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Experimental design methodologyapplication in the optimization of phytochemical compoundsextraction and antioxidant activity of *Thymelaea hirsuta* L.extracts

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Abstract

Thymelaea hirsuta L. is a medicinal plant known for its therapeutic virtues in traditional medicine. In this regard, the central composite design was implemented to improve the amounts of phenolic compounds extracted as well as the antioxidant compounds from *T. hirsuta* 's aerial parts. Three parameters namely the extraction temperature (24-60°C), the time (2-18 h) and the solvent (0-100 % methanol/ethyl acetate proportion) were used as independent variables. The second-order polynomial equation was carried out at different levels and a total of 16 experiments with two replicates at the center were conducted in order to obtain the optimal extraction conditions. Total phenolics (65.60±0.76 to 254.55±5.39 mg GAE/g dw), flavonoids content (61.59±1.34 to 125.99±4.78 mg QE/g dw), $ABTS^{\bullet+}$ (15.22±1.82 to 211.33± 3.21 mg/L), $DPPH^{\bullet}$ (19.03± 0.40 to 280.33± 2.08 mg/L) and FRAP (0.19±0.01 to 1.30±0.15 mM/L) antioxidant activities of the various extracts were determined and considered as responses. The optimal extraction conditions were obtained at a temperature of 46° C, a duration of extraction of 9h, and for a methanol proportion of 93 %. The corresponding predicted values were 187.79 mg GAE/g dw, 99.90 mg QE/g dw, 23.57 mg/L, 23.10 mg/L and 0.78 mM/L for TPC, TFC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP, respectively.

1. Introduction

All over the world, the interest in bioactive compounds is getting more and more important in several different fields such as food, pharmaceutical and cosmetic[1]. Furthermore, many researchesfocus on the bioactive molecule extraction like natural antioxidants that would be less toxic and more effective than synthetic antioxidants generally used against Oxidative Stress [2].Recently, phenolics present one of the main large secondary metabolites groupswhich are considered as natural antioxidant with a high ability to resist to oxidation[3]. Moreover, these compounds possess a large number of important pharmacological properties such as anti-inflammatory, anticancer and anti-ulcerative [4]. In this respect, phenolic compounds have attracted a lot attention for their antioxidant potential to the medicinal and nutritional uses. The selectivity ofphytochemical compoundsextraction is very affected by numerous environmental conditions and many other processing factors such as extraction, separation and purification. Among these, the extraction present one of the crucial steps in the individual separation and identification of phenolic compounds from solid samples[5]. Other than the extraction methods, the quality and quantity of these phytochemicals depends on the extraction time, the solvent, the temperature, the pH, the agitation rate, ratios and particle size[6].Thus, it is essential to improve and develop

the experimental extraction using response surface methodology (RSM). This methodologyis defined as an effective statistical and mathematical technique used to investigate the numerous factors impact and their interactions at a single time [7].

Here, we reported a study on the *Thymelaea hirsuta* L. Tunisian species to contribute to the well-known local medicinal plant valorization for their therapeutic virtues. This plant, commonly know as Methnane,is a shrub of the family of *Thymelaeaceae*. It is native from the Canary Island, Mediterranean region, north of central Europe and Eastern Central Asia. It has been traditionally used as antiseptic, ant-inflammatory and for the treatment of hypertension [8-9].Moreover, many biological properties of this plant extracts were also highlighted namely antimicrobial [9],antitumor [8], antihypoglycemic [10]. As it has been revealedthat it possesses a great anti-diabetic activity which justified itsutilization by theMoroccan population as decoction in the diabetes treatment [11-12]. Recent studies mentioned that the *T. hirsuta*'saerial parts exhibited a very notable antioxidant activity[13-14], mainly related to their phenolic and flavonoid content. Nevertheless, none of the previous works have evaluated the extraction factors impact on the recoveries of these phytochemical compounds.

Therefore, the main goal of thepresent investigation was to apply the RSM method in order to evaluate the optimal conditions of phenolic and flavonoid compounds extractionand their antioxidant property.

2. Material and methods

2.1. Chemicals and standards used

All chemicalsand standards employed in this study were purchased from Sigma-Aldrich.

2.2. Collection of plant material

T. hirsuta's aerial parts were gathered in October 2012 from Kasserine region which is located in west central of Tunisia (longitudeE 8°50′11″ and latitude N 35°10′03″). The identity of the plant material was confirmed by Dr. Mohamed Bousaid (Department of botany, National institute of applied sciences and technology, Tunis, Tunisia). After collection,the fresh samples of the plant were dried in the air and the shade. Then, they were powdered using anelectricgrinder.

2.3. Extraction process

2.3.1. Maceration

For the extraction process, 10g of plant material were placed into glass bottle containing 100 mL of extraction solvents under continuous agitation. The extraction temperature (24 -60 ± 1°C), time (2 -18 h) and solvent proportion (ethyl acetate and methanol(v/v) %) were predetermined using the experimental design summarized in table 1.

Table 1. Uncoded and coded Levels of variables for the experimental design

X_J	Independent parameters	Factor levels				
		-1.68	-1	0	1	1.68
X_1	Temperature (°C)	24	31	42	53	60
X_2	Time (h)	2	5	15	10	18
X_3	Methanol proportion (%)	0	20	50	80	100

Afterwards, the mixture was filtered using a filter paper.A rotary vacuum evaporator was used at 35°C in order to remove solvent. The crude extracts were kept in vials and stored at 4°C until further use. The extraction yield was calculated according the following formula:

$$\text{Yield } (\%) = \left(\frac{m}{M}\right) * 100$$

Where m: is the weight of residue in grams, M: is the weight of plant material in grams.

2.3.2. Experimental design for the Response Surface Methodology

The optimization of phenolics and flavonoids compounds extraction was carried out using RSM. Thefive-level three-factorrotatable central composite design (CCD) consisting of 16 experimental runs with two replicates at the center point. The independent variables were the temperature (X_1, °C), time (X_2, h) andmethanol proportion

(X_3, %, v/v methanol/ethyl acetate). As displayed in the table 1, five levels of values for each independentparameterwere presented in their original and coded forms.
Extraction yield, phenolics, flavonoids, $ABTS^{\bullet+}$, $DPPH^{\bullet}$and FRAP were chosen as the responses of the design experiments.A second-degree polynomial equation from RSM was used and given below:

$$Y = b_0 + b_1 * X_1 + b_2 * X_2 + b_3 * X_3 + b_{11} * (X_1 * X_1) + b_{22} * (X_2 * X_2) + b_{33} * (X_3 * X_3) + b_{12} * (X_1 * X_2) + b_{13} * (X_1 * X_3) + b_{23} * (X_2 * X_3) \quad (1)$$

where Y is the response (dependent variables); b_0, b_1, b_2, b_3, b_{11}, b_{22}, b_{33},b_{13},b_{12} andb_{23} are the regression coefficients for intercept, linear, quadratic and interaction terms; X_1, X_2 and X_3 are the non-coded values for temperature, time and solvent extraction, respectively.Based on the variance analysis, the regression coefficients of individual linear, quadratic and interaction terms were calculated.

2.4. Analyses of the response variables

2.4.1.Quantification of total phenolics content (TPC)

The amount of total phenols in each sample was determined by UV spectrophotometry as described by Ghazouani et al[15]. This method was based on a colorimetric oxidation/reduction reaction using a Folin -Ciocalteu as oxidizing reagent.In short, a volume of 100 µL of each sample and 500 µL Folin Ciocalteu reagent (0.2 N) were mixed. After 5 minutes of incubation at ambient temperature, 400 µL of sodium Carbonate solution (75 g/L prepared in water) were also added. Afterwards, the mixture was thoroughly shaken and allowed to stand in the darkness for 15min before measuring the absorbance at 360nm. The standard calibration curve was established by plotting the solution of Gallic Acid ranged from 0 to 30 mg/L. The total phenolics content were expressed as milligram of Gallic Acid equivalents (GAE) per g of dry weight (mg GAE/g dw).Triplicate measurements were realized for each sample.

2.4.2. Quantification of total flavonoids content (TFC)

The quantification of total flavonoids content was done by the colorimetric method as described by Sifaoui et al[16], with modifications. This method was based on the formation of a colored flavonoid-aluminum complex. Briefly, 250 µL of each sample were introduced into tube containing 1000 µL distilled water and mixed with 75 µLof a 15% sodium nitrite solution ($NaNO_2$). After 6 minutes of pre-incubation, 75 µL of a 10% Aluminum chloride solution($AlCl_3$) were also added and at the 6 th min,a volume of 1000 µL of a 4% sodium hydroxide solution (NaOH) was added.The distilled water was then added to reach a final volume of 2500 µL. The obtainedmixture was shaken vigorously and allowed to stand for 15 minutes. Afterwards, the absorbance was measured at 510 nm against water blank using a UV visible spectrophotometer. The estimation of the flavonoid content was determined from the calibration curve established by quercetin as standard. The results were expressed in milligram of quercetin equivalents per g of dry weigh (mg QE/g dw).Triplicate measurements were realized for each sample

2.4.3. DPPH• radical Scavenging Assay

The free radical scavenging activity of *T. hirsuta* extracts was evaluated using 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) of color dark purple, following the procedure outlined by Ghazouani et al [17].In this assay, a volume of 100 µL of each sample was mixed with 900 µL of freshly prepared methanolic $DPPH^{\bullet}$ solution and allowed to stand in the darkness for 25 minutes. Then, the absorbance was read at 524 nm against blank solution using a UV-vis spectrophotometer. The percentage of inhibition of the free radical scavenging activity of each extract was calculated as follow:

$$\%\ inhibition = (A\ (blank) - A(sample)/A\ (blank)) * 100$$

Where A_{sample} is the absorbance of sample with $DPPH^{\bullet}$solution and A_{blank} is the absorbance of $DPPH^{\bullet}$ solution without the extract.Results were expressed in terms of IC_{50} (mg/L), defined as the concentration of the test material required to scavenge 50% of $DPPH^{\bullet}$ radicals which calculated from the inhibition percentage of radical scavenging activity. Ascorbic acid was employed as standard.Triplicate measurements were realized for each sample.

2.4.4. ABTS• + radical Scavenging Assay

The $ABTS^{\bullet+}$ (2,2'-azinobis-3-ethylbenzothiazoline- 6-sulphonate) radical cation scavenging assay was performed based on the procedure as described by Ghazouani et al [18].The ABTS solution was prepared as follow: a

solution of ABTS (7 mM, prepared in distilled water) was mixed with a potassium persulfate($K_2S_2O_8$) solution (2.45 mM, dissolved in distilled water) in the ratio of 1:1 and the mixture were diluted with distilled water to obtain an absorbance ranging from 0.70 to 0.90. In order to perform $ABTS^{\bullet+}$ assay, 900 µL of ABTS solution were added to 100 µL of sample (extracts or standard) into same test tube. The mixture was vigorously agitated and incubated for 6 min in the dark at ambient temperature. Then, the absorbance of reaction mixture was measured at 734 nm using a UV-vis spectrophotometer.Results were expressed in terms of IC_{50} (mg/L) and calculated with the same formula cited previously in the $DPPH^{\bullet}$ scavenging assay. Ascorbic acid was served as standard. Triplicate measurements were realized for each sample.

2.4.5. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was employed to assess ferric reducing antioxidant power of *T. hirsuta* extracts according to the method described bySaoudi et al [19], with slightly modifications. In summary, the FRAP reagent solution was prepared as follow: the acetate buffer solution(300 mM, prepared in distilled water, pH =3.6) was mixed with 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM, dissolved in hydrochloric acid(40 mM)) and Iron(III) chloride ($FeCl_3$) solution (20 mM, prepared in distilled water) using a ratio of 10 :1 :1. Afterwards, 50 µL of each sample were mixed with 1500 µL of freshly FRAP reagent solution and incubated for 30 minutes in the darkness at 37 ºC. The absorbance of reaction mixture was measured at 595nm. The calibration curve was established usingIron(II) Sulfate Heptahydrate ($FeSO_4,7H_2O$) as standard. Results were expressedas$FeSO_4,7H_2O$ equivalent mM per gram of Dry Extract(mM /gDE). Triplicate measurements were realized for each sample.

2.5. Statistical analysis

All results were expressed as means ± standard deviations and all experiments were done in triplicate. The experimental data was analyzed by Nemrodw software.

3. Results and discussions

3.1. Fitting the model

The phenolic compounds extraction of *T. hirsuta* extracts was further improved through RSM approach. The Experimental data and responses were reported in Table 2. The independent factors used in the design were extraction temperature (X_1), extraction time (X_2) and methanol proportion (X_3). The extraction yield, TPC, TFC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP were the responses. In this investigation, a greater model fit was obtained. The determination coefficient R^2 value of yield was obtained to be 0.949, explaining 94.9% of variations in yield. For TPC and TFC, R^2 was equal to 0. 986 and 0.934, respectively. Among all the responses of antioxidant activity, the $ABTS^{\bullet+}$ assay had the most important R^2 (0.997) and the predicted values is equal to 0.992, which means that the model can be validated up to 99.2 % variability of the data. For $DPPH^{\bullet}$ and FRAP, The R^2 values were 0.967 and 0.955, respectively. All determination coefficients of responses are superior to 0.9, proving good representation of the variability of the factors by the models. It is important to notice that the model is considered accurate and reliable as the R^2 is superior to 0.75[16]. The 2D graphic of response surface was generated for each response and illustrated in Fig.1.

3.2. Response surface analysis for extraction yield

The extraction yields of *T. hirsuta* extracts recovered in all runs were given in Table 1. Results showed that the value ranged from 1.48 to 6.46 %. The maximum yield was obtained at a methanol proportion of 100 % and 42°C during 10 h. Among all independent variables studied, only linear effect of temperature ($\rho < 0.01$) and methanol proportion ($\rho < 0.001$) were found to produce statistically significant positive effect. Moreover, the quadratic effects of extraction temperature ($\rho < 0.01$) and time ($\rho < 0.01$) were observed.

According to the regression coefficient displayed in table 3, it can be clearly seen that the methanol proportion factor affects most the extraction yield compared to other factors. All the non-significant variables aside, the polynomial equation for extraction yield response follows as:

$$Y_{\text{Extraction Yield}} = 5.224 + 0.586\,X_1 + 1.112\,X_3 - 0.732\,{X_1}^2 - 0.787\,{X_2}^2 \qquad \textbf{(2)}$$

Table 2. Central composite design with responses of the independent factors to extraction conditions

Run	Independent factors				Response variable					
	Temperature (ºC)	Time (h)	Methanol proportion (%)		Yield (%)	Chemical composition		Antioxidant activity		
						TPC (mg GAE/g dw)	TFC (mg QE/g dw)	$ABTS^{\bullet+}$ (mg/L)	$DPPH^{\bullet}$ (mg/L)	FRAP (mM/L)
1	31	5	20		1.48	148.60 ± 0.54	82.20 ± 1.20	138.6 ±0.52	66.25 ±1.65	0.30 ± 0.00
2	31	5	80		3.53	150.31 ± 2.56	190.89± 2.74	21.73± 0.70	36.6 ±1.17	1.00 ± 0.06
3	53	5	20		2.95	83.41 ± 0.88	61.59± 1.34	176.62± 0.75	197.66 ±1.15	0.50 ± 0.02
4	53	5	80		5.69	203.06 ± 3.91	81.78± 1.46	31.5 ± 0.57	37.92 ± 0.67	0.75 ± 0.04
5	31	15	20		1.64	66.70 ± 3.17	83.85 ±1.20	89 ± 0.65	133.83 ±1.15	0.46 ± 0.00
6	31	15	80		3.14	203.82 ± 1.59	125.99± 4.78	23.76 ± 0.25	23.62 ± 1.88	1.30 ± 0.15
7	53	15	20		2.92	65.60 ± 0.76	62.35± 3.16	97.43 ± 1.88	220.5 ± 2.12	0.32 ± 0.01
8	53	15	80		4.31	254.55 ± 5.39	93.76± 3.66	21.86 ± 0.30	19.03 ± 0.40	0.76 ± 0.03
9	42	10	0		2.00	80.34 ± 0.44	64.18± 2.07	211.33 ±3.21	280.33± 2.08	0.19 ± 0.01
10	42	10	100		6.46	148.08 ± 2.60	102.37 ±1.20	33.12 ± 1.25	24.46 ± 2.65	0.81 ± 0.01
11	24	10	50		2.72	117.12 ±2.29	100.99± 1.20	18.63 ± 0.94	54.5 ± 1.73	0.64 ± 0.02
12	60	10	50		3.86	152.71 ±3.17	72.12 ±2.39	52.13 ± 0.75	80.16 ± 1.25	0.53 ± 0.02
13	42	2	50		3.05	120.12 ±1.52	75.31 ±1.55	83.125 ±2.83	149.60 ± 2.44	0.39 ± 0.01
14	42	18	50		3.22	134.68 ± 1.59	80.29± 1.58	15.22 ± 0.29	21.06 ± 0.11	0.97 ± 0.00
15	42	10	50		4.56	92.27 ± 2.37	86.13 ±2.39	24.87 ± 0.85	62.09 ± 1.00	0.57 ± 0.01
16	42	10	50		5.41	99.25 ± 3.08	100.80 ±2.07	20.95 ± 1.82	64.60 ± 0.14	0.56 0.05

TPC: Total Phenolics content, TFC: Total Flavonoids content, $DPPH^{\bullet}$=2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, $ABTS^{\bullet+}$= 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity, FRAP: Ferric reducing antioxidant power.

Table 3. Regression and determination coefficients of the predicted model to the response for phenolic and flavonoid compounds and antioxidant activities.

Model parameters	Yield (%)	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	$ABTS^{\bullet+}$ (mg/L)	$DPPH^{\bullet}$ (mg/L)	FRAP (mM/L)
Intercept, b_0	5.224***	92.770***	87.815***	22.587**	63.300 **	0.551**
Linear effects						
b_1	0.586**	7.894*	-11.789**	8.104**	17.937 *	-0.066*
b_2	-0.099 ^{NS}	1.392 ^{NS}	2.114 ^{NS}	-18.348***	-11.542 ^{NS}	0.093**
b_3	1.112***	44.625***	14.327***	-51.442***	-65.750***	0.240**
Quadratic effects						
b_{11}	-0.732**	15.911**	-0.802 ^{NS}	4.509*	1.519 ^{NS}	0.033*
b_{22}	-0.787**	13.254**	-3.897 ^{NS}	9.384**	2.756 ^{NS}	0.056*
b_{33}	-0.400 ^{NS}	15.955**	-1.962 ^{NS}	35.213***	26.290 **	0.003 ^{NS}
Interactions effects						
b_{12}	-0.148 ^{NS}	14.854**	0.624 ^{NS}	-5.160*	-7.206 ^{NS}	-0.073*
b_{13}	0.073 ^{NS}	22.566**	-3.529 ^{NS}	-4.820*	-26.044 *	-0.112**
b_{23}	-0.237 ^{NS}	24.244***	1.959 ^{NS}	15.150***	-10.661 ^{NS}	0.052*
R^2	0.949	0.986	0.934	0.997	0.967	0.955
Adjusted R^2	0.873	0.965	0.836	0.992	0.917	0.886

X_1: Temperature (°C), X_2: Time (h), X_3: methanol proportion (%), TPC: Total Phenolics content, TFC: Total Flavonoids content, $DPPH^{\bullet}$=2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, $ABTS^{\bullet+}$= 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity, FRAP: Ferric reducing antioxidant power, Levels of significance: *: $\rho < 0.05$, **: $\rho < 0.01$, ***: $\rho < 0.001$.

The polynomial equation demonstrated that the X_1 and X_3 have the positive linear effect. This indicated that increasing the temperature level and the solvent polarity enhance a better recovery of compounds which increases the yield. This might be related to the fact that the solubility of molecules present in plant depends on the polarity and chemical properties of solvent. By contrast, the quadratic regression coefficients for extraction temperature and time were found negative, suggesting that a significant decrease in extraction yield has been observed when the temperature and time increases (quadratic effect). The 2D response surface graph was drawn for temperature and time variables. This graph showed that the extraction yield is affected by temperature, which requires keeping the temperature below at a certain limit. This allows us to concluded that the increased temperature during the extraction directly influences on the mixture and leads to degradation of the thermolabile compounds which decreases the yield.

3.3. Response surface analysis for total phenolics content (TPC)

The results obtained during the optimization of the process showed that the amount of TPC varied considerably between 65.60 ± 0.76 and 254.55 ± 5.39 mg GAE/g dw. The highest amount was obtained with experiment number 8 (80 % of methanol proportion, 53° C and 15 h) while the lowest was obtained with experiment number 7(15 h, 53° C and 20 % methanol proportion). The linear effect of extraction temperature ($\rho < 0.05$) and solvent ($\rho < 0.001$) was found statistically significant.

For all independent variables tested, the quadratic effect of extraction temperature, time and solvent was statistically significant ($\rho < 0.01$). The polynomial equation for TPC response came as:

$$Y_{TPC} = 92.770 + 7.894\,X_1 + 44.625\,X_3 + 15.911\,X_1^2 + 13.254\,X_2^2 + 15.955\,X_3^2 + 14.854\,X_1X_2 + 22.566\,X_1X_3 + 24.244\,X_2X_3 \quad \textbf{(3)}$$

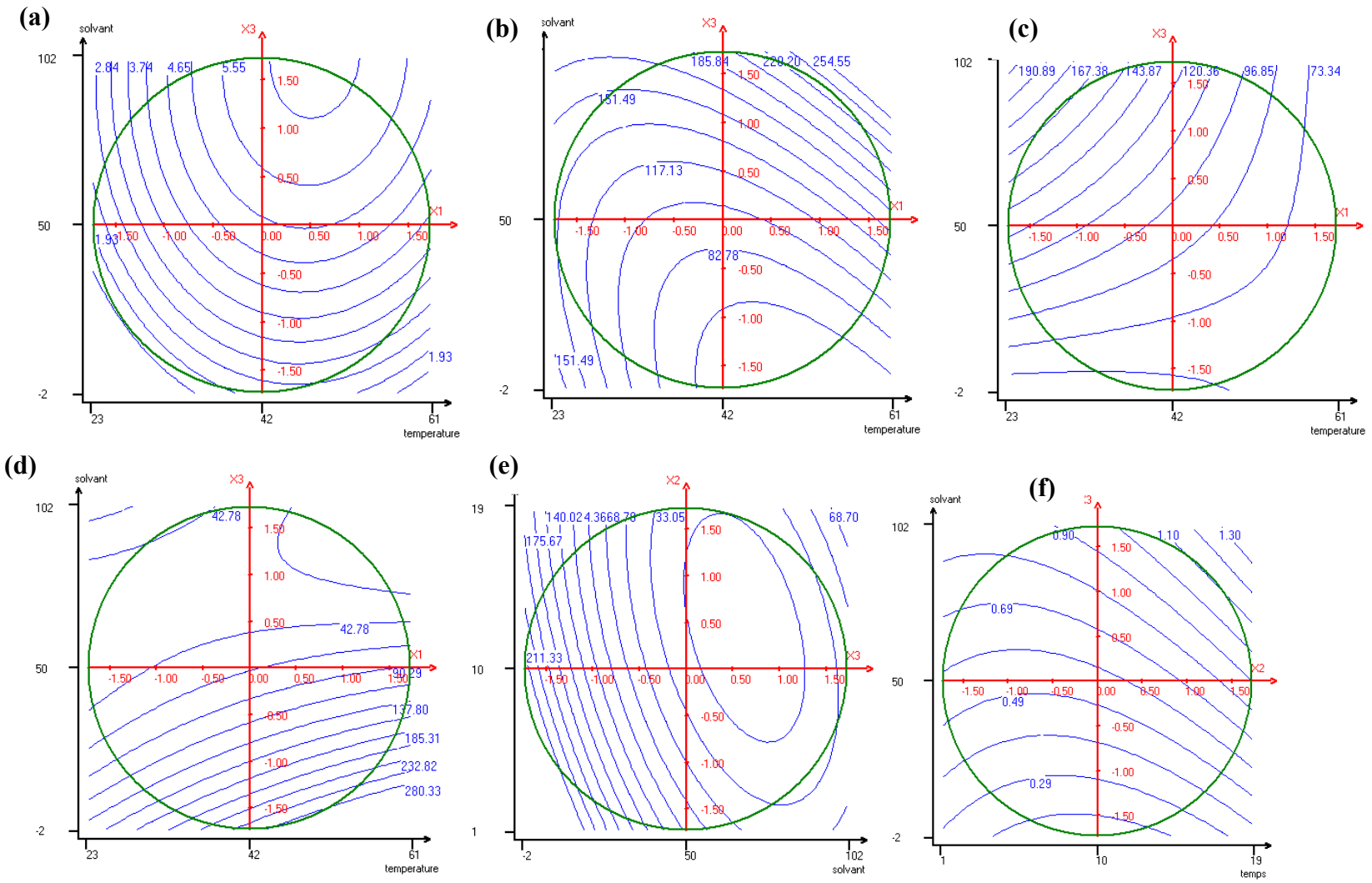

Fig 1. 2D response surface graphs for (a) Extraction Yield, (b) TPC, (c) TFC, (d) DPPH (e) ABTS $^{\bullet+}$, (f) FRAP

By referring to the regression coefficient, it appears that the methanol proportion was the most important factor which affects the TPC response, followed by the temperature.
The 2D response surface plot shown in fig1. illustrates the combined impact of extraction solvent and temperature. From this graph, it can be clearly observed that when the methanol proportion increased, TPC increased significantly ($\rho < 0.01$). This effect might be attributed to the solubility of phenolic compounds, which are strongly extractible by polar solvents. This suggestion was proved by previous study [20]. About the extraction temperature, a positive significant linear and quadratic effects were detected, indicating that the increase in temperature improves the TPC extraction. These findings are in agreement with previous studies, where it was shown that the phenolic compounds extraction enhances with increasing the extraction temperature [21-22]. In addition, a positive significant interactive was obtained between all variables used. From these results, it can be deduced that the most influential variables were extraction temperature and solvent composition. In literature, many researchers working with other plant have found in a similar result to those found in our work [23].

3.4. Response surface analysis for total flavonoids content (TFC)

Extraction of total flavonoids content from *T. hirsuta* aerial parts by a maceration technique under the experimental condition was significantly affected by extraction solvent ($\rho < 0.001$) and temperature ($\rho < 0.01$). Based on the regression coefficient displayed in table 3, it can be notice that the most difference in TFC might be due to the extraction solvent, followed by temperature. The response variables and the test variables are related by the following second-order polynomial equations:

$$Y_{TFC} = 87.815 - 11.789\,X_1 + 14.327\,X_3 \qquad \textbf{(4)}$$

The methanol proportion had a positive linear effect, which means that the use of higher methanol proportion lead to higher TFC in the extract. In contrast, extraction temperature had a negative effect. This can be explained by the simple fact that high temperature induces to the flavonoid compounds decomposition. Furthermore, none of interaction and quadratic terms of all variables have significant effect on TFC extraction. In litterature, many researchers have found that the flavonoid compounds extraction could be carried out at high temperatures. Although, some flavonoid compounds are thermo-sensitive mainly flavan-3-ol and derivatives as well as anthocyanin, which requires keeping the temperature extraction below at a certain limit [24-25]. To conclude, it is likely that our various extracts contained thermolabile flavonoids.

3.5. Response surface analysis for antioxidant activity

The measurement methods of antioxidant activity involve the oxidant mixture with an extract contained antioxidants capable to inhibit the radical's generation. These antioxidants can reduce the radicals by different mechanisms, which depends on the type of assay. Regarding the complexity of the process of oxidation, there is no universal and reliable method to confirm antioxidant power. In this respect, it is recommended to use at least two tests to obtain a complete prevision of the antioxidant effectiveness of a complex mixture. In this investigation, we chose to evaluate antioxidant activity in vitro by three chemical tests: $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and FRAP (ferric reducing antioxidant power) in order to optimize the extraction of antioxidants compounds. These methods showed a significant linear effect of extraction solvent on antioxidant activity. In the $ABTS^{\bullet+}$ assay, the obtained results showed that the IC_{50} ranging from 15.22 ± 1.82 to 211.33 ± 3.21 mg/L. In addition to the linear methanol proportion effect ($\rho < 0.001$), the extraction temperature ($\rho < 0.01$) and time ($\rho < 0.001$) have a linear impact statistically significant. The second-order polynomial equation of $ABTS^{\bullet+}$ activity is shown below:

$$Y_{ABTS\bullet+} = 22.587 + 8.104\,X_1 - 18.348\,X_2 - 51.442\,X_3 + 4.509\,X_1^2 + 9.384\,X_2^2 + 35.213\,X_3^2 - 5.160\,X_1X_2 - 4.820\,X_1X_3 + 15.150X_2X_3 \qquad \textbf{(5)}$$

Based on the regression coefficient (table 3), the extraction temperature affects most the $ABTS^{\bullet+}$ activity, which is proved by a positive linear effect compared to the extraction time and solvent. The increase of temperature from 24°C to 60°C induces the enhancement of the $ABTS^{\bullet+}$ activity significantly. Whereas the methanol proportion and time factors present a negative linear effect, suggesting that increasing the methanol level and

duration of extraction decreased the $ABTS^{\bullet+}$ activity. In this assay, it is important to note that the lowest value of IC_{50} corresponds to the highest activity. In fact, this allows us to suggest that the greatest activity is more favorable at high methanol proportion and extraction time and low temperature. The combined effect of extraction solvent and time shown in Fig. 1 (c) confirms the hypothesis cited previously.
Regarding the $DPPH^{\bullet}$ activity results (table 2), we can notice that the IC_{50} varying between 19. 03 ± 0.40 and 280.33 ± 2.08 mg/L. The linear effect of temperature ($\rho < 0.05$), time ($\rho < 0.05$) and extraction solvent ($\rho < 0.01$) was observed. The second-order polynomial equation for $DPPH^{\bullet}$ was given bellow:

$$Y_{\mathrm{DPPH}\bullet} = = \ 63.300 + 17.937\ \mathrm{X}_1 - \ 65\ .750\ \mathrm{X}_3 + 26.290\ \mathrm{X}_3^2 - 26.044 X_1 X_3 \qquad \textbf{(6)}$$

From this polynomial equation, it can be seen that the temperature extraction had a positive linear effect. This showed that increasing the temperature extraction from 24°C to 60°C leads the improvement of the $DPPH^{\bullet}$ activity significantly. The linear regression coefficients for methanol proportion were negative. As described previously in the $ABTS^{\bullet+}$ assay, our objective is to obtain a lower IC_{50}.This demonstrated that a greater $DPPH^{\bullet}$ activity could be achieved when the highest methanol proportion and lowest extraction temperature were selected. The combined impact of temperature and methanol proportion was illustrated in Fig.1 (d). A significant negative interaction between temperature and methanol proportion (X_1X_3) has also been observed ($p < 0.05$). According to the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ results, we can deduce that increasing temperature favored antioxidant compounds extraction by improving both the solubility of solute and the diffusion coefficient. These findings are in agreement with the report by Liu et al [26]
In the case of FRAP assay, the most significant linear variable on FRAP assay was extraction solvent with regression coefficient value 0.240 ($p < 0.01$), followed by duration of the extraction with regression coefficient equal to 0.093 ($p < 0.05$). The quadratic term of time and temperature had a significant effect ($p < 0.05$) on FRAP test. The following reduced second order models in terms of actual factors for FRAP assay as a function of temperature (X_1), extraction time (X_2) and methanol proportion (X_3) were obtained bellow:

$$Y_{FRAP} = \ 0.551\ 0.066\ \mathrm{X}_1 + 0.093\ \mathrm{X}_2 \ + \ 0.240\ \mathrm{X}_3 + \ 0.033\ \mathrm{X}_1^2 + \ 0.056\ \mathrm{X}_2^2 - 0.073\ \mathrm{X}_1 X_2 - 0\ .112\ X_1 X_3 \ + \ 0\ .052 X_2 X_3 \qquad \textbf{(7)}$$

As were seen in table 3, the extraction time and solvent possess a positive linear effect while the extraction temperature had a negative effect. This showed that when extraction carried out for longer time with a higher methanol proportion, FRAP value of the various extracts obtained by maceration technique increased. A negative linear regression coefficient of temperature was found significant. This revealed that an increase of temperature leads to a decrease in the FRAP values. Furthermore, increasing duration of extraction provides longer contact of plant materials with the extraction solvent which enhances the diffusion of antioxidant compounds. The extraction temperature and time also possess positive quadratic effect, which means that these factors played the dominant role in FRAP assay. The polynomial equation (7) indicates the presence a significant negative interaction between temperature and methanol proportion ($p < 0.05$) and between temperature and time ($p < 0.05$) while for time and methanol proportion a positive significant interaction appears. Based on these findings, it is important to note that the various extracts react differently to the three tests used. Such variation can be explained by their different mechanism of action for scavenging free radicals and impact of different variables on the extraction of antioxidant compounds.

3.6. Optimization and validation of the optimal conditions

Extraction of *T. hirsuta* aerial part was optimized in order to determine the maximum of TPC and TFC as well as the best antioxidant activity. Optimum extraction conditions were established according to the desirability function. To conclude, the model shows that the optimum extraction condition for all responses came as: a duration of extraction of 9h, an extraction temperature of 46° C and a methanol proportion of 93 %. Under these conditions, predicted TPC and TFC values were 187.79 mg GAE/g dw, 99.90 mg QE/g dw, respectively. In the case of antioxidant activity, the predicted value of $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP were 23.57 mg/L, 23.10 mg/L and 0.78 mM/L, respectively.

Conclusion

The RSM approach was successfully utilized to develop the phenolic compound extraction from *T. hirsuta* with enhanced yield as well as antioxidant activity using a CCD method. The effect of temperature, duration of extraction and solvent composition was clearly observed on the various responses. Among these factors, the temperature and methanol proportion played a key role on the TPC and TFC extraction as well as $DPPH^{\bullet}$ activity. Regarding $ABTS^{\bullet+}$ and FRAP activities, time and methanol proportion were the most important variables affecting extraction. The optimal conditions obtained for simultaneous maximum extraction of TPC were a temperature of 46° C, a duration of extraction of 9h, and a methanol proportion of 93 %. Under the optimized condition, predicted TPC value were 187.79 mg GAE/g dw. These results demonstrate that the *T. hirsuta* aerial parts were rich in natural phenolic compounds that could replace synthetic compound and used as a source of natural product.

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