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MODELING AND OPTIMIZATION OF BACs EXTRACTION FROM ALGERIAN *Rosmarinus Officinalis* L.VIA GREEN EXTRACTION

***Linda LOUCIF SEIAD, Soraya DEMIM**
Département de Chimie, Faculté des Sciences, UniversitéM'Hamed Bougara, Avenue de l'indépendance, 35000, Boumerdès, Algérie.
*loucifsiad_linda@univ-boumerdes.dz

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Abstract: *The aim of this study is to improve extraction of biologically active compounds (BACs) found in Rosmarinus officinalis* L. *from Algeria country using green extraction process. The effect of the main process variables (time, ethanol concentration, solid-to-liquid ratio) on maceration efficiency has been studied using Factorial Design (FD) to verify the single factors effects. Total Polyphenol Content (TPC) and Total Flavonoids Content (TFC) were measured to control the maceration efficiency in different experimental conditions. The iso-response curves and the response contours have been exploited. The best experimental results were 0.15 g/mL, 50 %, 1hour and 0.15 g/mL, 50 %, 72hours for TPC and TFC respectively. The values of TPC and TFC were 2.5 mg GAE/g DW and 1 mg QE/g DW respectively. These results were in perfect agreement with the expected theoretical model. The relationship between the TPC and TFC provides a high correlation coefficient (0.82).*

Keywords: *Rosmarinus officinalis* L., *BACs, green extraction, modelling.*

1. Introduction

Biologically active compounds (BACs) are known to have large properties. They can be used in food industry as antioxidants, coloring agents, preservatives, fortifiers, etc.[1-3]. BACs sources are essentially from Mediterranean aromatic plants [4], [5]. The targeted plant on this study, which is rosemary represent, one of them. It was reported that BACs products in rosemary have antifungal activity as well as antithrombotic, anti-inflammatory, antiulcerogenic and antidepressant effects [6]. According to the European Medicines Agency recommendations, rosemary essential oil (REO) can be used for many medical purposes. It can be used for peripheral circulatory disorders, alleviate muscular pain help, and dyspepsia [7]. It was also reported the use of rosemary extracts (RE) and REO for commercial purpose as a natural food biopreservative.
It is important to know that the antioxidant property is closely linked to phenolic acids and flavonoids [7-9]. They are the major classes of compounds with antioxidant activity among other vitamins (C and E). These natural antioxidants can be extracted from plants and substitute synthetic antioxidants, source of toxicity [5]. Many extraction techniques from plants are used: soxhlet extraction, maceration, ultrasound-assisted extraction, heated reflux extraction, accelerated supercritical fluid extraction and microwave-assisted extraction. Maceration technique is chosen because frequently it gives good results taken into account the principles of green chemistry.

Some parameters must be taken into consideration such as time, solid-to-liquid ratio, solvent, etc. and even the plant itself [10]. Optimizing the extraction technique in the BACs is the most important step. This is due principally to the plant matrices complexity and different physico-chemical properties of BACs. Another more rigorous aspect is the use of green chemistry. After having published the twelve principles of green chemistry and green engineering [11], [12], six new principles of green extraction were established [13]. Among these principles, alternative solvents were used (water or agro-solvents).

The present study investigates and discusses the valorization of Algerian plants *Rosmarinus officinalis* L. by improving the BACs extractions using an agro-solvent, taking into consideration the green extraction process. The lack of papers available about the plant used in this work has highly motivated this study. Factorial design (FD) was used to verify the single factors effect's (time, solvent concentration, and solid-to-liquid ratio) on extraction efficiency. TPC and TFC were measured to control the efficiency of maceration at different experimental conditions conducting to determine the interactions between those factors. The iso-response curves and the response contours have been explored to collect the maximum amount of data.

2. Matherials and methods

2.1. Characterization of plant matrix

Rosmarinus officinalis L. was collected in Algiers (Algeria) during the month of March. The leaves were removed from the stems and have been dried at room temperature for three weeks in order to preserve the maximum integrity of their molecules. The dried leaves were crushed into fine particles and ground to powder using a mortar then passed through a 1.12 mm sieve. The powder was stored in a cool and dark place, protected from light and moisture for subsequent use.

2.2. Reagents and chemicals

Folin–Ciocalteu reagent, Gallic acid and Quercetine were purchased from Sigma–Aldrich, Steinheim, Germany). EtOH of 96% purity (Rieldel-de Haen, Germany), sodium carbonate (BHD chemicals Ltd, England) and Aluminum chloride (Merck, Germany) were used.

2.3. Analysis

2.3.1. Total Polyphenols Content

TPC was determined by Folin-Ciocalteu according to Singleton et al. [14] expressed as Gallic acid equivalents (mg GAE/g DW). Absorbance was measured at 760 nm on a spectrophotometer (UV-VIS SECOMAM S250, France). The results were calculated using a standard Gallic acid curve of (1–30 µg/mL)**.**

2.3.2. Total Flavonoids Content

TFC estimated was determined by colorimetry according to the Miliauskas method [15], expressed as quercetin equivalents (mg QE/g DW). Absorbance was measured on a spectrophotometer (UV-VIS SECOMAM S250, France) at 430 nm. The results were calculated using a standard curve of quercetin (1 – 40 µg/mL).

2.4. Experimental design and statistical analysis

FD was used to study the effects of three variables: extraction time (X_1), ethanol concentration(X_2) and solid-to-liquid ratio (X_3). The low and high levels for each factor in the experimental design are given.

The responses (TPC and TFC) were expressed individually as a function of independent variables. The experimental design presented nine (9) combinations, including three replicates of the central point in order to estimate pure error and to assess the lack of fit for the proposed models. All experiments were performed randomly. A first-order polynomial equation (Eq.1) was used to express TPC (Y_1) and TFC (Y_2) of rosemary as a function of independent variables asfollow:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1X_2 + \beta_{13} X_1X_3 + \beta_{23} X_2X_3 \quad (1)$$

WhereYi represents the variables response, β_0 is the model constant, β_1, β_2 and β_3 are the linear coefficients.β_{12}, β_{13} and β_{23} are the interaction coefficients respectively. X_1, X_2 and X_3 are the levels of the independent variables. The model appropriateness was also evaluated by the coefficient model (R^2) and adjusted coefficient model (R^2_{Adj}) and also by the statically significant model (P-value model) properly tested by ANOVA method.

3. Results and discussion

3.1. Choice of solvent

In order to use environmentally friendly methods, the use of water ethanol and agro-solvents has been encouraged [16], [17]. However, using pure ethanol could dehydrate the vegetable cells, making the BACs diffusion process difficult from the plant material to the extracting liquid. A Combination of ethanol/water is then used in the actual study in order to adjust the polarity [18].

3.2. Fitting model

Three factors that may affect the experimental responses were selected as independent variables at three levels. Table 1 summarizes the minimum, central and maximum values for each factor and the different independent variables of the extracts. The experiments were performed according to the design of experiments shown in Tables 2. Observed and predicted responses on two levels are shown on the same table. Statistical Analysis of experimental data was performed by using "Modde 6" software [19].

Table 1.

Coded/ real levels used in Factorial Design of Algerian *Rosmarinus officinalis* L. maceration.

Coded levels	-1	0	+1
Time (hours) (X1)	1	36.5	72
Ethanol concentration (%) (X2)	50	65	80
Solid-to-liquid ratio (g/mL) (X3)	0.06	0.105	0 .15

The results obtained indicate that the TPC levels ranged from 1.35 mg GAE/g DW to 2.5 mg GAE/g DW confirmed the influence of the studied parameters (time, ethanol concentration, solid-to-liquid ratio). The best results were observed in run 5 at 2.5 mg GAE/g DW followed by 2.4 mg GAE/g DW in run 6. The TFC levels ranged from 0.45 mg QE/g DW to 1 mg QE/g DW. The best results related to the lower levels were observed in run 8 at 1 mg QE/g DW followed by 0.83 mg QE/g DW in run 1. These results indicate the presence of low content of polyphenols.

Table 2.

Factorial design of three variables with their experimental and predicted responses of Algerian *Rosmarinus officinalis* L. maceration.

Run	Coded variable			TPC (mgGAE/g DW)		TFC (mgQE/g DW)	
	X_1	X_2	X_3	Experimental	Predicted	Experimental	Predicted
1	-1	-1	-1	1.80	1.82	0.83	0.77
2	+1	-1	-1	1.65	1.66	0.49	0.49
3	-1	+1	-1	1.35	1.34	0.45	0.47
4	+1	+1	-1	1.60	1.62	0.59	0.59
5	-1	-1	+1	2.50	2.5	0.80	0.81
6	+1	-1	+1	2.40	2.3	0.75	0.73
7	-1	+1	+1	1.76	1.78	0.63	0.63
8	+1	+1	+1	2.15	2.14	1.00	0.99
9	0	0	0	1.93	1.91	0.70	0.69
10	0	0	0	1.90	1.91	0.69	0.69
11	0	0	0	1.92	1.91	0.68	0.69

It should be noted that some research have shown lower phenolic contents [20-22]. Further researches have given higher values [23]. They pointed out that the extract composition changes according to many factors (type of sample, location, and time... etc.). Other work has shown the influence of plant matrix and solvent [24]. Oliveira et al. have found that optimum yield reaches 89.8% using a hydroalcoholic solution of ethanol-water with 70% v/v, liquid-to-solid ratio of 5 mL/g and 55 min [25]. TPC levels range from 4.58 mg GAE/g DW to 28.06 mg GAE/g DW for 0% EtOH at pH 2. Bucic-Kojić et al. have found similar results with 50% EtOH [26].

Table 2 shows that experimental conditions, which give an extract with the best TPC (run 5); do not give the highest TFC. This is the reason to studied both TPC and TFC at the same time. The results also show that experimental data are closely to the predicted values.

The estimate goodness of fit results for all responses analyzed by ANOVA variance analysis, have been summarized in Table 3. The Data indicated that all the final models resulting from ANOVA analysis in terms of coded variables were significant at 95 % confidence level, with all p-values of regression ≤ 0.05 (from 0.0397 to 9.38 10^{-10} and 0.03011 to 5.957 10^{-9}, respectively for TPC and TFC). Lack of fit (LOF) were higher than 0.05.

Fig. 1(a) for TPC effects indicates that solid-to-liquid ratio has a higher effect. It is the most important factor (positive effect) followed by an important effect of ethanol concentration (negative effect) and by the interaction of ethanol concentration and time (positive effect). Interaction of solid-to-liquid ratio and time shows weak effect. Regarding the TFC shown in Fig. 1 (b), the interaction of ethanol concentration and time has a high effect. It is the most important factor. On the other side, ethanol concentration and time show weak effect. The reproducibility and models validity

values of TPC and TFC show a good agreement for all the responses. Solid-to-liquid ratio effect is positive for TPC and TFC because decreasing the solvent proportion increases the concentration gradient, which consequently leads to an increase in the diffusion of solid compounds in the solvent. The effect of ethanol concentration is negative in this case. There has to be a compromise between the amount of ethanol and water. Therefore, increasing ethanol concentration induces consequently a decrease in maceration time. Other research has shown that TPC yield increases with the concentration of ethanol, up to 50% the TPC yield decrease [27], [28]. On the other hand, time showed a weak effect because maceration process is carried at room temperature. A longer process causes phenolic compounds degradation [29] and this is not the case here. The fitted mathematical models (Y_1 andY_2) for TPC and TFC with 95% coefficient level were given in Eq. 2 and Eq. 3 respectively. The values of R^2 were 99.8% and 99.7% respectively.

$$Y_1 = 1.91 + 0.05\,X_1 - 0.19X_2 + 0.30\,X_3 + 0.11\,X_1X_2 + 0.02\,X_1X_3 - 0.06\,X_3X_2 \quad (2)$$

$$Y_2 = 0.69 + 0.01\,X_1 - 0.02\,X_2 + 0.10\,X_3 + 0.11\,X_1X_2 + 0.06\,X_1X_3 + 0.04\,X_3X_2 \quad (3)$$

From theses equations, the preponderant effect for the actual maceration process was solid-to-liquid ratio for Y_1 and Y_2. The high R^2_{Adj} values indicated that there is a good correlation and relationship between the experimental data and the obtained model data.

ANOVA data (Table 3) shows a p-value of LOF around 0.234 and 0.301 for TPC and TFC respectively. It confirms the high efficiency of fitting model suitability and explanation of experimental data, while coefficients of determination for its polynomial model equation are $R^2 = 0.998$, R^2_{Adj} of 0.996 and $R^2 = 0.997$, R^2_{Adj} of 0.993.

The experimental versus predicted response are shown in Fig. 2.

Table 3.
Analysis of variance (ANOVA) for the fitted model of Algerian *Rosmarinus officinalis* L. maceration

		TPC	TFC
DF	Model	6	6
	Lack of fit	2	2
	Pure error	2	2
Sum of Squares	Model	1.1568	0.2421
	Lack of fit	0.00153105	0.0463636
	Pure error	0.0466664	0.02
F value	Model	385.799	243.206
	Lack of fit	3.28084	2.31818
p value	Model	0.000	0.000
	Lack of fit	0.234	0.301
	R^2	0.998	0.997
	R^2_{Adj}	0.996	0.993

In the present study, the values of R^2 were 99.8% and 99.7% respectively for TPC and TFC. The values of R^2_{Adj} were 0.996 % and 0.993 % for TPC and TFC respectively. These results showed a close agreement between the experimental and the predicted values.

Histograms below (Fig. 1) present the factors effect and their interactions.

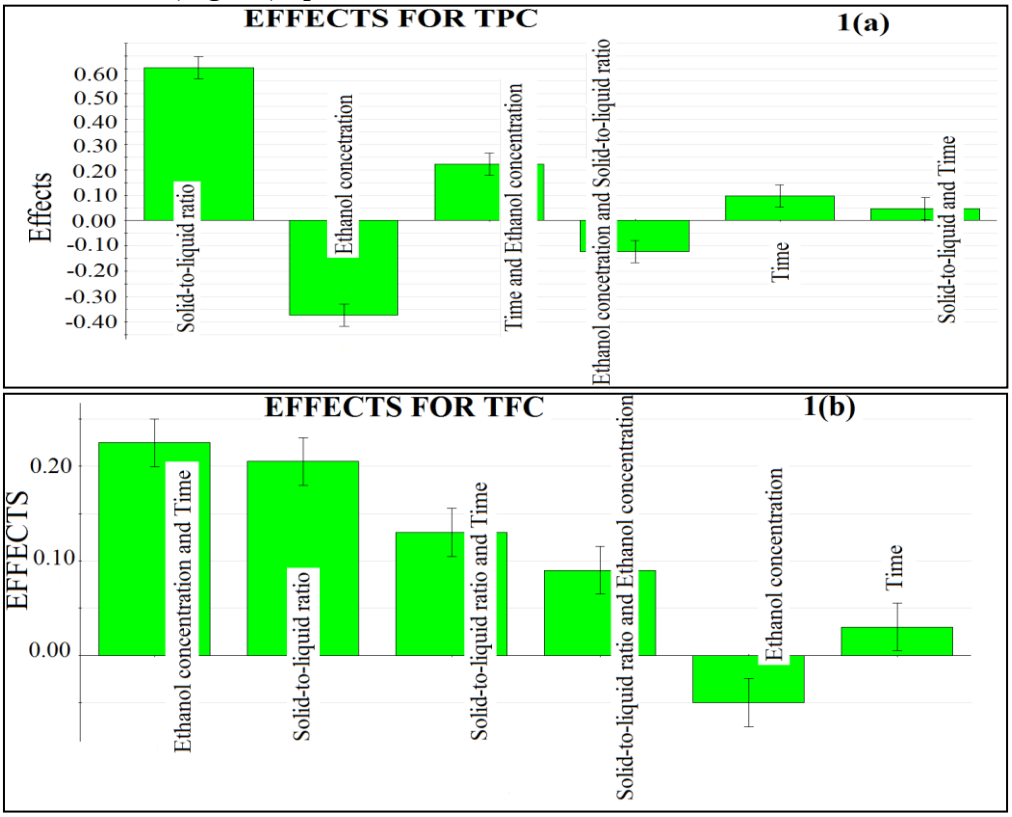
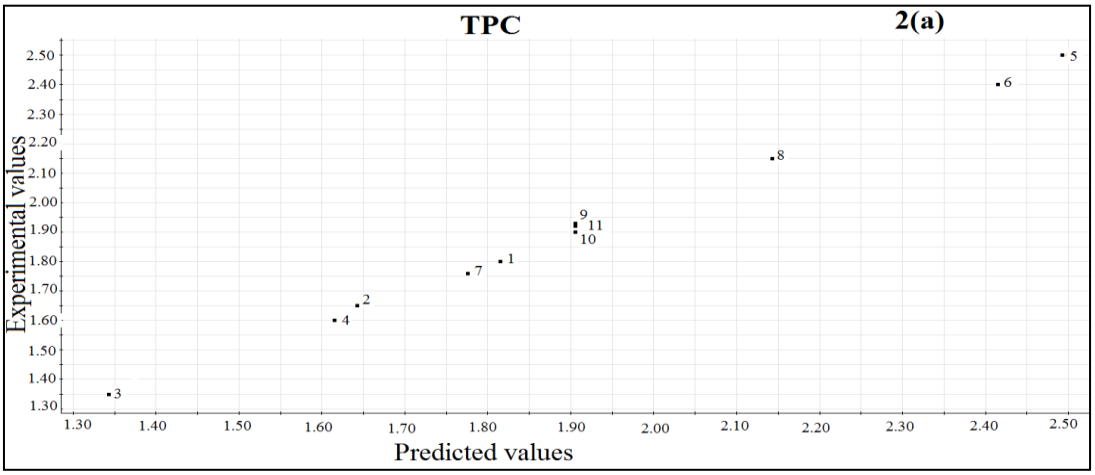


Fig. 1. Histogram of TPC and TFC effects from Algerian *Rosmarinus officinalis* L. maceration: (a) Histogram of TPC effects, (b) Histogram of TFC effects.

Fig. 2a and Fig. 2b reveal the presence of linear relationship between them with high correlation coefficient. It indicates normal distribution of error around the mean and a good applicability of model for interpreting the experimental data. These plots are required to check the normality assumption in fitting model.



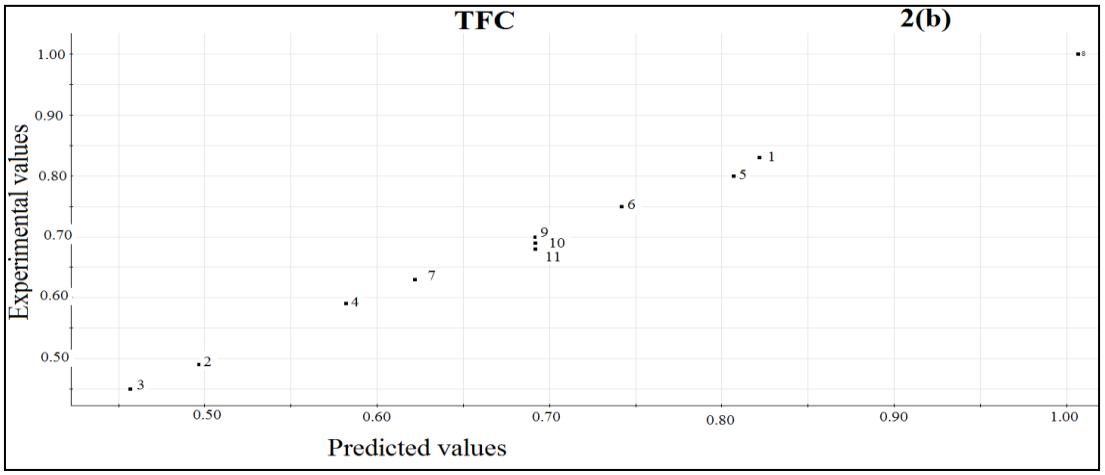


Fig. 2. Predicted values of TPC and TFC versus experimental values from Algerian *Rosmarinus officinalis* L. maceration: (a) Predicted values of TPC versus experimental values, (b) Predicted values of TFC versus experimental values.

3.3. Response surface and iso-response plots

3D surface plots serve to predict the response variation as a function of two chosen factors. In order to investigate the interactive effects of operational parameters on responses, three-dimensional response surface plots were generated by plotting the response on the *Z*-axis against two independent variables keeping the other independent variables at zero level. The advantage of this illustration is the possibility to visualize the evolution of responses according to two factors at the same time over the entire experimental range. Consequently, the estimated value of the desired parameter at any point in the field can be drawn directly. In figure 3 we represent surface response curves of TPC and TFC for the three factors by an illustration of simultaneous effect of two factors.

Fig. 3a and Fig. 3d illustrate simultaneous effect of time and solid-to-liquid ratio on TPC and TFC. It can be observed from Fig. 3a that solid-to-liquid ratio is less significant than other effects. The TPC increases by increasing thesolid-to-liquid ratio or time, but it decreases by increasing the ethanol concentration. Fig. 3d shows that TFC increases with increasing the time or solid-to-liquid ratio or ethanol concentration.

Fig. 4 represents the iso-response curves of TPC and TFC for the three factors. It illustrates the evolution of the TPC and TFC according to two factors simultaneously over the entire experimental range. It can be possible from these curves to draw directly the estimated value of the desired parameter at any point in the field of study.

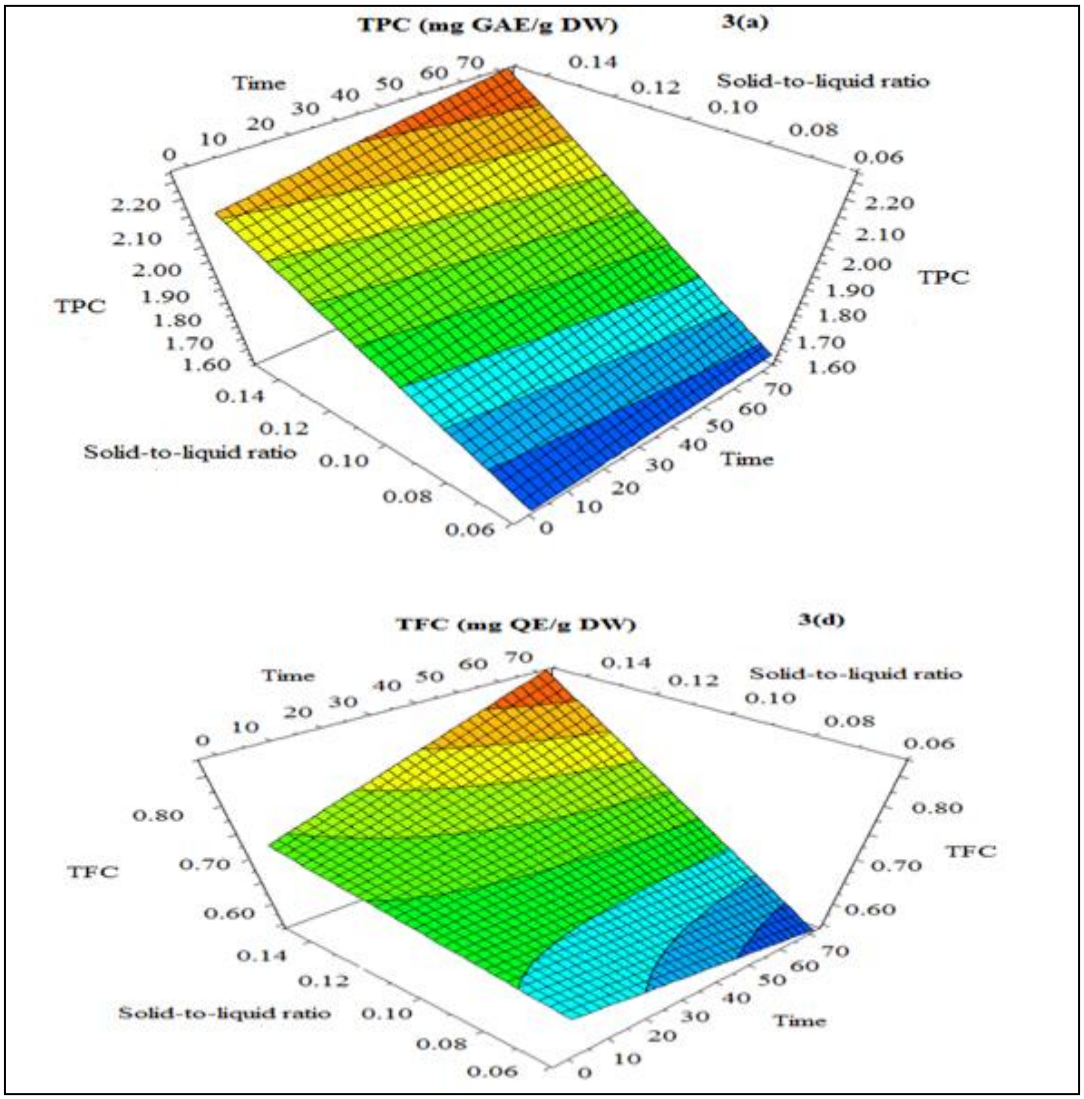


Fig. 3. Response surface plots for effects on TPC and TFC from Algerian *Rosmarinus officinalis* L. maceration: (a) time vs solid to liquid ratio for TPC, (d) time vs solid to liquid ratio for TFC.

Fig. 4a shows the pronounced efficiency zone for TPC which is around 2.361 mg GAE/g DW. In this zone, the solid-to-liquid ratio and ethanol concentration values are ranging from 0.14 g/mL to 0.15 g/mL and from 50% to 55 % respectively. Regarding the TFC, the pronounced efficiency zone is around 0.845 mg QE/g DW (Fig. 4d). The solid-to-liquid ratio and time values in this case are ranging from 0.145 g/mL to 0.15 g/mL and from 65 hours to 70 hours respectively. The results obtained were in good agreement with the experimental results found in this study.

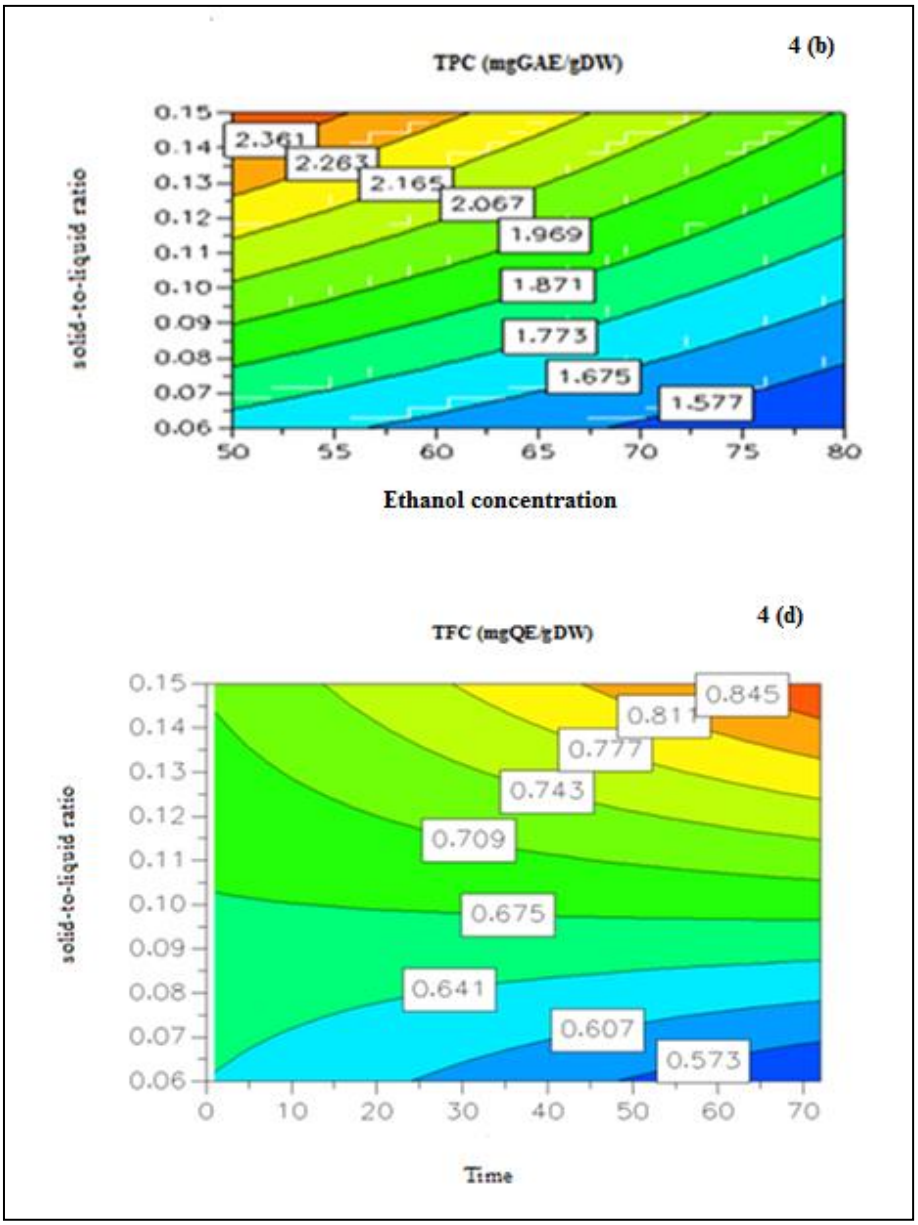


Fig. 4. Iso-response curves for efficiency zone of TPC and TFC from Algerian *Rosmarinus officinalis* L. maceration: TPC [(a) ethanol concentration Vs solid to liquid ratio] and TFC [(d) time Vs solid to liquid ratio]

3.4. Correlation between the responses of TPC and TFC

Since experimental conditions, which give an extract with the best TPC, do not givethe highest TFC. The relationships between those responses must be examined more deeply. The correlation coefficient between TPC and TFC on the extract was 0.83, suggesting that approximately 83 % of TPC in the extracts are TFC. This last may be related to the high selectivity of the solvent used in the extraction solution.

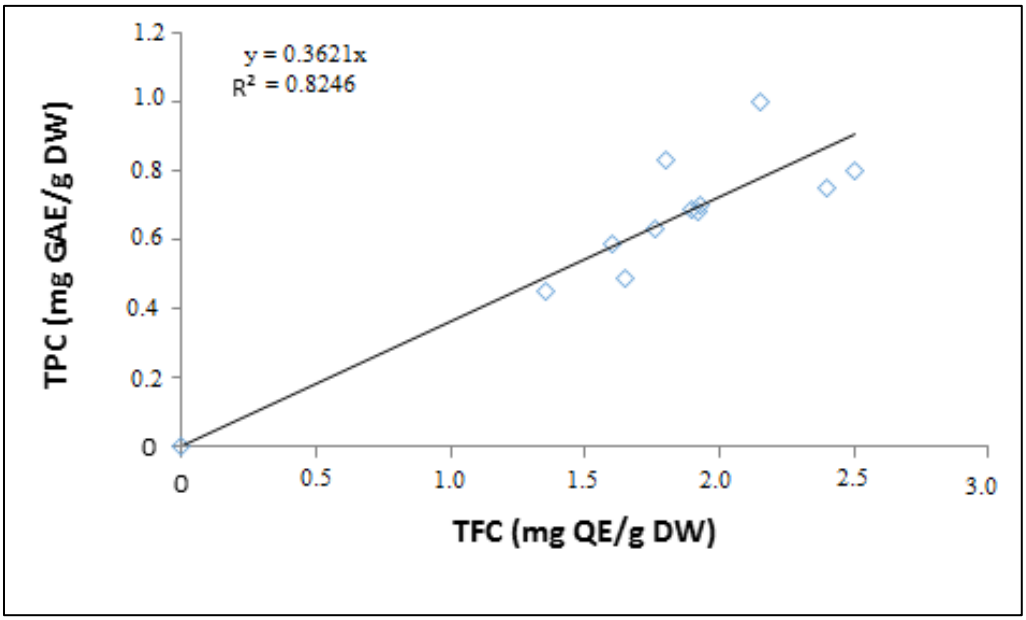


Fig. 5. Relationship between TPC and TFC of extracts from Algerian *Rosmarinus officinalis* L. maceration.

4. Conclusion

Algerian *Rosmarinus officinalis* L. leaf extract was obtained by green extraction process. The selection of the solvent is mainly related to the future use of the extract. Ethanol was used as solvent in this study. The effect of the main process variables (time, ethanol concentration, solid-to-liquid ratio) on TPC and TFC has been studied. The characteristic models released from each response are first-order linear models with interactions. The effect of each factor on the two selected responses shows that solid-to-liquid ratio represents the overriding factor for TPC and TFC. Time had less significant effects on TPC and TFC respectively. Indeed, the best experimental results for TPC and TFC were obtained for 1hour, 0.15 g/mL with 50% and for 72hours, 0.15 g/mL with 80% respectively. The value of TPC and TFC is 2.5 mg GAE/g DW and 1 mg QE/g DW. The relationship between TPC and TFC provides a good correlation coefficient (0.83). Finally, antioxidant and antibacterial activities of extract will be tested in prospect.

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6. References

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