JFBE 3(2): 101-109, 2020

Journal of Food and Bioprocess Engineering

Journal homepage: https://jfabe.ut.ac.ir

Original research

Sequential ultrasound-microwave assisted extraction as a green method to extract essential oil from *Zataria multiflor*

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ABSTRACT

Sequential ultrasound microwave associated extraction (SUMAE) is a combination of ultrasound waves and microwaves energies in which ultrasonic extraction is used as a pretreatment. It was performed to extract essential oil from *Zataria multiflora*. The extraction conditions were optimized by response surface methodology (RSM) and Central Composite Design (CCD). The results were compared with microwave associated extraction (MAE) method in terms of process yield, chemical composition, antioxidant activity and environmental impacts. The results show that the optimal conditions were ultrasound power of 150*W*, microwave power of 800*W*, and extraction time of 12 min. Under these conditions, the yield of the extracted essential oil was 0.812%, which was higher than that obtained by MAE method (0.6%). Regarding environmental impacts, the quantity of carbon dioxide emission was lower in case of SUMAE method (0.25 kg) compared to MAE method (0.54 kg) .This is also true for energy consumptions, which were 0.3125 kWh and 0.675 kWh for performed SUMAE and MAE, respectively, at the same power level. Furthermore, antioxidant activity of essential oil extracted by SUMAE was higher than that of MAE. Generally, the SUMAE not only did not damage the antioxidant properties, but also improved it and had less environmental damage. Consequently, it can be introduced as a green and safe method for the extraction of essential oils from *Zataria multiflora*.

Keywords: Antioxidant activity, Environmental impact, Microwave associated extraction, Optimization

Received 25 August 2020; Revised 9 September 2020; Accepted 12 September 2020

1. Introduction

Zataria multiflora is an aromatic and traditional medicinal herb, belonging to the family *Lamiaceae*. It grows wild in the mountainous areas, rocky and gravelly slopes and it is native to Iran. Because of the vast range of its medical benefits include antibacterial, antitumor, antifungal, and antioxidant properties, many researchers have encourage to study the efficient methods in extracting essential oils and extracts from this plant (Rahimi et al., 2019; Sajed et al. 2013). Generally, yield of extraction and composition of essential oil obtained from *Z. multiflora* depend on ecological and genetic features, and also postharvest processing factors. Besides, process of extraction plays a noteworthy role in the yield and in some case in its compounds (Basti et al., 2016; El Asbahani et al., 2015).

Khajenoori et al. (2009) studied extraction of essential oils from *Zataria multiflora Boiss* using subcritical water extraction and compared with the yield of soxhlet extraction and hydrodistillation extraction known as traditional methods. Khalili et al. (2018) also investigated two *Lamiaceae* family types: thyme and Melissa. They enhanced important ingredients in essential oils by using solvent-free microwave extraction. Gavahian et al. (2011) combined ohmic heating and distillation as a new system to get essential oils and extracts of *Zataria multiflora Boiss*. Hassanein et al. (2020) examined the use of microwave energy to extract essential oil from some *Lamiacease* species growing in Egypt. Their results recommended this technique as an effective extraction method. Kohari et al. (2020) also obtained essential oil from Mentha arvensis L. by solvent-free microwave extraction and compared the yield and composition with that of traditional methods. The results introduced solvent-free microwave extraction as a suitable method. However, research on *Zataria multiflora* and finding a suitable extraction method, especially among new methods, is still ongoing, and for this reason, this issue is the subject of the present study.

Owning the fact that the method of extraction of essential oil highly affects its efficiency and quality, various methods have been proposed for this purpose. Traditionally, the widest extraction method are based on hot water and known as hydro distillation (HD) or steam distillation (SD) without any organic solvent (Stahl-Biskup, 2002). The main disadvantages of these process are high

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https://doi.org/10.22059/jfabe.2020.308833.1064

energy consumption and long extraction time (Garcia-Vaquero et al., 2020; Wang et al., 2020). Consequently, innovative methods are needed to save both time and energy. Also, most of these new systems increase the yield of essential oil extraction (Lu et al., 2017). Thus, developing an environmentally friendly extraction technique to save time and energy is a significant need.

Recently traditional technologies are replaced by modern ones such as ultrasound and microwave extraction (Lu et al., 2017). Microwave-assisted extraction (MAE) is going to be a wide technology for extraction of essential oil or naturally occurring substance from various herbs and plants (Asofiei et al., 2016; Chemat et al., 2006; Mosayebi & Tabatabaei Yazdi, 2018; Pasandide et al., 2018). Moreover, ultrasound-assisted extraction (UAE) is an effective process that gives high valuable compounds and yield. So that researchers have used it to extract essential oils (Hashemi et al., 2018), polyphenols (Živković et al., 2018), pectin (Marić et al., 2018), and other volatile compounds (Abd Rahim et al., 2018; Alissandrakis et al., 2003; Guimarães et al., 2019; Wen et al., 2018). In addition, researches confirm that the combination of ultrasound and microwave extraction provides more efficiencies (Lianfu & Zelong, 2008). In other words, ultrasound-microwave assisted extraction (UMAE) combines ultrasound waves and micro waves energies to enhance yield and efficiency. During ultrasound extraction cavitation cause to disintegrate plant cells so chemical compounds pervade. On the other hand, microwaves make high temperature and it causes extraction acceleration (Mason et al., 2011). However, research on the extraction from herbs using this novel method is limited and needs further development to optimize and find the best conditions. In some cases, researches draw a comparison between traditional methods and the novel combination method.

Lianfu and Zelong (2008) optimized and compared extraction technologies including microwave-ultrasound extraction and ultrasound extraction to extract lycopene from tomatoes paste. Their results showed that the combined method is more effective than the ultrasound method. Zhao et al. (2010) used UMAE to maximize the performance of polysaccharides extracted from a fungus called *Ganoderma lucidum*. Their study confirmed high efficiency of using UMAE in the extraction of polysaccharides. In another study conducted by Chen et al. (2010) to optimize the extraction of polysaccharides from a fungus called Inonotus obliquus by UMAE and to evaluate its antitumor activity, good results were obtained for this combined method. These results showed that ultrasonic-microwave extraction of polysaccharides has high yield and efficiency compared to conventional hot water extraction. Liu et al. (2016) also optimized the extraction of pectin from *Jerusalem artichoke* by UMAE using citric acid. Based on the obtained optimal conditions, the efficiency of pectin in the combined method was higher than all other methods. Lu et al. (2017) optimized the extraction of oligosaccharide from lotus seeds using response surface methodology. Their results confirmed the efficiency and potential of UMAE method compared to other conventional hot water, ultrasonic or microwave extraction methods for oligosaccharide extraction. Wang et al. (2018) extracted black and white pepper essential oil using UMAE method and compared it with MAE and UAE and summarized the advantages and disadvantages of each of these methods. Yang et al. (2019) optimized the condition of UMAE process to extract potato pectin. The results showed that UMAE effectively increase the extraction efficiency. Wang et al. (2020) optimized conditions for extraction and determined antioxidant and antibacterial activities of flavonoid compounds from *Eucommia ulmoides* leaves. Gharibzahedi et al. (2019) applied UMAE process to obtain pectin from *Ficus carica* L. and investigated bioactivity of extracted pectin. Generally, according to earlier studies, UMAE method is more efficient in terms of both yield and time than the other methods.

Based on our knowledge, there is no study on the extraction of essential oils from *Zataria* with help of UMAE. On the other hand, despite the benefits mentioned for UMAE method, there is a general concern that whether microwave irradiation and ultrasound will affect the quality of the extracted essential oils or not. Therefore, in this work a new technique called sequential ultrasound-microwave associated extraction (SUMAE) was proposed. SUMAE is a combination of ultrasound waves and micro waves energies in which ultrasonic extraction is used as a pretreatment. Here, SUMAE was used for the extraction of essential oil from dried *Zataria multiflora* and optimized it. Thus, central composite design (CCD) has been developed to study the importance of different factors. Moreover, a comparison study with MAE method was done on the process yield, chemical composition, antioxidant activity *Zataria multiflora* essential oils. Environmental impacts of these two methods were also compared.

2. Material and Methods

2.1. Materials

Z. multiflora leaves were purchased from Daru Kesh Pajuhan Company, Isfahan, Iran. They were properly dried in the shade, packed in a dark glass jar, and stored in a dry and cool place. 1,1-diphenyl-2-picryl-hydrazyl (DPPH.), 3-(2-Pyridyl)-5, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) were purchased from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Methanol was purchased from Merck.

2.2. Essential oil extraction by SUMAE method

Fifteen grams of *Z. multiflora* leaves were weighed and placed in a 500*ml* beaker. Dried leaves were mixed with distilled water in a ratio of 1:20 (g/ml). The mixture was placed in an ultrasonic homogenizer (Topsonic, UHP-400, Iran). Ultrasound generator equipped with a 12 mm diameter tip, frequency 20 ± 1kHz, Maximum power 400 W with different sonication power. In the present work, this step was selected as pre-treatment. According to preliminary tests and earlier studies (Guandalini et al., 2019; Morsy, 2015; Zhao et al., 2017), ultrasound extraction time was fixed at 20 min. Also, the temperature was controlled during the operation so that it did not fall below 70ºC. After that the mixture was transferred to a 500 ml round-bottom flask and placed into the microwave. The microwave oven (Samsung, ME341, 2450 MHz, South Korea) was modified for microwave-assisted extraction process. A schematic of the modified microwave setup is shown in Fig. 1. Here, time, temperature, and power can be controlled during the extraction. They will be discussed in detail in the experimental design section. As shown in Fig. 1, in the setup Clevenger apparatus was placed exactly on the top of the flask. Essential oils were raised into Clevenger and cooled in the condenser. Different levels of time and power were supplied and the temperature was fixed at 102ºC during the extraction process. The essential oils were weighted, collected in brown bottles, and stored in a cool and dry place until quality tests.

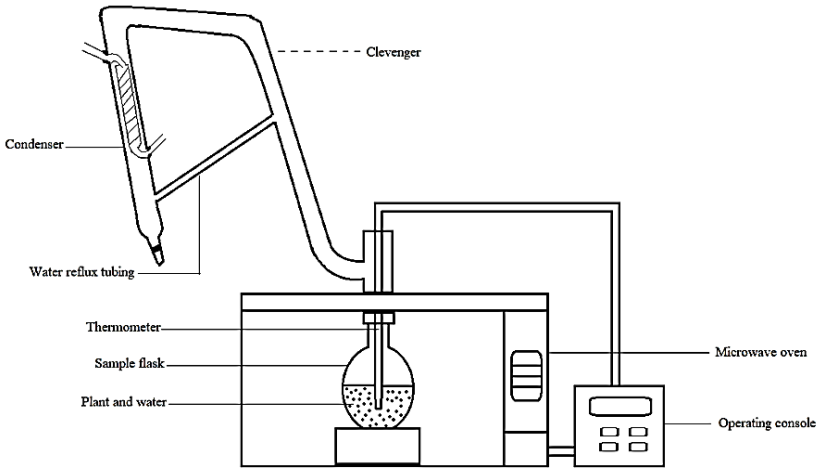

Fig. 1. Schematic diagram of microwave extractor.

Table 1. Levels of independent variables employed in the CCD.

Factors	Coded symbols	Actual symbols	Levels				
			-1.68179	-1	0	1	1.68179
Ultrasound power (W)	x_1	X_1	100	150	250	350	400
Microwave power (W)	x_2	X_2	300	400	600	800	900
Extraction time (min)	x_3	X_3	5	10	15	20	23

Table 2. Central composite design matrix and results for essential oil yield using SUMAE method.

Run	Independent variables						Dependent variable
	x_1	(X_1)	x_2	(X_2)	x_3	(X_3)	Yield (%)
1	0	250	-1	400	-1	10	0.402461
2	0	250	1	800	-1	10	0.780507
3	-1	150	-1	400	1	20	0.12277
4	1	350	1	800	1	20	0.653192
5	-1	150	-1	400	-1	10	0.391169
6	1	350	1	800	-1	10	0.74880
7	0	250	-1	400	1	20	0.145186
8	-1.68179	100	1	800	1	20	0.720443
9	0	250	-1.68179	300	0	15	0.211745
10	1	350	1.68179	900	0	15	0.877355
11	-1	150	0	600	-1.68179	5	0.540815
12	-1	150	0	600	1.68179	23	0.287836
13	0	250	0	600	0	15	0.577768
14	0	250	0	600	0	15	0.536754
15	0	250	0	600	0	15	0.501284
16	1.68179	400	0	600	0	15	0.524788
17	1	350	0	600	0	15	0.563893
18	0	250	0	600	0	15	0.492475
19	0	250	0	600	0	15	0.53415
20	0	250	0	600	0	15	0.539634

2.3. Experimental design

In the present study, response surface methodology (RSM) depending on Central Composite Design (CCD) was applied to investigate the optimal extraction condition in terms of yield. Three independent variables at five levels were selected for this purpose which are ultrasound power (X_1), microwave power (X_2), and microwave extraction time (X_3) (Table 1 and 2). The ultrasound power was varied between 150 and 350W. While the microwave power was ranged from 400 to 800W with the extraction time of 10 to 20min. The most important parameter in the process is essential oil extraction yield, which was selected here as dependent variable (response) in the experimental design. The complete design consisted of 20 experimental points including 6 center points.

The experimental yields were analyzed by multiple regressions to fit the following quadratic polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is response, $\beta_\circ$, β_i, β_{ii} and β_{ij} are regression coefficients for the center point of the system, coefficients of the linear, quadratic and interactive terms, respectively. X_i and X_j refer to coded independent variable. The effects of process variables were analyzed statistically by using analysis of variance (ANOVA). Also, the adequacy of the model equation for predicting the optimum response values was validated with experimental results.

2.4. Essential oil extraction by MAE method

In order to do a comparative study, the extraction of *Zataria* essential oil was also conducted using the MAE method, based on the obtained optimized conditions of SUMAE method. Thus, samples were made up of 15g of *Zataria* leaves and 300 mL of distilled water. Then the mixture was transferred into a microwave extractor and irradiated based on the extraction conditions obtained from optimized SUMAE method. Extraction was performed in triplicates.

2.5. Antioxidant capacity

The antioxidant activity of the essential oils of *Z. multiflora* obtained by MAE and SUMAE were assessed by monitoring their ability in quenching the stable free radical DPPH according to the method of Danh et al. (2012) with slight modifications. Briefly, 1.0 ml methanolic stock solution of essential oils with different ratio (ranged from 0.15 to 0.99$\mu g/ml$) were mixed with 1.0 ml of 90 µM DPPH and adjusted with 95% methanol to final volume of 4 ml. The resulted solutions were protected from light damage. The blank was also made with the same chemicals, except the essential oil sample. Thirty minutes later, the absorbance of the blank and mixture solution was measured at room temperature. All measurements were read at 517 nm with a spectrophotometer (UNICO, UV/V is 2100). The DPPH radical scavenging activity was calculated as a percentage inhabitation (I) using the following formula:

$$I(\%) = 100 \times \frac{A_{blank} - A_{sample}}{A_{blank}} \quad (2)$$

where, A_{blank} is the absorbance of the blank, and A_{sample} is the absorbance in the present of sample.

2.6. Extracted oil composition

The composition of the extracted oil was determined by Gas chromatography-Mass spectrometry identification (GC-MS) using Agilent Technologist 7890 series coupled with a Agilent HP-5ms GC column (with 30 m length and 0.25 µm internal diameter) and a mass selective detector (MSD), 5975 series in central lab of Agriculture and Natural Resources University of Khuzestan. GC-MS was applied using the following condition: carrier gas of *He*; constant flow rate of 0.8 ml/min; injection volume of 1 µl; injection temperature of 290ºC; and Ion-source temperature of 280ºC. The initial temperature of the oven was 50ºC for 5 min, followed by a gradient rate of 3 ºC/min until it reaches to 240ºC, and then with an increase of 15 ºC/min to 280ºC held for 10 min. Total running time for the GC-MS process was 75 min.

The essential oil components were characterized by comparison of their retention time and mass spectra with the available literature data and computer matching with Wiley 7n and National Institute of Standards and Technology (NIST5.0) libraries produced with the computer controlling the GC-MS system, and literature. The spectral data of the unknown component was compared with the spectrum of the known components stored in the library. Finally, the name of the components of the test materials was ascertained.

Table 3. Analysis of variance (ANOVA) for regression model of essential oil extraction yield.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value
Model	0.7821	9	0.0869**	144.49	< 0.0001
X_1-Ultrasound power	0.0000	1	0.0000**	0.0273	0.8719
X_2-Microwave power	0.4823	1	0.4823^{ns}	801.93	<0.0001
X_3-Microwave time	0.0693	1	0.0693**	115.20	< 0.0001
X_1 X_2	0.0021	1	0.0021^{ns}	3.56	0.0884
X_1 X_3	8.607E-08	1	8.607E-08*	0.0001	0.9907
X_2 X_3	0.0088	1	0.0088^{ns}	14.59	0.0034
X_1^2	0.0000	1	0.0000^{ns}	0.0707	0.7957
X_2^2	0.0010	1	0.0010^{ns}	1.69	0.2228
X_3^2	0.0171	1	0.0171**	28.43	0.0003
Residual	0.0060	10	0.0006		
Lack of Fit	0.0013	5	0.0003^{ns}	0.2880	0.9010
Pure Error	0.0047	5	0.0009		
Cor Total	0.7881	19			

* significant at $p < 0.05$
** significant at $p < 0.01$
^{ns} not significant

2.7. Environmental impact of applied techniques

In regard to environmental impact, in the present study electrical consumption and CO_2 emission of the applied techniques were also analyzed. Thus, the electrical consumption (EC) was calculated as the electrical power for a time, as follows (Drinić et al., 2020):

$$EC = P \times t \quad (3)$$

where *EC*, *P* and *t* are electrical consumption (kWh), electrical power (kW), and time (h), respectively.

According to (Farhat et al., 2017) to obtain 1 kWh from coal or fuel, 800 gr of CO_2 will be released into atmosphere during the combustion of fossil fuel. Therefore, CO_2 emission can be calculated by the following formula:

$$E_{CO_2} = \frac{EC \times 800}{1000} \quad (4)$$

where E_{CO2} is CO_2 emission (*kg*) and *EC* is the electrical consumption (*kWh*).

3. Results and Discussion

3.1. Model fitting and statistical analysis

In order to determine the optimum conditions for the yield of essential oils from *Z. multiflora*, RSM was employed by using CCD. Independent variables include microwave power (W), ultrasound power (W), and extraction time (min), the range of which was identified in the preliminary experiments. The experimental data and the process variables for the yield of the essential oil obtained from the extraction of *Z. multiflora* using SUMAE system at different extracted conditions are presented in Table 2. As indicated in Table 2, the yield of essential oils varied from 0.21 to 0.87% (w/w), depends on operating conditions. Analysis of variance (ANOVA) was accomplished to investigate the adequacy of the quadratic model and identify the effect of each factor. Table 3 gives the results of the statistical analysis along with the lack of fit, pure error and cor total for each response. Table 3 clearly reveals that model F-values of all variables are significant and the chance that large F-value is due to noise is less than 0.01%. Moreover, linear effects of microwave time and power were significant on the yield ($p < 0.0001$). Whereas the linear effect of ultrasound power was not significant. Only the mutual interaction between time and power of microwave was significant at the 0.05% level, while the mutual interactions between other factors were not significant. Quadratic effect of microwave power was also significant on the yield of extraction oil at the 0.01% level. Although the quadratic effects of the others were not significant. The lack of fit F-value of 0.2880 implies the lack of fit is not significant relative to the pure errors. The non-significant lack of fit is good and emphasize that the model is appropriate to use to predict the response.

The results of model fit were summarized in Table 4. The quality of the fitting procedure was checked by means of coefficient of determination (R^2), adjusted and predicted R^2, adequate precision and coefficient of variation (C.V.). According to Table 4, coefficient of determination (R^2), which measures the conformity of the model, is high enough ($R^2 > 0.95$). It means that more than 95% of the stability changes are related to independent variables and less than 5 percent of changes is unjustifiable. Meanwhile, the predicted R^2 is in reasonable agreement with the adjusted-R^2. In addition, the high values of adequate precision (> 4) are desirable and indicate an adequate signal. It is noted that adequate precision measures the ratio of signal to noise. Moreover, the last parameter (C.V.) is relatively low, which indicates the accuracy and reliability of the model.

Table 4. Regression coefficients, R^2, adjusted R^2, predicted R^2, adequate precision and C.V. for the response resurface model.

β_0	56.768
β_1	0.486
β_2	0.455
β_3	9.705
β_{11}	0.000258715
β_{22}	0.000278845
β_{33}	-0.001743
β_{12}	-0.00106162
β_{13}	0.000256851
β_{23}	0.041
R^2	0.9924
R^2 (Adj.)	0.9855
R^2 (Pred.)	0.9840
Adequate precision	42.9249
C.V. (%)	4.83
β_i	For the main linear effects
β_{ii}	For the quadratic effects
β_{ij}	For the interaction effects
1	Ultrasound power
2	Microwave power
3	Microwave time

3.2. Effect of process variables on essential oil yield

The results demonstrated that the essential oil extraction yield was positively proportional to the main effects of extraction time, ultrasound power and microwave power and interaction effects between extraction time and the others, while it was negatively affected by the interaction between ultrasound and microwave powers. Moreover, extraction yield was positively affected by quadratic terms of ultrasound and microwave powers, whereas it is negatively proportional to quadratic term of time (Table 4).

The positive effect of ultrasonic power can be explained in terms of the ultrasonic amplitude. When the plant subjected to ultrasonic waves, cell wall appears to collapse due to the shock wave and liquid jets that are produced by cavitation. This cavitation of liquid media occurs as a consequence of ultrasonic wave compression and rarefaction process. The process of compression and rarefaction depends upon the strength of the ultrasonic waves. Thus the more the ultrasonic amplitude, the more the number of cavity, which in turn results in the maximum extraction yield (Luque-Garcıa & Castro, 2003; Maran & Priya, 2015; Vinatoru, 2001). However, this effect is not so significant compared to the effect of microwave power. As their interaction has an effect about zero on the yield.

The enhanced extraction yield by increasing microwave power is related to the direct effects of microwave energy on plant materials. Microwave radiation loosens the matrix of cell wall, resulting in quickly and thoroughly opening up the skin tissues (Kratchanova et al., 2004). More electromagnetic waves was

transported to biomolecules via ionic conduction and dipole rotations, resulting in energy absorbed within the solvent and plant material and then quickly generating molecular movement on the extraction system and increasing the yield of the extraction (Gfrerer & Lankmayr, 2005). This parameter in combination with time also has a positive effect on the process. Because irradiation time is one of the main factors influencing essential oil extraction yield, and a suitable irradiation period must be chosen to ensure optimal extraction. The extraction yield was found to be proportional to the extraction time whereas its quadratic effect is negatively affected the extraction yield. However, prolonged exposure to microwave power leads to structural degradation of the extracted compounds, thereby reducing the extraction yield (Zheng et al., 2011). In economic terms, shorter time leads to more economic extraction.

As shown in Table 2, the highest yield of 0.87% (w/w) was related to the extraction time of 15min, microwave power of 900W, and ultrasonic power of 350 W. This yield is almost 82% higher than the result reported by Lucchesi et al. (2004) for thyme extraction obtained by using solvent free microwave extraction method. Comparing the reported conditions, their microwave power of 500 W was lower than that of the current study.

However, their reported microwave irradiation time was 30 min. Furthermore, the essential oil yield of SUMAE of this study was higher than the extract yield of MAE method. This suggests that ultrasound pre-treatment has assisted in the extraction of essential oil. The effects of process variables are shown in Fig. 2 by plotting three-dimensional response surface plots.

Generally, as numerical optimizer calculates, optimum parameters in extraction of essential oil of *Zataria multiflora* by SUMAE method were microwave power of 800 W, ultrasound power of 150 W, and extraction time of 12 min, in which essential oil extraction yield is 0.812%.

3.3. Comparison between MAE and SUMAE methods

In order to evaluate the efficiency of the proposed SUMAE method, extraction was also performed by microwave method. Compared with MAE, the application of SUMAE had positive effect on the yield of essential oil obtained. This means that using an ultrasound pre-treatment, the yield of essential oil increased from 0.6 to 0.87% at the same microwave power (900 W) and substantially shorter extraction time (23 min for MAE vs. 15 min for SUMAE). On the other words, extraction time decreased by using ultrasound power factor compared with the MAE. The higher efficiency of the SUMAE can be explained by synergistic effects of ultrasonic and microwave on the cell walls of plant resulting in a significantly increased in extraction yield of the essential oil. This proves that SUMAE can be an appropriate extraction technique to extract essential oil from *Z. multiflora* since it provides the maximum extraction yield at the shortest extraction time.

3.4. DPPH analysis

The most common test used in determination of the antioxidant activity of plant extracts is 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) free radical test (Brand-Williams et al., 1995). It is based on the reduction of alcoholic DPPH$^{\bullet}$ solutions in the presence of a hydrogen donating antioxidant. DPPH$^{\bullet}$ solutions demonstrate a strong absorption band at 517 nm appearing a deep purple color. After a certain time (thirty minutes in our study), the remaining DPPH$^{\bullet}$ is measured, which inversely corresponds to the radical scavenging activity of the antioxidant (Molyneux, 2004). The IC_{50} values of the obtained essential oils by two mentioned methods and BHT as a standard antioxidant are shown in Table 5. Lower IC_{50} value specifies higher antioxidant activity. According to the results, antioxidant activity of essential oil extracted by SUMAE was superior to that of MAE. Therefore, the SUMAE treatment does not cause any deterioration of the antioxidant activities and it can be introduced as a safe and green method for the extraction of essential oil from *Zataria multiflora*.

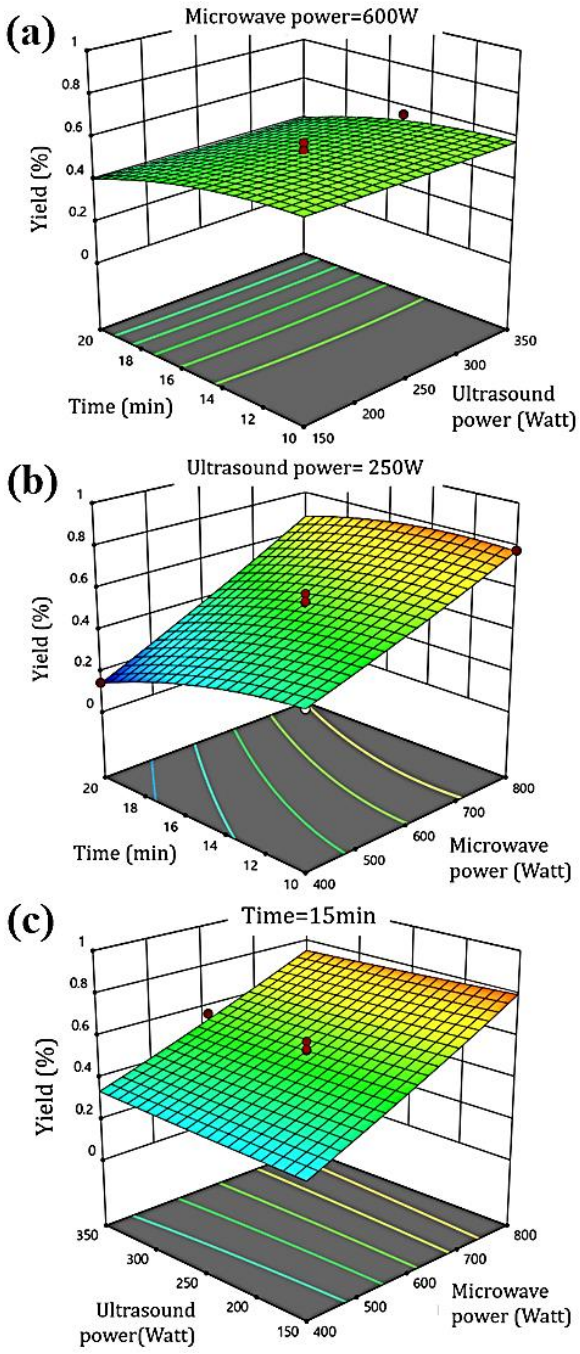

Fig. 2. Response surface and contour plots for the effect of independent variables on total essential oil extraction yield.

Table 5. Antioxidant effects of essential oils of *Zataria multiflora* obtained by MAE and SUMAE methods.

Antioxidant	IC_{50}[a]
essential oil by MAE[b]	157.0425 (153.25_161.15)
essential oil by SUMAE[c]	107.2625 (106.65_108.54)
BHT[d]	88.8175 (88.54_89.21)

[a] IC_{50}, Concentration ($\mu g/ml$) for a 50% inhibition
[b] MAE, microwave-assisted extraction
[c] SUMAE, Sequential ultrasound-microwave assisted extraction
[d] BHT, Butylated Hydroxy Toluene

Table 6. Chemical composition of Z. multiflora essential oils obtained by MAE and SUMAE methods.

No.	Compound	RT[a]	Area%	
			MAE[b]	SUMAE[c]
1	ρ-Cymene	13.894	0.33	0.36
2	1,8-Cineole	14.212	0.61	0.51
3	γ-Terpinene	15.617	0.31	-
4	cis-sabinene hydrate	16.069	0.41	0.14
5	Pyridine	17.628	0.14	0.12
6	Linalool	17.761	0.2	0.22
7	Camphor	19.823	0.15	0.14
8	Borneol	20.939	1.44	1.17
9	Terpinen-4-ol	21.484	0.64	0.64
10	α-Terpinol	22.182	0.26	0.30
11	Glutaconic anhydride	22.469	-	0.20
12	Piperazine	22.807	-	0.19
13	Catechol	24.223	-	0.28
14	Carvacrol methyl ether	24.674	0.26	0.40
15	Thymoquinone	24.992	-	0.37
16	Nerol	25.320	0.28	0.54
17	Thymol	27.228	2.06	2.01
18	Carvacrol	27.710	90.87	86.14
19	2,5-Diethylphenol	28.140	-	0.31
20	Piperitone	29.135	-	0.13
21	Phenol, 2- methoxy- 3-(2- propenyl)-	29.812	-	0.34
22	Geranyl acetate	30.951	-	0.11
23	β-caryophyllene	32.366	-	0.38
24	Norephedrine	34.766	-	0.12
25	β-Acoradiene	35.515	-	0.11
26	Phenylephrine	36.079	-	0.32
27	γ-Cadinene	36.294	-	0.23
28	δ-Cadinene	36.664	0.27	0.42
29	Chloracetamide	37.433	-	0.14
30	Phenylephrine	38.981	-	0.23
31	β-Cubebene	41.207	0.93	1.14
32	2- Bromoethanol	41.720	-	0.14
33	Methamphetamine acetylated	42.879	-	0.10
34	Adamantane	51.135	-	0.26
35	Allantoicacid	52.263	-	0.30
36	Benzenemethanol, alpha.-(1- aminoethyl)-	57.648	-	0.13
	Identification (%)		99.16	97.83

[a] RT, Retention time
[b] MAE, , microwave-assisted extraction
[c] SUMAE, sequential ultrasound-microwave assisted extraction

3.5. *Chemical composition of the essential oil*

Specified components of the extracted essential oils of *Zataria multiflora* obtained by SUMAE technique are summarized in Table 6 and compared with the ones extracted by MAE method. We have found 36 compounds in essential oils extracted by the SUMAE whiles it was equal to 16 for MAE method and they were all also found in SUMAE extracted analysis. However, finding new component in the combined method confirms the effectiveness of combining ultrasound with microwave extraction. According to Table 6, the prominent compounds determined in essential oils extracted by MAE were carvacrol (90.87%), thymol (2.06%), and borneol (1.44%). Comparatively, the main constituents of the essential oils extracted by SUMAE technique were carvacrol (86.14%), thymol (2.01%), borneol (1.17%), and β-Cubebene (1.14%). carvacrol as the primary chemical component found in the extracted oils, is a monoterpenoid phenol. This vital substance is a powerful antimicrobial compound and has been used for the prevention and protection against infectious diseases (Memar et al., 2017). carvacrol and thymol are generally known as powerful antioxidants, anti-stress and anti-inflammatory agents.

Other researchers (Gavahian et al., 2011; Golmakani & Rezaei, 2008; Khalili et al., 2018) have also found similar components in their analysis. However, in the present study, the amount of the obtained carvacrol was higher than the one reported by Golmakani and Rezaei (23.9±0.6%) (Golmakani & Rezaei, 2008). In some reports, linalool has been identified as the main ingredient (Gavahian et al., 2011), while the amount of this substance in the composition obtained in the present experiments was not significant. It is noted, yields and chemical compounds of the essential oils extracted from medicinal plants can wildly differ because of season and ontogenetic variations and also these different results may be due to the different geographic and climate conditions (Morales et al., 2002).

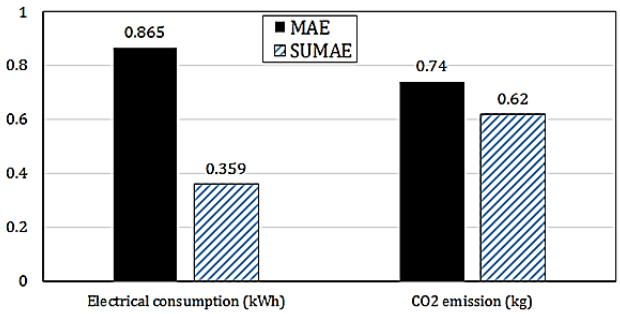

Fig. 3. Energy consumption and environmental impact of the two extraction methods; MAE: microwave associated extraction; SUMAE: sequential ultrasound microwave associated extraction.

3.6. *Energy and environment impact*

The electrical energy consumption required to perform MAE extraction was 0.675 kWh, while in SUMAE at levels of 900W it was 0.3125 kWh, which is lower than the amount consumed for MAE extraction. Regarding environmental impact, the calculated quantity of carbon dioxide emission to the atmosphere was higher in the case of MAE (0.54 kg) compared to SUMAE (0.25 kg).

In order to more accurately compare environmental impacts, since the yield of essential oils obtained by MAE and SUMAE methods was not the same, the total electrical consumption and CO_2 emission were calculated for 1% of extracted essential oil. Thus, electrical consumption for yield of 1% was 0.865, and 0.359 kWh, also CO_2 emission was 0.74, and 0.62 kg for MAE and SUMAE, respectively (Fig. 3). Therefore, using SUMAE method

led to save energy about 141% in comparison with MAE method, and emission of CO_2 was reduced almost 20%, as well.

It is noted, electrical consumption and CO_2 emission are straightly related to extraction time. For heating 300 ml of water and 15 g of dried *Z. multiflora* leaves to reach the fixed extraction temperature, it was 45 min in the extraction with MAE method with microwave power of 900 W. The yield of essential oil obtained by these conditions was 0.78%. On the other hand, in SUMAE method for the same ratio of water and plant material, it was recorded about 15 min. While, Essential oil obtained from the novel method was 0.87%. Therefore, a significant saving in the extraction time was clearly observed in sequential UMAE procedure. Generally, it can be concluded that the proposed SUMAE was a cleaner process for the extraction of essential oil of *Zataria multiflora*.

4. Conclusion

In this study, SUMAE of essential oil of *Z. multiflura* was investigated, and presented as an appropriate and 'environmentally friendly' method for essential oils extraction. As a consequence of SUMAE, a reduced extraction time and a noticeable energy saving obtained, compared to the MAE technique. Response surface methodology was used to determine the optimum process parameters that could obtain a maximum yield of essential oil from *Z. multiflura* during the sequential ultrasound-microwave assisted extraction. The results showed that the optimum conditions of SUMAE for essential oil of *Z. multiflura* were: ultrasonic power of 150 W, microwave power of 800 W, and extraction time of 12 min. Furthermore, SUMAE technique produced essential oils with higher quantities of valuable compounds due to their GC analyses. In addition, the antioxidant activities for essential oils obtained by both methods were evaluated. The results showed that essential oil obtained by SUMAE had higher antioxidant activities compared to that of by MAE. The comparison of these two methods indicated that SUMAE overcomes the shortcomings of MAE and will be a more beneficial extract process.

Acknowledgment

The authors would like to thank Jundi-Shapur University of Technology for providing the equipment used in the study.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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