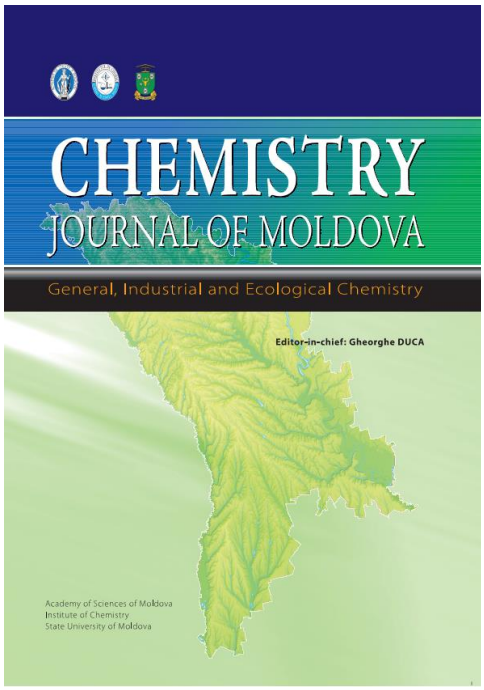

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OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF ANTIOXIDANT PHENOLICS FROM ALGERIAN *TRIFOLIUM TOMENTOSUM* L. USING RESPONSE SURFACE METHODOLOGY

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Abstract. The current study aims to maximize the recovery of antioxidant phenolics from Algerian *Trifolium tomentosum* L. using an innovative green process: ultrasonic assisted extraction (UAE). Firstly, four different solvents were used: 50% acetone, 50% ethanol, 50% methanol, and 100% ethyl acetate, as well as four different extraction methods: maceration, refluxed extraction, Soxhlet extraction, and ultrasonic assisted extraction (UAE). The classification of the best solvent (50% ethanol) and most effective extraction method (UAE) on the basis of quantified total phenolic content (TPC) led to the second part, which focused on optimizing the UAE using response surface methodology (RSM) and a Box Behnken design (BBD). Algerian *Trifolium tomentosum* L. extract demonstrated intriguing total phenolic and flavonoid contents (TPC and TFC) greater than 30 mg GAE/g dw and 6 mg QE/g dw, respectively, and potential total antioxidant capacity (TAC), closer to 20 mg AAE/g dw, under the optimal conditions with 70% ethanol concentration, an extraction time of 30.4545 minutes, and an extraction temperature of 75°C. Based on these findings, Algerian *Trifolium tomentosum* L. optimized extract can be used as a green natural ingredient in cosmetic formulations as well as a food preservative.

Keywords: *Trifolium tomentosum* L., antioxidant phenolics, green process, ultrasonic, response surface methodology.

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Introduction

Plants are an important source for drug discovery and development because they often include high amounts of bioactive secondary metabolites. Crude plant extracts may contain different chemicals such as alkaloids, terpenoids, and phenolics, all of which contribute to their biological properties. Non-polar extracts, for instance, can exhibit interesting cytotoxic effects as they contain considerable levels of some terpenoids, like diterpenes and sesquitepenes [1,2]. On the other hand, phenolic compounds, particularly flavonoids, are emerging as promising bioactive ingredients with novel bioactivities like antioxidant, antimicrobial, anti-inflammatory, antiproliferative, antihemolytic, neuroprotective, and photoprotective abilities [3-6].

Clover is a common name for plants in the *Trifolium* genus (Fabaceae family), which contains around 255 herbaceous, perennial, and annual species that occur in the Mediterranean region, East Europe, Eurasia, the highlands of eastern Africa, and western North America [7]. The Algerian natural flora includes 37 species of clover [8].

The most common clovers are red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.). People have long used red clover to treat bronchitis, burns, sedation, polycystic ovarian syndrome, heart conditions, and as an anti-diabetic and laxative. In Russia and Ukraine, white clover's biological properties have piqued interest due to their potential medicinal uses in folk medicines for bronchial asthma, headaches,

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analgesics, antitoxins, diuretics, wound healing remedies, epilepsy, pulmonary tuberculosis, and gynecological diseases. Phytochemical studies on *Trifolium* species have revealed a high concentration of bioactive secondary metabolites, like phenolics, flavonoids, isoflavonoids, coumarins, and saponins [9].

The present work focused on Algerian clover, *Trifolium tomentosum* L. The first step of this investigation is to attain an effective extraction process. For that purpose, various methods (maceration, refluxed extraction, Soxhlet method, ultrasound-assisted extraction) and conditions (different solvents) were compared in terms of the amount of total phenolic content (TPC) in the issued extracts. Then, using a surface response methodology (RSM) and a Box Behnken design (BBD), we attempted to optimize ultrasound-assisted extraction (UAE), since that was the best extraction method for the prepared extract, by investigating the influence of process parameters (extraction time, extraction temperature, and solvent concentration) on responses (TPC, TFC, and TAC).

Experimental

Plant material and chemicals

Trifolium tomentosum L. plant material was collected from El Kala, province of El Taref (Northeastern from Algeria). Polyphenol standards (gallic acid and quercetin) were purchased from Sigma-Aldrich (St. Louis, MO) and used for calibration curves. Other chemicals: ammonium molybdate tetrahydrate, ascorbic acid, the Folin-Ciocalteu reagent, and aluminium chloride were purchased from Sigma-Aldrich (Steinheim, Germany). The solvents and all standards used were of analytical grade.

Extraction procedures and preliminary study

Maceration

Dried and powdered samples (1 g) were macerated separately with 30 mL of each solvent (50% ethanol, 50% methanol, 50% acetone, and 100% ethyl acetate) for 24 h at room temperature. No additional stirring was applied; after this period, the mixture was filtered through Whatman No. 1 paper and diluted for further analysis (TPC) [10,11].

Refluxed extraction

Dried and powdered samples (1 g) were refluxed with 30 mL of each solvent (50% ethanol, 50% methanol, 50% acetone, and 100% ethyl acetate) for 2 hours at a temperature of 75°C. Then the extract was filtrated (after cooling) through Whatman No. 1 paper and diluted for further analysis (TPC) [11,12].

Soxhlet extraction

Dried powdered plant samples (10 g) were extracted continuously with 300 mL of each solvent (50% ethanol, 50% methanol, 50% acetone, and 100% ethyl acetate) for 6 h at a maximum temperature of 75°C using a Soxhlet apparatus. After the extraction time was completed, the Soxhlet extract was filtered (after cooling) and diluted for further analysis (TPC) [11].

Ultrasonic extraction

Dried powdered plant samples (1 g) were extracted with each solvent (50% ethanol, 50% methanol, 50% acetone, and 100% ethyl acetate) by considering the ratio (30/1; v/m) using an ultrasonic cleaning bath (ultrasounds-H, 50/60 hz, 720W, Ctra. Nll Km: 585.1 Abrera (Barcelona), Spain). The sonication was performed for 60 minutes at room temperature. Following extraction and cooling, samples were filtered through Whatman No. 1 paper and subjected to analysis their TPC, after adequate dilution [11,12].

Optimization of ultrasound-assisted extraction method

The independent parameters were selected by considering the results of the preliminary tests and previously published papers on *Trifolium* species [10-15]. In all experimental runs, 1g of powdered *Trifolium tomentosum* L. was mixed with 30 mL of the extraction solvent in screw-cap tubes and sonicated at different times and temperatures as required by the experiment (Table 2) using ultrasonic cleaning bath equipment (ultrasounds-H, 50/60 hz, 720W, Ctra. Nll Km: 585.1 Abrera (Barcelona), Spain). After extraction and cooling, the samples were filtered through Whatman No. 1 paper and tested for TPC, TFC, and TAC.

Experimental design

A Box Behnken design (BBD) was implemented on three independent variables across three levels, with six axial points and six replicates of the central point, totalling 18 extractions [16]. Table 1 displays the coding values, levels, and real values. Table 2 illustrates the parameters used in the experiment and the Y responses for each test, as well as their respective averages.

Table 1

Estimated optimal conditions, predicted values, and experimental values of investigated responses.

Independent variables	*Code*	*Factors levels*		
		-1	0	1
Ethanol concentration (%)	X_1	30	50	70
Extraction time (Min)	X_2	15	30	45
Temperature (°C)	X_3	25	50	75

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By employing the obtained results, the regression coefficients can be calculated, and the equation that best fits each test performed in this study can be generated using the Eq.(1) [16].

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \alpha_{ij} X_i X_J \qquad (1)$$

where, Y represents the predicted response value (TPC, TFC, TAC), while X_1, X_2, and X_3 are independent variables (solvent concentration, extraction time, and extraction temperature);
α_0 signifies the theoretical mean value of the response when all variables are at level 0;
α_i denotes linear regression coefficients;
α_{ii} denotes quadratic regression coefficients;
α_{ij} represents interaction regression coefficients.

Determination of bioactive content (TPC and TFC)

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu test. As a whole, 300 µL of diluted extract was combined with 1200 µL of Folin-Ciocalteu reagent (diluted 1:10). After 5 minutes, 1500 µL of 7.50% Na_2CO_3 solution was added to the mixture. The tubes were kept at room temperature in the dark for two hours.

A UV/Visible spectrophotometer was used to determine the absorbance at 765 nm. All assays were performed in triplicate. The TPC was calculated using a calibration curve based on gallic acid (15.62–200 µg/mL). The findings were presented in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/ g dw) [17].

The total flavonoid content (TFC) was determined with the aluminium trichloride ($AlCl_3$) method. In brief, 1500µL of 2% $AlCl_3$ solution was added to 1500µL of each appropriate diluted extract. After 10 minutes, the mixture's absorbance was measured at 430 nm. TFC was calculated using a standard curve with varying concentrations (1.95–40 µg/mL) of quercetin under identical conditions. The TFC was expressed as milligrams of quercetin equivalents per gram of dry weight (mg EQ/g dw). The experiment was conducted three times [18].

Determination of total antioxidant capacity (TAC)

The total antioxidant activity (TAC) of the testing extracts was assessed using Prieto, P. *et al.* phosphomolybdate method. In summary, 100 µL of each dilution from each experimental sample was added to 1000 µL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After 90 minutes of incubation at 95°C, the absorbance was measured against a blank at 695 nm. The sample's TAC was measured in milligrams of ascorbic acid equivalent per gram of dry weight (mg AAE/g dw). All assays were carried out in triplicate [19].

Statistical analysis

Multiple regression analysis was executed using Minitab Release 19 (Minitab Inc., State College, PA, USA). The models were used to generate response surfaces in Statistica 10 (Statsoft, France). A one-way analysis of variance (ANOVA) was applied to compare the effects of selected variables on the responses. The coefficient of determination (R^2) was calculated, and model adequacy was assessed by dividing the residual sum of squares into pure error and lack-of-fit. Minitab Release 19 (Minitab Inc., State College, PA, USA) was used for optimization as well.

Results and discussion

Preliminary assays

The current research was started by conducting preliminary tests to determine the most efficient extraction method and solvent for *Trifolium tomentosum* L. A total of 16 extracts were prepared by comparing three traditional extraction methods (maceration, reflux, and Soxhlet) to the environmentally friendly ultrasonic extraction process (Figure 1).

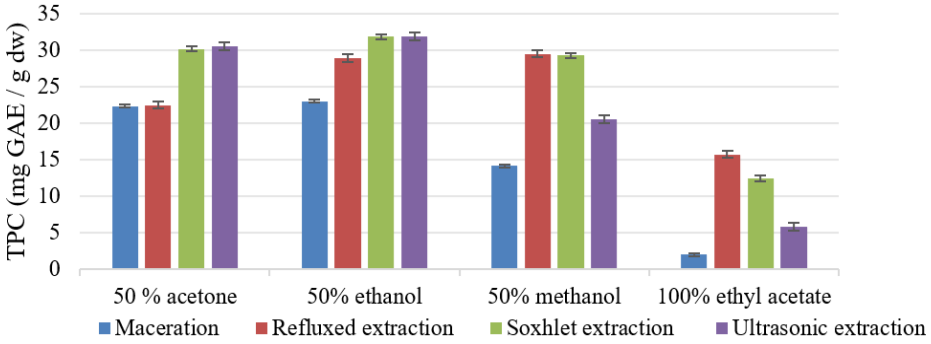

Figure 1. Effect of solvent and extraction methods on *Trifolium tomentosum* L.'s total phenolics (TPC).

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Was used four different solvents: 50% acetone, 50% ethanol, 50% methanol, and 100% ethyl acetate. The solvent-to-solid ratio (30/1; v/m) remained consistent across all experiments.

In general (Figure 1), the extraction methods tested in our study were efficient with all solvents except those containing 100% ethyl acetate. For 50% methanol, the reflux and Soxhlet methods produced similar results of TPC (29.54±0.355 and 29.32±0.215 mg GAE/g dw, respectively). The ultrasonic extraction method yielded a medium TPC value (20.55±0.01 mg GAE/g dw). Maceration resulted in a lower value (14.18±0.26 mg GAE /g dw) than previous methods. For 50% ethanol, reflux, Soxhlet, and ultrasound allowed the extracts with the highest TPC values (ranging from 28.95±0.505 to 31.95±0.5 mg GAE/g dw), while the maceration method produced the lowest value (23.02±0.18 mg GAE/g dw). For 50% acetone, maceration and reflux provided comparable TPC values (22.34±0.21 and 22.51±0.04 mg GAE/g dw, respectively), as well as Soxhlet and ultrasound (30.20±0.3 and 30.55±0.45 mg GAE/g dw). TPC quantification with 100% ethyl acetate yielded consistently lower results than with other solvents tested.

According to the findings of these preliminary studies, increasing the extraction temperature resulted in an increase in total phenolic content (TPC), as demonstrated by comparing the levels of TPC obtained using heated methods (reflux and Soxhlet) and cold methods (maceration). This phenomenon could be explained by improved mass transfer at high temperatures due to the increased solubility and diffusivity of polyphenols in the solvent [6,20,21]. However, when ultrasonic-assisted extraction and maceration results were compared, it was discovered that ultrasound waves increased TPC extraction yield. Ultrasounds exhibit a mechanical effect; by compressing and expanding ultrasonic waves, cavitation improves solvent penetration into the sample matrix and increases the contact surface between the solid and liquid phases, leading to greater diffusion and mass transfer [20-24].

Even though 50% methanol was the best extractable solvent (TPC= 29.54±0.355 mg GAE/g dw), when employed with the reflux method, 50% ethanol offered higher levels of total phenolic content (TPC) using maceration, Soxhlet, and ultrasonic-assisted extraction processes. The extraction yield of phenolic compounds has been shown to be higher as a mixture of solvents, like water and ethanol, is used, due to the different optimum amounts of extractability of compounds varying in polarities for the various solvents in the mixture. Ethanol was preferred due to its high affinity for phenolic compounds and its environmental friendliness [6,20,23].

As concluded results from the first step, of this work, the ultrasonic-assisted extraction method for 60 minutes with 50% ethanol produced the highest phenolic content (TPC) (31.95±0.5 mg GAE/g dw), followed by Soxhlet (31.89±0.055 mg GAE/g dw) for 6 hours, reflux (28.95±0.505 mg GAE/g dw) for 2 hours, and finally maceration (23.02±0.18 mg GAE/g dw) for 24 hours. Ultrasonic-assisted extraction resulted in higher polyphenol level in shorter periods of time, resulting in a significant reduction in energy consumed. As an outcome, ethanol was chosen as a suitable extraction solvent, and an ultrasound-assisted procedure was selected as the extraction method for the next experiments. Our findings are in line with those recently reported by Gligor, O. *et al.* [11], as well as many previous studies [6,11,20,22,24]. Several investigations have proven that ultrasonic-assisted extractions possess multiple benefits in the extraction procedure. However, extended ultrasound treatment combined with high temperatures can degrade bioactive compounds, lowering their antioxidant quality [6,20-22]. Thus, optimizing experimental parameters such as solvent mixture, time, power, ultrasonic frequency, and temperature can reduce the degree of degradation, implying that optimization studies improve both product quantity and quality [6,20-22,24].

Box Behnken design (BBD) results

The chosen working parameters were *X1* (solvent concentration), *X2* (extraction time), and *X3* (extraction temperature). In order to maximize the total phenolic and flavonoid contents (TPC and TFC) and antioxidant capacity (TAC) of *Trifolium tomentosum* L. extracts, the impact of selected parameters on the ultrasonic-assisted extraction (UAE) process was studied by response surface methodology (RSM).

Across the experimental design, each response (TPC, TFC, and TAC) was carried out. Table 2 displays the decoded values and results from 18 experiments. Algerian *Trifolium tomentosum* L. extracts ranged in total phenolic content (TPC) from 20.027 to 32.43 mg GAE/g dw, total flavonoid content (TFC) from 3.48 to 6.12 mg QE/g dw, and total antioxidant capacity (TAC) from 15.104 to 19.438 mg AAE/g dw.

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Effect of process variables on responses (TPC, TFC, and TAC)

Table 2 displays the TPC, TFC, and TAC of *Trifolium tomentosum* L. extracts obtained using ultrasonic extraction. The experimental data was analysed using regression, and model coefficients were tested for significance in phenolic compound extraction. ANOVA for the response (TPC) yielded an R^2 of 98.08%, indicating a good fit between the model and the experimental results (Table 3).

The experimental data collected after 18 runs permitted us to predict all of the responses as a function of ethanol concentration, extraction time, and temperature. For the extraction of TPC, the Eq.(2) represents the relationship between the process variables.

According to the coefficients of the above equation and p-values in Table 3, all parameters (linear, quadratic, and interaction except for those between extraction time and temperature) had a significant effect on total phenolic content (TPC) extraction ($p< 0.0001$).

Table 3 also displays a satisfactory correlation between predicted and experimental data, with an R^2 value of 98.59% of the response (TFC). TFC is significantly affected by linear and interaction effects of all parameters ($p< 0.0001$) as follows in Eq.(3).

The quadratic effects of all parameters had no significant effect on TFC ($p> 0.05$).

For the TAC, the ANOVA analysis in Table 3, in addition to the equation model Eq.(4) of this response, revealed significant effects of linear, quadratic, and interaction terms of almost tested parameters ($p< 0.0001$), with negative effects for linear terms and positive effects for quadratic terms. Furthermore, the model has an R^2 correlation coefficient of 97.95%, indicating that it accurately represents the experimental results.

$$TPC = 41.19 - 0.6580X_1 + 0.0693X_2 - 0.1571X_3 + 0.006600{X_1}^2 + 0.001712{X_2}^2 + 0.000654{X_3}^2 - 0.005804X_1X_2 + 0.004215X_1X_3 - 0.000182X_2X_3 \quad (2)$$

$$TFC = 4.201 + 0.0127\,X_1 + 0.0081X_2 - 0.0447X_3 - 0.000182\,{X_1}^2 - 0.000338{X_2}^2 - 0.000179{X_3}^2 - 0.000990X_1X_2 + 0.000954X_1X_3 + 0.001504X_2X_3 \quad (3)$$

$$TAC = 35.97 - 0.3487X_1 - 0.4700X_2 - 0.1801X_3 + 0.002987{X_1}^2 + 0.003456{X_2}^2 + 0.002273{X_3}^2 + 0.003929X_1X_2 - 0.000738X_1X_3 - 0.000065X_2X_3 \quad (4)$$

Table 2

Box Behnken design (BBD) of three variables and three levels and the resulted responses: TPC, TFC, and TAC.

Entry	*UAE Independent variables*			*Investigated responses*		
	X1 (%)	*X2 (Min)*	*X3 (°C)*	*TPC (mg GAE/ g dw)*	*TFC (mg QE/ g dw)*	*TAC (mg AAE/ g dw)*
1	50	45	75	25.788	5.976	16.674
2	70	30	25	22.75	3.480	19.057
3	50	30	50	23.80	4.812	15.723
4	50	30	50	23.76	4.800	15.676
5	50	30	50	23.70	4.800	15.723
6	70	15	50	31.45	4.980	17.771
7	30	30	25	25.38	4.044	17.057
8	30	30	75	26.63	4.776	18.200
9	70	30	75	32.43	6.120	18.723
10	50	30	50	23.70	4.764	15.533
11	30	45	50	25,58	4.896	15.104
12	50	45	25	20.027	3.516	16.295
13	50	30	50	23.73	4.788	15.628
14	70	45	50	23.600	4.908	18.676
15	50	30	50	23.802	4.776	15.580
16	50	15	25	23.161	4.356	18.961
17	30	15	50	26.465	3.780	18.914
18	50	15	75	29.195	4.560	19.438

GAE: gallic acid equivalents; QE: quercetin equivalents; AAE: ascorbic acid equivalents; dw: dry weight; X_1: ethanol concentration; X_2: extraction time; X_3: extraction temperature.

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Table 3

ANOVA for design by the target responses (TPC, TFC, and TAC).

Source of variation	*DF*	*TPC*			*TFC*			*TAC*		
		Sum of Squares	*F-Value*	*p-Value*	*Sum of Squares*	*F-Value*	*p-Value*	*Sum of Squares*	*F-Value*	*p-Value*
Model	9	162.782	203.99	0.000[a]	8.03843	62.17	0.000[a]	39.5194	42.40	0.000[a]
Linear	3	98.489	370.26	0.000[a]	5.37822	124.79	0.000[a]	12.0958	38.93	0.000[a]
X_1	1	4.766	53.76	0.000[a]	0.49601	34.53	0.000[a]	3.0653	29.60	0.001[b]
X_2	1	29.170	328.98	0.001[b]	0.32805	22.84	0.001[b]	8.6840	83.85	0.000[a]
X_3	1	64.553	728.05	0.000[a]	4.55416	317.02	0.000[a]	0.3465	3.35	0.105[ns]
Quadratic	3	34.380	129.25	0.000[a]	0.12487	2.90	0.102[ns]	21.3179	68.61	0.000[a]
X_1*X_1	1	30.411	342.98	0.000[a]	0.02325	1.62	0.239[ns]	6.2296	60.15	0.000[a]
X_2*X_2	1	0.647	7.30	0.027[c]	0.02520	1.75	0.222[ns]	2.6384	25.48	0.001[b]
X_3*X_3	1	0.730	8.23	0.021[c]	0.05474	3.81	0.087[ns]	8.8061	85.03	0.000[a]
Interaction	3	29.913	112.45	0.000[a]	2.53534	58.83	0.000[a]	6.1056	19.65	0.000[a]
X_1*X_2	1	12.128	136.78	0.000[a]	0.35284	24.56	0.001[b]	5.5578	53.67	0.000[a]
X_1*X_3	1	17.766	200.37	0.000[a]	0.91012	63.35	0.000[a]	0.5454	5.27	0.051[ns]
X_2*X_3	1	0.019	0.21	0.569[ns]	1.27238	88.57	0.000[a]	0.0024	0.02	0.883[ns]
Error	8	0.709			0.11492			0.8285		
Lack of fit	3	0.699	108.89	0.000[a]	0.11336	121.12	0.000[a]	0.7983	44.09	0.001[b]
Pure error	5	0.011			0.00156			0.0302		
Total	17	163.491			8.15335			40.3479		
R^2		99.57%			98.59%			97.95%		
R^2 *(adj)*		99.08%			97.00%			95.64%		

DF: degree of freedom; [a]*statistically significant at p< 0.0001;* [b]*statistically significant at p< 0.01;* [c]*statistically significant at p< 0.05;* [ns]*not significant.*

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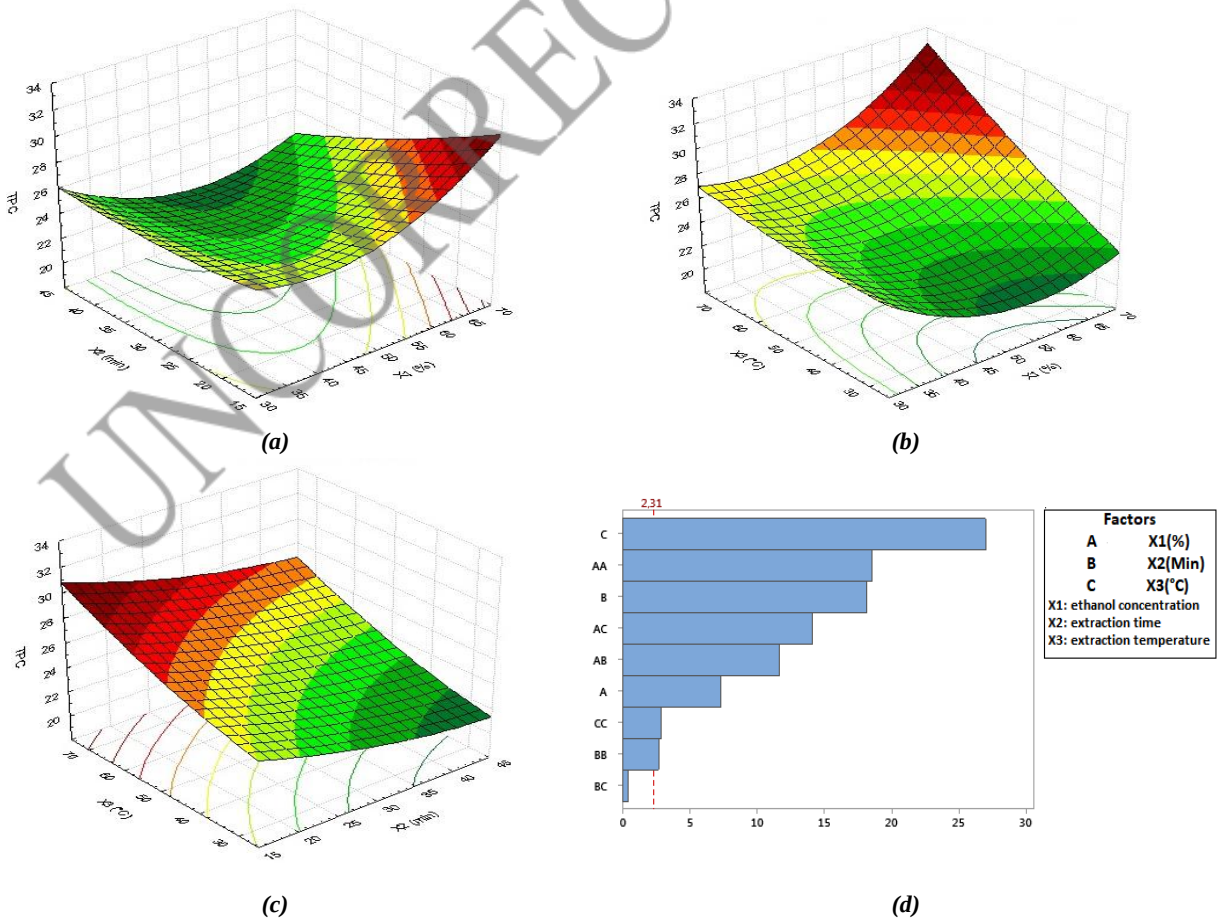

(a) ***(b)*** ***(c)*** ***(d)***

Figure 2. Response surface plots indicating combined effects of UAE variables on TPC (mg GAE/g dw): ethanol concentration and time *(a)*; ethanol concentration and temperature *(b)*; time and temperature *(c)*; Pareto chart (α= 0.05) *(d)*.

Based on the Pareto results in Figure 2*(d)*, extraction temperature had the greatest influence on TPC, followed by squared terms of ethanol concentration, extraction time, the interaction of ethanol concentration and extraction temperature, ethanol concentration after squared terms of extraction temperature, and finally squared terms of extraction time. The quadratic terms of all extraction conditions had significant positive effects, indicating that yield evolution had reached a minimum, whereas the interaction terms had both negative and positive effects.

The surface plot analysis in Figure 2 agrees with the multiple regression analysis. TPC increases with ethanol concentration for shorter periods of time, as shown in Figure 2*(a)*; higher phenolic content was obtained with ethanol concentrations ranging from 60 to 70% and extraction times ranging from 15 to 30 minutes. The highest phenolic concentration (TPC= 31.45 mg GAE/g dw) was detected for 15 minutes, along with the highest ethanol concentration (70%) at 50°C.

Figure 2*(b)* depicts the way extraction temperature and ethanol concentration affect TPC over a 30 minutes period. According to this analysis, TPC levels peaked between 55 and 75°C, when the ethanol concentration ranged from 55-70%. The greatest TPC value (TPC= 32.43 mg GAE/g dw) was achieved under the following experimental conditions: 70% ethanol concentration, 30-minute extraction time, and 75°C temperature. Regarding the effects of temperature extraction and time (Figure 2*(c)*), extraction with 50% ethanol at a higher temperature (55-75°C) for a period of 15 to 40 minutes produced the highest levels of TPC. Notably, the extractable TPC is inversely proportional to the extraction time.

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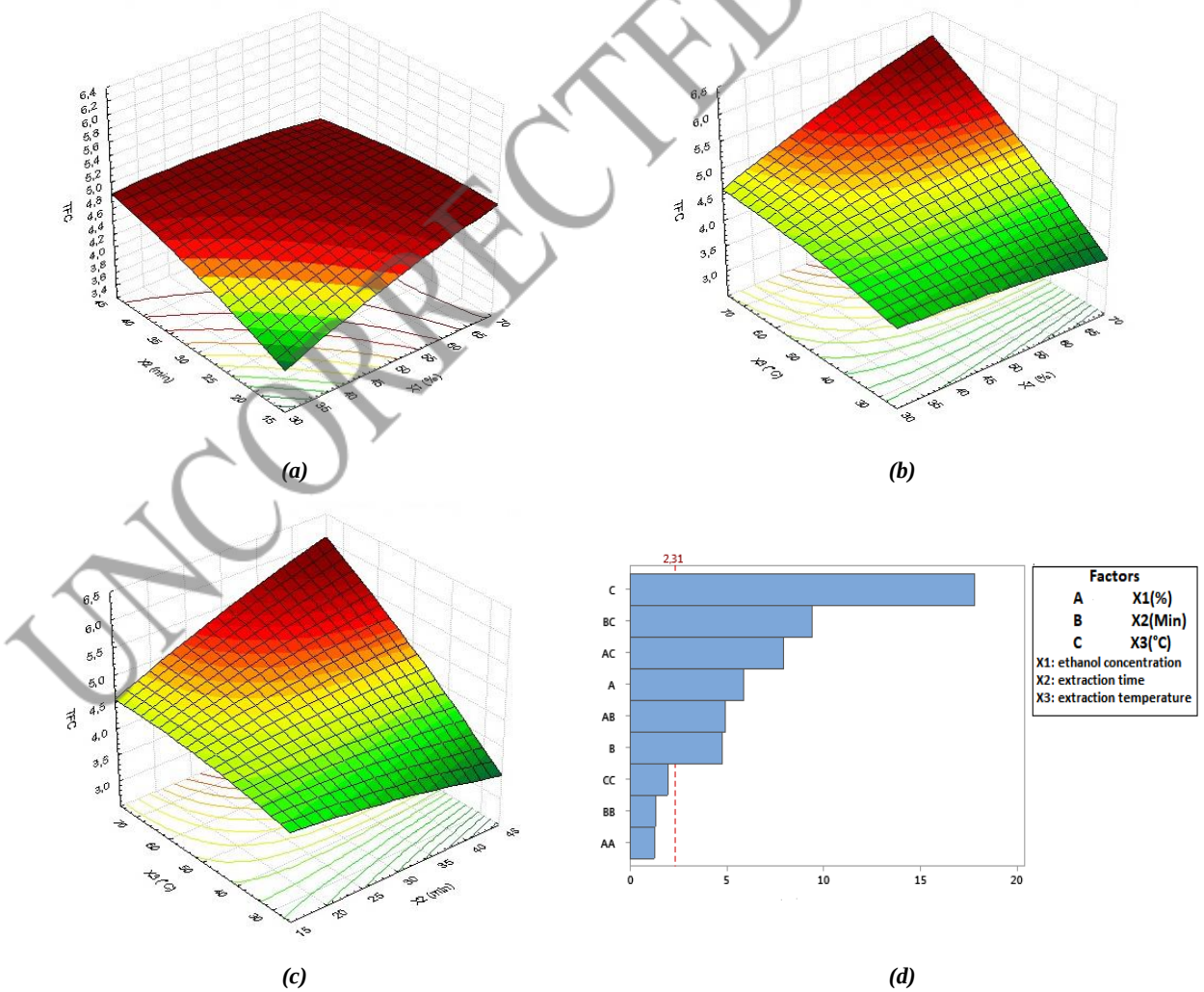

(a) *(b)* *(c)* *(d)*

Figure 3. Response surface plots indicating combined effects of UAE variables on TFC (mg QE/g dw): ethanol concentration and time *(a)*; ethanol concentration and temperature *(b)*; time and temperature *(c)*; Pareto chart (α= 0.05) *(d)*.

Similarly, to TPC, the study conducted via Pareto chart in Figure 3*(d)* discloses that the amount of TFC was considerably affected by the linear effect of extraction temperature as well as their interaction with both ethanol concentration and extraction time. The linear effects of ethanol concentration and extraction time, as well as their interaction, rank second. As shown in Figure 3*(a)*, combining a higher ethanol concentration (60-70%) with a longer time (40-45 min) led to the highest yield of TFC. In contrast to TPC, the increase in TFC is directly proportional to the extraction time. In a comparable manner, higher temperatures (60-75°C), coupled with a higher ethanol concentration (55-70%) or a longer extraction time (30-45 minutes), resulted in an increase in TFC. The maximum TFC (6.12 mg QE/g dw) was extracted under the same conditions as the highest level of TPC: 70% ethanol concentration and 30 minutes of extraction time at 75°C.

Figure 4*(d)* shows a Pareto chart that summarizes TAC's data. TAC was strongly influenced by both quadratic terms of extraction temperature and linear terms of extraction time. For ethanol concentration, the quadratic terms came first, followed by the interaction terms with extraction time and the linear terms. Then, quadratic terms of extraction time were identified. The figure 4*(d)* shows that the interaction between ethanol concentration and extraction temperature, the interaction between extraction time and temperature, and the linear temperature term were all insignificant. All quadratic parameter terms have significantly positive effects, indicating a minimum TAC in the studied range. On the other hand, the negative effects of ethanol concentration and extraction time, as well as their interaction, seem to suggest a lower ethanol concentration combined with a shorter extraction time would be used to mitigate the negative effects on TAC.

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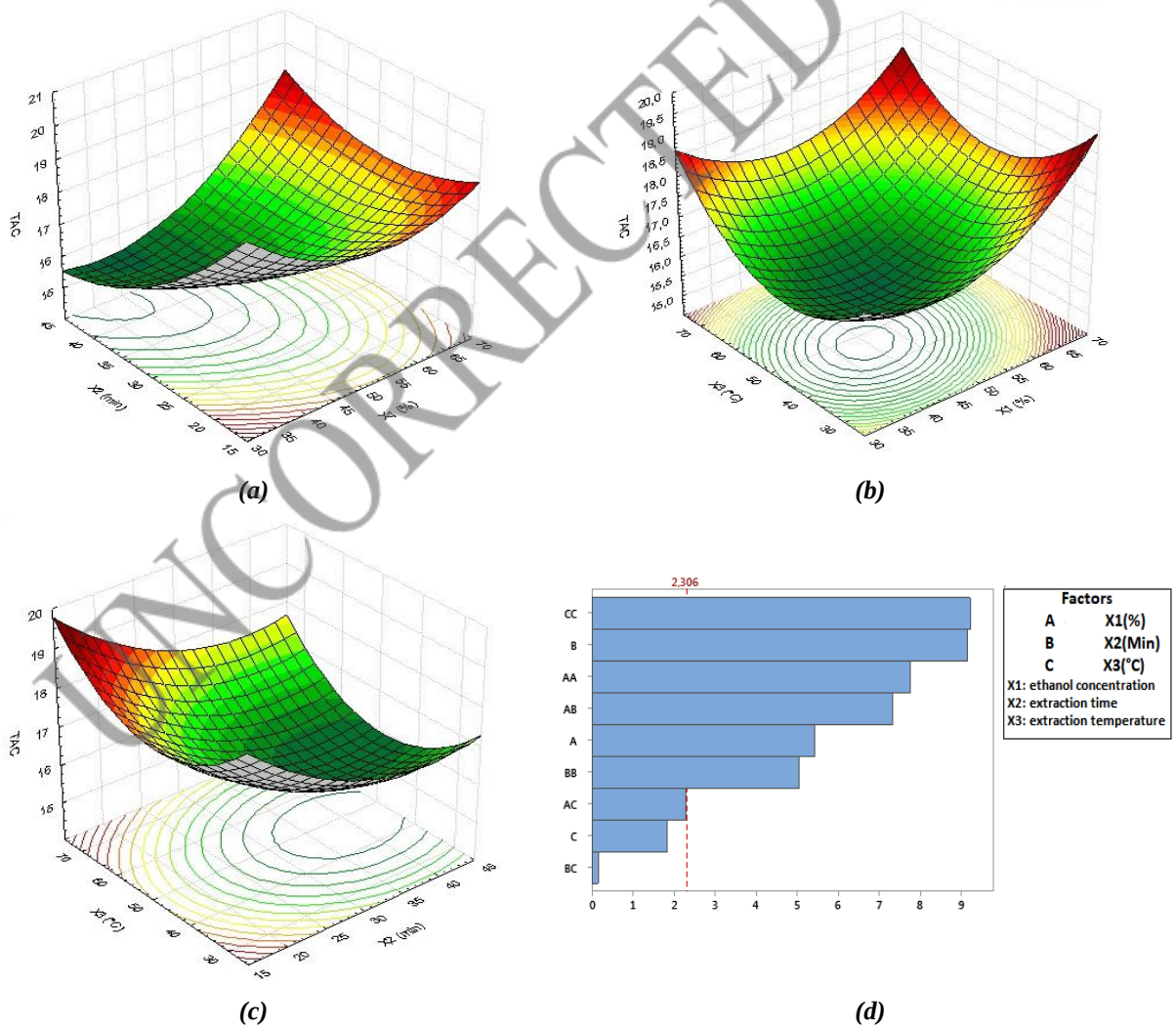

(a) *(b)* *(c)* *(d)*

Figure 4. Response surface plots indicating combined effects of UAE variables on TAC (mg AAE/g dw): ethanol concentration and time *(a)*; ethanol concentration and temperature *(b)*; time and temperature *(c)*; Pareto chart (α= 0.05) *(d)*.

Table 4

Estimated optimal conditions, predicted values, and experimental values of investigated responses.

UAE conditions	*Optimum values*	*Response variables*					
Time (Min)	30.4545	*TPC (mg GAE/g dw)*		*TFC (mg QE/g dw)*		*TAC (mg AAE/ g dw)*	
Temperature (°C)	75	*predicted*	*Experimental**	*Predicted*	*experimental*	*Predicted*	*Experimental*
Ethanol concentration (%)	70	32.4052	32.3300±0.06	6.0997	5.9800±0.04	18.7212	18.6995±0.02

**Values are the means ±SD of three independent replicates.*

The representation of the TAC response surface backs up the preceding conclusions. Figure 4*(a)* shows that the lowest TAC (15.104 mg AAE/g dw) was obtained at 50°C with a 30% ethanol concentration and a 45 minutes extraction time. Figure 4*(b)* depicts the effects of extraction temperature and ethanol concentration on TAC, implying that the higher these variables, the higher the values of TAC. At 30 minutes, a higher temperature (75°C) and a higher ethanol concentration (70%) resulted in the highest level of TAC (18.723 mg AAE/g dw). Figure 4*(c)* emphasizes the effects of temperature extraction and time on TAC, confirming the Pareto chart results (Figure 4*(d)*). Extraction with 50% ethanol for shorter periods of time at higher temperatures produced the highest TAC value (19.438 mg AAE/g dw at 75°C for 15 minutes). Although this value did not correspond to the highest concentration of TPC and TFC, it was found in extracts with high TPC and TFC levels. It has been demonstrated that the TAC of plant extracts can be affected not only by the abundance of polyphenols and flavonoids but also by the quantity and type of antioxidant compounds that have reducing potential [25].

It is important to note that previous research on *Trifolium* species has found that their extracts have a wide range of biological activities [9,26], including antioxidant capacity, which has been more recently extensively studied [11-15,27]. These potentials could be attributed to their polyphenolic profile, and our findings are completely consistent with previously obtained data [6,10,11-15,20-22,24-27].

Optimization of extraction conditions by RSM

To validate the model's predictive capacity, experimental confirmation was carried out under the optimized conditions obtained. The optimal conditions and predicted values were calculated using a desirability function.

Table 4 recaps the optimal UAE conditions for maximum TPC, TFC, and TAC responses, as well as the predicted and experimental responses. The experimental confirmation was carried out three times under the optimized conditions obtained from RSM.

The measured values agreed with those predicted by the desirability function. The high level of correlation recorded confirmed the predictability of the response models for assessing TPC, TFC, and TAC of *Trifolium tomentosum* L. As a result, the model could be successfully used to extract antioxidant polyphenols from *Trifolium tomentosum* L.

To the best of our knowledge, no research or comparative work has been conducted on the extraction of total phenolic contents (TPC) and total flavonoid contents (TFC) from *Trifolium tomentosum* L., as well as the assessment of total antioxidant capacity (TAC), either through conventional extraction or green extraction. Thus, our work was ranked as the first report on those findings. Besides, it is necessary to highlight that our work is the first to detail an ultrasound-assisted extraction optimization by surface response methodology (RSM) not only for Algerian *Trifolium tomentosum* L.'s extracts but also for all *Trifolium* genus.

Conclusion

In this study, the efficacy of ultrasonic-assisted extraction of polyphenols (TPC) with an ethanol-water mixture from Algerian *Trifolium tomentosum* L. versus conventional methods was first demonstrated. Even though the same solid-to-liquid ratio was used in all tested extraction processes, the duration of the ultrasound-assisted green procedure and hence the energy input were drastically reduced without affecting the quantity of TPC.

The second step involved using response surface methodology (RSM) to optimize the extraction conditions: ethanol concentration (%), extraction time (min), and extraction temperature (°C) in order to maximize the targeted responses: TPC, TFC, and TAC. The statistical and graphical data provided show that the extraction temperature was the most influential parameter in

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the extraction of all responses. The optimal UAE parameters for maximum responses in this experimental design process were a 70% ethanol concentration, an extraction time of 30.4545 minutes, and an extraction temperature of 75°C. As a result, optimized extracts prepared from Algerian *Trifolium tomentosum* L. by ultrasound may serve as a natural antioxidant alternative for photoprotective formulations or as a food preservative, supporting green chemistry principles and sustainability.

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