International Journal of Organic Chemistry, 2014, 4, 292-308
Published Online December 2014 in SciRes. http://www.scirp.org/journal/ijoc
http://dx.doi.org/10.4236/ijoc.2014.45032

Optimization of Extraction Conditions of Some Phenolic Compounds from White Horehound (*Marrubium vulgare* L.) Leaves

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Received 12 October 2014; revised 28 November 2014; accepted 13 December 2014

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Abstract

This research was aimed to optimize the extraction conditions of three phenolic compounds: total phenolics, flavonoids and condensed tannins, from White Horehound's leaves (*Marrubium vulgare* L.). Distilled water and different organic solvents such as: methanol, ethanol and acetone, were used, with various concentrations (20% - 80%, v/v), temperatures (20˚C - 60˚C) and extraction times (30 - 450 min). Results showed that the maximum total phenolics amounts (293.34 ± 14.60 mg gallic acid equivalent/g dry weigh), were obtained with 60% aqueous methanol at 25˚C for 180 min; total flavonoids (79.52 ± 0.55 mg catechin equivalent/g dry weigh) with 80% aqueous methanol at 20˚C for 450 min, and condensed tannins (28.15 ± 0.80 mg catechin equivalent/g dry weigh) with 60% aqueous acetone at 50˚C and for 180 min. ANOVA test showed the significant effect (*$P < 0.001$) of the extraction conditions tested on phenolic compounds. The Principal Component Analysis (PCA) exhibited the positive effect of low temperatures on total phenolics and flavonoids extraction, and the effect of high temperatures on the condensed tannins extraction. The Response Surface Methodology (RSM) provided predicted values of extraction conditions and maximum polyphenols amounts similar to those obtained experimentally.**

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How to cite this paper: Bouterfas, K., Mehdadi, Z., Benmansour, D., Khaled, M.B., Bouterfas, M. and Latreche, A. (2014) Optimization of Extraction Conditions of Some Phenolic Compounds from White Horehound (*Marrubium vulgare* L.) Leaves. *International Journal of Organic Chemistry*, **4**, 292-308. http://dx.doi.org/10.4236/ijoc.2014.45032

Keywords

***Marrubium vulgare*, Phenolic Compounds, Optimization, Extraction Conditions**

1. Introduction

Polyphenols constitute one of the most common and widespread groups of substances in flowering plants, occurring in all vegetative organs, as well as in flowers and fruits. These molecules are involved in many physiological processes such as cell growth, root formation, seed germination and fruit ripening [1]. Moreover, these compounds are considered secondary metabolites involved in the chemical defense of plants against predators, pathogens, environmental stresses and in plant-plant interferences [2].

Nowadays, phenolic compounds represent a unique and a functional place, composed of bioactive products, present in plant-derived foods and beverages and included in the formulations of well-marketed cosmetic and parapharmaceutical products [3]. Furthermore, polyphenols exhibit various biological activities such as anticancer [4], antioxidant [5], antimicrobial [6] and anti-inflammatory activities [7]. Therefore, in recent years, the determination of phenolic compounds concentrations in fruits [8] [9], vegetables [10] and some aromatic and medicinal plants [11] [12] has been of increasing interest in the scientific community as well as among health professionals and business partners.

It is well known that the content of phenolic compounds could be influenced by environmental conditions, such as season [13] [14], sampling period and geographic origin [15], precipitations and temperatures [16], and soil type [17]. Additionally, there are several experimental factors that can influence the rate of extraction and the quality of extracted bioactive phenolic compounds. These factors include extraction method, solvent type used and concentration [18], particle size of medicinal plants, temperature and pH of extraction [19], extraction time [20], number of extractions repetition [1] and solvent-to-sample ratio [21].

In order to recover bioactive compounds from plant raw materials, extraction is widely used and it constitutes the first important step [22]. Different solvents and techniques are used for the extraction of polyphenols from plants [23] [24]. However, there is no one standard extraction method used to extract phenolic compounds from plant materials because of their complexity and their interaction with other bioactive compounds [25]. Furthermore, each plant material has its unique properties, in term of phenolic extraction; different plants may require different extraction conditions to achieve the optimum recovery of phenolic compounds [26].

The family Lamiaceae includes aromatic and medicinal plants, which are used in traditional medicine, although Lamiaceae species are well known for their volatile oil content, their therapeutic activities and other properties. This reflects the existence of other chemical components, such as the polyphenols. *Marrubium vulgare* L. commonly known in Europe as "White horehound", and in the Mediterranean region as "Marute" or as "Merriouet" in Algeria, is a perennial herb of Lamiaceae family, naturalized in North and South America, and Western Asia [27]. In Algeria, *M. vulgare* is used in folk medicine to treat several digestive diseases, diarrhea, as well as diabetes, rheumatism, acute or chronic bronchitis, cough, asthma and other respiratory infections [28]. Earlier, phytochemical investigation of *M. vulgare* have led to the characterization of a very complex metabolic pattern, containing, among other secondary metabolites, diterpenes [29], phenyl propanoids esters [30], tannins [31] and flavonoids [32]. Several activities, traditionally attributed to *M. vulgare*, were approved by intensive modern research and clinical trials, such as hypoglycemic [33], vasorelaxant and antihypertensive [29], analgesic [34], antidiabetic [35], anti-inflammatory [36], and antioxidant properties [37].

However, to the best of our knowledge, optimizing the extraction of phenolic compounds from *M. vulgare* leaves, using different extraction conditions and response surface methodology (RSM), has not been reported yet. Hence, the purpose of the current study was to investigate the effects of different extracting conditions (organic solvent type, concentration of organic solvent, temperature and time) on the extraction of phenolic compounds (total phenolic content, TPC; total flavonoid content, TFC; and condensed tannins content, CTC) from *M. vulgare* leaves.

2. Material and Methods

2.1. Plant Material

Leaves of *M. vulgare* were collected in April 2012, from Tessala Mountain (north-western Algeria, semi-arid

climate) at the level of a station which latitudinal coordinates 35˚16'33"N, and longitudinal 0˚46'27"W, altitude 596 m. The identification of plant specimen was done by Professor Z. Mehdadi and put at the Laboratory of Vegetal Biodiversity: Conservation and Valorization (Faculty of Natural and Life Sciences, Djillali Liabes University of Sidi Bel-Abbes, Algeria).

Upon arrival at the laboratory, samples were thoroughly rinsed with distilled water, dried in the dark for three weeks at room temperature and crushed with the cutting mill. The powdered samples were packaged into a linear-low-density polyethylene (LLDPE) film and stored in dark at room temperature for further experiments.

2.2. Extract Preparation

Two grams of leaf powder *M. vulgare* were extracted using 20 ml of extraction solvent with different concentrations, introduced in conical flask of capacity 100 ml sealed with parafilm and wrapped with aluminum foil to prevent solvent loss and exposure to light. Therefore, the mixture was stirred at 150 rpm in bath water with controlling temperature at a constant speed (level 8) for a particular duration. After completing the extraction process, the *M. vulgare* extract was filtered using Whatman No. 1 filter paper into amber bottle for analysis without storage overnight, in order to obtain a clear crude extract solution.

To determine the optimal conditions for phenolic extraction from *M. vulgare* leaves, the extraction conditions were set according to the experimental design described below:

- *Organic solvents type*: three kinds of organic solvents (methanol, ethanol and acetone) were selected. Distilled water was tested as control. The selection of the three extraction solvents is made firstly after several (about one hundred) referenced protocols in terms of quantification of phenolic compounds and secondly from the viewpoint of the availability of the chemicals used;
- *Solvent concentrations*: four concentrations (20%, 40%, 60% and 80%; v/v) were prepared in distilled water;
- *Extraction temperatures*: six temperatures (20˚C, 25˚C, 30˚C, 40˚C, 50˚C and 60˚C) were used;
- *Extraction times*: six different times (30, 90, 180, 270, 360 and 450 min) were chosen.

2.3. Total Phenolic Contents (TPC)

TPC of *M. vulgare* extracts was determined using Folin-Ciocalteu reagent, according to method suggested by Li *et al*. [38] and slightly modified by Chew *et al.* [39]. Crude extracts were diluted 50 times with deionized water prior to analysis. 1 ml of diluted extract was mixed with 1 ml of diluted Folin-Ciocalteu reagent (10 times diluted with deionized water). After incubating the mixture at room temperature for 4 min, 0.8 ml of 7.5% (w/v), sodium carbonate anhydrous solution was added. The mixture was then vortexed for 10 s and incubated in the dark at room temperature during 2 h. The absorbance of the mixture was measured against blank at 765 nm using UVi light spectrophotometer. Gallic acid with different concentrations (0-100-200-300-400-500 mg/l) was used to calibrate the standard curve. The calibration equation for gallic acid was $Y = 0.0042X - 0.0178$ ($R^2 = 0.9992$). Each crude extract was analyzed in triplicate and the results were expressed in milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW). Data were expressed as mean ± standard deviation.

2.4. Total Flavonoids Contents (TFC)

TFC was determined using procedures described by Tan *et al.* [21]. The crude extract was diluted 10 times. An amount of 1.25 ml deionised water followed by 75 µl of 5% sodium nitrite (NaNO2), was added to 0.25 ml of diluted crude extract in an aluminium foil-wrapped 15 ml test tube. The mixture was left standing for 6 min before adding 150 µl of 10% (w/v) aluminium chloride (AlCl3). The mixture was left standing for 5 min before adding 0.5 ml of 1 M sodium hydroxide (NaOH) and 275 µl of deionised water. The tip of the test tube was covered with parafilm and then mixed using vortex mixer for approximately 10 s. The absorbance of the mixture was determined at 510 nm versus the prepared blank using Uvi light spectrophotometer. Catechin with different concentration (0 - 100 - 200 - 300 - 400 - 500 mg/l) was used for calibration. The calibration equation for catechin was calculated as follow: $Y = 0.0035X - 0.0062$ ($R^2 = 0.9995$). Each crude extract was analyzed in triplicate and the results were expressed in milligrams of catechin equivalents per gram of dry weight (mg CE/g DW). Data were expressed as mean ± standard deviation.

2.5. Total Flavonoid Contents (TFC)

TFC was determined using procedures described by Tan *et al.* [21]. The crude extract was diluted 10 times. An

amount of 1.25 ml deionised water followed by 75 µl of 5% sodium nitrite ($NaNO_2$), was added to 0.25 ml of diluted crude extract in an aluminium foil-wrapped 15 ml test tube. The mixture was left standing for 6 min before adding 150 µl of 10% (w/v) aluminium chloride (AlCl3). The mixture was left standing for 5 min before adding 0.5 ml of 1 M sodium hydroxide (NaOH) and 275 µl of deionised water. The tip of the test tube was covered with parafilm and then mixed using vortex mixer for approximately 10 s. The absorbance of the mixture was determined at 510 nm versus the prepared blank using Uvi light spectrophotometer. Catechin with different concentration (0 - 100 - 200 - 300 - 400 - 500 mg/l) was used for calibration. The calibration equation for catechin was calculated as follow: $Y = 0.0035X - 0.0062$ ($R^2 = 0.9995$). Each crude extract was analyzed in triplicate and the results were expressed in milligrams of catechin equivalents per gram of dry weight (mg CE/g DW). Data were expressed as mean ± standard deviation.

2.6. Condensed Tannins Contents (TFC)

CTC assay was performed according to the method described by Chew *et al*. [39]. 0.5 ml undiluted crude extract was firstly mixed with 3 ml of vanillin reagent (4%, w/v, in absolute methanol), followed by addition of 1.5 ml of concentrated HCl (37%). After that, the mixture was stored in the dark at room temperature for 15 min. The absorbance of mixture was measured at 500 nm against blank using Uv light spectrometer. Each undiluted crude extract was measured in triplicate. Catechin with different concentrations (0 - 100 - 200 - 300 - 400 - 500 mg/l) was used for calibration of standard curve. The calibration equation for catechin was calculated using the formula $Y = 0.0021X - 0.0143$ ($R^2 = 0.997$). Each crude extract was analyzed in triplicate and the results were expressed in milligrams of catechin equivalents per gram of dry weight (mg CE/g DW). Data were expressed as mean ± standard deviation.

2.7. Statistical Analysis

In order to control the influence of extraction conditions (solvent type, solvent concentration, extraction time, and extraction temperature) on the mean concentrations of each phenolic compound, ANOVA, with more classification criteria, using Fisher's least significant difference test and the significant differences at the 5% level, were calculated. The difference was considered as not significant when $P > 0.05$, significant when $^{*}P \leq 0.05$, and highly significant for $^{**}P \leq 0.01$ and extremely significant for $^{***}P \leq 0.001$. These analyzes were performed using Minitab 16. Tukey's test was also performed for pair-wise comparisons at the 5% level.

To determine possible correlation among polyphenols concentrations and extraction conditions, PCA was used. This statistical tool is dedicated for data exploration, which allows the reduction of the number of quantitative variables to a small number of components. Moreover, using PCA, interrelationships between different variables could be seen, and detected and sample patterns, groupings, similarities or differences could be interpreted [40]. A matrix is prepared using XLSTAT 2012 software, by taking polyphenols concentrations as observations and extraction conditions as variables.

Optimal conditions for the extraction of phenolic compounds from *M. vulgare* leaves were obtained using response surface methodology (RSM) [41] [42]. This method is adopted for each type of phenolic compound with the solvent giving the highest experimental concentration. The independent variables studies were solvent concentration (X_1), extraction temperature (X_2) and extraction time (X_3); while the dependent variable (Y: response variable) measured was the contents of phenolic compound. To obtain the best combination of independent variables giving a maximum percentage of adjusted R^2, a best subsets regression is performed using Minitab 16 software. Thus, the regression equation of the model is obtained by an analysis of general regression. Finally, the optimum polyphenols contents and extraction conditions are obtained by introducing the regression equation in Maple 6 software. The experimental and predicted values of polyphenols contents and extraction conditions were compared, in order to determine the validity of the model.

3. Results and Discussion

Results of total phenolic, total flavonoids, and condensed tannins amounts are shown in **Figure 1** and **Figure 2**. The influence of extraction's conditions used on phenolic compounds amounts obtained using ANOVA, with more classification criteria, is presented in **Table 1**.

Table 1. Analysis of variance (ANOVA) with more classification criteria of quantified phenolic compounds.

Source	Degree of freedom	Sum of Squares	Mean squares	F-value	*P*-value
		40326.1^{TPC}	20163.1^{TPC}	262.15^{TPC}	<0.0001***
{1}Solvent type	2	2326.12^{TFC}	1163.06^{TFC}	274.61^{TFC}	<0.0001***
		43.14^{CTC}	21.57^{CTC}	UD^{CTC}	<0.0001***
		107832.8^{TPC}	26958.2^{TPC}	350.49^{TPC}	<0.0001***
{2}Solvent concentration	4	5132.83^{TFC}	1283.21^{TFC}	302.98^{TFC}	<0.0001***
		257.24^{CTC}	64.31^{CTC}	UD^{CTC}	<0.0001***
		45983.2^{TPC}	9196.6^{TPC}	119.57^{TPC}	<0.0001***
{3}Extraction time	5	111.09^{TFC}	22.22^{TFC}	5.25^{TFC}	<0.0001***
		592.16^{CTC}	118.43^{CTC}	UD^{CTC}	<0.0001***
		553938.7^{TPC}	110787.7^{TPC}	1440.39^{TPC}	<0.0001***
{4}Extraction temperature	5	39821.11^{TFC}	7964.22^{TFC}	1880.44^{TFC}	<0.0001***
		10589.43^{CTC}	2117.89^{CTC}	UD^{CTC}	<0.0001***
		31097.1^{TPC}	3887.1^{TPC}	50.54^{TPC}	<0.0001***
{1}*{2}	8	1654.30^{TFC}	206.79^{TFC}	48.82^{TFC}	<0.0001***
		10.91^{CTC}	1.36^{CTC}	UD^{CTC}	<0.0001***
		7922.1^{TPC}	792.2^{TPC}	10.30^{TPC}	<0.0001***
{1}*{3}	10	54.86^{TFC}	5.49^{TFC}	1.30^{TFC}	=0.235^{NS}
		2.59^{CTC}	0.26^{CTC}	UD^{CTC}	<0.0001***
		14801.5^{TPC}	1480.1^{TPC}	19.24^{TPC}	<0.0001***
{1}*{4}	10	2422.48^{TFC}	242.25^{TFC}	57.20^{TFC}	<0.0001***
		0.00^{CTC}	0.00^{CTC}	UD^{CTC}	<0.0001***
		35664.5^{TPC}	1783.2^{TPC}	23.18^{TPC}	<0.0001***
{2}*{3}	20	599.22^{TFC}	29.96^{TFC}	7.07TFC	<0.0001***
		100.97^{CTC}	5.05^{CTC}	UD^{CTC}	<0.0001***
		48446.9^{TPC}	2422.3^{TPC}	31.49^{TPC}	<0.0001***
{2}*{4}	20	7603.54^{TFC}	380.18^{TFC}	89.76^{TFC}	<0.0001***
		233.26^{CTC}	11 .66^{CTC}	UD^{CTC}	<0.0001***
		18241.9^{TPC}	729.7^{TPC}	9.49^{TPC}	<0.0001***
{3}*{4}	25	767.42^{TFC}	30.70^{TFC}	7.25^{TFC}	<0.0001***
		92.06^{CTC}	3.68^{CTC}	UD^{CTC}	<0.0001***
		8775.9^{TPC}	219.4^{TPC}	2.58^{TPC}	<0.0001***
{1}*{2}*{3}	40	314.37^{TFC}	7.86^{TFC}	1.86^{TFC}	=0.003**
		1.26^{CTC}	0.03^{CTC}	UD^{CTC}	<0.0001***
		18002.2^{TPC}	450.1^{TPC}	5.85^{TPC}	<0.0001***
{1}*{2}*{4}	40	3061.91^{TFC}	76.55^{TFC}	18.07^{TFC}	<0.0001***
		0.00^{CTC}	0.00^{CTC}	0.00^{CTC}	<0.0001***
		5440.4^{TPC}	108.8^{TPC}	1.41^{TPC}	=0.050*
{1}*{3}*{4}	50	272.27^{TFC}	5.45^{TFC}	1.29^{TFC}	=0.116^{NS}
		0.00^{CTC}	0.00^{CTC}	UD^{CTC}	<0.0001***
		46098.9^{TPC}	461.0^{TPC}	5.99^{TPC}	<0.0001***
{2}*{3}*{4}	100	1176.72^{TFC}	11.77^{TFC}	2.78^{TFC}	<0.0001***
		176.67^{CTC}	1.77^{CTC}	UD^{CTC}	<0.0001***
		15383.0	76.09	UD^{TPC}	<0.0001***
{1}*{2}*{3}*{4}	200	847.06	4.24	UD^{TFC}	<0.0001***
		0.00	0.00	UD^{CTC}	<0.0001***

UD: undefined (the denominator of Fisher's test is null or undefined).

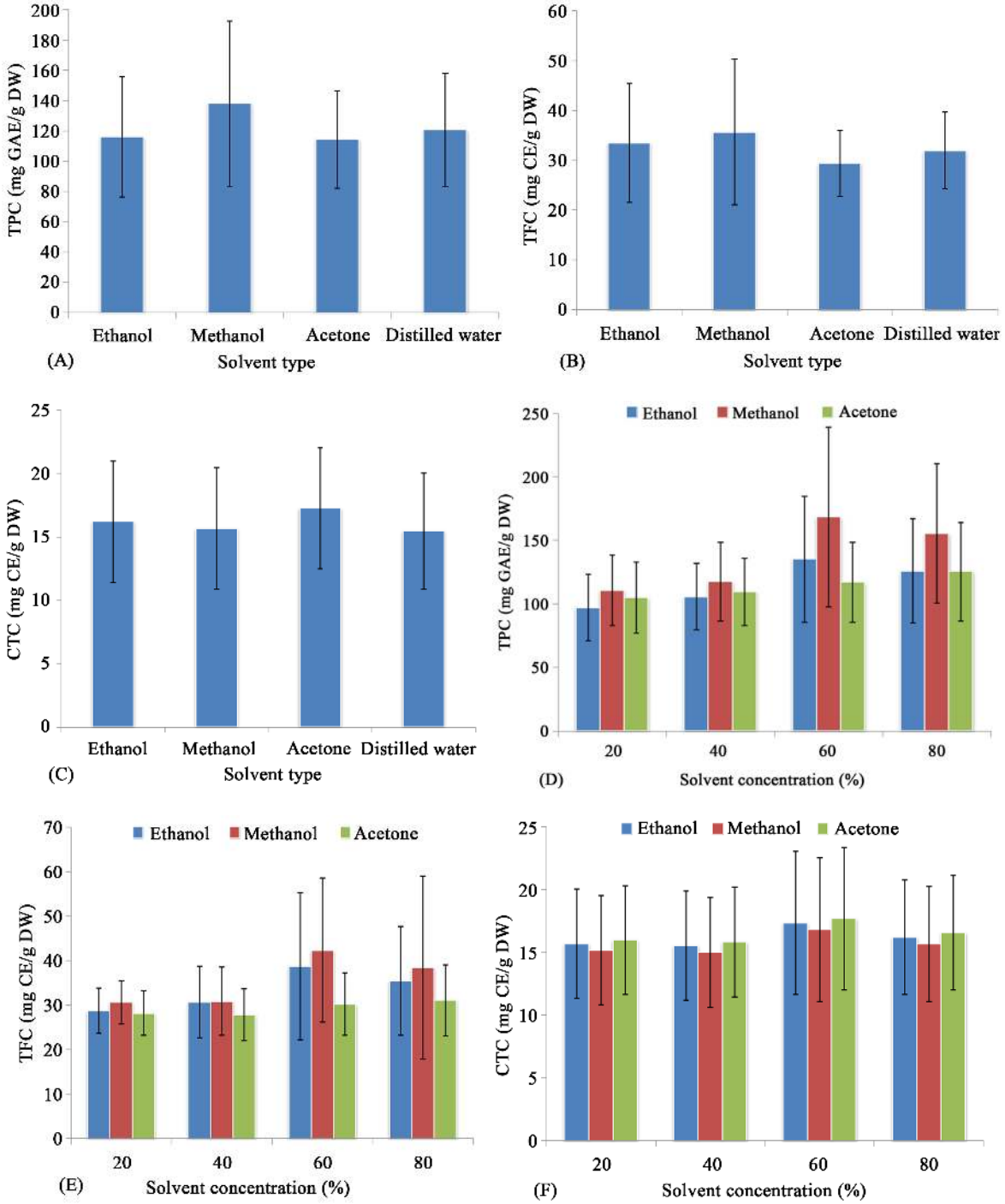

Figure 1. Effects of solvent type ((A), (B), (C)) and solvent concentration ((D), (E), (F)) on TPC, TFC and CTC extracted from *M. vulgare* leaves.

3.1. Effect of Solvent Type on Extraction of Phenolic Compounds

The choice of extraction solvents is important for complex food samples. They allow determining the amount and the type of phenolic compounds to extract. Organic solvents, particularly acetone, ethanol and methanol are the most commonly used in polyphenols extraction from botanical materials [43].

Our results showed that methanol (137.79 ± 54.62 mg GAE/g) was significantly high effective ($^{***}P < 0.001$)

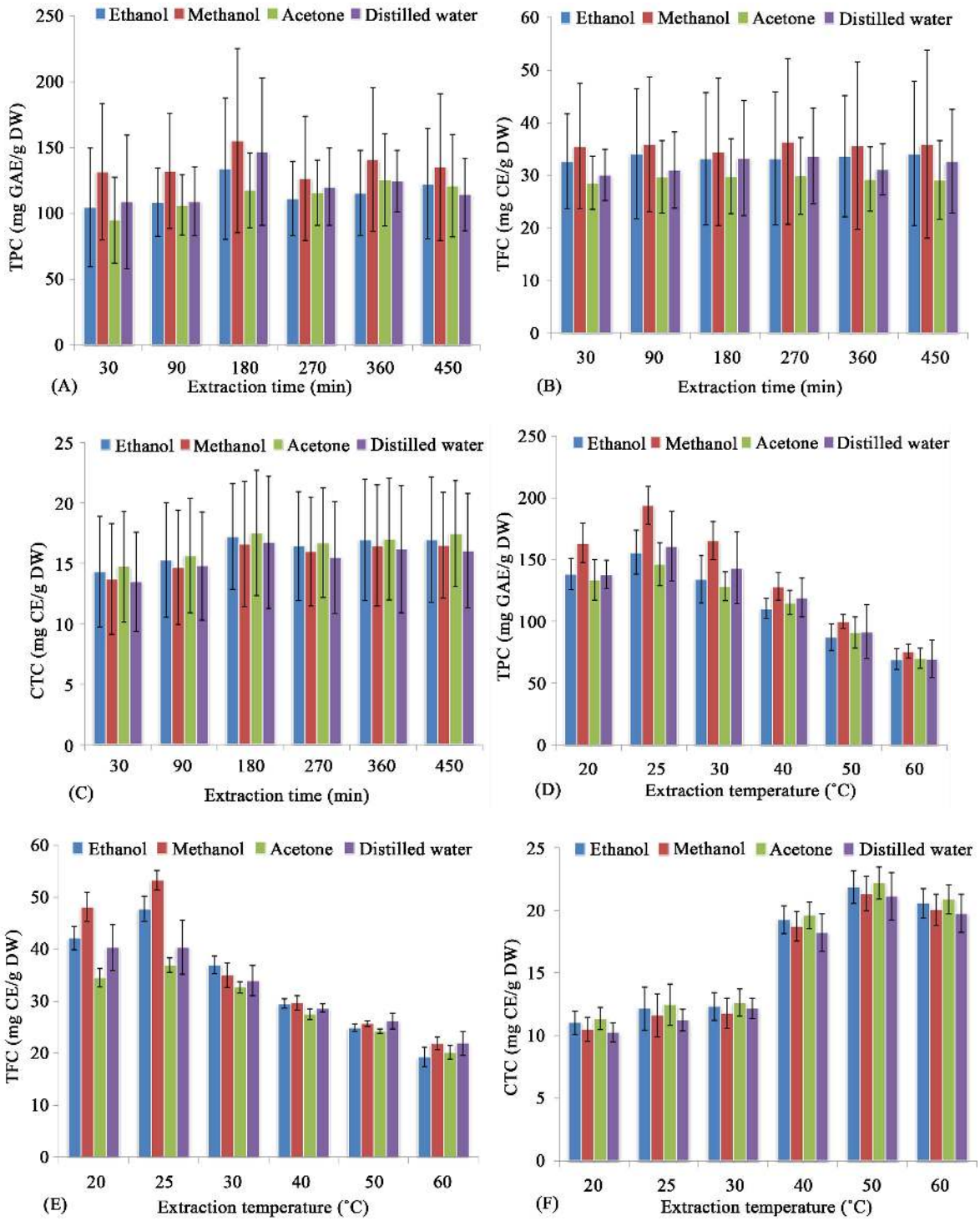

Figure 2. Effects of extraction time ((A), (B), (C)) and extraction temperature ((D), (E), (F)) on TPC, TFC and CTC extracted from *M. vulgare* leaves.

when compared with all other solvent systems used in extracting TPC from *M. vulgare* leaves, as shown in **Figure 1(A)**. Methanol has been considered as an ideal solvent for the TPC extraction from green walnut fruits [44], *Zingiber officinalis* leaves [45], *Tamarix aphylia* leaves [46] and *Artemisia annua* leaves [47]. Several studies confirmed that TPC depends on organic solvent polarity and methanol is one of the most suitable solvents for

the extraction of TPC from plants [48] [49]. Mohammedi and Atik [46] reported that the high polarity of methanol, estimated at 6.6, compared to other organic solvents, plays a key role in increasing phenolic solubility. However, Kong *et al.* [50] found that TPC in methanolic extracts, obtained from *Pouteria campechiana* fruit parts, was about 23 % to 45 % lower than the ethanolic extracts.

Methanol permitted to obtain the highest extraction levels of TFC (35.64 ± 14.61 mg CE/g) from *M. vulgare* leaves (**Figure 1(B)**). Furthermore, a significant difference ($^{***}P < 0.001$) was found between the type of organic solvents used. Similar results were observed for *Moringa oleifera* and *Aloe barbadensis* leaves [51], *Euphorbia helioscopia* leaves, steams and flowers [52]. Our results agree with previous studies, which found that ethanol was less effective than methanol for extracting TFC [53] [54]. In contrast, Settharaksa *et al.* [55] have found that using water to extract solvent from some Thai medicinal plants, was better than using ethanolic and methanolic solvents to extract TFC. Ghasemzadeh *et al.* [45] reported that extraction of TFC from *Zingiber officinale* leaves, using methanol, was about 3 times higher when using acetone and 4 times higher when using hexane. Spigno *et al.* [56] suggested that high level of TPC obtained using methanol, could be explained by the fact that this solvent allows a good solubility of flavonoids' hydroxyl groups.

A maximum level of CTC (17.24 ± 4.77 mg CE/g) was reached using acetone, as shown in **Figure 1(C)**. There was a high significant difference between the four tested solvents ($^{***}P < 0.001$). Our results agree with those of Trabelsi *et al.* [18] who found that maximum CTC level, from *Limoniastrum monopetalum* leaves, was obtained when acetone solvent was used. Similarly, in other studies performed by Wina *et al*. [57] and Mazandarani *et al.* [58], acetone was found to be the most effective solvent extracting CTC from *Acacia mangium* barks and *Onosma dichroanthum* roots, respectively. According to Antwi-Boasiako and Animapauh [59], the four solvents tested in the current study, especially acetone and methanol were the best extraction solvents of CTC from the barks of three tropical hardwoods. Additionally, Uma *et al.* [60] showed that acetone was the most effective solvent for extraction of condensed tannins as tannins have a relatively high molecular weight.

3.2. Effect of Solvent Concentration on Extraction of Phenolic Compounds

Mixtures of alcohols with different proportions of water have shown to be more effective in extracting phenolic compounds compared to mono-component solvent system [25]. Addition of small quantity of water to organic solvent usually leads to a more polar medium, which facilitates the polyphenols extraction [56].

We observed that maximum rate of TPC (168.41 ± 70.60 mg GAE/g), extracted from *M. vulgare* leaves, was obtained when aqueous methanol was used at 60% (**Figure 1(D)**). However, there were high significant differences ($^{***}P < 0.001$) in TPC among the various concentrations. Our results are in line with those found by Chan *et al.* [61] and Chew *et al*. [39] who found that 60% aqueous methanol gave the best effectiveness extracting TPC from *Citrus hystrix* peels and *Centella asiatica* leaves, respectively. Yilmaz and Toledo [62] reported that aqueous mixtures of methanol, ethanol or acetone were better than a mono-component solvent for the extraction of TPC from Muscadine seeds.

The highest value of TFC (42.40 ± 16.22 mg CE/g), as shown in **Figure 1(E)**, was obtained, when we used 60% of aqueous methanol and was significantly different ($^{***}P < 0.001$) compared to the other studied concentrations. In contrast, aqueous methanol at 80% was the best concentration for *Calendula officinalis* flowers [63] and aqueous ethanol at 80% from the leaves of *Limoniastrum monopetalum* [18], *Bauhinia monandra* [64] and *Callicarpa nudiflora* [41]. Musa *et al.* [65] confirmed that the mixture of an aqueous solvent (distilled water) with an organic solvent (methanol and ethanol) improves the flavonoids yield comparing to water or organic solvent used separately.

Sixty percent (60%) acetone in water gave the highest value of CTC, which was 17.68 ± 5.69 mg CE/g (**Figure 1(F)**). Moreover, there were high significant ($^{***}P < 0.001$) differences in CTC values among the different concentrations used. Our data are in agreement with those reported by Downey and Hanlin [66] on grape skin. In other studies, it has been noticed that 80% aqueous acetone was the best concentration for CTC extraction from *Limonium densiflorum* shoots [67], 60% aqueous methanol from *Cichorium intybus* L. roots, leaves, stems and seeds [68], and 80% aqueous ethanol from the Chinese chestnut [69].

3.3. Effect of Extraction Time on Extraction of Phenolic Compounds

Extraction time represents another key parameter in optimizing the phenolic compounds extraction. In the literature, this parameter might be as short as few minutes or long up to 24 hours, depending on the phenolic com-

pounds present in samples [70].

In this study, extraction time showed a significant effect ($^{***}P < 0.001$) on the extraction of TPC from *M. vulgare* leaves. As shown in **Figure 2(A)**, the highest value of TPC (155.16 ± 69.83 GAE/g) was recorded using methanol for 180 min. Chan *et al*. [61] demonstrated that 180 min represented the best extraction time for TPC from the peels of *Citrus hystrix*. In other studies, the best extraction time of TPC was estimated to 18 hours for black tea, *Camellia sinensis* [49], 90 min from grape pomace extracts [71], 45 min from *Areca catechu* seeds [72] and *Azadirachta indica* leaves [70]. Increased time of extraction beyond 180 min (270, 360 and 450 min) induced a loss in TPC. Dent *et al*. [73] highly recommended that the extraction time, should not exceed 3 h for the extraction of TPC from *Salvia officinalis* leaves. Several authors stated that more the extraction time is long, less the content of polyphenols is obtained. This could be the result of loss of phenolic compounds, via oxidation, which might polymerize into insoluble compounds [26] [74]. Therefore, extraction time of TPC depends, not only, on maceration or agitation times, but also on several factors such as filtration time or the time spent during the evaporation of solvents [75].

The maximum of TFC concentration (39.95 ± 17.90 mg CE/g) was obtained using methanol for 450 min as illustrated in **Figure 2(B)**. Extraction time showed significant effect ($^{***}P < 0.001$) in TFC. However, 3 hours was considered to be the best extraction time for TFC from *Callicarpa nudiflora* leaves [46] and from some thyme varieties [76]. Additionally, 30 min has been shown to be the most favorable extraction time for TFC from *Gynura medica* leaves [77].

180 min was the longest extraction time, using acetone, for CTC (**Figure 2(C)**) with a maximum rate of 17.51 ± 5.18 CE/g. A high significant difference ($^{***}P < 0.001$) was obtained in CTC among different extraction times. Our results agree with those obtained by Zhekova and Pavlov [76] on some thyme varieties. In other works, the best extraction time was of 120 min for mangosteen fruits [78], 150 min for *Parkia clappertoniana* husks [79], 80 min for *Cichorium intybus* different organs [67], and 20 min for *Punica granatum* peels [80].

3.4. Effect of Extraction Temperature on Extraction of Phenolic Compounds

The effectiveness of extraction process of phenolic compounds is largely regulated by different experimental parameters particularly by the extraction temperature [81]. An increase of temperature is mainly due to an increase of the diffusion rate and solubility of the extracted substances. On the other hand, it should be taken into account, that some important biological active substances, such as TPC are damaged at high temperatures [82].

The extraction of TPC, as shown in **Figure 2(D)**, was optimal when methanol was used at 25˚C. In this case, 194.16 ± 15.45 mg GAE/g DW were obtained with high significant difference ($^{***}P < 0.001$) and between different temperatures. Similar to our finding, 25˚C was the most optimal extraction temperature for TPC from *Lawsonia inermis* leaves [60] and *Azadirachta indica* leaves [70]. Extension of extraction temperature beyond 25˚C led to an important decrease in TPC. However, it should be noticed that increasing the extraction temperature, beyond certain values, might promote possible concurrent decomposition of phenolic compounds, which were already mobilized at lower temperature. Furthermore, this elevation of temperature can even lead to the breakdown of phenolic compounds that remained in the plant matrix [83] [84]. Hence, heating may affect the polyphenolic composition in many cases; therefore, high-temperature drying should be avoided as much as possible [2]. Usually, TPC extraction is used at room temperature (≈25˚C) to avoid the degradation of phenolic compounds [25]. However, in other experiments, the optimal extraction temperature for TPC was found more elevated and estimated to 40˚C for *Citrus hystrix* peels [61], 100˚C for *Areca catechu* seeds [72], 65˚C for *Centella asiatica* leaves [39] and *Orthosiphon stamineus* stems and leaves [85], and 90˚C for *Moringa oleifera* leaves [86].

A maximum of TFC rate (53.31 ± 1.83 CE/g) was obtained at 25˚C using methanol as solvent (**Figure 2(E)**). In the current investigation we found that extraction temperature significantly ($^{***}P < 0.001$) affected the TFC extraction. However, in other researches, the highest extraction temperature was fixed at 90 °C for *Callicarpa nudiflora* leaves [41] and 60˚C from some thyme varieties [76]. Like TPC, the extension of extraction temperature higher than 25˚C led to an important decrease in TFC. However, the temperature conditions during the extraction procedures of flavonoids have to be carefully adjusted because of the possibility of thermal degradation of flavonoid derivatives, especially hydroxyl groups [87] [88]. In addition, mild heating was also found to have the ability to soften the plant tissues, to weaken the cell wall integrity, and thus to favor the release of bound phenolic compounds [56] [89].

As shown in **Figure 2(F)**, the maximum of CTC rate (22.18 ± 1.27 CE/g) was reached when acetone was used as a solvent and when the extraction temperature was about 50˚C. We observed a significant difference ($^{***}P < 0.001$) in CTC between the extraction temperatures. Our results agree with those of Zam *et al*. [80] on *Punica granatum* peels. In other studies, the best extraction temperature was slightly elevated compared to those we obtained: 80˚C for mangosteen fruits [78], 70˚C *Parkia clappertoniana* husks [79] and 60˚C for some thyme varieties [73]. Unlike the obtained results for TPC and TFC, the extension of extraction temperature beyond 25˚C led to a significant increase in CTC. Al-Farsi and Lee [90] reported that elevated temperature could stimulate the CTC extraction by increasing both diffusion coefficient and solubility of condensed tannins in extraction solvent. Besides that, intense heat from solvent allowed the release of cell wall phenolics and bounded phenolics by breaking down cellular constituents [91] and consequently, increasing the phenolic yield in extract. Moreover, Juntachote *et al*. [89] reported that elevated extraction temperature would increase, on one hand, the mass transfer of condensed tannins, and on the other hand, it would reduce the solvent viscosity and surface tension and, promotes the extraction of phenolic compounds.

3.5. Principal Component Analysis (PCA)

PCA aimed to diminish the size of data collected into a reduced number of components to examine the possible grouping of phenolic compounds according to the different extraction conditions. The first factor, PC1 presented 85.55% of variance accounted for, whereas, the second one, PC2 presented 10.86%. With the two first PCs, the explained variance accumulated was of 96.41%. This great value means that nearly all the variance contained in the original data was explained by just using the first new coordinates. By layering variables projection circle on the observations scatter plot, two groups were obtained as shown in the PCA plot (**Figure 3**):

- Group 1 (Gr1) on the positive side of PC1, formed by TPC and TFC with 0.932 and 0.958 as contributions, respectively. These phenolic compounds are related with ethanolic, methanolic and aqueous extracts at low temperatures 20˚C and 25˚C; ET1, ET2, MT1, MT2, DWT1, DWT2 with respective contributions of 2.285; 3.040; 3.578; 4.596; 2.253 and 2.618;

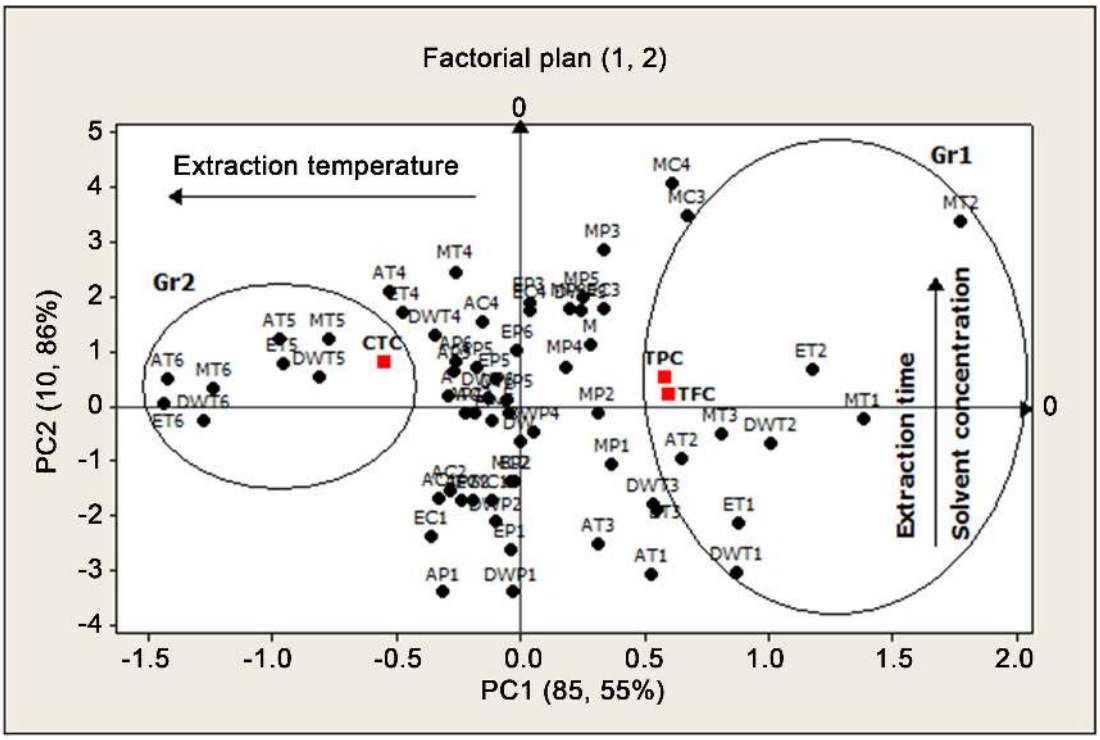

Figure 3. Principal component plot of PC1 and PC2. Gr: group; PC1: first principal component; PC2: second principal component; M: methanol; E: ethanol; DW: distilled water; A: acetone, C: concentration in % (1: 20; 2: 40; 3: 60; 4: 80); T: temperature in ˚C (1: 20; 2: 25; 3: 30; 4: 40; 5: 50; 6: 60); *P*: time in min (1: 30; 2: 90; 3: 180; 4: 270; 5: 360; 6: 450).

- Group 2 (Gr2) on the negative side of PC1, constituted by CTC with a contribution of −0.883. This phenolic compound was associated to ethanolic, acetonic and aqueous extracts at high temperatures 50˚C and 60˚C; ET5, ET6, AT5, AT6, DWT5, and DWT6 with contributions of 2.473; 3.705; −2.506; −3.661; −2.089 and −3.290, respectively.
- Moreover, considering the contributions of observations and variables on PC1, we defined two gradients:
- A horizontal gradient, moving from right to left of PC1, formed by the extraction temperature used, which explains the position of TPC and TFC right of PC1, correlated with low temperatures and the location of CTC to the left of PC1 in conjunction with high temperatures;
- A vertical gradient from the bottom to the top of PC1, constituted by the organic solvent concentration and the extraction time. This gradient explains the position of the three phenolic compounds measured at the top of PC1, correlated with high solvent concentrations (60% for TPC and CTC, 80% for TFC) and high times (180 min for TPC and CTC, 270 min for TFC).

3.6. Response Surface Methodology (RSM)

The Response Surface Methodology (RSM) was plotted to study the effects of extraction conditions on phenolic compounds extraction from *M. vulgare* leaves. 3 D response surface plots are illustrated in **Figure 4**. The regression equation of the model selected, for each phenolic compound measured and their adjusted R^2 was as follow:

- TPC = 323.205 − 827.094 X1 − 4.2516 X2 + 0.015029 X3 + 843.115 X12 + 17.1341 X1X2 − 13.5293 X2X12 − 0.0615286 X1X22, with adjusted R2 = 82.03%,
- TFC = 32.4239 + 31.9683 X1 + 0.42576 X2 + 0.0308808 X3 + 98.4907 X12 − 0.0108914 X22 − 2.96617 X1X2 − 0.00163763 X3X2 − 0.00122396 X1X3X2 + 0.0702683 X3X12 − 2.22016 X2X12 + 2.10557e-5 X3X22 + 0.0492972 X1X32, with adjusted R2 = 77.61%,

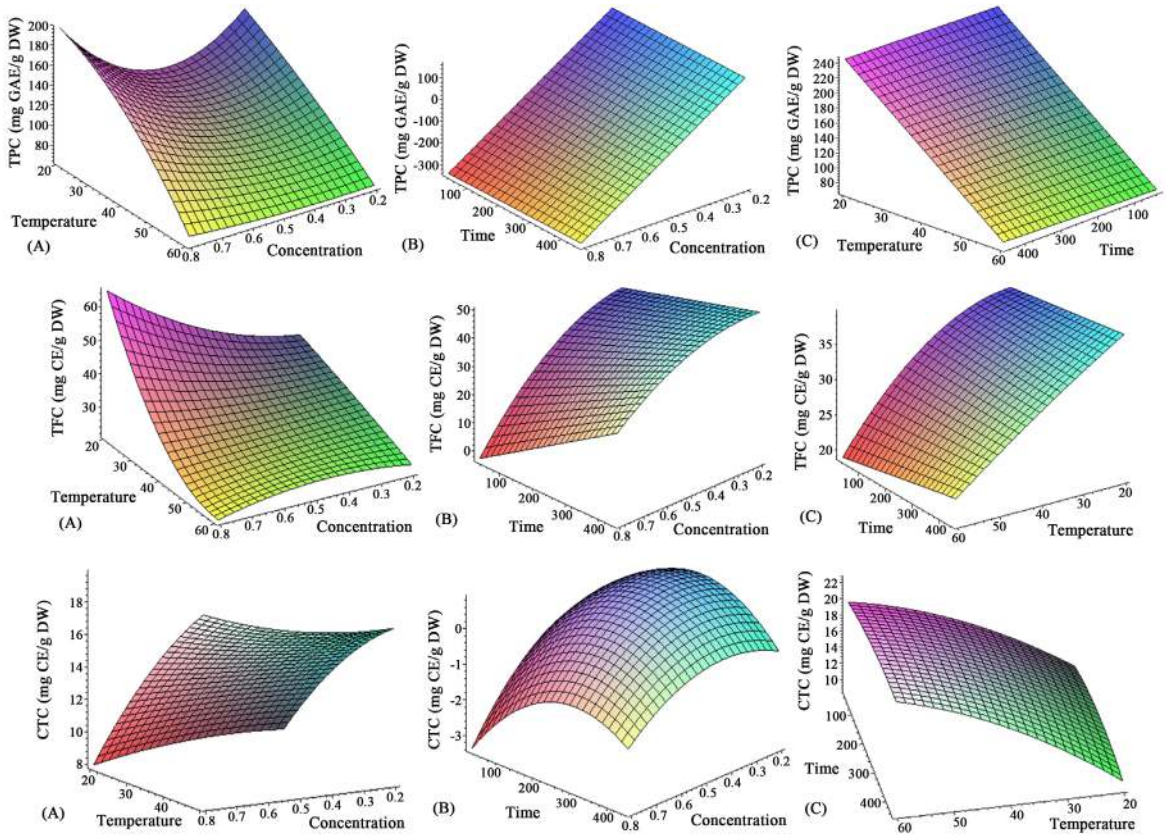

Figure 4. The 3D response surface plots representing the effects of (A) solvent concentration and extraction temperature; (B) solvent concentration and time extraction and (C) extraction temperature and extraction time on the extraction of phenolic compounds from *M. vulgare* leaves.

- CTC = −2.19999 + 7.81089 X1 + 0.603279 X2+ 0.01403 X3 − 12.395 X12 − 0.00405521 X22 − 2.96468e − 5 X32 − 0.220734 X1X2 + 0.00680008 X3X12 + 0.388604 X2X12 + 2.36161e − 7 X2X32, with adjusted R2 = 90.62%.

The comparison between the maximum experimental values of phenolic compounds and extraction conditions, and the predicted ones (**Table 2**), showed that the two sets of values were close. Indeed, the maximum value of TFC (experimental: 79.52 mg CE/g; predicted: 79.46 mg CE/g) and CTC (experimental: 28.15 mg CE/g, predicted: 20.81 mg CE/g) were obtained by the same experimental and predicted extraction conditions (TFC: aqueous methanol 80% at 20˚C and for 450 min; CTC: 60% at 50˚C and for 180 min). Concerning TPC, the maximum values of experimental and predicted contents and extraction conditions were different. The experimental values were of 293.34 mg GAE/g, obtained with aqueous methanol 60% at 25˚C and for 180 min. However, the predicted ones were of 204.75 mg GAE/g, obtained with aqueous methanol 80% at 20˚C during 167 min. Both, accounting experimental and predicted results indicated that the experimental model was valid. This implied that there was a high fit degree between the values observed in experiment and those predicted from the regression model. Hence, the response surface modeling could be applied effectively to predict extraction of phenolic compounds from *M. vulgare* leaves.

4. Conclusions

The results obtained in our study indicate that the experimental conditions tested (organic solvent type, solvent concentration, extraction time and extraction temperature) influence notably the values of TPC, TFC and CTC extracted from *M. vulgare* leaves, with extremely significant differences ($^{***}P < 0.001$).

We were able to define the optimum extraction conditions too, for obtaining higher values of some phenolic compounds from *M. vulgare* leaves. Indeed, the optimum level of TPC (293 ± 14.60 mg GAE/g) was obtained using 60% aqueous methanol at 25˚C for 180 min, TFC (79.52 ± 0.55 mg CE/g) using 80% aqueous methanol at 20˚C for 450 min, and CTC (28.15 ± 0.80 mg CE/g) with 80% aqueous acetone at 50˚C for 450 min. These levels highlight the richness of *M. vulgare* in these secondary metabolites. We conclude that this species remains poorly studied compared to other species belonging to the same taxonomic family (Lamiaceae) as thyme and sage.

Our results provide some confirmations on the effect of the variability of extraction procedures and assay on the amount of phenolic compounds recorded and reinforce previous works in this context.

This work is far from exhaustive as other experimental conditions can be tested, as the solid-to-solvent ratio, the number of extractions and the nature of the extraction method. Also, other extraction solvents (ethylene glycol, acetic acid and ethyl acetate), fractional extraction for many times or multistage extraction can be taken into consideration in our future works.

The impact of environmental conditions as the geographical region, altitude, exposure and the harvest season on the levels of polyphenols in *M. vulgare* is a promising perspective to this study and is still under investigation in our laboratory.

Acknowledgements

This research is a part of the Algerian National Project Research (entitled "Flora of Tessala Mountains: inventory, valorization and conservation", code 1/u22/397), financially supported by the Algerian Ministry of High

Table 2. Optimum values of experimental and predicted polyphenols contents and extraction conditions.

	Polyphenols concentrations		Solvent type		Solvent concentration (X_1)		Extraction temperature (X_2)		Extraction time (X_3)	
	EV	PV	EV	PV	EV	PV	EV	PV	EV	PV
TPC	293.34	204.75	Methanol		60	80	25	20	180	167
TFC	79.52	79.46	Methanol		80	80	20	20	450	450
CTC	28.15	20.81	Acetone		60	60	50	50	180	180

EV: experimental values; PV: predicted values.

Education and Scientific Research. Also, the authors are grateful to Dr. Amel Latifi, Bacterial Chemistry Laboratory (Marseille, France), for valuable editorial assistance during the drafting of this manuscript.

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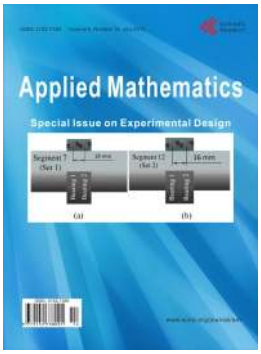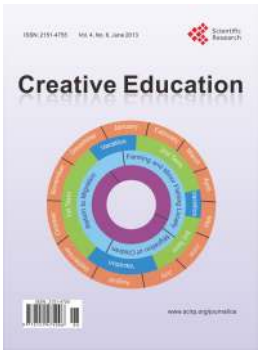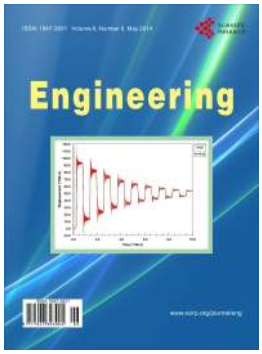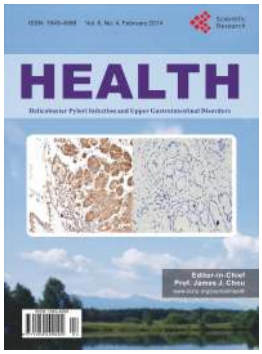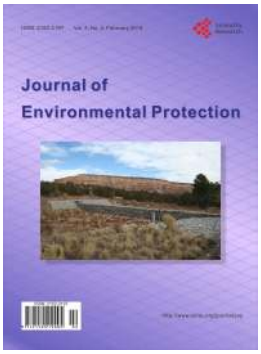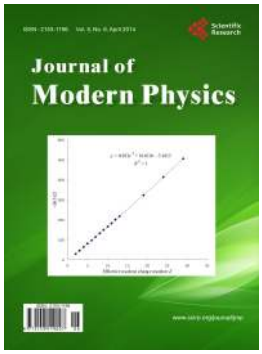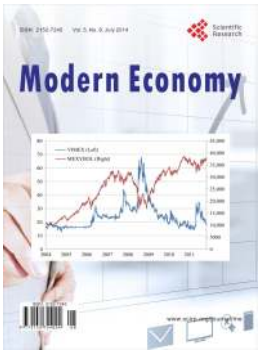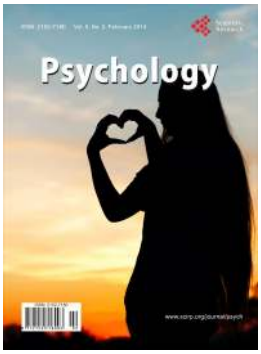
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