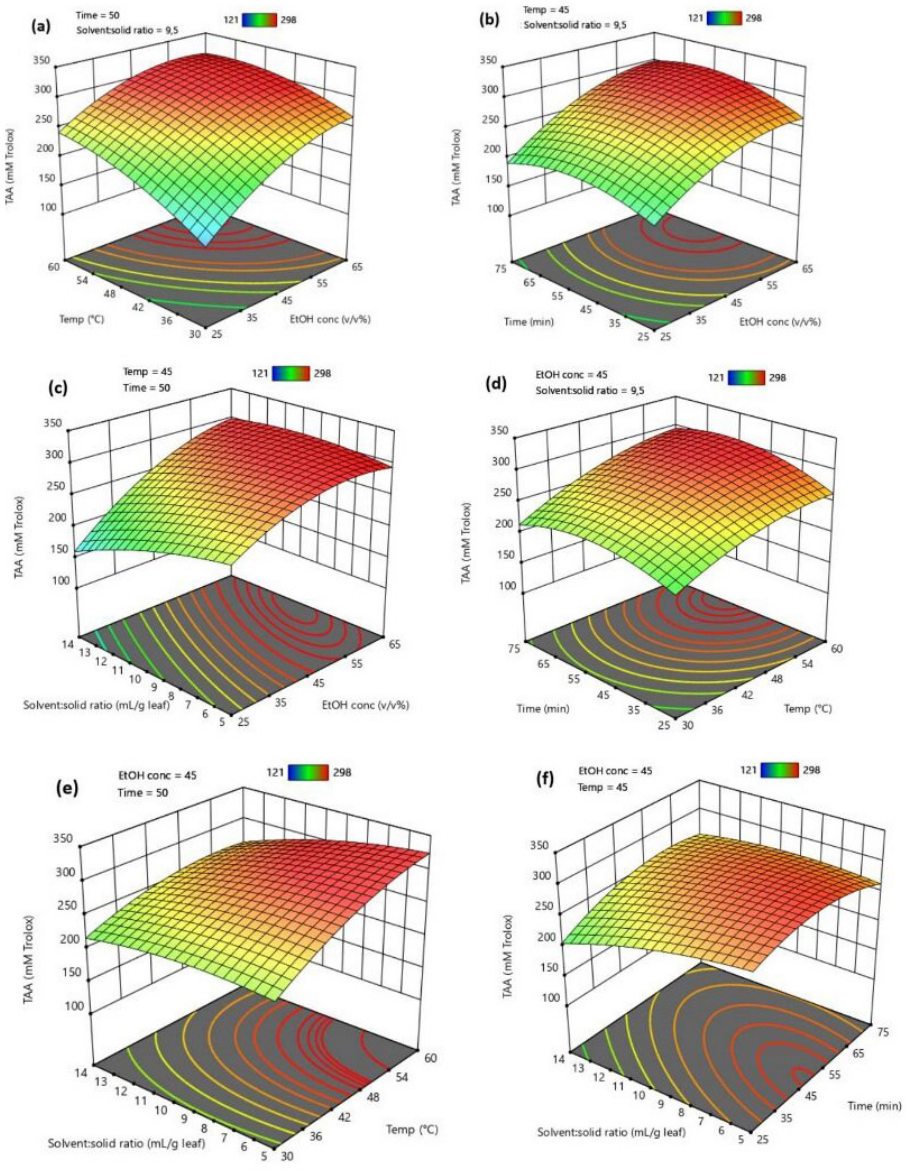

Figure 4. Response surface plots of TAA of olive leaves extracts in EtOH/H_2O hydroalcoholic solvent extraction as affected by (a) EtOH conc. and temperature (b) EtOH conc. and time (c) EtOH conc. and solvent:solid ratio (d) temperature and time (e) temperature and solvent:solid ratio (f) time and solvent:solid ratio.

(UAE), respectively; however, for TAA (DPPH), the opposite was true (Yao et al. 2019).

Temperature (Temp)

Temp ranges of 30-60 °C were chosen for both MeOH and EtOH solvent systems in this research. In MeOH solvent system, the linear term of Temp was significant (*p=0.0256*) for HT. For TAA, the linear term of Temp was not significant (*p=0.9602*) while the quadratic term of Temp was significant (*p=0.0089*). The interaction term of Temp-time (X_2X_3) (*p<0.0001*) and Temp-SSR (X_2X_4) (*p=0.0095*) were significant on HT. While Fig. 2 (a) shows the change in TAA value as a function of Temp and MeOH concentration, there is no *p*-value in the ANOVA results.

In the EtOH solvent system, the linear term of extraction Temp was both significant for HT and TAA (*p< 0.0001*). Similarly, the quadratic term of Temp was significant for both HT (*p= 0.0150*) and TAA (*p<0.0001*). The binary relationship of Temp with EtOH:H_2O concentration (*p<0.0001*,

p<0.0001), Temp and time (*p=0.0002*, *p<0.0001*) were significant for both HT (Fig. 3(a) and Fig. 3(d)) and TAA (Fig. 4(a) and Fig. 4(d)). The interaction term of Temp and SSR was significant for TAA (*p<0.0001*), as shown in Fig. 4(e). Also, Fig. 3(e) shows the surface plot for the relationship of Temp and SSR for HT however it was not shown in the ANOVA table (Table IIIb). Similar positive effect of Temp on HT was found in the study of Irakli et al. (2018) who found that TPC rose as the Temp increased from 25 to 60°C in the UAE for phenolic compounds from OLLs. It was found that 60°C was the most effective temperature for acquiring phenolic compounds, including HT. However, no significant difference was found for TAA with increasing Temp (Irakli et al. 2018).

Similar results about HT, which acts in a Temp-dependent manner, were also obtained by Yao et al. (2019), who chose a temp of 60°C for the study of the optimization of UAE of total flavonoids and HT from OLLs. Furthermore, Stamatopoulos et al. (2014) found that high amounts of HT appeared when Temp particularly ≥ 60 °C. Similar to our results, an interaction term of Temp and time generated a significant effect on HT content. In terms of TAA, independent of time, a reduction in Temp resulted in a greater value of DPPH and FRAP (Nakilcioğlu-Taş & Ötleş 2019). Similarly, as the temperature climbed from 40˚C to 60˚C, the IC_{50} reduced by 2.3±0.6 times (Wissam et al. 2016). Because of the high sensitivity of phenolics to heat, it is critical to define an upper limit to prevent the destruction of these thermosensitive bioactive compounds (Wissam et al. 2016).

Time (T)

Phenolic compounds generally rise steadily over time, resulting in increased extraction efficiency (Şahin et al. 2015). However, after a crucial period, the degree of chemical reactions, particularly phenolic oxidation, may increase, resulting in a fall in TAA level (Wissam et al. 2016, Şahin et al. 2015, Candrawinata et al. 2014). Time must thus be carefully considered and adjusted in order to protect polyphenols and antioxidants. The use of a modest extraction time has additional benefits in that it reduces solvent loss through vaporization as well as the process's energy requirements (Nakilcioğlu-Taş & Ötleş 2019, Wissam et al. 2016).

OLL bioactive substances were extracted using hydroalcoholic solutions for 25 to 75 minutes. The important linear effect of time was acquired for HT (*p=0.0054*), and TAA (*p=0.0027*) in methanolic aqueous extract. The binary relationship of time and Temp (*p<0.0001),* time and SSR (*p<0.0001*) were significant for HT, whereas these interactions were not significant for TAA. Fig. 1(c) and Fig. 1(d) show the effect of time with Temp and time with SSR for HT, respectively.

In hydroethanolic extraction, the effects of the extraction time on HT (*p=0.0001*) and TAA (*p<0.0001*) were found statistically significant. The interaction effect of time and EtOH:H_2O concentration (*p=0.0012, p<0.0001*), time and Temp (*p= 0.0002, p<0.0001*), time and SSR (*p<0.0001*, *p<0.0001*) were significant for both HT and TAA, respectively. The response surface plots given in Figs. 3(b), 3(d), 3(f) and Figs. 4(b), 4(d) and 4(f) show these interactions, respectively. Also, the quadratic term of time was significant for both HT (*p< 0.0001*) and TAA (*p< 0.0001*).

The linear term of time and the interaction term of time and Temp on HT extraction were found to be statistically significant, which is consistent with our findings. It was reported that the content of HT increased by 9.80 % when the period at 40 °C extended from 30 to 60 minutes. Contrary to our findings, time did not significantly influence the change in TAA (Nakilcioğlu-Taş & Ötleş 2019). Yao et al. (2019) reported that the HT efficiency was highest in

conditions where the ultrasonic effect was greater than 50 min. In another study, TAA was found to be constant between 10 and 30 min but decreased further as time increased. However, yields of other phenolic compounds, including HT, did not change after 10 min of UAE (Irakli et al. 2018).

Solvent:solid ratio (SSR)

In (MeOH:H_2O) SE, the linear term of SSR had no statistically significant effect on both HT and TAA (*p>0.05*) (Table IIIa). Although there was no significant relationship of SSR with the other variables for TAA; SSR and MeOH concentration (*p=0.0476*), SSR and Temp (*p=0.0095*) (Fig. 1a), SSR and time (*p< 0.0001*) were found significant for HT. The surface plot for the interaction between SSR and MeOH concentration is shown in Fig. 1(b), whereas the surface plot for the interaction between SSR and time on HT is shown in Fig. 1(d). Contrary to (MeOH:H_2O) SE, the linear term of SSR was significant on both HT and TAA (*p<0.0001*) in (EtOH:H_2O) SE. Similarly, the quadratic term of SSR was significant for both HT (*p=0.0005*) and TAA (*p<0.0001*), respectively. The interaction terms of SSR and EtOH concentration ($X'_1 X'_4$) (*p<0.0001*) and the SSR and time ($X'_3 X'_4$) were significant (*p<0.0001*) on HT and TAA. The changes in HT and TAA as a function of these variables were demonstrated by 3D response surface plots in Fig. 3(c), Fig. 3(f) and Fig. 4(c) and Fig. 4(f), respectively. Additionally, the interaction terms of SSR and Temp for TAA were significant (*p<0.0001*) which is shown in Fig. 4(e). Also, Fig. 3(e) shows the interaction between SSR and Temp, however, no significant value was found for HT in Table IIIb.

SSR and extraction temperature had a strong interactive impact. It should be noted that extremely high extraction temperatures may result in higher solvent loss owing to vaporization. The solvent evaporation changes SSR according to the solvent's boiling point (Wissam et al. 2016, Khemakhem et al. 2017, Irakli et al. 2018). The determination of the optimum SSR is also important because it has a direct effect on the extraction process's cost due to solvent consumption (Stamatopoulos et al. 2014). In the results of Stamatopoulos et al. (2014), who improved the multistage extraction approach in which OLLs were previously steam blanched, a solvent-solid ratio of 7:1 was proposed. Similarly, Bilek (2010) reported that the optimal point in the SE of phenolics from OLLs was seven times the SSR. In the study of Goldsmith et al. (2014), an SSR of 1:60 g/mL was suggested since less extraction solvent was consumed.

Optimization

The primary goal of the optimization study was to find the hydroalcoholic SE conditions that gave the maximum extraction yield for HT amount and TAA. To establish the ideal parameter values for the MOO process, the maximizing total DF model was created. The experiments were performed in random order using a CCD with six center points, the mean values of the triplicate trials were calculated, and the design and results are given in Table IV(a,b). A quadratic polynomial model for each response was done.

$$yi = \beta_0 + \sum_{j=1}^{4}\beta_j x_j + \sum_{j'=2}^{4}\sum_{j=1}^{j'-1}\beta_{jj'} x_j x_{j'} + \sum_{j=1}^{4}\beta_{jj} x_j^2 + \varepsilon i, \quad i = 1,2 \tag{10}$$

The experimental region, after coding, was xj = [−α +α], j = 1, 2, 3, 4. Our aim in this study was to maximize the responses *y*1 and *y*2 and minimize the standard errors, under the constraints that following [*ymin* , *ymax*] and r=0.2 imposed for all the two individual DFs in Eq (1). The restrictions applied were given below:

Table IV. Experimental design and observed responses for MeOH/H_2O and EtOH/H_2O hydroalcoholic solvent extraction.

a) MeOH/H_2O hydro-alcoholic binary solvents system.

	Std	x1	x2	x3	x4	Y_1	Y_2	Ty	L7G	OLE	A7G	Lut
		MeOH conc (%v/v)	Temp. (°C)	Time (min)	Solvent: solid ratio (mL/g leaf)	HT (mg/g dw)	TAA (mM Trolox)	(mg/g dw)	(mg/g dw)	(mg/g dw)	(mg/g dw)	(mg/g dw)
	1	65(+1)	60(+1)	75(+1)	5(-1)	0.083±0.001	472±1.99	0.81±0.080	0.005±0.001	12.56±0.31	0.21±0.025	0.08±0.009
	2	65(+1)	60(+1)	25(-1)	5(-1)	0.983±0.121	485±1.89	0.39±0.002	0.016±0.002	6.54±0.22	0.18±0.017	0.08±0.010
	3	65(+1)	30(-1)	75(+1)	14(+1)	1.069±0.125	490±1.75	0.12±0.010	0.024±0.005	4.08±0.18	0.05±0.006	0.04±0.003
	4	25(-1)	60(+1)	25(-1)	14(+1)	0.04±0.001	284±0.85	0.06±0.002	0.012±0.007	1.94±0.08	0.04±0.007	0.01±0.000
	5	65(+1)	30(-1)	25(-1)	14(+1)	0.098±0.002	411±1.01	0.05±0.002	0.012±0.008	3.96±0.11	0.04±0.005	0.01±0.000
	6	25(-1)	30(-1)	75(+1)	5(-1)	0.049±0.001	385±0.98	0.32±0.018	0.031±0.007	6.11±0.18	0.25±0.022	0.04±0.002
	7	25(-1)	60(+1)	75(+1)	14(+1)	0.09±0.002	345±0.87	0.10±0.011	0.001±0.000	2.33±0.09	0.10±0.011	0.04±0.002
	8	25(-1)	30(-1)	25(-1)	5(-1)	0.035±0.001	321±0.76	1.48±0.115	0.093±0.008	4.49±0.16	0.13±0.012	0.01±0.001
	9	12(-1.68)	45(0)	50(0)	9.50 (0)	0.001±0.001	210±0.65	0.14±0.011	0.015±0.002	1.22±0.05	0.04±0.006	0 (nd)
	10	79(1.68)	45(0)	50(0)	9.50 (0)	1.145±0.121	498±0.98	0.21±0.012	0.006±0.001	5.55±0.12	0.08±0.007	0.03±0.002
	11	45(0)	20(-1.67)	50(0)	9.50 (0)	0.11±0.003	397±0.75	0.06±0.003	0.015±0.002	2.15±0.08	0.05±0.004	0 (nd)
	12	45(0)	70(+1.67)	50(0)	9.50 (0)	0.29±0.0025	405±0.98	0.24±0.012	0.003±0.000	4.16±0.14	0.11±0.012	0.06±0.008
	13	45(0)	45(0)	8(-1.68)	9.50 (0)	0.005±0.001	195±0.57	0.001±0.00	0 (nd)	1.02±0.08	0.004±0.000	0 (nd)
	14	45(0)	45(0)	92(+1.68)	9.50 (0)	0.297±0.020	398±0.75	0.15±0.012	0.01±0.003	5.47±0.21	0.16±0.014	0.07±0.005
	15	45(0)	45(0)	50(0)	2(-1.67)	0.074±0.003	359±0.77	0.11±0.008	0.004±0.000	3.51±0.18	0.08±0.005	0.02±0.001
	16	45(0)	45(0)	50(0)	17.00(+1.67)	0.223±0.019	309±0.71	0.05±0.001	0.005±0.000	2.35±0.11	0.07±0.006	0.01±0.000
	17-22	45(0)	45(0)	50(0)	9.50 (0)	0.175±0.012	345±0.74	0.16±0.02	0.013±0.010	3.31±0.14	0.11±0.021	0.04±0.010
Desirability	Opt(Obs)	63.30	36.00	62.00	11.80	0.809±0.110	451±2.32	0.09±0.003	0.018±0.006	9.25±0.23	0.11±0.017	0.05±0.009
	Opt(Pred)	63.30	36.00	62.00	11.80	0.721	442					

Analytical results are the average of triplicates (mean ± sd).

b) EtOH/H_2O hydro-alcoholic binary solvents system.

	Std	x'1	x'2	x'3	x'4	Y'_1	Y'_2	Ty	L7G	OLE	A7G	Lut
		EtOH conc (%v/v)	Temp. (°C)	Time (min)	Solvent:solid ratio (mL/g leaf)	HT (mg/g dw)	TAA (mM Trolox)	(mg/g dw)	(mg/g dw)	(mg/g dw)	(mg/g dw)	(mg/g dw)
	1	65(+1)	60(+1)	75(+1)	5(-1)	0.01±0.000	293±1.11	1.42±0.02	0.68±0.05	7.90±0.11	0.35±0.02	0.11±0.030
	2	65(+1)	60(+1)	25(-1)	5(-1)	0.02±0.001	281±0.98	1.00±0.01	0.21±0.02	4.54±0.07	0.18±0.01	0.091±0.010
	3	65(+1)	30(-1)	75(+1)	14(+1)	0.098±0.009	272±0.77	2.89±0.06	0.419±0.04	2.64±0.03	0.047±0.00	0.032±0.002
	4	25(-1)	60(+1)	25(-1)	14(+1)	0.01±0.001	129±0.23	0.07±0.01	0.11±0.01	0.90±0.01	0.05±0.00	0.01±0.001
	5	65(+1)	30(-1)	25(-1)	14(+1)	0.07±0.002	222±0.39	1.996±0.03	0.321±0.02	1.30±0.02	0.029±0.00	0.02±0.002
	6	25(-1)	30(-1)	75(+1)	5(-1)	0.03±0.000	121±0.21	0.15±0.02	0.137±0.01	0.70±0.01	0.073±0.01	0.03±0.004
	7	25(-1)	60(+1)	75(+1)	14(+1)	0.19±0.010	178±0.18	0.02±0.00	0.199±0.02	2.60±0.03	0.088±0.01	0.023±0.003
	8	25(-1)	30(-1)	25(-1)	5(-1)	0.06±0.002	156±0.17	0.057±0.01	0.115±0.01	0.98±0.01	0.11±0.01	0.028±0.003
	9	12(-1.68)	45(0)	50(0)	9.50 (0)	0.05±0.003	130±0.11	0.001±0.00	0.005±0.00	1.18±0.02	0.07±0.00	0.006±0.00
	10	79(1.68)	45(0)	50(0)	9.50 (0)	0.021±0.001	292±0.41	1.01±0.04	0.385±0.02	3.92±0.05	0.067±0.00	0.04±0.001
	11	45(0)	20(-1.68)	50(0)	9.50 (0)	0.05±0.002	180±0.12	1.00±0.03	0.311±0.03	1.75±0.02	0.05±0.00	0.011±0.00
	12	45(0)	70(+1.68)	50(0)	9.50 (0)	0.25±0.020	298±0.38	1.24±0.04	0.452±0.05	3.79±0.06	0.12±0.01	0.06±0.002
	13	45(0)	45(0)	8(-1.68)	9.50 (0)	0.001±0.000	198±0.13	1.01±0.02	0.332±0.04	0.80±0.01	0.003±0.00	0.002±0.00
	14	45(0)	45(0)	92(+1.68)	9.50 (0)	0.05±0.002	235±0.14	1.11±0.02	0.348±0.04	5.20±0.08	0.09±0.01	0.049±0.001
	15	45(0)	45(0)	50(0)	2(-1.68)	0.14±0.015	288±0.13	0.66±0.01	0.033±0.004	3.40±0.05	0.06±0.01	0.025±0.002
	16	45(0)	45(0)	50(0)	17.00(+1.68)	0.04±0.002	225±0.12	1.01±0.05	0.335±0.05	1.90±0.02	0.044±0.00	0.019±0.001
	17-22	45(0)	45(0)	50(0)	9.50 (0)	0.14±0.01	285±1.05	1.25±0.05	0.442±0.08	3.10±0.05	0.10±0.01	0.041±0.005
Desirability	Opt(Obs)	43.80	52.00	58.00	9.40	0.175±0.004	297±0.817	1.28±0.04	0.475±0.06	4.89±0.07	0.11±0.01	0.07±0.006
	Opt(Pred)	43.80	52.00	58.00	9.40	0.172	298					

Analytical results are the average of triplicates (mean ± sd).

for MeOH:H_2O solvent system:

$$y1 \geq 0.001 \qquad y2 \geq 195$$

for EtOH:H_2O solvent system:

$$y1 \geq 0.001 \qquad y2 \geq 121$$

In the DF described by the geometric mean (Eq (5)), the predicted values were confirmed using least-squares predictions. For MeOH:H_2O hydroalcoholic SE, the optimal solution was **(X_1, X_2, X_3, X_4)**= (**63.30 36.00 62.00 11.80**) with *DF*=0.722 while for EtOH:H_2O hydroalcoholic SE, it was **(X'_1, X'_2, X'_3, X'_4)=** (**43.80 52.00 58.00 9.40**) with *DF*=0.891.

A maximum value of DF (0.722) for MeOH:H_2O solvent system is reached for Y_1(HT) = 0.820 and Y_2(TAA) = 455. The point DF (0.722) was chosen as the optimal solution for experimental validation. The results of the confirmation run are presented in Tables II and V. The optimum conditions in terms of controllable variables were [**63.30 36.00 62.00 11.80**] (X= [X_1,X_2,X_3,X_4]). Under such conditions, the responses confirmed by experiment were Y_1(HT) = 0.809±0.110 mg/g dw and Y_2(TAA) = 451±2.32 mM Trolox. Similarly, the maximum value of DF (0.891) for EtOH:H_2O solvent system was attained for Y'_1(HT) = 0.179 and Y'_2(TAA) = 298. This point was the optimal solution for experimental validation and the results of the confirmation run are given (Table II and Table V). The optimum conditions for controllable variables were as follows: [**43.80 52.00 58.00 9.40**] (X= [X'_1, X'_2, X'_3, X'_4]). The responses confirmed by experiment under such conditions were Y'_1(HT) = 0.175±0.004 mg/g dw and Y'_2(TAA)= 297±0.817 mM Trolox. The optimum point determined by the MeOH:H_2O solvent system was more appropriate for the enhanced extraction of a higher amount of HT and TAA.

Figs. 5 and 6 indicate a close link between the experimental and estimated values, with no significant (*p> 0.05*) difference between them.

Method development and validation

HPLC method development and validation were performed for six important bioactive compounds (HT, Ty, L7G, OLE, A7G and Lut) of

Table V. Best compromise solution with observed and predicted response values for HT and TAA and comparison of Desirability.

	Method	Real values of independent variables X= [X_1,X_2,X_3,X_4]	Observed response values HT	TAA	Predicted response value HT	TAA
			(mg/g dw)	(mM Trolox)	(mg/g dw)	(mM Trolox)
MeOH/H_2O hydroalcoholic solvent extraction	Desirability (0.722)	[63.30 36.00 62.00 11.80]	0.809±0.110	451±2.32	0.820	455
EtOH/H_2O hydroalcoholic solvent extraction	Desirability (0.891)	[43.80 52.00 58.00 9.40]	0.175±0.004	297±0.817	0.179	298

X_1: MeOH or EtOH conc; X_2: Temperature; X_3:Time; X_4: solvent:solid ratio, Analytical results are the average of triplicates (mean ± sd).

the OLL MeOH and EtOH aqueous extracts. The proposed HPLC method was evaluated in terms of accuracy, precision, linearity, range, limit of detection (LOD) and limit of quantitation (LOQ) (Singh 2013, Julia et al. 2011, Khan et al. 2012). Table II summarizes the method's performance characteristics.

The accuracy was determined by comparing the measured and added concentrations (ICH 2005, Gonzalez et al. 2009, Singh 2013). After a spike was introduced to a blank sample, the percentage of analyte recovered was calculated. Accuracy was achieved at three concentrations covering the method range. In the evaluation of the mean recovery, the rule of being within 100%±5.0 over the entire studied range was valid (Al-Rimawi 2014, Green 1996, Winslow & Meyer 1997). The mean recovery and the RSD for each compound were calculated. It was determined that the validated method has good recovery for HT (97.32%±0.51), Ty (98.85%±1.02), L7G (97.98%±0.88), OLE (95.85%±1.45), A7G (98.33%±0.79), and Lut (98.43%±0.92) with a low RSD value (Table II).

Precision, defined as a measure of repeatability, was described as repeatability and intermediate precision in this study (Al-Rimawi 2014). Reproducibility is also known as intra-assay precision, defined as three replicates of each concentration and extract, and it is expressed as the RSD of the replicate (Singh 2013). RSD for repeat injections of the standard solutions with three concentrations were (0.5, 5 and 10 mg L^{-1}) for Ty, L7G, Lut (5, 10 and 25 mg L^{-1}) for HT, A7G and (50, 500 and 1000 mg L^{-1}) for OLE. They were determined to be 1.01, 0.70, 0.55, 1.70, 0.70 and, 0.35 for the analyzed compounds, respectively. The RSD of replicates was not higher than 1.5 (Al-Rimawi 2014, Huber 1998) demonstrating that the method is repeatable. Intermediate precision (three replicates of each concentration and extract, 3 days), called inter-day precision, measures the reproducibility of the result performed by the same method, on the same sample, in the same laboratory, but by different operators and on different days, to confirm that the method will produce the same results in the same laboratory after development (Al-Rimawi 2014). The intermediate precision of the method was assessed by determining the % recovery of the analyzed compounds by another analyst on a different day at three concentration levels: (0.5, 5 and 10 mg L^{-1}) for Ty, L7G, Lut; (5, 10 and 25 mg L^{-1}) for HT, A7G and (50, 500 and 1000 mg L^{-1}) for OLE. The RSDs were discovered to be 2.00, 2.25, 1.99, 4.25, 2.25, 1.80 for the analyzed

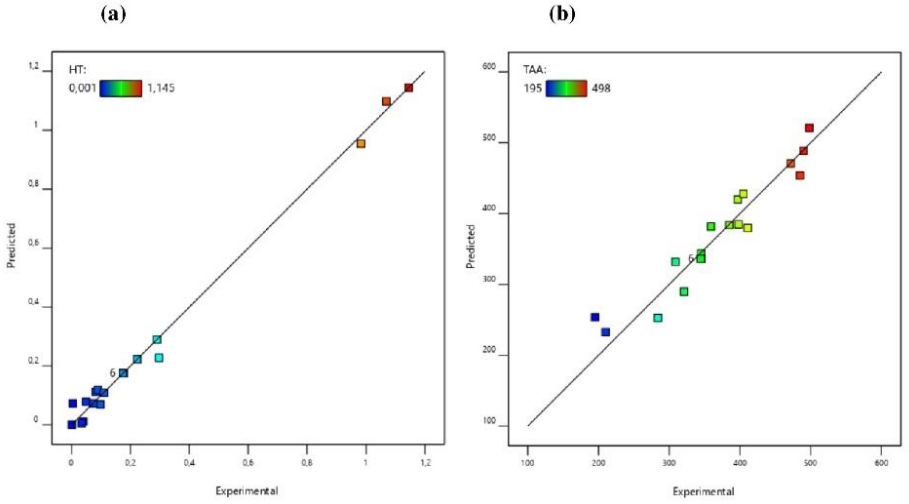

Figure 5. In MeOH solvent system comparison of experimental and predicted values of (a) HT (b) TAA.

compounds, respectively. Table II displays the inter-day and intra-day precisions (RSDs) for the bioactive chemicals studied.

In this work, the calibration equations' correlation coefficients (r) for six analytes were more than 0.9921 (Table II). The significance of the deviation of tthe calibration line's intercept was statistically evaluated by setting limits of confidence for the intercept, usually at the 95% level and the lack of fit was found to be insignificant. A one-way ANOVA with post test Tukey was employed to evaluate this set of data (Singh 2013, Miller & Miller 2005, Shabir et al. 2007).

In the validation of HPLC method to determine the quantification of HT, Ty, L7G, OLE, A7G, and Lut in OLL extract, the linearity was demonstrated over the range of 0.5 to 100 mg L^{-1}, 0.01 to 20 mg L^{-1}, 0.01 to 20 mg L^{-1}, 2.5 to 1750 mg L^{-1}, 0.25-50 mg L^{-1}, 0.010-20 mg L^{-1}, respectively. The estimation of LOD and LOQ was calculated using the formulas LOD = 3.3 Sa/b and LOQ = 10 Sa/b, based on signal-to-noise ratios of 3 and 10, respectively, where Sa is the standard deviation of the response and b is the slope of the calibration curve. The LOD and LOQ for six bioactive compounds of OLL extract ranged between $0.4.10^{-3}$- 0.012 mg L^{-1} and $1.7.10^{-3}$-0.042 mg L^{-1}, respectively (Table II). As a consequence, the observed low LOD and LOQ values demonstrated that the approach is suitable for the detection and quantification of substances at low concentrations. The suggested HPLC technique may be utilized to determine valuable compounds such as HT, Ty, L7G, OLE, A7G and Lut in OLL extracts, as shown in Table II.

Individual phenolic amounts in OLLs

The leading bioactives (Ty, L7G, OLE, A7G, and Lut) in OLL were also determined by HPLC analysis in the present study. The DOE was studied over a wide range of intervals, and in these intervals these phenolic compounds of OLL extracts were determined. Ty, L7G, OLE, A7G, and Lut were determined in (MeOH:H_2O) SE in the range of 0.001-1.48 mg/g dw, 0-0.093 mg/g dw, 1.02-12.56 mg/g dw, 0.004-0.25 mg/g dw, 0-0.08 mg/g dw, whereas in (EtOH:H_2O) SE, the phenolics were determined as 0.001-2.89 mg/g dw, 0.005-0.68 mg/g dw, 0.70-7.90 mg/g dw, 0.003-0.35 mg/g dw, 0.002-0.11 mg/g dw, respectively Table IV (a,b). Additionally, Ty, L7G, OLE, A7G, and Lut were found at the optimum extraction points for both solvent systems. They were 0.09±0.003, 0.018±0.006, 9.25±0.23, 0.11±0.017, 0.05±0.009 in the optimum (MeOH:H_2O) SE points, whereas they

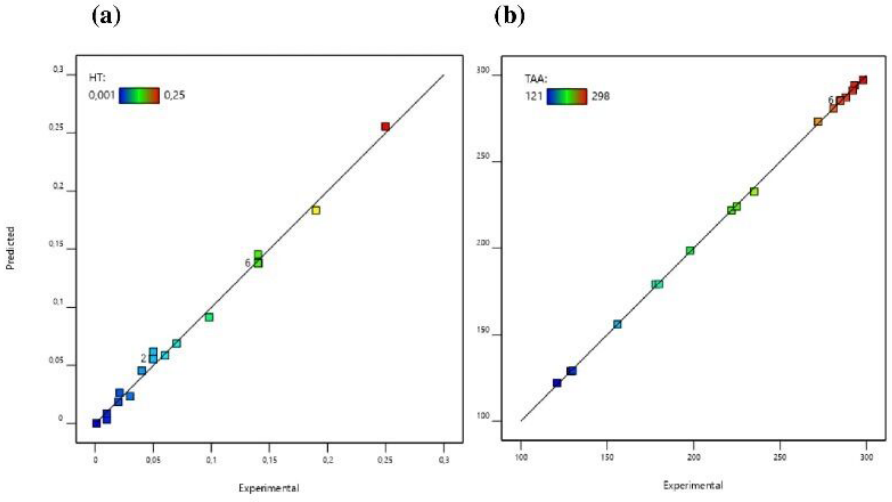

Figure 6. In EtOH solvent system comparison of experimental and predicted values of (a) HT (b) TAA.

were 1.28±0.04, 0.475±0.06, 4.89±0.07, 0.11±0.01, 0.07±0.006 mg/g dw in the optimum (EtOH:H_2O) SE points.

The findings of the studies on bioactive chemicals in OLLs were compared to the literature. Water, acetone, methanol, ethanol, and aqueous alcohol mixtures have been reported to be the solvents typically utilized in the extraction of polyphenols from olive leaves (Abaza et al. 2015). Our results are generally in agreement with previous findings, which indicated OLE to be the main phenolic component in both MeOH and EtOH hydroalcoholic extracts. Hayes et al. (2011) found six major phenolic compounds including OLE (1151.5±57.2 µg/ml), L7G (25.6±0.6µg/ml), A7G (15.9±0.7µg/ml), HT (10.2±0.1µg/ml) and Ty (15.6 ±0.1 µg/ml) in OLL-MeOH extract obtained by conventional extraction. OLE was the major component with 24.5% and followed by HT (1.5%), L7G (1.4%), verbascoside (1.1%), Ty (0.7%), A7G (1.4%) in olive leaf extract in the results of Benavente-Garcia et al. (2000). Similarly, the dominant phenolic and flavonoid components in OLL extracts were found to be OLE and L7G. Their levels were 65.57±0.70 g/kg and 1.32±0.03 g/kg for Soxhlet extraction, and 69.91±1.53 g/kg and 1.82±0.04 g/kg for UAE extraction, respectively. Wang et al. (2018) discovered several flavonoids in OLLs extracted by the UAE at optimum conditions using MeOH. A7G and Lut were found in concentrations ranging from 1.00±0.02–2.06±0.04 mg/g and 0.07±0.001–0.60±0.006 mg/g, respectively. In another study, Lut was determined as 1.42±0.04 mg/g in the OLL by SE using 50% MeOH (Haghi & Hatami 2010). Under ideal circumstances, OLE, A7G, and L7G were determined to be 2610±632 mg/kg, 1072±38 mg/kg, 970±43 mg/kg, respectively, employing a dynamic ultrasound-assisted method using an EtOH-H_2O combination as an extractant (Japón-Luján et al. 2006). Similarly, Xie et al. (2015) suggested that a 75% EtOH combination may be an appropriate solvent for extracting OLE. Under ideal conditions, it was recovered at 7.08 ± 0.05% by ultrasound-assisted and reduced-pressure extraction (URPE) from OLL. Unlike in other searches, OLE was not determined to be the dominant form; instead, the ideally obtained extract had substantial amounts of luteolin 7-O-glucoside and apigenin 7-O-rutinoside. This discrepancy has been attributed to varietal diversity and environmental factors (Mylonaki et al. 2008). Another factor influencing the extraction of phenols is the solvent's variable polarity and solubility (Mohamed & Khan 2013). When EtOH extracts are compared to water extracts, the flavonoid concentration of ethanol extracts is shown to be greater (Quirantes-Piné et al. 2013, Herrero et al. 2011). Water, methanol, and ethanol were tested as solvents by Sifaoui et al. (2016) who found that the methanolic extract had the greatest extraction yield while the aqueous extract had the lowest. EtOH provided a lower concentration of antioxidant phenolics than methanol. In our study, high amount of HT and OLE were quantified in MeOH aqueous extract compared to EtOH aqueous extract which were also in accordance with TAA results higher in MeOH:H_2O extracts. However, more phenolic compounds (secoiridoids, simple phenols, phenolic acids, and flavonoids) should be identified in order to assess the components' synergistic effects on the extract's total antioxidant activity.

CONCLUSIONS

Olive leaf is a byproduct of olive processing that is widely known for its numerous health advantages, including its high antioxidant activity. The extraction procedure was modeled and optimized using chemometric approaches to acquire the highest amount of HT and TAA in olive leaf extract. Under RSM, a Box-Wilson-CCD/

small factorial design was employed for the extraction's DOE. MOO was solved using Pareto solutions, and DF was used to find the ideal input variable values. Under optimal conditions with MeOH:H_2O and EtOH:H2O solvent systems, the maximum quantity of HT and TAA was extracted from OLLs. The MeOH:H_2O solvent solution determined higher HT and TAA levels at the optimal point. Furthermore, OLE was shown to be the major component in both extracts, with a greater concentration in MEOH:H_2O extracts. In addition, a simple and reliable HPLC method for the isolation and quantification of Ty, L7G, OLE, A7G, and Lut was developed and validated. The obtained results could be a promising alternative for the valorization of olive leaves, which are a low-cost source of natural antioxidants. By carefully selecting the process conditions, it is possible to produce olive leaf extracts with strong antioxidant capacities for large-scale applications in the food, pharmaceutical, and cosmetics industries. It is critical to support the literature with experiments utilizing various extraction methods on various olive varietals in order to produce efficient, simple and low-cost procedures.

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