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Optimization of the extraction of phenolic compounds and antioxidant activity from the roots of *Waltheria ovata* using the response surface methodology

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Abstract

Waltheria ovata is a medicinal plant belonging to the *Sterculiaceae* genus. Natural products of *Waltheria ovata* could be used in the food industry as natural antioxidants due to its high content of polyphenols according to the literature. The main objective in this research was to optimize the extraction of phenolic compounds and the antioxidant activity from *Waltheria ovata* roots using response surface methodology (RSM). The total phenolic content in different extracts was determined by spectrophotometric method (Folin-Ciocalteu reagent) and the antioxidant activity by using DPPH assay. To optimize the conditions for total phenolic content and antioxidant activity were used three independent variables: solvent/sample ratio (1:10, 1:20 and 1:30 g/mL), temperature (40, 50, and 60°C) and time (40, 50 and 60 mins). The results showed that total phenolic content and antioxidant activity in the experiments ranged from 8.7 to 12.1 mg GAE/g and 76.1% to 96.7%, respectively. The coefficients of determination (R^2 values) for phenolic content and antioxidant activity were 0.86 and 0.91, respectively. Under the optimum conditions of 1:20 g/mL, 60°C and 55 mins of extraction, the values for total phenolic content and antioxidant activity were 0.448±0.02 mg GAE/g and 87.00±2.0%, respectively. These data showed that the experimental responses were reasonably close to the predicted responses (0.444 mg GAE/g and 84.67%). Therefore, the results showed that *Waltheria ovata* can be used as antioxidant in foods.

1. Introduction

Natural antioxidants obtained from plant materials (flowers, leaves, roots, fruits and other parts) by different extraction techniques are mainly polyphenols (anthocyanins, phenolic acids, flavonoids, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin A, E and C) and other compounds (Pisoschi *et al*., 2016). Currently, there has been an increase of new methods to study the mechanisms of natural antioxidants from plant-based extracts or to measure its activity for use in the food, cosmetics and pharmaceutical industries (*Xu et al*., 2017).

Phytomedicine appears as a good alternative in modern times, where different types of chronic diseases such as cancer, diabetes, cardiovascular diseases, among others, are increasing day by day. In the traditional medicine from Ica, Peru, *Waltheria ovata* Cav. (Family: Sterculiaceae) is considered a rich source of antioxidants and other phytochemicals that have been reported to overcome some of the degenerative diseases that affect humans (Herrera-Calderon *et al*., 2016). *Waltheria ovata* is a shrub with open foliage. It has wrinkled pale bluish-green leaves. The stems are dark red and flowers are small of yellow color. It is known as "lucraco" and roots are used in the treatment of diarrhea, pain, inflammation, and spasm (Herrera-Calderon *et al*., 2018). In addition, *Waltheria ovata* exhibits anti-carcinogenic, anti-bacterial, antioxidant, analgesic and anti-inflammatory properties (Bussmann and Glenn, 2010). In traditional medicine, infusion of leaves and flowers is used to combat pathology conditions respiratory like cold and cough. Among the inhabitants of Ica, its use is very widespread lucraco root cooking, to combat inflammation of the prostate.

The total phenolic content and antioxidant activity of ethanolic extract of lucraco can be affected by many factors including solvent, solvent/sample ratio,

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temperature and extraction time. Under different conditions in the laboratory, where multiple variables may influence the effect of tested products, response surface methodology (RSM) is an effective technique to determine the most favorable conditions of the independent variables in view of a profitable process (Lee *et al*., 2013).

RSM was published by Box and Wilson in 1951 as a topic of chemical engineering (Şenaras, 2019). Generally, RSM is used to determine different factors that influence response and their interaction (Uysal *et al*., 2017; Kaleem *et al*., 2019). The advantages of using RSM are the evaluation of the effects of certain process variables and their interaction on response variables, less laboriousness, more speed, less cost, less number of required experiments and less time consumption. Furthermore, in many articles, RSM linked to foods and biotechnological processes proved to be a helpful tool, which allows process optimization to be conducted effectively (Bassani *et al*., 2014; Hou *et al*., 2016).

The objective of the present work was to optimize the phenolic content and antioxidant activity under three factors such as solvent/sample ratio, temperature and extraction time of *Waltheria ovata* roots using the RSM.

2. Materials and methods

2.1 Plant material

W. ovata roots were collected, in April 2018 from *Los Aquijes* district, Ica, Peru. Authentication of the specimen was preserved (51-USM-2015), which was deposited at the Museum of Natural History of the Universidad Nacional Mayor de San Marcos, Lima, Peru.

2.2 Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), Folin-Ciocalteu (FC) reagent, gallic acid, from Sigma-Aldrich (St. Louis, MO, USA) were used. Analytical grade ethanol, sodium carbonate and other chemical reagents were purchased from Merck Peruana (Ate, Lima, Peru).

Waltheria ovata was converted to powder using an electric grinder, packaged in polyethylene bags and kept in a dark place at room temperature.

2.3 Extraction procedure

The ethanolic extract of the root powder was obtained under different treatments of the central compound rotatable design for three independent variables (Table 1).

The different extracts soaked with 96% ethanol were filtered through Whatman No. 1 filter paper and then centrifuged at 10,000 rpm for 10 mins at 4°C until further use (Fattahi and Rahimi, 2016).

Table 1. Independent variable levels in experimental design for response surface analysis

Symbol	Independent variable	Factor level				
		-1.68	-1	0	1	1.68
X_1	Extraction time (min)	33	40	50	60	67
X_2	Solid/liquid ratio (g/mL)	1 : 03	1 : 10	1 : 20	1 : 30	1 : 37
X_3	Temperature (°C)	33	40	50	60	67

2.4 Total polyphenols content

TPC was estimated by spectrophotometric technique (Ainsworth and Gillespie, 2007). Thus, 300 µL of sample extract was put into three tubes. Then, 1800 µL distilled water was added to each test tube, and 450 µL of eight-time diluted Folin-Ciocalteu reagent was added and allowed to stand for 5 mins at room temperature. Finally, 450 µL of 20% (w/v) sodium carbonate was added to react for 60 mins. The absorbance was estimated at 760 nm with UV-VIS spectrophotometer. A standard curve of gallic acid (1–7.5 µg/mL) was used to measure the TPC and was expressed as gallic acid equivalent milligram per gram of dry extract (mg GAE/g).

2.5 Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of *Waltheria ovata* was determined according to the method developed by Aguilar-Felices *et al*. (2019). Antioxidant activity (AA) was expressed as percent of inhibition of DPPH radical (%).

$$\text{Antioxidant activity (\%)} = [(A0 - A1) / A0] \times 100$$

Where A0 is the absorbance of the control reaction (DPPH) and A1 is the absorbance of the sample, corrected by the absorbance of the sample (blank).

2.6 Statistical optimization

2.6.1 Experiments one factor at a time

To evaluate the effect of each factor under extraction treatment on TPC and antioxidant activity of the extract from *W. ovata* roots, time (40, 50 and 60 mins), material/solvent ratio (1: 10; 1: 20; 1: 30 g/mL), and temperature (40, 50, and 60°C) were investigated as single factor experiments.

2.6.2 Experimental design

Response surface methodology was used to find the optimal condition of extraction. The three variables

mentioned in the previous section were coded as X_1-X_3 according to Equation (1) and examined at five levels (Table 1).

$$x_i = \frac{2 \cdot (X_i - X_{icp})}{\Delta X_i} \quad (1)$$

where xi is the coded level of the ith variable, Xi is the level of the ith natural variable, Xicp is the ith natural variable at the center point, and ΔXi is the step change value of the ith natural variable.

Table 2 presents the matrix of the three-variable central composite rotatable design with twenty experimental runs that were performed in a random order, including five genuine replicas at the center point.

The behavior of the analyzed responses is described by the following quadratic polynomial model:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum \sum \beta_{ij} x_i x_j \quad (2)$$

where Y is the response value; β_0 is a constant; βi is the linear regression coefficient; βii is the quadratic regression coefficient; βij is the interaction regression coefficient; xi and xj are the independent variables encoded.

2.6.3 Statistical analysis

The regression coefficients of the model were obtained by a multiple regression analysis on the experimental data using the least-squares method. The data analyses were performed using Statistica® (software system, StatSoft, Tulsa, USA), version 10.0. Differences were considered significant at p-values < 0.05.

3. Results and discussion

The results of the central compound rotatable design to optimize the extraction process for the content of phenolic compounds and antioxidant activity of *Waltheria ovata* roots are presented in Table 2.

Figure 1 shows the Pareto chart for each response with all possible standardized effects at a 0.05 level of significance.

The effect of the variables (temperature, time and solid/liquid ratio) and their interactions are represented in Pareto charts. When the histograms which represent each variable cross the vertical line, they are considered as significant. According to Figure 1(a), total phenolic content is positively affected by temperature and time but negatively influenced by the quadratic effect of solid /liquid ratio. In Figure 1(b), antioxidant activity is positively affected by temperature, time and solid /liquid ratio and negatively influenced by the interaction between the variable: time and solid/liquid ratio.

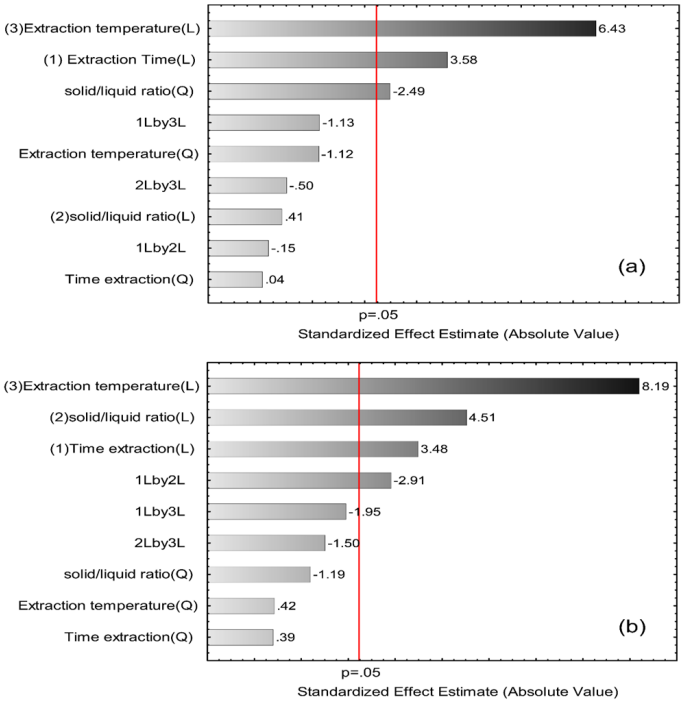

Figure 1. Pareto chart of the standardized effects for (a) total phenolic content (TPC) and (b) antioxidant activity (DPPH assay).

This implies that high levels of temperature and time favored both responses because they are variables that improve extraction yield in the leaching process. Meanwhile, the solid/liquid ratio had different effects on the responses. The quadratic effect for the total phenolic content implied that a concave surface was generated as a function of this variable (Figures 2(a) and (b)). Thus, this response was favored up to the level of the central point, and higher values of this variable decreased the total phenolic content. This is evident that the leaching of these compounds occurred with the increase in the dry matter of the roots until saturation. Then, a higher solid content generated diffusion restrictions for the leaching of phenolic compounds. In contrast, the high level of the solid/liquid ratio favored antioxidant activity. This phenomenon means that other compounds with antioxidant activity than phenolic compounds were extracted under these conditions, implying that the extraction conditions can modulate the isolation of fractions of antioxidant compounds.

An analysis of variance (ANOVA) for each reduced model was required to confirm the results shown in Figure 1 and the adequacy of the model. These results are presented in Tables 3 and 4. As can be seen, the data provided are consistent with the Pareto charts.

F and p values give the significance for the coefficient terms of the model. Thus, the largest F-value and the smallest p-value imply that the term is most

Table 2. Matrix of the central composite rotatable design and its experimental values in the total phenolic content (TPC) and antioxidant activity (AA)

Run	Factors			Experimental values		Predicted values	
	X_1	X_2	X_3	TPC (mg GAE/g)	AA (%)	TPC (mg GAE/g)	AA (%)
1	-1	-1	-1	0.242±0.01	45.50±1.5	0.242	44.56
2	-1	-1	1	0.419±0.01	78.00±1.0	0.384	74.87
3	-1	1	-1	0.239±0.02	70.00±2.0	0.242	69.07
4	-1	1	1	0.368±0.02	83.00±1.0	0.384	90.02
5	1	-1	-1	0.346±0.01	71.00±1.2	0.332	68
6	1	-1	1	0.445±0.01	83.21±1.5	0.422	86.16
7	1	1	-1	0.310±0.01	69.23±2.0	0.332	74.38
8	1	1	1	0.411±0.01	80.23±2.0	0.422	83.19
9	-1.68	0	0	0.318±0.03	67.23±1.0	0.323	66.8
10	1.68	0	0	0.433±0.03	85.98±1.5	0.431	80.76
11	0	-1.68	0	0.261±0.02	57.50±1.5	0.315	64.73
12	0	1.68	0	0.365±0.01	85.30±2.0	0.315	82.83
13	0	0	-1.68	0.263±0.01	58.00±2.0	0.251	57.34
14	0	0	1.68	0.431±0.02	95.36±2.0	0.446	90.22
15	0	0	0	0.381±0.03	75.01±1.5	0.377	73.78
16	0	0	0	0.382±0.02	75.32±1.0	0.377	73.78
17	0	0	0	0.384±0.02	76.00±1.0	0.377	73.78
18	0	0	0	0.404±0.02	75.20±1.0	0.377	73.78
19	0	0	0	0.379±0.03	73.00±2.0	0.377	73.78
20	0	0	0	0.332±0.01	69.52±1.0	0.377	73.78

Table 3. Analysis of variance of the quadratic model for optimization of total phenolic content from *W. ovata* roots

Factor	SS	df	MS	F-value	Probability-p[a]
Time extraction (L)	0.014106	1	0.014106	17.19021	0.000989
Solid/liquid ratio (Q)	0.006884	1	0.006884	8.38923	0.011725
Extraction temperature (L)	0.045495	1	0.045495	55.4424	0.000003
Extraction temperature (Q)	0.001392	1	0.001392	1.69659	0.213754
Time extraction (L) x Extraction Temp (L)	0.00141	1	0.00141	1.71806	0.211038
Error	0.011488	14	0.000821		
Lack of fit	0.008663	9	0.000963	1.70367	0.289208
Pure error	0.002825	5	0.000565		
Total sum of squares	0.080279	19			

$R^2 = 0.86$. F-value > $F_{0.05}$ (1, 14) tabular = 4.60. [a] Significant for p-values < 0.05.

Table 4. Analysis of variance of quadratic model for optimization of antioxidant activity from *W. ovata* roots by using DPPH assay

Factor	SS	df	MS	F-value	Probability-p[a]
Time extraction (L)	235.435	1	235.435	13.20106	0.003032
Solid/liquid ratio (L)	395.612	1	395.612	22.18234	0.000408
Extraction temperature (L)	1305.821	1	1305.821	73.21862	0.000001
Time extraction (L) x Solid/liquid ratio (L)	164.258	1	164.258	9.21009	0.009576
Time extraction (L) x extraction temperature (L)	73.751	1	73.751	4.13526	0.062936
Solid/liquid ratio (L) x Extraction temperature (L)	43.758	1	43.758	2.45355	0.141269
Error	231.849	13	17.835		
Lack of fit	202.577	8	25.322	4.3252	0.06175
Pure error	29.272	5	5.854		
Total sum of squares	2450.483	19			

$R^2 = 0.86$. F-value > $F_{0.05}$ (1, 14) tabular = 4.60. [a] Significant for p-values < 0.05.

significant to the model. Hence, the linear effects of X_1, X_2, and X_3 for the TPC and AA responses, the quadratic effect of X_2 for the TPC response, and the X_1X_2 interaction for the AA response had p-values less than 0.05, proposing that the model could be used to predict these responses.

Lack of fit gives the variation of the data around the fitted model. For the TPC model, the F and p values were 1.70 and 0.289, respectively. For the AA model, the F and p values were 4,325 and 0.0617, respectively. This implies that the lack of fit is not significant for both responses.

The goodness of the reduced model fit was verified by the coefficient of determination R^2, which were 0.86 and 0.91 for TPC and AA, respectively. Approximately, $100R^2$ = 86% and 91%, of the variability of the observed responses can be explained by the fitted models in the coded form of Eqs. (3) and (4), which were obtained by multiple regression on the experimental data using the least-squares method as shown below for TPC:

$$y = 0.377 + 0.032x_1 - 0.022x_2^2 + 0.058x_3 - 0.01x_3^2 - 0.013x_1x_3 \quad (3)$$

whilst for antioxidant activity (AA) the regression coefficients of the model are stated as:

$$y = 73.78 + 4.152x_1 + 5.382x_2 + 9.778x_3 - 4.531x_1x_2 - 3.036x_1x_3 - 2.339x_2x_3 \quad (4)$$

Note that; X_1: time extraction, X_2: solid/liquid ratio and X_3: temperature extraction

3D response surface plots help to understand the effect of the study variables on the response variable as shown in Figure 2. As can be seen, the increase in temperature in the range of 50 - 67°C favored the extraction of the phenolic compound content and antioxidant activity. However, a further increase in temperature could cause a decrease in the phenolic compound content and thus in antioxidant activity. Likewise, the extraction time between 60 and 70 mins increased the extraction of phenolic compounds and antioxidant activity. Figures 2(a) and (c) show that the material/ solvent ratio had a maximum response value at the central level for the phenolic compound content, while it had a linear effect for antioxidant activity. Therefore, the validation experiment was carried out under the experimental conditions that favored both responses as shown in Table 5. The results indicate a good prediction of the model in the experimental region.

The antioxidant activity of plants is generally attributed to phenolic compounds and their derivatives such as phenolic acids, flavonoids, isoflavonoids, flavanones, flavones, anthocyanins, among others. However, several factors play a critical role during the isolation of phenolic compounds with antioxidant activity from plant materials (Liyana-Pathirana and Shahidi, 2005; Ghafoor *et al*., 2009). Numerous reports confirm that aqueous mixtures of organic solvents are the most suitable for extraction of phenolic compounds from plant sources, in our study the solvent was 96% ethanol and showed a better phenolic content compared to a water-ethanol (3:7) extraction. Although, this can vary, depending on the plant material and type of species (Ilghami *et al*., 2015; Chen *et al*., 2018). Based on many reports and studies of the toxicity of solvents, ethanol was selected as the main solvent instead of methanol, propanol and other which is considered much safer to extract phytochemicals as antioxidants (Belwal *et al*., 2016; Wani *et al*., 2017).

A relation between extraction time and TPC was established, wherein the increase in time of exposure showed the highest TPC. The extraction temperature only influenced on the antioxidant capacity when it was determined by DPPH assay but in others such as ABTS and FRAP were not significant (Cacace and Mazza, 2003). The interaction between temperature and extraction time could be explained by considering that at higher temperatures, and more time in the extraction process, the ethanol has a higher capacity to solubilize polyphenols and surface tension and solvent viscosity decreases with temperature, which will improve the penetration into the matrix by the solvent used.

4. Conclusion

Extraction is the main stage in the recovery and isolation of polyphenols and other antioxidant compounds from various plant-based materials, but the effective extraction of these compounds depends on the appropriate selection of the levels of various physical and chemical variables that interact with each other. Thus, the response surface methodology is a tool to maximize the content of these compounds in the extraction of *W. ovata* roots. Experimental conditions for maximum extraction were as follows: 60°C, solid/ solvent ratio 1:20 g/mL, and time of 60 mins. Under these optimum conditions, TPC was 0.448±0.02

Table 5. Comparison between experimental and predicted values under optimum conditions for total phenolic content (TPC) and antioxidant activity (AA)

Response variable	Optimum conditions			Experimental values	Predicted values
	Time (min)	Solid/liquid ratio (g/mL)	Temperature (°C)		
TPC (mg GAE/g)	60	1:20	60	0.448±0.02	0.444
AA (%)	60	1:20	60	87.00±2.0	84.67

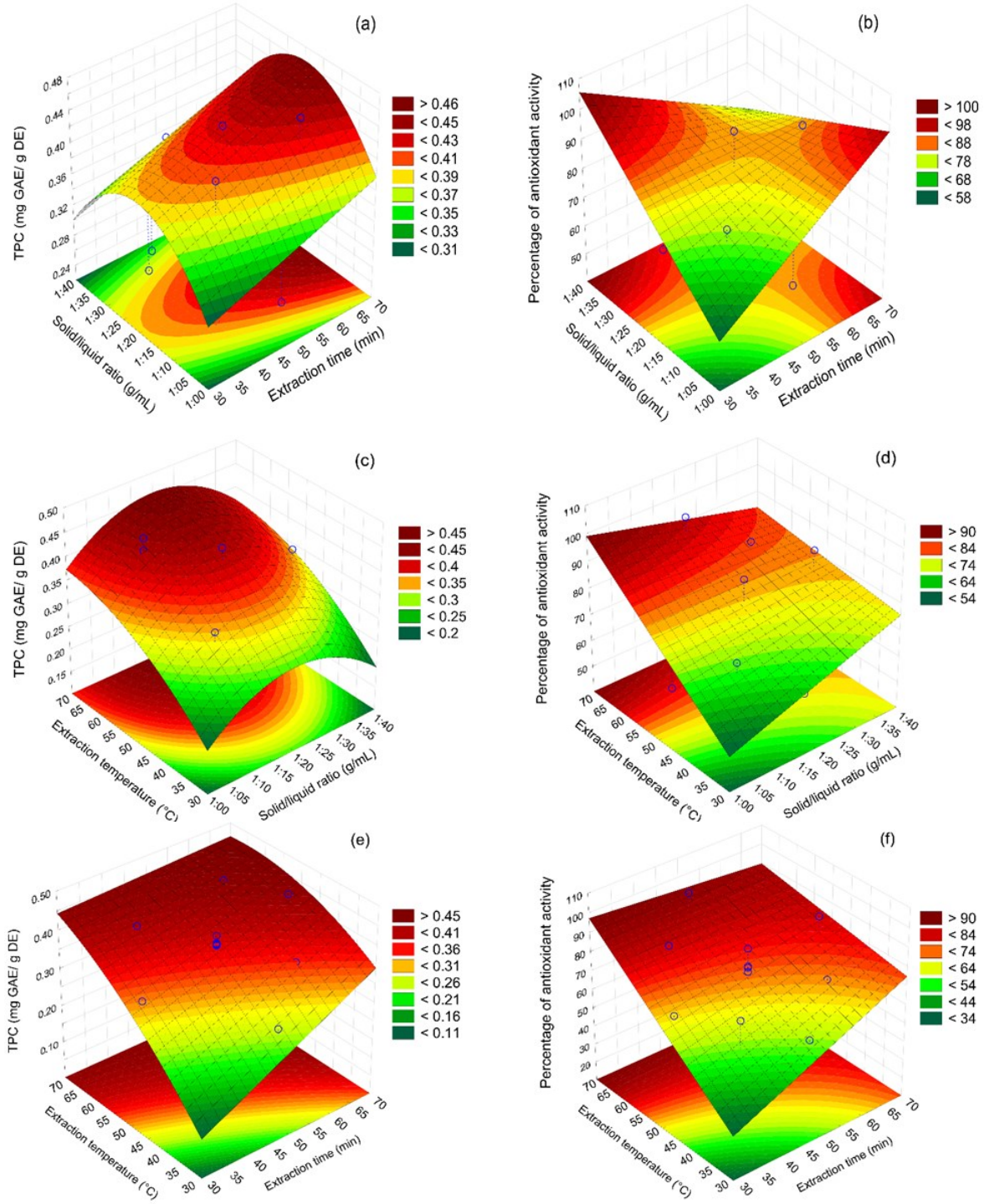

Figure 2. Response surfaces and contour plots for TPC: Total phenolic content (mg GAE/g) and AA: antioxidant activity (%). Extraction temperature was 60°C (Figures (a) and (b)). Extraction time was 60 mins (Figures (c) and (d)) and solid/liquid ratio was 1:20 (Figures (e) and (f))

mgGAE/g and DPPH radical scavenging activity was 87.00±2.0%. The results of the validation experiment are consistent with those predicted by the models.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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