Journal of Medicinal Plants and By-products (2022) Special Issue: 107-115

Original Article

How Quantity of Bioactive Compounds of *Zataria multiflora* Differ Using Traditional or Modern Extraction Methods

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Article History: Received: 19 February 2021/Accepted in revised form: 18 May 2021

Abstract

For obtaining bioactive compounds, decoction of *Zataria multiflora* Boiss. (ZM) plant materials was interactively evaluated and compared with the modern method of ultrasound-assisted extraction (UAE). The interactive effects of several parameters including; liquid to solid (L/S) ratio, extraction time, extraction method, and plant material size were studied and optimized using the response surface methodology (RSM). Interpretation of the model outputs revealed that total phenolic content (TPC) of the resulting extracts is mostly affected by the interactive effect of plant material size and the extraction time, while rosmarinic acid (RA) content is only affected by the extraction method. Decoctions was found to have higher RA content (21.19 (mg/g E)) in comparison to UAE extractions (11.64 (mg/g E)), statistically significant at ($p \leq 0.05$). In addition, UAE as the modern method of extraction showed no privilege over the conventional method in the case of RA and TPC extraction. However, results suggest higher efficiency of UAE compared to decoction for extraction of antioxidant compounds by water. Eventually, ZM decoction obtained by the optimal extraction conditions was chemically characterized for the first time by the aid of LC-MS/MS chromatography. Naringenin, which is almost exclusively found in *Citrus* fruits, besides luteolin-7-O-rutinoside and a natural derivative of citric acid were shown to be the major constituents of ZM decoction.

Keywords: Response surface methodology, LC-MS/MS, Rosmarinic acid, Decoction, *Zataria multiflora*

Introduction

Decoctions are the most popular methods of making herbal beverages in various traditional medicine contexts [1,2]. Decoctions are aqueous preparations of plant materials boiled in water that can be absorbed quickly. Of all the traditional types of preparations, decoctions are reported to have the strongest action [3]. However, lack of scientific evidence and insufficient research data on water extractions from medicinal plants have created a barrier to their commercialization and informed use. Water-based herbal preparations could be obtained by simple instruments at home (decoction) or could be made by innovative techniques that enhance solid-liquid extraction such as ultrasound-assisted extractions [4]. In all cases, the quality and quantity of the final product may vary due to intrinsic (i.e. quality of raw materials) or extrinsic factors (i.e. extraction parameters) [5]. According to the WHO guideline (World Health Organization, 2018), apart from the solvent, particle size of the herbal material, the solid to solvent ratio, extraction time and the extraction method are influencing extraction parameters, which have to be evaluated and optimized. For a long time, water extractions of Shirazi thyme have been routinely the first choice of people to relieve a range of symptoms and complaints such as gastrointestinal tract disorders, sore throat, cough, cold, and respiratory problems such as congestion [6,1]. Shirazi thyme (*Zataria multiflora* (ZM)) [7], which is a popular food spice from *Lamiaceae* family [8] is also known for its significant medicinal effects such as; antioxidant, antibacterial, antiviral, antidiabetic, antifungal, and

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cytotoxic effects on human breast cancer cells [7,9,10]. The main ways of ZM consumption are oral use of its water extraction in form of decoction [11] and also steam inhalation of the plant leaves boiled in hot water. Despite the well-established effectiveness of ZM decoction and its common use [12,6], it is not chemically characterized yet and there is no information on its chemical constituents. In addition, there has been no attempt to evaluate how this conventional extraction method could vary or differ from modern extraction techniques in terms of quality or bioactive constituents. Rosmarinic acid is one of the most important bioactive compounds of ZM leaves [13], which possess many demonstrated pharmacological effects including antioxidant, antibacterial, and antiviral properties [14]. Therefore, ZM decoctions rich in rosmarinic acid can provide a wide range of nutritional and health benefits for the consumers. In the present work, two conventional (decoction) and modern (ultrasound-assisted extraction (UAE)) approaches were evaluated for extraction of rosmarinic acid (RA) by water using a response surface methodology (RSM). Antioxidant capacity and total phenolic content (TPC) were also assessed and compared for the obtained extracts. Subsequently, the interactive effects of different extraction parameters were interpreted using the graphical or numerical outputs of RSM model and their optimum ranges were also determined. Eventually, the chemical constituents of ZM decoction were identified by the aid of liquid chromatography–mass spectrometry (LC-MS/MS), which provides the necessary basis for further applications of ZM in food and pharmaceutical industries.

Material and Methods

Plant Material, Chemicals, and Reagents

The plant material was purchased from a local store, and an expert botanist authenticated the plant samples. The plant materials were thoroughly washed and air-dried, away from direct sunlight. Acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany). Ultrapure water obtained by an aqua MAX ultra-water purification system (Young Lin purification system) was used for HPLC and LC–MS/MS analyses. Folin–Ciocalteu reagent, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, sodium hydroxide, formic acid, naringenin, rosmarinic acid, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation and Extraction Procedures

In this work, herbal materials were milled into coarse and fine powders using two methods of ‘crushing’ and ‘grinding’, respectively. In the crushing procedure, the dried plant material was simply crushed using a mortar and pestle, and then it was passed through a 3 mm mesh size sieve so that the particle size is maintained uniform. While in the grinding method, an electric coffee grinder was applied for pulverization of plant material to obtain a fine powder. Then, the plant powder consecutively passed through a 0.2 mm mesh size sieve. The stock plant materials obtained by each of the methods were separately kept in a glass container in a cool and dry place until used. Different extraction procedures were carried out using UAE and decoction methods with different parameters. Details for each of the extraction runs are described in Table 1. Decoction was performed by adding water to the ZM powder, heated and boiled for the specified time (Table 1). For the UAE process, an ultrasonic bath (Elma sonic) equipped with a cooling coil connected to a cryostat was applied to provide the ultrasonic frequency of 37 kHz and ultrasonic power effective of 200 W and the extractions were carried out at 60 ˚C for the specified time according to Table 1.

After each extraction procedure, the solid residue was removed by centrifugation (4000 rpm for ten minutes) before filtration through Whatman No. 4 paper. The water extracts were then lyophilized and were kept in a refrigerator until analysis.

Evaluation of TPC, and Antioxidant Capacity

The antioxidant capacity of the extracts was evaluated based on the previously described method [15]. DPPH was dissolved in methanol to obtain a concentration of 80 µg/ml. The concentrations of 1.6–1000 (µg/ml) from extracts were prepared by dissolving them in pure water. A volume of 100 µl of each sample solution was mixed with DPPH (100 µl), and reduction of the DPPH radical was determined by measuring the absorption at 517 nm after 30 min. Each extracted sample was analyzed in triplicate. The DPPH inhibition percentage was calculated using the following equation:

$$\% \text{ Inhibition} = (AB - AS) / AB \times 100 \quad (1)$$

where, AB and AS are the absorbance of the mixture of water and DPPH solution (100 µl each) and the test solutions at 517 nm, respectively. The IC_{50} value (µg/ml) for each sample was then determined graphically by plotting the inhibition percentage as a function of the extract concentrations. TPC assay was performed according to the microplate-based methods described by Herald *et al*. [16]. Briefly, 75 µl of de-ionized water and 25 µL of the sample or standard compound followed by 25 µl of water diluted F–C reagent (1:1) were added to each well. After 6 min, 100 µl of Na_2CO_3 (75 g/l) was added. The microplate was placed in darkness for 90 min. Then a Synergy HTX Multi-Mode microplate reader (BioTek) was applied for absorbance measurement at 765 nm. The extracts were evaluated in a final concentration of 1.0 mg/ml. All extracts or standards were assayed in triplicate. To generate the calibration curve, gallic acid

(12.5–400 µg/ml) was applied as the standard compound. TPC results were finally reported as mg of gallic acid-equivalent per gram of the extract's dry weight (mg GAE/g).

HPLC-DAD and LC-MS/MS Analyses

The extracts were dissolved in water and were filtered through a 0.2 µm syringe filter, prior to HPLC-DAD analysis. Aqueous formic acid (0.1%) (A) and HPLC-grade acetonitrile (B) were used as the mobile phases. The chromatographic data were acquired from Agilent 1200 Series HPLC system equipped with a photodiode array detector (G1315A) using a C_{18} Agilen column (5 µm, 100 Å, 250 × 4.6 mm). Detection was carried out at 320 nm and quantification was performed using a calibration curve for rosmarinic acid (25–500 µg/mL, R^2 = 0.9993). The results were expressed as milligram of rosmarinic acid per gram of extract dry weight (mg RA/g E). The applied gradient profile for HPLC with the flow rate of 0.8 ml/min was as follows; % B increased from 10 to 30% in 15 min, from 30 to 50% between 15 and 22 min, and was kept at 100% between 28 and 35 min.

LC-MS/MS analysis was performed in a triple Quad LC-MS/MS System (AB SCIEX) using a binary gradient solvent mode. The following gradient was used: from 15 to 25% B (0–5 min), from 25 to 35% B (5–10 min), from 35 to 50% B (10–25 min), from 50 to 100% B (25–28 min), return to 15% B until 35, with the same column and mobile phase as for HPLC min. The flow rate was established at 0.5 ml/min and MS analysis was operated in a scan acquisition range from 100 to 1000 *m*/*z*. Gas flow 8 l/min, nebulizer pressure 38 psi, dry gas 7 l/min, and dry temperature 220 °C. MS/MS analysis was performed based on the previously determined accurate mass and fragmentation using different collision energy ramps to cover a range from 15 to 50 eV.

Response Surface Methodology

The impact of the affecting extraction parameters (extraction method, extraction time, plant material size, and liquid to solid (L/S) ratio) was assessed using a D-optimal design to visualize the response surface models. D-optimal designs [17] are a class of response surface models that can reduce the costs of experimentation by estimation of parameters with fewer experimental runs without bias and with minimum variance. In this study, the affecting parameters including extraction method type (UAE or decoction) and plant material size (crushed or milled) as the qualitative variables in addition to extraction time and L/S ratio as the quantitative variables were considered and optimized. The D-optimal design was applied not only to optimize the extraction procedure but also to explore and understand the interactions between the extraction parameters and their impacts on the model responses (TPC, antioxidant capacity, and rosmarinic acid content).

Table 1 D-optimal design codes and the responses

Run	A:L/S ratio	B:Time	C:Method	D:Material size	TPC (mg GA/g Extract) [a]	Antioxidant capacity [IC_{50} (µg/ml)] [a]	RA (mg/gE) [a]
1	-1	0	UAE	Crushed	85.15±1.02	29.67±1.58	12.71±0.98
2	-1	-1	UAE	Crushed	75.54±1.08	27.21±0.44	10.70±0.13
3	-1	-1	Decoction	Grinded	99.05±2.61	26.86±1.39	22.84±0.39
4	1	1	UAE	Grinded	80.95±1.76	22.12±0.66	10.93±0.18
5	-1	1	Decoction	Crushed	102.69±2.69	30.57±1.23	19.36±0.18
6	-1	1	Decoction	Crushed	94.95±2.30	30.53±1.71	18.72±0.46
7	1	1	Decoction	Crushed	107.41±2.17	29.75±1.56	23.83±0.72
8	1	-1	Decoction	Crushed	83.74±3.01	31.13±2.49	17.66±0.21
9	0	1	Decoction	Grinded	96.29±1.65	30.03±0.56	19.49±0.55
10	1	1	Decoction	Crushed	100.84±2.43	28.40±1.63	21.94±0.83
11	-1	-1	UAE	Grinded	89.14±1.17	24.75±1.51	7.95±0.23
12	1	-0.5	Decoction	Grinded	98.31±1.25	31.24±0.97	20.75±0.40
13	1	1	UAE	Grinded	86.75±0.97	23.48±0.06	14.20±0.62
14	1	-0.5	UAE	Crushed	80.79±2.25	25.87±1.05	11.33±0.31
15	0	-0.5	Decoction	Grinded	98.67±1.92	30.17±2.17	25.51±0.51
16	-1	0.5	Decoction	Grinded	98.37±2.14	31.47±0.89	20.92±0.95
17	0	-1	UAE	Grinded	89.87±1.92	24.36±0.46	10.58±0.15
18	1	-0.5	UAE	Crushed	81.83±1.86	25.97±1.81	12.98±0.70
19	-1	0.5	UAE	Grinded	81.98±1.33	26.46±0.92	13.16±0.54
20	-1	-1	UAE	Grinded	95.36±2.85	25.85±2.10	11.14±0.67
21	-0.5	0	Decoction	Crushed	91.34±2.44	32.84±1.84	22.07±1.02
22	0	1	UAE	Crushed	98.03±2.36	26.47±1.43	12.38±0.80

[a] Values are means±SD (n=3) and are significant at ($P<0.05$)

Table 2 Original and coded values of the independent variables of the design matrix

Independent variables	Symbols	Levels of variables				
Extraction method	C	UAE	Decoction			
Plant material size	D	Crushed	Grinded			
		Coded levels (original values)				
Extraction time (min)	B	-1 (5)	-0.5 (10)	0 (20)	0.5 (30)	1 (35)
Liquid to solid (L/S) ratio	A	-1 (10)	-0.5 (20)	0 (30)	0.5 (40)	1 (50)

All design descriptions are in terms of coded values of the variables. The highest value of the original variable is coded as (+1) and the lowest value is (-1). The independent variables and their related levels and codes are shown in Table 2.

Results and Discussion

Process Variables and Model Fitting

In order to study and compare the effects of the considered variables on the extraction process and to obtain the optimum range for each variable, response surface methodology was applied. The interactive effects of four variables including; liquid to solid (L/S) ratio (variable A), extraction time (variable B), extraction method (variable C), and plant material size (variable D) were studied using a D-optimal design with 22 experimental runs. The design matrix along with the corresponding responses of each run are listed in Table 1. In this design, extraction method and plant material size were the qualitative variables, with two levels for each one. Decoction and UAE were considered as two levels of "extraction method" variable. In the preparation of decoction, water temperature reaches the boiling temperature and therefore it is likely that some unstable compounds will be decomposed by the heat and destroyed. Therefore, UAE method was also investigated as a versatile technique and innovative technology for enhancement of the extraction process working at lower temperatures. In a liquid medium, the efficiency of UAE is mainly attributed to the phenomenon of cavitation. The bubbles' implosion near the surface of the sample collapses the cell structure, increases solvent penetration into the plant material, and therefore facilitates the extraction process [18]. Although, the number of cavitation bubbles may increase in higher temperatures but when the temperature is near the solvent's boiling point, the effects of the bubbles are reduced [19]. Therefore, in this study, the extraction temperature for UAE was kept at 60 °C employing a thermostated system, which can also help preventing thermal decomposition of bioactive components. For plant material size, we considered both the common way of plant preparation by ordinary people (crushing) and the scientifically supported method (grinding) [20]. In the common method, well-dried plant materials are crushed by a simple instrument such as a mortar and pestle to obtain smaller particles or a coarse powder of plant material. However, in the grinding technique, relatively expensive equipment such as a grinder is applied to homogenize the plant to obtain a fine powder. To evaluate how the resulting extract could vary in relation to the "plant material size", both plant preparation methods were considered and examined. The other two variables of the liquid to solid (L/S) ratio and extraction time are also relevant to the efficacy of the extraction [20], which were assigned as quantitative variables with five levels for each one (based on the literature [21]) to evaluate a wide variation range. Short extraction time is one of the advantages commonly associated with UEA in literature [19]. On the other hand, extraction efficiency could be improved by elongation of extraction time in certain cases [22]. Therefore, the "extraction time" was studied in the range of 5 to 35 minutes. For a successful extraction, intended compounds must be dissolved in the solvent until an equilibrium concentration is reached. Frequently, the employed L/S ratios range from 10:1 to 50:1 (v:w) [21], but this value has to be studied for each raw material to assess the influence. Therefore, different "L/S ratios" were investigated in the range of 10:1 to 50:1 (v:w). Twenty-two experimental runs of the design were carried out (each with specified parameters (Table 1)). The extractions were compared with respect to multiple responses including; rosmarinic acid content, TPC, and antioxidant capacity. Table 1 shows all experiments of D-optimal design and the corresponding responses. The best model fitted for each one of the design responses was selected based on the best ANOVA attributes. The resulting models for the three responses are as follows:

TPC = 90.76 + 3.34 B + 4.10 C - 1.69 D + 6.50 BD - 1.27 CD (Eq. 1)

(R^2= 0.90, Adj R^2= 0.87, standard deviation= 3.15, P-value< 0.0001, P-value for lack of fit: 0.92)

Antioxidant capacity (IC_{50}) = 29.45 - 0.65 A + 0.11 B + 2.15 C + 0.64 D - 0.70 AB + 0.78 AC - 2.09 B^2 (Eq. 2)

(R^2= 0.94, Adj R^2= 0.91, standard deviation= 0.87, *P*-value< 0.0001, P-value for lack of fit: 0.26)

Rosmarinic acid content = 16.41 + 4.77 C (Eq. 3)

(R^2= 0.86, Adj R^2= 0.85, standard deviation= 2.04, *P*-value< 0.0001, P-value for lack of fit: 0.29)

The model terms (A, B, C, and D) in the above equations are codes for the corresponding variables listed in Table 1. According to the model statistics, all the obtained equations (Eq. 1 to Eq. 3) are significant based on the *P*-values less than 0.05 and the models are well fitted to the data based on the non-significant lack of fits.

Evaluation of TPC Model and Variables Influences

The statistical model for TPC response is a two-factor interaction model, which describes the relative impact of the variables on TPC based on the variable coefficients. According to the coefficients in Eq. 1, the total phenolic content of the extracts is mostly influenced by the interaction between plant material size (D) and extraction time (B) while the extraction method (C) takes the second place. Examination of TPC response model for both UAE and decoction methods (Fig. 1 (parts a, and b)), showed that increasing the extraction time (even more than 35 min) when using crushed plant material resulted in higher total phenolic contents of the extracts. However, when grinded plant powder was applied, longer extraction times in both extraction methods (UAE and decoction) led to lower TPC values (Fig. 1 (parts a, and b)). It is clear that smaller particle sizes of plant material (milled) with higher surface area result in a fast and enhanced mass transfer of phenolic compounds from plant material to water [18]. Nevertheless, it takes longer for phenolic compounds to be extracted from crushed material. However, increasing the time of extraction from milled plant material not only did not improve the TPC but also degraded the compounds due to longer exposure to ultrasonic waves in UAE or excessive temperatures in the decoction procedure. According to Eq. 1, the second most influencing variable in TPC model is the extraction method (C). Precise evaluation of the responses in Table 1 revealed that experimental runs corresponding to the decoction method resulted in extracts with higher values for TPC, with the mean value of 97.42 (mg GA/g Extract) for decoction versus 85.94 (mg GA/g Extract) for UAE runs (statistically significant (*P*-value < 0.05)). This implies that the conventional method of decoction overcomes UAE method in terms of extraction of phenolic compounds. This could possibly be explained by higher temperature of water in decoction process in comparison with UAE procedure. The dielectric constant of water decreases at high temperatures [23] so that water will be able to act like a less polar solvent and solubilize more of nonpolar molecules including more phenolic compounds.

Antioxidant Capacity Model and Variables Impacts

For antioxidant capacity (Eq. 2), the most influencing variables in the extraction process were shown to be extraction method (C) and time (B^2) based on their larger coefficients. The lower mean value of IC_{50} values corresponding to UAE experimental runs (25.65 (µg/ml)) in comparison with the mean value for decoction extractions (30.27 (µg/ml)) shows a higher antioxidant capacity of the extracts obtained by this technique. Statistical significance of the differences among the mean values was further confirmed by t-test with *P*-value <0.05. The superiority of UAE over conventional methods in terms of extraction of antioxidant compounds is also reported in other studies [24]. Our findings in this regard suggest the presence of thermolabile antioxidant compounds in water extracts of ZM leaves, which are destroyed at the higher temperature of the decoction process. Since decoctions turned out to be richer than UAE extracts in terms of TPC, it could be concluded that not necessarily all the antioxidants present in water preparations of ZM leaves are phenolic compounds. Parts c and d of Fig. 1 depict the interactive effects of the (L/S) ratio and the extraction time on the response (antioxidant capacity) in decoction and UAE methods, independently. Evaluation of the time influence for both decoction and UAE methods from Fig. 1 (parts c and d) shows a similar trend. According to Fig. 1 (parts c and d), an increase in the extraction time leads to the antioxidant capacity decrease (higher IC_{50} values) until a turning point is reached at around 20 minutes. Beyond that value, longer times lead to lower IC_{50} values (higher antioxidant capacity). This finding could be interpreted by the presence of antioxidant compounds with different natures in ZM extracts [25]. It can be suggested that the first class of antioxidant compounds, which are extracted in the initial moments of the extraction process, degrade with excessive temperatures or sonication. On the other hand, the second class of antioxidant compounds is strictly bound to the plant cell structures and is extracted in longer times over 20 minutes, which causes improvement in antioxidant capacity.

Evaluation of a Model for Rosmarinic Acid Content

Different model fitting calculations for the rosmarinic acid response revealed that none of the four extraction variables except for “extraction method” showed any appreciable effect on the response as indicated by their higher than 0.05 *P*-values. The response model for rosmarinic acid with only one variable (extraction method) showed to be a well-fitted model based on the model statistics (see Eq. 3). Decoction extraction proved to be an effective method, capable of yielding an average value of 21.19 (mg/g E) for rosmarinic acid in decoctions versus the average value of 11.64 (mg/g E) of RA in UAE extractions (confirmed by t-test, *P*<0.05). This finding suggests that the high temperature of water in decoction is more effective than the ultrasound effects of UAE for extraction of rosmarinic acid from ZM.

Optimization of the Extraction Process

At first, we applied numerical optimization technique by design expert software to find the optimum conditions of

the variables to obtain the best of all responses simultaneously [24]. The extraction time of 5 min, the (L/S) ratio of 10, the extraction method of decoction, and milled plant material were found as the optimal conditions for multi-response optimization of the extraction process. Applying these conditions, the respective obtained values for antioxidant capacity, TPC and rosmarinic acid content responses were 26.51±1.23, 99.83±1.89, and 21.02±1.02, which are in close agreement with the predicted values of 27.93, 100.98 and 21.19. Therefore, under the obtained optimal conditions, all responses of TPC, antioxidant capacity and rosmarinic acid content can be obtained at the maximum possible level at the same time.

Chemical Characterization of ZM Decoction

The base peak chromatogram (BPC) of ZM water extract, obtained under multi-response optimized conditions, is shown in Fig. 2. Peak characteristics and tentative identifications are presented in Table 3, and the identifications are described in the supplementary material.

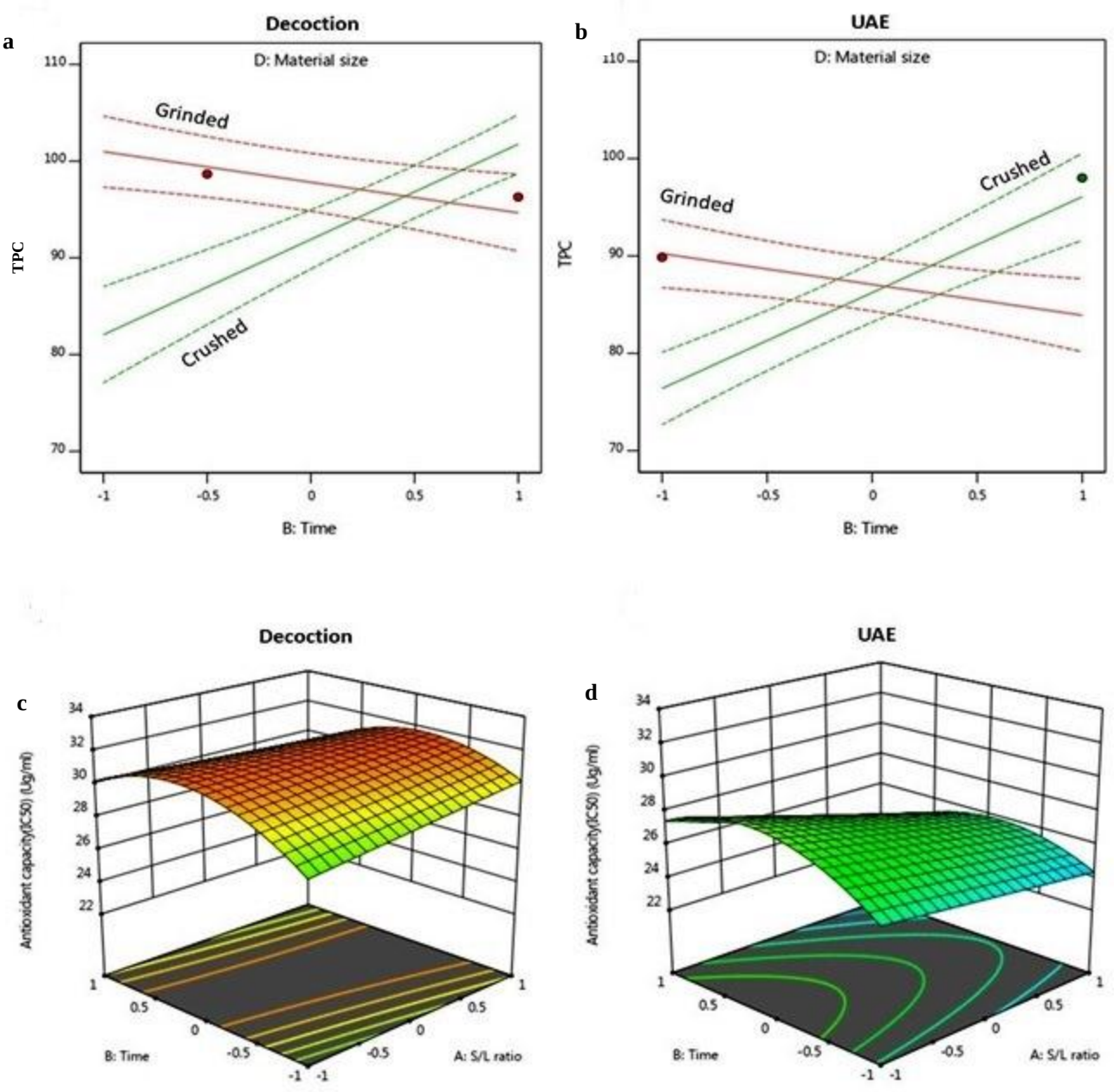

Fig. 1 Interaction plots for "BD" term in TPC model for decoction (a) and UAE (b) methods and 3D surface plots of decoction (c) and UAE (d) "method" term in antioxidant capacity model

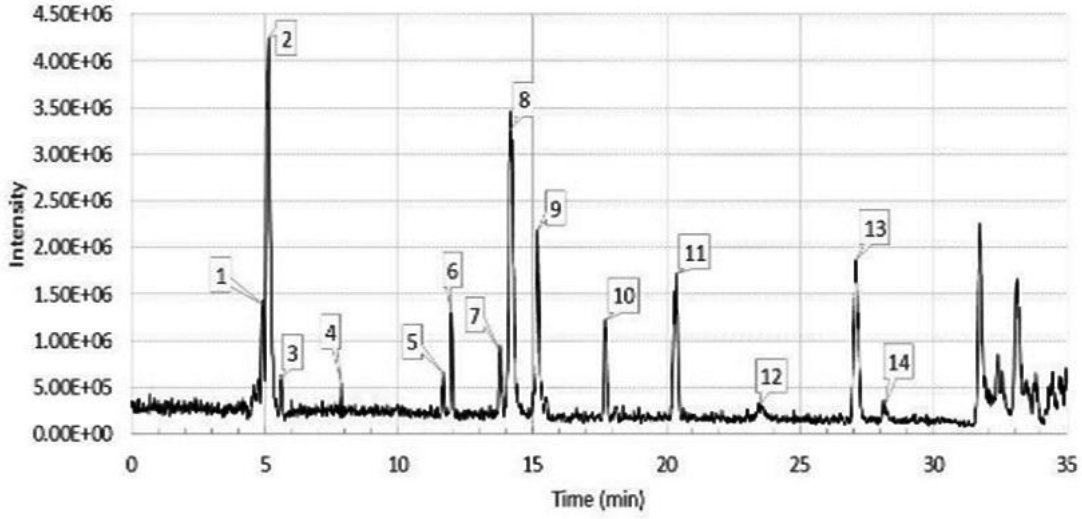

Fig. 2 Base peak chromatogram (BPC) chromatogram of decoction of *Z. multiflora* Boiss. The compounds are as follows: 1) Caffeoyl-O-hexoside, 2) Citric acid derivative, 3) Malic acid, 4) Unidentified, 5) Apigenin 6,8-di-C-glucoside (vicenin-2), 6) Medioresinol, 7) Myricetin-3-O-rhamnoside (myricitrin), 8) Luteolin-7-O-rutinoside, 9) Astragalin (Kaempferol-3-Oglucoside), 10) Rosmarinic acid, 11) Dihydrokaempferol (Aromadendrin), 12) Sakuranetin (Naringenin 7-methyl ether), 13) Naringenin, 14) Galangin

Table 3 Tentative identification of phenolic compounds in water extract of *Z. multiflora* Boiss.

Peak No	RT (min)	λ_{max}	[M-H] (*m/z*)	MS/MS Fragments (m/z)	Identification	Chemical class
1	4.90	-	387	341, 179	Caffeoyl-O-hexoside	Monosaccharide derivative
2	5.16	210, 256, 352	405	191	Citric acid derivative	Tricarboxylic acid derivative
3	5.57	192, 210, 270	133	115	Malic acid	Dicarboxylic acid
4	7.74	194, 276, 346	447	316, 191	Unidentified	-
5	11.68	212, 276, 338	593	473, 165	Apigenin 6,8-di-C-glucoside (vicenin-2)	Trihydroxyflavone
6	12.01	216, 272, 336	387	207	Medioresinol	Lignan
7	13.78	212, 230, 284	463	322, 137	Myricetin-3-O-rhamnoside (myricitrin)	Flavonoid
8	14.26	202, 270, 282, 344	593	285	Luteolin-7-O-rutinoside	Flavonoid
9	15.24	266, 270, 344	447	285	Astragalin (Kaempferol-3-Oglucoside)	Flavonol
10	17.74	208, 288, 330	359	161, 197	Rosmarinic acid *	Carboxylic acid
11	20.40	230, 285, 325	287	259, 125	Dihydrokaempferol (Aromadendrin)	Flavonoid
12	23.51	-	285	243, 165	Sakuranetin (Naringenin 7-methyl ether)	Flavonoid
13	27.11	216, 288	271	151, 119	Naringenin *	Flavanone
14	28.21	265, 360	269	147	Galangin	Flavonoid

*Confirmed with authentic standard

Chemical characterization was performed based on the HPLC-PDA-MS/MS information and comparison with the literature and further confirmation with the available standards. Thirteen compounds were identified, three of which were phenolic acids (citric acid derivative, malic acid, and rosmarinic acid), eight flavonoids (vicenin-2, myricitrin, luteolin-7-O-rutinoside, astragalin, aromadendrin, sakuranetin, naringenin, galangin), one saccharide, and one lignin (medioresinol). In general, the identification results revealed that apart from rosmarinic acid and a natural derivative of citric acid, flavonoids are the predominant constituents of ZM decoction. While carboxylic acids are the major compounds present in the methanolic extract of ZM [13]. It is very interesting that naringenin, a bioflavonoid (flavanone), which occurs almost exclusively in *Citrus* fruits, and tomatoes [26] is an abundant flavonoid identified in ZM decoction. Therapeutic effects of naringenin in the cure of different diseases such as cancer, diabetes, and cardiovascular diseases have been already reported [27]. Therefore, ZM decoction could be considered and examined for obtaining drug formulations and healthcare products with high water solubility.

Conclusion

Decoction is the most common form of using herbal medicines. However, there is not enough scientific evidence regarding the chemical characterization of decoction in terms of bioactive compounds. Relevant scientific research works can pave the way for obtaining the key requirements for safe and informed use and for widespread industrial applications. In this study, a response surface methodology was applied to evaluate

and compare the conventional (decoction) and the modern (UAE) technique in terms of bioactive components. Interpretation of the obtained results revealed that for both the decoction and UAE methods the proper time to extract phenolic compounds is highly dependent on the particle size of the plant material. While longer extraction time can improve the content of phenolic compounds for the coarse powder, elongation of the extraction time causes a decrease in TPC when using the fine plant powder. Although UAE is known for its short extraction time, decoction turned out to perform better than UAE in extraction of phenolic compounds, when using fine plant powder in less than 5 minutes. In addition, among the studied extraction parameters of liquid to solid ratio, extraction time, extraction method, and plant material size, "extraction method" showed to be the only significant parameter that affects the extraction of rosmarinic acid from ZM. The amount of rosmarinic acid obtained by the decoction method is almost twice of that by the UAE method. This suggests the higher efficiency of boiling water in decoction in comparison with the ultrasound effects of UAE for RA extraction. Therefore, UAE as the modern method of extraction showed no privilege over the conventional method in the case of RA extraction. However, UAE method turned out to be a slightly better method for extraction of antioxidant compounds, which could be possibly due to the lower temperature applied in UAE. Considering the small difference between two extraction methods in terms of IC_{50} values, ZM decoction is still a good source of antioxidant compounds. In general, the decoction of ZM fine powder (milled) with water to a solid ratio of 10 for 5 minutes provides the conditions for having a decoction with the optimal antioxidant capacity, rosmarinic acid, and total phenolic contents, at the same time. ZM decoction is a valuable source of significant bioflavonoids such as naringenin and luteolin-7-O-rutinoside and a natural derivative of citric acid that turned out to be the major components of ZM decoction. Naringenin, and rosmarinic acid are invaluable phytochemicals of ZM decoction with well-established antioxidant, antitumor, antiviral, antibacterial, and cardioprotective effects. Considering the rich content of ZM decoction in terms of the precious bioactive compounds, it is worth being further investigated for additional pharmacokinetic and pharmacodynamic studies for evaluation of the bioavailability and the commercialized formulation. One of the major problems encountered with the formulation development of new therapeutic entities is low aqueous solubility. However, ZM decoction does not have this defect. Simple preparation and no need for complex processes and sophisticated equipment make ZM decoction a useful drink that can become a useful drug for treatment or relief of many discomforts if standardized. Therefore, the obtained results in this study could be informative not only for food but also for pharmaceutical industries and researchers.

Acknowledgments

This research was financially supported by research council of Shiraz University of Medical Sciences under the grant number of (98-01-64-20174).

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