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OPTIMIZATION OF EXTRACTION OF PHENOLIC AND ANTIOXIDANT CONTENTS FROM OLIVE LEAVES USING COMPOSITE CENTRAL DESIGN

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ABSTRACT
Recently, the interest towards phenol compounds has progressed. Olive leaves have been used from the past as cure against several pathologies. Still, the extraction of the bioactive molecules from this plant material should be investigated. In this study, a Response surface methodology (RSM) was applied to predict the optimum conditions for extraction of phenolic and antioxidant compounds from olive leaves. Two independent variables, solvent composition and temperature, were investigated. The experimental data were fitted to a second-order polynomial equation. Optimal extraction conditions were as follows: solvent concentration of 80% for ethanol and 75% for methanol, extraction temperature of 40°C for ethanolic extract and 50°C for methanolic extract. Under these conditions, the experimental yield of extracted polyphenols was 51 mg EAG g^{-1} dp and 56 mg EAG g^{-1} dp as for the experimental antioxidant activity was 90% and 92% respectively for ethanolic and methanolic extracts.

KEY WORDS: RSM, Olive leaves, extraction, phenolic compounds, antioxidant activity.

1. INTRODUCTION

Recently, many human illnesses such as cancer, heart and cerebrovascular diseases are correlated to Reactive Oxygen Species (ROS). In fact, those molecules are generated from auto-oxidation and thermal oxidation of different metabolism like the lipids (Frang *et al*., 2002; Briante *et al*., 2003; Huang *et al*., 2005). A wide array of enzymatic and non-enzymatic endogenous antioxidant defense systems has been evolved to compensate for the generation of ROS (Sies, 1997). Recently, Common natural antioxidants include tocopherols, carotenoids, flavonoids, and polyphenols - compounds have received considerable attention especially for they function as chemo preventive agents against oxidative damage (Carrasco-Pancorbo *et al*., 2005; Perez-Bonilla *et al*., 2006). Among the plants rich in antioxidant compounds the olive tree (*Olea europeaea,* Oleaceae) has historically provided huge economic and dietary benefits to the Mediterranean basin (Japòn-Lujàn *et al*., 2006). In fact, olive oil as well as leaves have been used for medical purposes, and were introduced recently into the Pharmacopoea PhEur 5. Olive Leaf Extract (OLE) has also been used by native people of these areas in folk medicine to treat fever and other diseases such as malaria (Gucci *et al*., 1997; Fernández-Escobar *et al*., 1997; Ciafardini and Zullo; 2002). Recently, several works have confirmed the pharmacology effects of (OLE). Among those reports some have shown that (OLE) has the capacity to lower blood pressure in animals (Samuelsson, 1951) to increase blood flow in the coronary arteries (Zarzuelo *et al*., 1991) to relieve arrhythmia, and to prevent intestinal muscle spasms (Benavente-Garcia *et al*., 2000). Recent studies have focused on the olive leaves contents and their extraction as high-added value compounds (Da Silva *et al*., 2006; Japòn-Lujàn *et al*., 2006; Ranalli *et al*., 2006). Solvent extraction is a favorable process since heat sensitive materials can be recovered at low temperatures. In order to optimize the recovery of the bioactive compounds, the parameters affecting the extraction process should be investigated. This study aims to investigate the efficiency of extraction of polyphenols and antioxidants from olive leaves. Central Composite Design (CCD) was used to investigate the effects of two independent variables - namely solvent composition and temperature (°C) for maceration - on phenols and antioxidant content.

2. MATERIALS & METHODS

2.1. Samples

The Swebea Elgia variety was used to determine the experimental conditions for the phenolic and antioxidant extraction method.

2.2. Extraction protocol

The olive leaves collected during the maturing fruit season of 2009/2010 were ground to a fine powder using a mill.

1.2.1. Maceration

Each shell dry powder sample (0.5 g) with 18% of humidity was macerated with 5mL of extraction solvents in a capped glass tube on an agitating plate (IKA ®KS 130 basic, Chine) at a constant speed (0.03g) for 24 hours. The glass tube was wrapped in aluminum foil to prevent light degradation during extraction. Extraction solvents used were methanol, ethanol and distilled water. Afterwards, a rotary vacuum evaporator (Heidolph, Germany) set at

40°C was used in order to remove the solvents. In this experiment many solvents were used including ethanol and methanol, in both their pure and aqueous forms.

1.2.2. Experimental design used

A response surface methodology was used to determine the influence of two independent variables and the optimal conditions of phenolic and antioxidant activity extraction. Two different methods of extraction were performed: maceration and soxhlet. The effect of the variables solvent composition (X1) and temperature (X2) in phenol and antioxidant activity was investigated. Each variable were coded at five levels: -1.41, -1, 0, 1, 1.41.

The variables were coded according to the following equation:

$$Xi= (x_i - x_0)/ \Delta x_i$$

Where xi is the (dimensionless) coded value of the variable Xi, X0 is the value of Xi at the centre point, and ΔX is the step change. The coded and uncoded (actual) levels of the independent variables are given in Table 1.

TABLE 1: Uncoded and coded levels of independent variables used in the RSM design

Symbols	Independent variables	Coded levels				
		-1,41	-1	0	1	1,41
X_1	Solvent proportion (%)	60	66	80	94	100
X_2	Temperature (°C)	20	26	40	54	60

Phenol yield (Y1) and percentage of inhibition DPPH (Y2) were taken as the response of the design experiments. A central composite design (CCD) was constructed to allow for the fitting of the second-order model. Ten experiments, each with two replications, were carried out at the center points to calculate the pure error. Once the experiments were performed, the response variable (extraction yield) was fitted to a second-order model in order to correlate the response variable to the independent variable. The general form of the second-order polynomial equation is as follows:

$$Y= \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j + \xi$$

Where Y is the response (dependent variable); β_0 the constant coefficient; βi, βii and βij the coefficients for the linear, quadratic and interaction effects, respectively; Xi and Xj the factors (independent variables); and ξ the error.

According to the analysis of variance, the regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were then used to make statistical calculations to generate dimensional and contour maps from the regression models. Design-Expert (v.8.0.2 trial, Stat-Ease, Inc., Minneapolis, USA) software was used to analyze the experimental data. P values of less than 0.05 were considered to be statistically significant.

1.3. Determination of total polyphenol contents

Total quantity of polyphenols of the olive leaf extract was determined using the Folin–Ciocalteu procedure (Hagerman *et al*., 2000; Amrani *et al*., 2009). 1 ml of diluted extract (at a dilution of 1:50 in distillated water) was mixed with 0.5 ml of Folin–Ciocalteu reagent. 1.25 ml of 200 g kg^{-1} aqueous sodium carbonate solution was added to the mixture after 5 minutes. Samples were then shaken in a vortex mixture and incubated at 30°C for 90 minutes. The absorbance of blue colored mixtures was recorded at 725 nm against a blank containing 1 ml of water, 0.5 ml of Folin–Ciocalteu reagent and 1.25 ml of aqueous sodium carbonate solution (200g kg^{-1}). The amount of total polyphenols was expressed as mg Equivalent Gallic Acid g^{-1} dry powder. For the gallic acid, the curve absorbance versus concentrations is described by the equation y = 43,48x - 0,036 (R^2 = 0,997). All measurements were done in triplicate.

1.4. DPPH radical scavenging activity

The diphenyl-1-picrylhydrazyl (DPPH) assay was performed according to the method of Brand-Williams *et al.* (1995) with a few modifications. 0.5 ml of extract solution at different concentrations made in methanol was added to 0.5 ml of DPPH solution (0.07 mg ml^{-1} in methanol) and mixed vigorously. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a Cary 50 UV–vis spectrophotometer (Varian, Inc., CA, USA) with methanol as a blank. BHT was used as positive control. The level of percentage scavenging of DPPH by the extracts was calculated according to the following equation:

$$\% \text{ Radical scavenging} = ((AC-A)/AC)\times 100$$

Where A_C is the absorbance of the control and A is the absorbance of sample.

3. RESULTS & DISCUSSION

Response surface methodology (RSM) has been used first to reduce the number of experimental trials and as result to decrease the experiment cost and secondly to study the effect of multiple parameters and the interaction between them. As an effective statistical technique for optimizing complex processes, RSM has been used frequently in order to optimize the extraction of several molecules such as protein (Ahmad *et al*., 2012), polysaccharides (Wang *et al*., 2012), antioxidant (Hossain *et al*., 2011), phenols compounds (Stévigny *et al*., 2007). In this study, RSM was applied to optimize the extraction of phenol and antioxidant compounds from olive leaves.

3.1. Fitting the models

3.1.1. Maceration with ethanol

The yield of total phenol compounds (Y1) and antioxidant activity (Y2) in ethanolic olive leaf extracts obtained from all the experiments are listed in Table 2. The experimental data was used to calculate the coefficients of the second-order polynomial equation and Table 4 summarizes the regression coefficients and results of the ANOVA- the significance of the coefficients of the models. For any of the terms in the model, a large regression coefficient and a small *p-value* to compare the *F value* would indicate a more significant effect on the respective response variables. As for "Lack of Fit F-value" there are a 31.64% and 17.40% respectively for Y1 and Y2 chance that a "Lack of Fit F-value" this large could occur due ANOVA showed that the resultant second-order polynomial model adequately represented the experimental data with the coefficient of multiple determinations (R^2) of 0.983 and 0.960 for the response of total phenolic compound yield and antioxidant activity, respectively. Values of "*p*" less than 0.05 indicate that the model terms are significant. In this case A, B, A^2, B^2 are significant model terms.

TABLE 2: Experimental design and responses of the dependent variables to the ethanolic extract parameters

Run order	EtOH	Temperature	Total phenolic (TP) mg GAE g^{-1} dm	Antioxidant activity (AA) %
	X_1%	X_2°C	(Y_1)	(Y_2)
1	66	26	24.10	63.00
2	94	26	35.52	73.28
3	66	54	28.00	71.00
4	94	54	43.17	78.00
5	60	40	25.11	66.90
6	100	40	39.59	77.00
7	80	20	34.00	74.58
8	80	60	38.78	80.00
9	80	40	50.17	89.00
10	80	40	51.50	90.00

3.1.2. Maceration with methanol

The value of responses (extraction yield of phenolic compounds (Y1) and antioxidant activity (Y2)) at different experimental combinations for coded variables is given in Table 3. ANOVA showed that the resultant second-order polynomial model adequately represented the experimental data with the coefficient of multiple determinations (R^2) of 0.991 and 0.996 for the response of total phenolic compound yield and antioxidant activity, respectively. The Model F-values of 85.77 and 178.03 respectively for Y1 and Y2 implies that the model is significant. There is only a 0.04% and 0.01% chance that a "Model F-Value" this large could occur due to noise.

TABLE 3. Experimental design and responses of the dependent variables to the methanolic extract parameters

Run order	MetOH	Temperature	TP	AA
	X1(%)	X2°C	(Y1) mg GAE g^{-1} dm	(Y2) %
1	65.86	25.86	24.33	60.34
2	94.14	25.86	32.19	64.90
3	65.86	54.14	39.64	75.19
4	94.14	54.14	42.92	79.30
5	60.00	40.00	22.74	58.23
6	100.00	40.00	30.00	67.12
7	80.00	20.00	28.00	65.39
8	80.00	60.00	52.00	90.11
9	80.00	40.00	55.00	90.24
10	80.00	40.00	56.00	89.50

3.2. Response surface analysis of total phenolic compound

Response surface analysis (RSA) of the data in Tables 2 and 3 demonstrates that the relationship between the total phenolic compound and extraction parameters is quadratic with good regression coefficients 0.983 and 0.991, respectively, for ethanol and methanol. By applying multiple regression analysis on the experimental data, the response variables and the test variables are related by the following second-order polynomial equations:

$$Y_{ethanol\ solvent} = 50.835 + 5.884\ x_1 + 2.289\ x_2 - 9.661\ x_{11} - 7.641\ x_{22}$$

$$Y_{methanol\ solvent} = 55.500 + 2.676\ x_1 + 7.498\ x_2 - 14.169\ x_{11} - 7.354\ x_{22}$$

The regression analysis for both responses indicated that the results were highly significant (P <0.001), which suggests that they adequately explain the responses observed.

The results of the analysis of variance, goodness-of-fit and the adequacy of the models are summarized in Table 6. The determination coefficients for ethanolic extracts (R^2 = 0.983) and for methanolic extracts (0.991) indicate that only 2.0% for ethanolic extract and 1.0 % for methanolic extract of the total variations was not

explained by the model. The values of the adjusted determination coefficients (Adj R^2 = 0.995; Adj R^2=0.985) also confirmed that the model was highly significant.

TABLE 4: Analysis of variance of the experimental results of the Ethanolic extraction

	Phenol content					Antioxidant activity				
Variables	SS[a]	DF[b]	MS[c]	*F*-Value	*p*	SS[a]	DF[b]	MS[c]	*F*-Value	*p*
Model	817.60	5	163.52	46.66	0.0012	644.87	5	128.97	19.40	0.0066
A	276.92	1	276.92	79.03	0.0009	124.53	1	124.53	18.73	0.0124
B	41.91	1	41.91	11.96	0.0259	51.94	1	51.94	7.81	0.0491
A B	3.52	1	3.52	1.00	0.3732	2.69	1	2.69	0.40	0.5593
A^2	426.64	1	426.64	121.75	0.0004	421.30	1	421.30	63.36	0.0013
B^2	266.88	1	266.88	76.16	0.0009[a]	219.54	1	6.65	33.02	0.0045
Residual	14.02	4	3.50	-	-	26.60	4	8.70	-	-
Lack of fit	13.13	3	4.38	4.95	0.3164	26.10	3	0.50	17.40	0.1740
Pure error	0.88	1	0.88	-	-	0.50	1	-	-	
Correlation total	831.62	9	-	-	-	671.46	9	-	-	

a Sum of squares.
b Degree of freedom.
c Mean square.
A Means significance (values of p less than 0.05).

TABLE 5: Analysis of variance of the experimental results of the Methanolic extraction

	Phenol content					Antioxidant activity				
Variables	SS[a]	DF[b]	MS[c]	*F*-Value	*p*	SS[a]	DF[b]	MS[c]	*F*-Value	*p*
Model	1439.17	5	287.83	85.77	0.0004	1426.17	5	285.23	178.03	<0.0001
A	57.28	1	57.28	17.07	0.0145	56.40	1	56.40	35.20	0.0040
B	449.72	1	449.72	134.01	0.0003	515.36	1	515.36	321.66	<0.0001
A B	5.24	1	5.24	1.56	0.2794	0.051	1	0.051	0.032	0.8676
A^2	917.73	1	917.73	273.48	<0.0001	853.95	1	835.95	532.99	<0.0001
B^2	247.21	1	247.21	73.67	0.0010	171.78	1	171.78	107.22	0.0005
Residual	13.42	4	3.36	-	-	6.41	4	1.60	-	-
Lack of fit	12.92	3	4.31	8.62	0.2442	6.13	3	2.04	7.47	0.2613
Pure error	0.50	1	0.50	-	-	0.27	1	0.27	-	-
Correlation total	1452.59	9	-	-	-	1432.58	9	-	-	

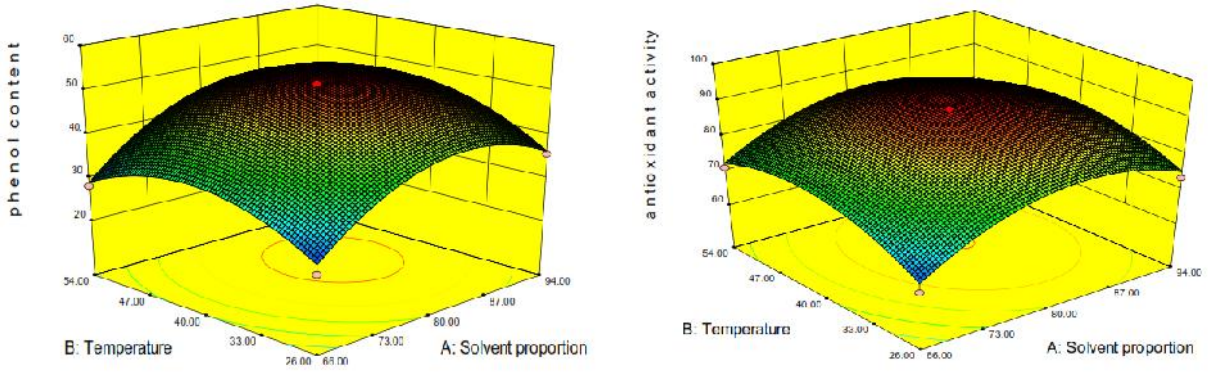
a Sum of squares.
b Degree of freedom.
c Mean square.
A Means significance (values of p less than 0.05).

TABLE 6: Analysis of variance for the fitted quadratic polynomial model of phenol and antioxidant extraction by both solvent ethanol and methanol

Item	Solvent	Responses	S.D.	Mean	C.V.%	Press	R^2	R^2_{Adj}	R^2_{Pred}	Adeq. precision
Value	Ethanol	Phenol yield	1.87	36.99	5.06	96.92	0.9831	0.9621	0.8835	19.063
		Antioxidant activity	2.58	76.28	3.38	187.57	0.9604	0.9109	0.7206	12.406
	Methanol	Phenol yield	1.83	38.28	4.79	93.90	0.9908	0.9792	0.9354	23.144
		Antioxidant activity	1.27	74.03	1.71	44.72	0.9955	0.9899	0.9688	31.710

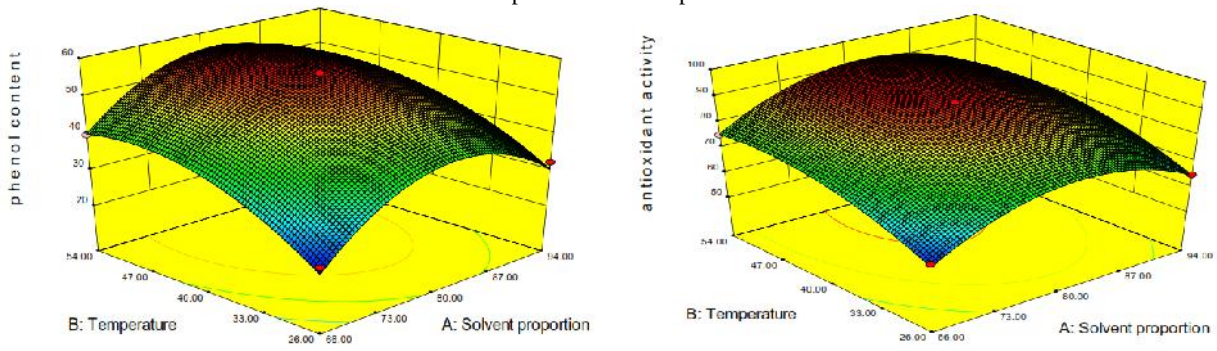
Fig 1(a) and 2(a) is a response surface plot showing the effect of solvent concentration and temperature extraction on the total phenol content. Both variables were shown as a positive linear and a negative quadratic effect on the phenols content. The total of phenols extract first increased and then decreased when the variables increased, revealing that a medium concentration of solvent (ethanol or methanol) and a moderate temperature were favorable for extracting phenols from olive leaves. The optimal condition for ethanolic maceration determined by RSA was at an ethanol concentration of 80% and a temperature of 45°C with a maximal yield of total phenolic compounds of 51mg Equivalent Gallic Acid (EGA) g^{-1} of dry powder (dp). Similar results were observed by Altıok *et al.* (2008), the high content of phenolic content was obtained using 70% ethanol as extraction solvent. For methanolic maceration best results were yielded with a methanolic concentration of 78% and temperature of 50°C with a maximal yield of total phenolic compounds of 56 mg EGA g^{-1} of dry powder. The solvents used in this study had a clear ability to extract phenolic substances from olive leaves. The results obtained showed that 78% methanol was the more adequate extraction solvent. This return was in accordance with previous studies which reported that binary-solvent (alcohol-water) systems were more favorable in the extraction of phenolic compounds from plant samples as compared to mono-solvent systems (pure alcohol). This

ascertainment could be explained by the rising of solvent polarity with water addition. As a result, the polar constituents would be extracted more easily (Lang and Wai, 2001; Spigno *et al*., 2007; Chew *et al*., 2011). It has been shown in previous studies that extraction of phenolic compounds from a sample is directly related to the compatibility of the compounds with the solvent system according to the ‘‘like dissolves like” principle (Zhang *et al*., 2008).



a **b**

FIGURE 1: Response surface for total phenolics (TP) (a) and antioxidant activity (AA) (b) , as function of ethanol composition and temperature



a **b**

FIGURE 2: Response surface for total phenolics (TP) (a) and antioxidant activity (AA) (b) , as function of methanol composition and temperature

Heat has been found to enhance the recovery of phenolic compounds as described by Durling *et al.* (2007) and Silva *et al.* (2006) Increased temperature promotes solvent extraction by enhancing both diffusion coefficients and the solubility of polyphenol content (Amrani, 2009). However, the negative quadratic effects of extraction temperature on phenol content show the thermo-sensitivity of some compounds in these extracts. Actually, it should be noted that enhancing the temperature beyond certain values could lead to solvent evaporation increasing therefore the industrial extraction cost. High temperatures could also decompose phenolic compounds already extracted or even involve the breakdown of these molecules still remaining in the plant matrix (Chan *et al*., 2009; Saha *et al*., 2011).

3.3. Response surface analysis of antioxidant activity

Response surface analysis (RSA) of the data in Tables 2 and 3 demonstrates that the relationship between the antioxidant activity and extraction parameters is quadratic with good regression coefficients of 0.996 and 0.993, respectively, for ethanol and methanol. By applying multiple regression analysis on the experimental data, the response variables and the test variables are related by the following second-order polynomial equations:

$$Y_{ethanol} = 89.500 + 3.945\ x_1 + 2.548\ x_2 - 9.600\ x_{11} - 6.930\ x_{22}$$

$$Y_{methanol} = 89.870 + 2.655\ x_1 + 8.026\ x_2 - 13.668\ x_{11} - 6.130\ x_{22}$$

Fig 1(b) and 2(b) is a response surface plot showing the effect of solvent concentration and temperature extraction on the antioxidant activity. Fig 1(b) and 2 (b) shows that the DPPH scavenging activities of antioxidants significantly increased with the increase in extraction temperatures from 20°C to 45 °C ($P < 0.05$) to reach a value of 90% for ethanol and 92% for methanol which was similar to the trends of phenol contents. Beyond a temperature of 55°C the antioxidant activity decreases as shown by the negative quadratic effect of the temperature on the responses. This decrease can be attributed to the decrease of phenol content in the extracts. From a holistic view of the results of the different assays, it can be concluded that the results of total phenol content and DPPH revealed a very good correlation.

Many authors agree with the fact that an increase in the working temperature favors extraction by enhancing both the solubility of solute and the diffusion coefficient, but that beyond a certain value phenolic compounds can be denatur (Juntachote *et al*., 2006; De Faveri *et al*., 2008).

Nevertheless, it is well known that extraction of phenolic compounds from herbs and spices is dependent on others factors such as the method and time of extraction, particle size and solvent to herb/spice ratio. Accordingly, Taamalli *et al*. (2012) reported that pressurized liquid extraction (PLE) was the suitable method to extract phenol compounds from olive leaves among different method; however the phenolic profiles were mainly influenced by the solvent. For nowadays, the use of supercritical method, to extract olive leaf phenol molecules, has widely increased as alternative of conventional solvent however, an optimization could be necessary (Xynos *et al*., 2012).

3.4. Verification of predicted extraction parameters

The suitability of the model equation for predicting the optimum response values was verified using the optimal condition. The experimental value (Table 7) was 51.23 and mg Equivalent Gallic Acid (EGA) g^{-1} for the yield of total phenolic compounds - and 89% - 90% for antioxidant activity of ethanol and methanol extracts, respectively - which were close to the values predicted by the regression models.

TABLE 7. Regression coefficients of the predicted qudratic model for the response variables, total phenolics (TP) and antioxidant activity (AA)

	Ethanol		Methanol	
Model parameters	TP	AA	TP	AA
Intercept	50.835	89.500	55.500	89.870
Linear				
A	5.884	3.945	2.676	2.655
B	2.289	2.55	7.498	8.026
Quadratic				
A^2	-9.661	-9.600	-14.169	-13.668
B^2	-7.641	-6.930	-7.354	-6.130
Interaction				
AB	0.983	0.820	-1.145	-0.113

TABLE 8: Optimum conditions, predicted and experimental value of response at that condition

Optimum condition			TP mg GAE g^{-1} dm		AA	
Solvent	Solvent proportion	Temperature	Experimental	Predicted	Experimental	Predicted
Ethanol	80 %	40°C	51.23± 1.25	49.05	89%± 0.60	86.42
Methanol	75 %	50°C	55.25± 2.32	51.44	90% ± 1.65	86.65

[a]Mean ± standard deviation (N)

4. CONCLUSION

In the present study, an RSM was applied to describe and predict the extraction process of bioactive compounds from olive leaves. The variables chosen, namely solvent concentration and extraction temperature, all have a positive linear influence and negative quadratic effect on phenol content and antioxidant activity. The optimal conditions obtained by RSM for production of phenols content and antioxidant activity include the following parameters: solvent concentration of 80% for ethanol and 75% for methanol, extraction temperature of 40°C for ethanolic extract and 50°C for methanolic extract.

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