Ultrasound-assisted extraction of thymol from *Zataria multiflora* Boiss.: Optimization by response surface methodology and comparison with conventional Soxhlet extraction

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This study focuses on the extraction of thymol from the medicinal plant *Zataria multiflora* Boiss. leaves by ultrasound technique and by the Soxhlet method. The effects of several parameters including time, temperature, solid-to-solvent ratio, ultrasound power, and duty cycle on the amount of thymol extracted from *Z. multiflora* leaves were investigated and optimized using response surface methodology (RSM). The optimized circumstances for the extraction were as follows: extraction time 30 min, temperature 34°C, ultrasound power 340 watts, solid-to-solvent ratio 1:35 g/ml and duty cycle 20%. The amount of thymol extracted at the above-mentioned parameters was 7.195 mg/g which was consistent with the predicted amount of 7.31 mg/g by RSM. Furthermore, Peleg's model was used to predict the kinetics of ultrasound-assisted extraction (UAE) of thymol. The results proved the good agreement of the model with the experimental data. Moreover, UAE was found to be more efficient, faster and easier to manipulate compared to the Soxhlet method. To the best of researchers' knowledge, this is the first report on the extraction of essential oils from *Z. multiflora* using ultrasound technique.

Keywords: Ultrasound-assisted extraction; *Zataria multiflora* Boiss.; Thymol; Response surface methodology.

INTRODUCTION

Oil essences are natural aromatic compounds of plants, which are widely utilized in perfume, pharmaceutical and food industries [1]. Essences are commonly prepared from different parts of fresh or dried herbal organs. These compounds have physiological and medicinal properties, the most common and well known of which are antimicrobial, antifungal, analgesic and antioxidant [2].

Essences, especially Thyme essential oils, are useful in preserving food. *Z. multiflora* Boiss. (Shirazi Thyme) is one of the species of thyme classified in the Mints group. It belongs to the southwestern part of Asia, Iran, and is the most important species of thymes after *Thymus vulgaris L.*, [2, 3]. Thymol is the most important compound of this plant, which accounts for 30-70% of oil essence. It is a white aromatic substance with antibacterial and antiseptic properties [4]. Anti-bacterial feature of thymol is the cause for thyme essence's anti-bacterial trait. For this reason, it is commonly used in mouthwashes and toothpastes. In addition, thymol can reduce bacterial resistance to antibiotics and eliminate fungi and it can function as a general medical disinfector [5-7]. Thymol's anti-fungal effect has led to its use in alcoholic solutions to treat fungal infections, as well as intestinal infections in the United States [8]. Also Middle Easters consume *Z. multiflora* to reduce and remove internal parasites [9].

In recent years, in order to obtain valuable compounds from plants and to prevent their decomposition, various technologies have been evaluated to provide an efficient extraction method. Conventional methods are divided into three categories, namely distillation, solvent extraction and cold compression [10]. These methods, which have been repeatedly used since long time ago, have many drawbacks. The mechanism of the aforementioned methods is that when the plant is placed in the solvent, the compounds enter the solvent due to the concentration gradient introduced inside and outside. This process sometimes takes up to a month to reach the equilibrium [11]. Other disadvantages of these methods include degradation of volatile and unsaturated compounds, low yield, and solvent toxicity [2].

Today, for the extraction of various compounds in various industries, such as food industry, ultrasound technology is used, the driving force of which is the cavitation phenomenon [12, 13]. In this process, due to the oscillation of the bubbles and their collapse, the solvent faster targets the plant's material and causes an increase in the temperature. This results in tissue destruction, as well as increased extraction efficiency and mass transfer rate [14-16].

Many studies have been carried out on the extraction of oil essences from *Z. multiflora*. For instance, the comparison made between the supercritical fluid extraction of *Z. multiflora* essence

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and steam distillation revealed that although supercritical fluid extraction takes less time than the steam distillation (half an hour vs. 1 hour), the amount of extracted thymol equals 44.6% of that in steam distillation. Furthermore, the resulting mixtures from the two methods slightly differed, but with the supercritical fluid method under higher pressure and by changing extraction parameters, there will be better selectivity while compared to steam distillation [17]. Additionally, Golmakani and Rezaei (2008) have illustrated that the extraction of *Z. multiflora* essence by Microwave-Assisted hydrodistillation (MAHD) took less time than hydrodistillation (1 hour *vs*. 4 hours) [3]. In addition, the resulting mixtures have been more similar, but the MAHD method yielded a higher output and costed less. Moreover, using a subcritical water extraction method carried out by Khajenoori *et al.* (2009) [2] it has been shown that this method is more efficient and provides more valuable oxygenated compounds of essence compared to Soxhlet extraction (a conventional method used in the present study). Considering all above mentioned approaches, it is demonstrated that despite novel methods' supremacy over the traditional ones, these differences are insignificant.

According to the literature review on extraction by ultrasound method and the importance of thymol, which is a valuable compound of *Z. multiflora*, the main objective of the present study is to evaluate the efficiency of this method and calculate the maximum amount of extracted substances. In order to optimize the extraction of the valuable compound of *Z. multiflora* by ultrasound waves, the effective parameters including temperature, power, time, duty cycle and solvent-to-solid ratio were investigated by a 3-level and 5-factor Box–Behnken design of response surface methodology (RSM). In addition, since minimizing time and expenses is an all-the-time concern in industry, kinetic studies play a significant role in extraction processes allowing predicting of their speed. Despite the broad considerations about ultrasound-assisted extraction, there is a huge gap concerning the kinetics of this process. That is why the kinetics of extracting thymol out of *Z. multiflora* using ultrasound-assisted Peleg's equation, and the RSM model were investigated. Eventually, the obtained results were compared with those of the Soxhlet method.

MATERIALS AND METHODS

Plant materials and chemicals

In June 2017, dried leaves of *Z. multiflora* were procured from the Bamu area in the Zagros mountains located in Fars province (south of Iran). Moisture was measured according to a standard method using a laboratory oven at 150 ºC to reach constant weight at 5 wt% (dry basis). Samples were powdered using a mill and were kept in a refrigerator until use. *n*-Hexane (extra pure) was purchased from Daejung (Korea). Thymol (Roth, Datteln, Germany) was used as a reference for identifying the thymol peak.

Soxhlet extraction

Soxhlet extraction of 10 g *Z. multiflora* powder was done for 6 hours using 250 ml of hexane. The extract was filtered using a filter paper under vacuum (Whatman No. 1, England) and was centrifuged at 3600 rpm for 15 min. Hexane was then removed by a rotary evaporator (HAHNSHIN, Korea) at 35 °C. The extracted essential oil was transferred into a volumetric flask, using three rinses of hexane prior to the GC. **10** µl of naphthalene solution was added to the concentrate as an internal standard and 1 µl of this mixture was directly injected into the GC (Fig. 1).

Ultrasound-assisted extraction (UAE)

To extract thymol from *Z. multiflora* leaves, ultrasound equipment (Adecco, Iran) (Fig. 2) was used at a constant frequency of 20 KHz. It has a capacity of 800 watts with a titanium alloy flat tip probe (diameter 13 mm). Based on the design of the experiment (DOE), 5 g of *Z. multiflora* powder was mixed with different volumes of hexane. The effects of solid-to-solvent ratio (1:15-1:35 g/ml), temperature (20-40 ºC) extraction time (15-30 min), ultrasound power (150-350 watt), and duty cycle (20-80%), were examined. After conducting every test, the sample obtained was filtered and prepared for GC analysis, as mentioned in the previous section.

Experimental design

In order to optimize the conditions for the extraction of thymol from *Z. multiflora*, the response surface method was used as an effective statistical model. This allows determination of the favorable conditions of UAE. As previously reported, studying the effects of variables at the same time is more efficient than when the variables are separately examined [18, 19].

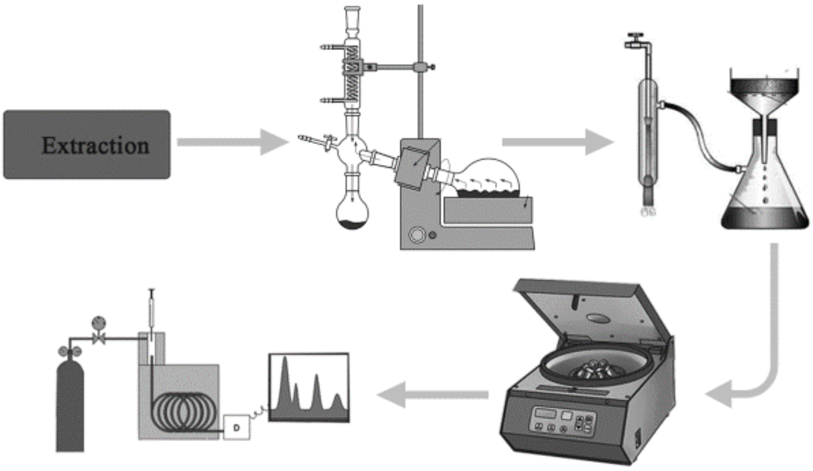

Figure 1. Schematic representation of the ultrasound-assisted extraction process.

Box-Behnken is a spherical design of the RSM family, which includes one central point and several midpoints [20, 21]. A 5-factor 3-level version of Box-Behnken design was used to attain the highest efficiency. One of the features of this model is minimizing the effect of kinematic errors. The plan includes 46 tests. To prevent errors and minimize the effects of unexpected changes, the tests were done randomly. The five independent variables include solid-to-solvent ratio (X1), temperature (X2), extraction time (X3), ultrasound power (X4), and duty cycle (X5) (Table 1), while the amount of thymol extracted is the response variable. The entire test design matrix is shown in Table 2. Statistical analysis was performed on the experimental data and the thymol response obtained from different extractions of the *Z. multiflora* was entered into a quadratic polynomial equation (Equation 1). This equation shows the relationship between the dependent and independent variables:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_J + \sum_{i=1}^{n} b_{ii} X_i^2 \qquad (1)$$

where Y is the thymol response expected, b_0 is a constant of model, b_i denote linear coefficients, b_{ij} are interactive coefficients, b_{ii} are second-order coefficients, and X_i and X_j are independent variables. Statistical significance of this equation was investigated by analysis of variance (ANOVA). Furthermore, the quality of the fitted model and significance of each coefficient were evaluated through R^2 and P-value, respectively.

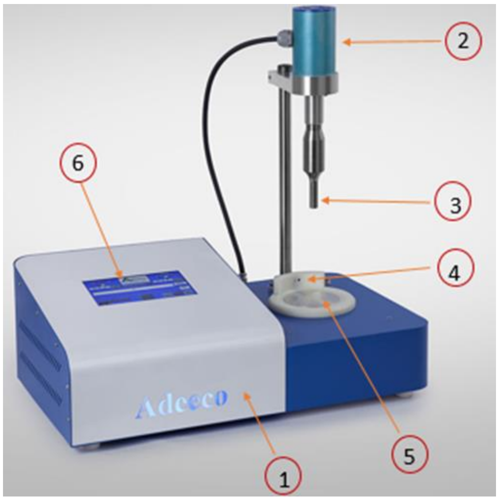

Figure 2. Ultrasound equipment: (1) ultrasonic generator; (2) ultrasonic transducer; (3) titanium probe; (4) IR sensor; (5) temperature control module; (6) touch screen.

Table 1. Coding of independent variables and their levels for Box-Behnken design (BBD).

Parameter	Symbol	Levels		
		-1	0	1
Solid-to-solvent ratio (g/ml)	X_1	1:15	1:25	1:35
Temperature (ºC)	X_2	20	30	40
Extraction time (min)	X_3	15	22.5	30
Ultrasonic power (watts)	X_4	150	250	350
Duty cycle (%)	X_5	20	50	80

Table 2. Box-Behnken design matrix with experimental results.

Run	Parameters					Response
	Ratio X_1	Temp. X_2	Time X_3	Power X_4	Duty cycle X_5	Y Thymol value (mg/g)
1	15.00	30.00	22.50	350.00	50.00	4.83
2	25.00	30.00	22.50	150.00	20.00	5.257
3	25.00	30.00	15.00	350.00	50.00	5.63
4	15.00	30.00	22.50	250.00	80.00	4.96
5	25.00	30.00	22.50	150.00	80.00	5.49
6	35.00	30.00	15.00	250.00	50.00	4.9
7	25.00	30.00	22.50	250.00	50.00	5.01
8	15.00	30.00	30.00	250.00	50.00	4.63
9	15.00	40.00	22.50	250.00	50.00	4.345
10	35.00	30.00	22.50	250.00	80.00	5.1
11	25.00	20.00	30.00	250.00	50.00	4.48
12	25.00	20.00	22.50	250.00	80.00	4.45
13	35.00	20.00	22.50	250.00	50.00	4.24
14	25.00	30.00	22.50	350.00	20.00	6.13
15	25.00	30.00	30.00	350.00	50.00	5.86
16	25.00	30.00	15.00	250.00	20.00	4.5
17	15.00	20.00	22.50	250.00	50.00	3.3
18	25.00	40.00	22.50	150.00	50.00	5.09
19	25.00	30.00	22.50	250.00	50.00	4.985
20	35.00	30.00	22.50	250.00	20.00	5.64
21	25.00	30.00	22.50	250.00	50.00	4.98
22	25.00	20.00	22.50	150.00	50.00	3.99
23	25.00	40.00	22.50	350.00	50.00	5.56
24	25.00	20.00	22.50	250.00	20.00	4.36
25	25.00	30.00	30.00	150.00	50.00	5.46
26	35.00	40.00	22.50	250.00	50.00	4.84
27	25.00	40.00	22.50	250.00	80.00	5.315
28	25.00	30.00	30.00	250.00	20.00	6.2
29	25.00	30.00	30.00	250.00	80.00	5.01
30	25.00	40.00	15.00	250.00	50.00	4.95
31	25.00	30.00	15.00	250.00	80.00	5.85
32	25.00	40.00	22.50	250.00	20.00	5.22
33	35.00	30.00	22.50	150.00	50.00	4.78
34	25.00	30.00	22.50	250.00	50.00	4.99
35	25.00	30.00	22.50	350.00	80.00	5.95
36	25.00	30.00	22.50	250.00	50.00	5.02
37	25.00	30.00	15.00	150.00	50.00	4.79
38	15.00	30.00	22.50	150.00	50.00	4.63
39	25.00	30.00	22.50	250.00	50.00	4.995
40	15.00	30.00	22.50	250.00	20.00	4.35
41	15.00	30.00	15.00	250.00	50.00	4.15
42	25.00	20.00	22.50	350.00	50.00	4.86
43	35.00	30.00	22.50	350.00	50.00	5.95
44	35.00	30.00	30.00	250.00	50.00	5.34
45	25.00	40.00	30.00	250.00	50.00	5.12
46	25.00	20.00	15.00	250.00	50.00	3.85

Gas chromatographic analysis

The compounds of *Z. multiflora* were proven by means of gas chromatography (GC). Separation and identification of the essence compounds were carried out by a GC (ACME 6100, 6000 series) equipped with a TRB-WAX capillary column (60 m × 0.25 mm i.d.) with a 0.5 μm polyethylene glycol film. Helium (Roham Gas Co, Iran, 99.999%) was used as the carrier gas with a constant flow rate of 1 ml/min. Both detector and injector temperatures were set at 250 °C. The volume of each sample injected was 1.0 μl and the total time spent for each injection was 20 min. The GC temperature program was as follows: oven temperature increased from 60 °C to 200 °C with a rate of 4 °C/min using splitless mode injection. Naphthalene was used as internal standard, and the amount reported as relative peak area is based on this (peak area of thymol to peak area of naphthalene (p_T/p_N)).

Extraction kinetics

Mathematic modeling can play a crucial role in designing extraction processes, predicting their rate, and controlling them. So different empirical and semi-empirical equations have been presented such as the Peleg's equation [22]. This equation is widely used for solid-liquid extraction of biological material out of plants. The present model equation is as follows:

$$C_t = C_0 + \frac{t}{K_1 + K_2 t} \quad (2)$$

where C_t is the concentration of thymol at time *t* (mg of thymol/g of dried sample), K_1 is Peleg's initial rate constant (min.g of dried sample/mg of thymol), $K_2 t$ is Peleg's capacity constant (g of dried sample/mg of thymol) and C_0 is initial concentration of thymol.

Supposing that the initial concentration of thymol is zero, as a fresh solvent is used, the equation will be as follows:

$$C_t = \frac{t}{K_1 + K_2 t} \quad (3)$$

According to the equation, K_1 and K_2, respectively, the slope and the intercept of $\frac{1}{C_t}$ *vs.* $\frac{1}{t}$ are calculated.

RESULTS AND DISCUSSION

Analysis of response surface methodology

Extracting thymol from *Z. multiflora* was done using UAE, entering the resulting lab data in the quadratic equation. The significance of model coefficients and compatibility of the design have also been reviewed using variance analysis (ANOVA). The obtained results show that linear coefficients for all independent variables as well as all quadratic coefficients are significant. The observed value for all effects is as $p < 0.0001$. The results also demonstrate a significant interaction between independent variables, except for temperature, and duty cycle, time and solvent-to-solid ratio. Eliminating non-significant variables, the final predictive equation coefficients will be as follows (Table 3).

Variance analysis was conducted for the regression equation, validating the model using ANOVA. This analysis proves that while F-value=1048.54 ($p < 0.0001$), the model is significant.

Table 3. Predicted equation coefficients.

Factor	Coefficient
b_0	-7.98027
A=time	+0.19606
B=temp	+0.42275
C=power	-0.013230
D=s/s	+0.21840
E=duty cycle	+0.060220
time * temp	-1.53333E-003
time * power	-1.46667E-004
time * duty cycle	-2.82222E-003
temp * power	-1.00000E-004
temp * s/s	-1.11250E-003
power * s/s	+4.16667E-006
power * duty cycle	+2.42500E-004
s/s * duty cycle	-3.44167E-005
time^2	-9.58333E-004
temp^2	+1.33444E-003
power^2	-4.87438E-003
s/s^2	+3.69813E-005
duty cycle^2	-3.19521E-003

Model quality was assessed using the R^2 correlation coefficient and the value for lack-of-fit. P-value of lack-of-fit ($p > 0.05$) and F-value show that this factor which is used to verify model adequacy is not significant. Correlation coefficient equaled 0.9788 (R^2), signifying that more than 97.8 % of the changes are explainable by the regulated model. R^2_{adj} coefficient which shows the level of correlation between predicted and real attained values equaled 0.9479. In addition, the predicting functions of the model considering variance inflation factor (VIF) have not been decreased (< 4) . Given the results discussed above, it is arguable that this model describes well the connection between response and the independent variables.

Effects of independent variables on extraction yield in the RSM model

The effects of various parameters on total thymol content in the form of three-dimensional graphs of surface response are depicted in Figs. 3a-3h. In each diagram, changes in the amount of the extracted thymol, shown in the vertical axis, are displayed in terms of the two specific variables, which are independent of each other, while other parameters are kept constant. Fig. 3a depicts the interactive effect of temperature and time. As it can be seen, the highest amount of thymol is extracted at 30 ºC and 30 min. The effects of time and power are shown in Fig. 3b. It is seen that in the first 30 min, with increasing power (about 350 watts), the amount of thymol extracted is still rising. In another study, (see Fig. 3c) the interaction between duty cycle and time parameters was examined. In fact, a decrease in duty cycle from 80 to 20% causes an increase in thymol extraction. Furthermore, the interaction between power and temperature is shown in Fig. 3d. As seen, with increasing power, more thymol is extracted.

In this way, with increasing power from 150 to 250 w, the slope of the extraction is negligible; however, from 250 to 350 w, the process of increasing extraction is considerable. Thymol extraction changes in terms of temperature variations are such that before reaching 30º, the extraction is initially increased and then reduced. The interactive effect of temperature and solid-to-solvent ratio is shown in Fig. 3e. As can be seen, by increasing the solvent-to-solid ratio, thymol extraction is increased, so that in the range of 1:15 to 1:30, the slope of the extraction increase is greater than that at 1:30 to 1:35. In fact, increasing the solid-to-solvent ratio from 1:30 to 1:35 has no significant effect on thymol extraction. The effect of the interaction between power and the solid-to-solvent ratio is shown in Fig. 3f. As it can be seen, the extraction of thymol increases with increasing both the power and the solid-to-solvent ratio.

In Figs. 3g and 3h, respectively, the interactive effects of power and of the solid-to-solvent ratio with the duty cycle are shown. Again, with the reduction of the duty cycle, and the increase in the power and the solid-to-solvent ratio, the extraction of thymol from *Z. multiflora* is increased. The striking feature of the graphs is that with increasing power from 250 to 350 watts and reducing the duty cycle from 50 to 20%, the extraction is significant. The results show that all five parameters studied on the thymol extraction are effective.

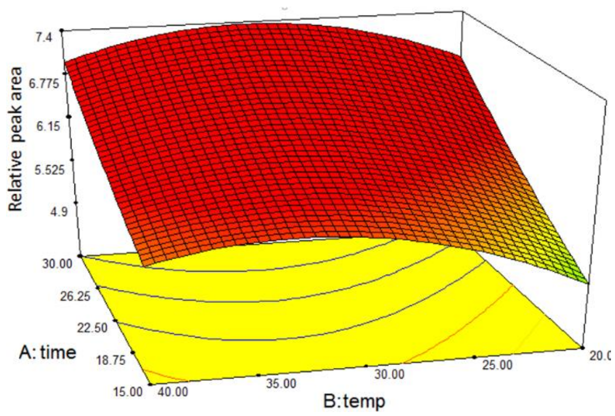

Figure 3(a). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: time and temperature.

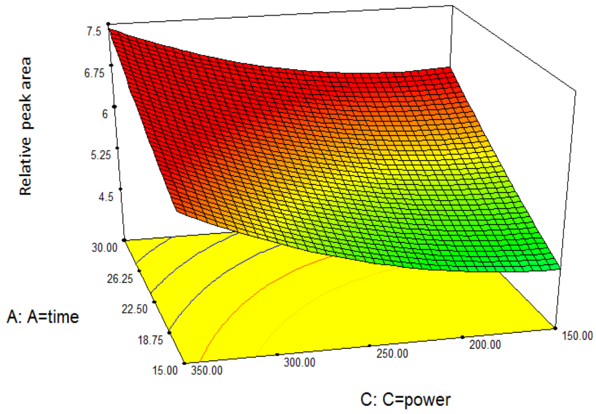

Figure 3(b). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: time and power.

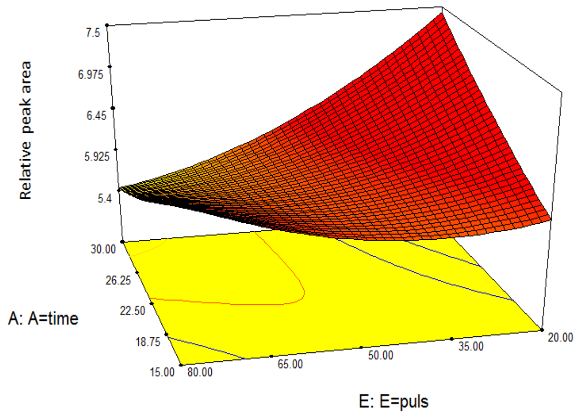

Figure 3(c). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: time and duty cycle.

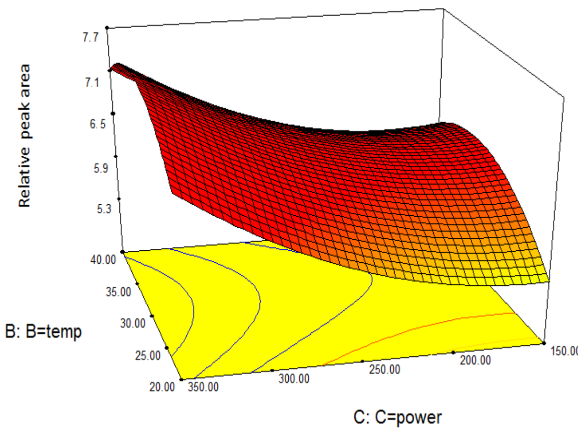

Figure 3(d). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: temperature and power.

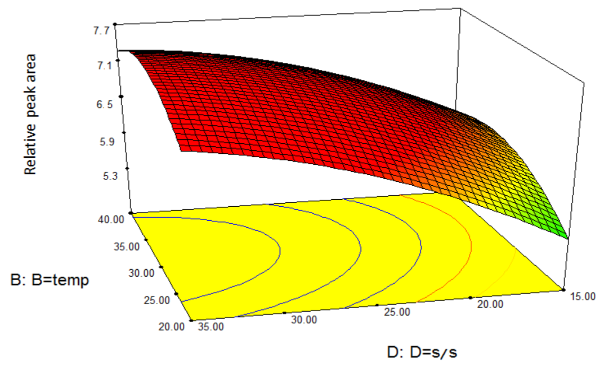

Figure 3(e). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: temperature and solvent-to-solid ratio.

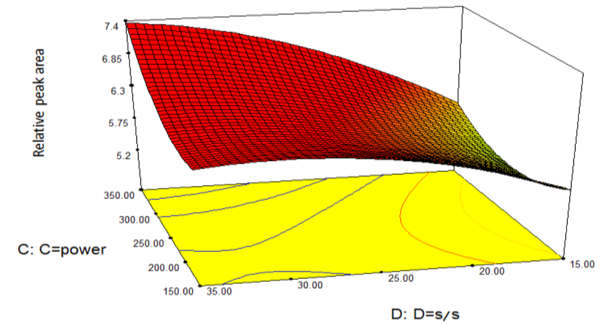

Figure 3(f). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: power and solid-to-solvent ratio.

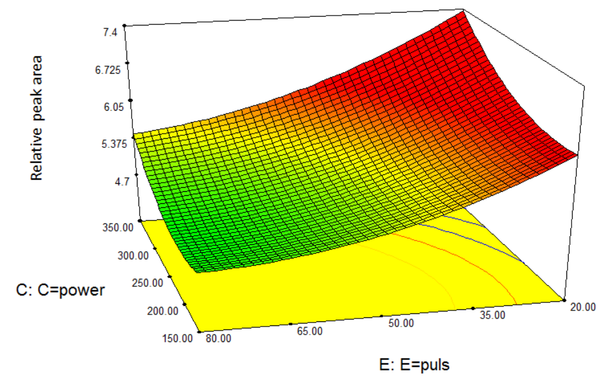

Figure 3(g). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: power and duty cycle.

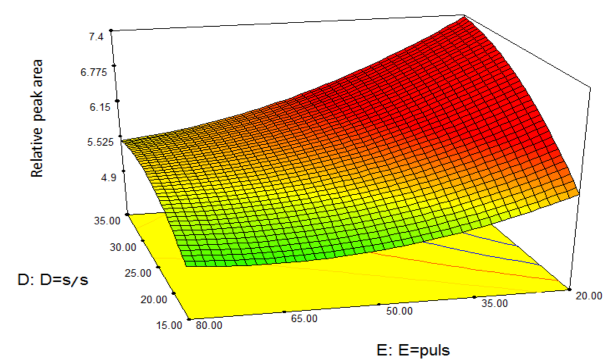

Figure 3(h). Response surface analysis for thymol from *Z. multiflora* with ultrasound-assisted extraction: solvent-to-solid ratio and duty cycle.

Validation of the predicted value

Different methods have been used to optimize the process. The most common method is to change one of the parameters while retaining the rest in constant levels. Such methods are time consuming, difficult, and impractical due to numerous experiments needed, moreover that these methods do not guarantee optimal situations in most cases [18, 23, 24]. As a result of this, RSM predictions have been applied and are known for attaining the best possible circumstances needed for maximum extraction efficiency. After designing the experiment, the predicted values were examined for results in optimal situation. According to the results, optimal conditions are as follows: time: 30 min, temperature: 34 ºC, ultrasound power: 340 watts, solid-to-solvent ratio: 1:35, and duty cycle: 20%. According to the optimal extraction conditions, the experimental amount of thymol achieved was 7.195 which was acceptable in comparison with the predicted value of 7.31 using the polynomial model.

Therefore, it can be concluded that the model developed can successfully predict thymol extraction from *Z. multiflora* using UAE.

Comparison of ultrasound-assisted extraction with Soxhlet extraction method

To represent the benefits of utilizing UAE over conventional methods, a comparison was done between the results of this experiment and those of the Soxhlet extraction method. As shown in table 4, the amount of thymol obtained using UAE for 30 min, is higher than that using Soxhlet extraction for 6 hours, so the efficiency of the former is higher. The reason for this is the destruction of plant’s cellular texture due to the use of high-frequency waves which cause cavitation resulting in mass transfer to the solvent. Finally, having spent less time, showing higher efficiency, lower temperature, and less consumption of solvent compared to traditional methods such as Soxhlet extraction, UAE proves to be more efficient [25-27].

Effect of time

Since minimizing costs has always been an important point regarded by industries, in this experiment, the kinetics of the process of extracting thymol under optimal circumstances (described in the former section), was analyzed. In this section, six experiments with constant values for temperature, power, solid-to-solvent ratio, and duty cycle parameters were done while varying the time parameter.

Table 4. Comparison of Soxhlet extraction with UAE.

Extracting method	Temperature	Time	Thymol value, (mg/g)
Ultrasonic	34 ºC	30 min	7.195
Soxhlet	68 ºC	6 h	5.216

Empirical and predicted results concerning the amount of extracted thymol are demonstrated in Fig. 4, in which the bullets are the empirical results and the line shows the values predicted by the Peleg's equation. It can be observed that the model can perform the prediction well. Using the data obtained from the previous section, we have analyzed the RSM predicting procedure. Based on the results, it can be perceived that more than 85% of the extracted thymol is obtained during the first 20 min (Fig. 5). Also, the findings of this section show that the model resulted from the RSM can predict the process of thymol extraction with proper accuracy.

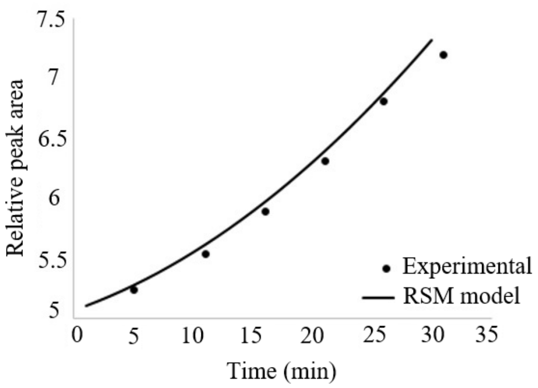

Figure 4. Experimental data of thymol extraction from *Z. multiflora* and comparison with Peleg model.

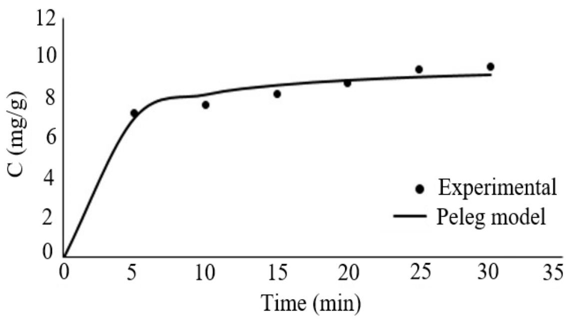

Figure 5. Kinetic extraction of thymol from *Z. multiflora* and comparison with RSM model.

CONCLUSION

In this study, UAE was successfully applied using RSM to extract thymol from *Z. multiflora* Boiss. Five factors, namely time, temperature, power, solid-to-solvent ratio, and duty cycle were examined and it was revealed that these parameters have significant effects on thymol extraction. The optimal circumstances were reported to be 30 min, 34 ºC, 340 watts of power, solid-to-solvent ratio of 1:35 g/ml, and a duty cycle equaling 20% with extracted thymol value of 7.195 mg/g as compared to the predicted value of 7.31 mg/g using the polynomial model. This shows that this model works well in predicting the amount of thymol extracted from *Z. multiflora*. In comparison to the Soxhlet method, it is shown that UAE significantly reduces the time of extraction (30 min *vs.* 6 h). Reviewing the kinetic extraction, it was shown that under optimal conditions it is possible to extract more than 85% of thymol in less time; moreover, Peleg's model showed a good agreement with the experimental results. Generally, it can be argued that as a modern technology, UAE is an appropriate and easy way of extracting essences.

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