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Ultrasonic irradiation in a continuous flow batch-mode system for essential oil extraction and in vitro anti-inflammatory evaluation

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ABSTRACT

Thymus daenensis Celak, an endemic species of the Lamiaceae family, is widely used in traditional Persian medicine in Iran. In this study, a novel approach is presented to enhance the extraction of essential oil (EO) from dried aerial parts of the plant by integrating an online ultrasound-assisted extraction (UAE) technique into a continuous flow batch-mode system coupled with a Clevenger apparatus. A response surface methodology (RSM) was applied to evaluate the interactions among key variables—temperature, extraction time, and feed-to-solvent (F/S) ratio. The optimal extraction conditions were determined to be 30.06 °C, an F/S ratio of 14.52 g·L^{-1}, and an extraction time of 53.96 minutes. Under these conditions, the EO yield obtained via UAE was 10.51 mg·g^{-1} of plant material, representing a 23.8% increase compared to the yield from the conventional method (8.49 mg·g^{-1}). Chemical composition analysis of the EO revealed that carvacrol (57.82%) and thymol (22.20%) were the predominant constituents. Ultrasound treatment also induced significant morphological alterations in the plant cell structure compared to traditional extraction techniques. Moreover, the EO extracted under optimal UAE conditions exhibited a notable anti-inflammatory effect, achieving 89% inhibition of albumin denaturation at a concentration of 900 µg·mL^{-1}.

Keywords: *Albumin protein denaturation, Anti-inflammatory, Response surface methodology (RSM), Thyme, Ultrasound-assisted extraction*

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Introduction

Essential oils (EOs) are complex natural and aromatic compounds derived from various parts of the plants. The main EO constituents are phenols, terpenoids, polyphenols, lectins, polyacetylenes, alkaloids, and polypeptides, which possess antioxidant, antibacterial, antifungal, antiviral, and cytotoxic activities (1-3). Plant EOs have a wide range of medicinal properties, making them ideal candidates to be utilized in the food, pharmaceutical, and cosmetic industries (4). *Thymus daenensis* Celak is a member of Lamiaceae family that is commonly recommended in traditional medicine owing to its pharmacological and biological properties (5, 6). Iranian *Thymus*-derived EOs and their constituents have a wide range of uses as natural food preservatives, as well as therapeutic properties (7-11).

Various methods like hydro-distillation, steam-distillation, solvent extraction, and hydro-diffusion have been mainly utilized for the extraction of EO from aromatic plants (12). Although they are easily accessible and inexpensive, some disadvantages such as lower yield and hazardous solvent residues have been recognized. Additionally, the quantity and quality of EOs are impacted by the loss of more volatile and heat-sensitive EO components the longer the extraction procedure lasts. More efficient unconventional approaches such as pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), pulsed electric field extraction (PEFE), and ultrasound-assisted extraction (UAE) have been reported (13-15). These methods can operate, at high temperatures or pressures, with minimal use of organic solvents and minimal extraction time. UAE uses solvents and ultrasonic energy to extract target ingredients from different plant matrices (16, 17).

Ultrasound is a mechanical wave with a frequency of > 20 kHz that propagates through a fluid medium, displacing molecules from their original position (18). The propagation of ultrasonic waves is accompanied by alternating high and low-pressure cycles in liquid media which results in cavitation phenomena (19). The implosion of the cavitation bubbles can also generate liquid jets at a high velocity of up to 280 $m.s^{-1}$, resulting in shear forces that mechanically rupture the cell wall and membrane, and eventually promote the release of the cell components (20). The UAE method has become more common than traditional extraction methods due to a number of benefits, including an increased extraction yield, shorter duration of extraction, lower energy consumption, and less damage to active compounds (21, 22). In this regard, Palmieri et al., (2020) compared various conventional and new techniques for extraction of bioactive molecules including maceration, soxhlet, UAE, and rapid solid-liquid dynamic from *Thymus vulgaris* L (23). (thyme), *Cannabis sativa* L. (industrial hemp) and *Coriandrum sativum* L. (coriander). Based on the report, the extracts obtained by the unconventional Rapid Solid-Liquid Dynamic Extraction (RSLDE) and UAE techniques were shown higher extraction yield. Another study reported the extraction kinetics of thymol extract using UAE (24). They showed that UAE can disrupt the structural surface of the leaves and extract compounds from internal structures of the leaves into the solvent medium.

A purposeful flow system can control the capacity of acoustic cavitation caused by ultrasound waves to increase the efficiency of extraction and the mass transfer rates between two phases (25). Rahimi et al. (25, 26) developed a novel ultrasound-assisted continuous flow approach to improve the contact area between two immiscible liquid phases by the injection strategy. On the other hand, the extraction variables can have a significant impact on the EO extraction efficiency. For EO extraction to yield more, the important variables must be identified and optimized. As a result of its capacity to concurrently examine the effects of numerous variables and their interactions,

the response surface methodology (RSM) is frequently used to refine and enhance complicated extraction conditions (27, 28). There is no report considering the optimization process of extraction towards obtaining active EO compounds particularly from *T. daenensis* using an efficient UAE system.

This study aims to develop a system for the extraction of EO from the aerial parts of *T. daenensis* using a continuous flow technique with the aid of ultrasonic waves. In terms of time, EO quality, and quantity, this method is compared to conventional approach. The chemical profile of the extracted EO was determined by GC-MS analysis. The influence of EO extraction variables (UAE time, UAE temperature, and F/S ratio) on EO yield was evaluated using the RSM. The microscopic structural changes in the aerial leaves of *T. daenensis* after ultrasonic treatment and traditional extraction were employed to explain the morphology changes. Finally, to look into anti-inflammatory activity of *T. daenensis* EO, the Mizushima method was exploited to study the inhibition of albumin denaturation, and the outcomes were compared with those of conventional diclofenac sodium.

Material and methods

Plant materials and chemicals

T. daenensis has been attained and endorsed by Shahid Beheshti University's Department of Phytochemistry, Medicinal Plants and Drugs Research Institute (Herbarium No MPH-1942, Tehran, Iran). The aerial parts of the plant were dried at room temperature before being ground by hand into a fine powder, and sieved through a 2.00 mm mesh sieve (No. 10). The chemicals employed in this research were classified as analytical grade and did not need to be purified further. Merck Co. provided diclofenac sodium, hydrochloric acid 1 N (HCL), and phosphate buffer saline (PBS) (Germany). Darou Pakhsh Pharmaceutical MFG Co, Ltd provided human serum albumin (HSA) for the anti-inflammatory assay (Iran, Tehran). Distilled water was also used for the injections throughout the tests.

Instrumentation

The performance of two-phase systems is greatly enhanced by expanding the surface contact between the phases. The "active zone" is the region below the ultrasonic horn's point where the strongest and most irregular velocity component fluctuations occur, allowing for the highest level of energy dissipation. Furthermore, the high temperature and pressure created by the power of ultrasound near the sonotrode's tip produce more active hydroxyl radicals (OH•) than in other zones, suggesting that this region is practically active chemical zone. As a result, this active point can be considered a suitable place for the introduction of a mixture of solid and liquid phases, where maximum energy dissipation occurs.

Fig. 1 illustrates a schematic diagram of a continuous-flow batch-mode system aided by an ultrasonic horn combined with a Clevenger device for extracting EO from the aerial parts of *T. daenensis*. *T. daenensis* powder (30, 45, and 60 g) sieved with a 10-mesh sieve was combined with 4L of water in a round bottom flask. A 500 mL working capacity sono-tank was used to hold the water and solids mixture. Using a magnetic compressor, it was recirculated over the procedure (SPC Magnet pump/Model: MD - 70RZ/Voltage: 220V/Speed: 2860 rpm) with a maximum capacity of 40 L. min^{-1}. The pretreatment procedure was turned off after the sonication was finished. Later on, the tank temperature was raised up to the boiling point of the aqueous solvent, and the hydro-distillation (HD) was carried out with the help of a Clevenger device attached to the system. The Clevenger device has a 500W electric heating jacket. Preliminary experiments revealed that 3 hours was the ideal extraction period for conventional HD. The extracted EOs were dried using anhydrous sodium

chloride. The collected EOs were then weighed and kept in sealed containers at 4°C for future research. In terms of mg of EOs per g of dried *T. daenensis* powder, the EO yields were reported. It should be mentioned that each extraction process was carried out 3 times and the mean values of the extraction yields were reported. Comparable plant samples, serving as controls, were examined under similar conditions without ultrasonic irradiation.

Fig 1. Schematic representation of continuous flow-flow batch mode operation of ultrasound-assisted extraction system include: (1) Organic phase tank, (2) Flask Heater, (3) Clevenger, (4) Cold water in, (5) Condenser, (6) Water out, (7) Valve – outlet, (8) Suction pump, (9) Sono –reactor, (10) Ultrasonic transducer, (11) Valve – inlet, and (12) Power supply.

Experimental design

The one-factor-at-a-time (OFAT) method in conventional optimization is a time-consuming, labor-intensive, and expensive procedure. Additionally, the conventional approach does not reveal information about the interactions between different process variables on the process output. For identifying the ideal operational variables in technological processes, the statistical design of experiments (DOE), including Box-Behnken Design (BBD), orthogonal array design, and Central Composite Design (CCD) is used frequently (29-31). The effect of extraction variables on the yield of EO was evaluated using a three-factor, three-level Box–Behnken design (BBD). The most effective variables to examine the extraction efficiency in the UAE system were UAE time (X_1), UAE temperature (X_2), and feed-to-solvent ratio (X_3). Based on the preliminary studies, the acceptable ranges for all three process variables are shown in Table 1.

Table 1. Experimental ranges and levels used for the optimization of the extraction process.

Factors	Symbol coded	Range and levels		
		-1	0	1
Time (min)	X_1	30	60	90
Temperature (ºC)	X_2	25.00	42.50	60.00
Feed to solvent ratio (F/S, g.L^{-1})	X_3	7.50	11.25	15.00

To calculate the relationship between the determined EO extraction yield and the extraction variables, the following equation (1) was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the yield of EO (mg EO of *T. daenensis* /g dry weight of plant); β_0 is the intercept coefficient, β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22}, and β_{33} are the squared coefficients, and β_{12}, β_{13}, and β_{23} are the interaction coefficients; showing the linear, quadratic and interaction effects of the variables. Design-Expert Software {Design, 2021

#129120} was applied to determine the predicted responses. The statistical experimental design was then utilized in additional validation tests to make sure it was feasible.

Scanning electron microscopy

Scanning electron microscopy (SEM, Hitachi, SU3500) was applied to ascertain the impact of ultrasound treatment on the structural alterations of T. *daenensis* leaf samples. Two samples of *T. daenensis* leaves are prepared: a sample was taken from a control sample of untreated *T. daenensis* leaves (extracted with the conventional method), and the other from UAE treated under the optimal circumstances for operation. Under high vacuum conditions, the SEM was employed with a 20.0 kV accelerating voltage, and a working distance of 8-9 mm. Having fixed in the sample container, each sample was coated with 10 nm gold in a sputter coater.

Phytochemical measurements by Gas chromatography–mass spectrometry (GC–MS)

GC analysis was performed using a TRACE GC gas chromatograph (Thermo Quest, Finnigan, Manchester, UK) with a DB -5 column of fused silica (30 m × 0.25 mm; 0.25 µm film thickness). The temperature in the oven was maintained at 60 °C for 5 min before being increased to 250°C at a rate of 5 °C.min^{-1}. The temperatures for the injector and detector (FID) were 250°C and 280°C, respectively; nitrogen was used as the carrier gas at a linear rate of 1.1 mL.min^{-1} and a split ratio of 1:10. Gas chromatography/mass spectrometry was used to identify the volatile components in *thyme* EO (GC-MS). The GC-MS analyzes (mass spectroscopy, GC-MS, TRACCE MS, Thermo Quest-Finnigan) were carried out using the following temperature and column program: The temperature on the transmission line was 250°C, the carrier gas was helium with a linear flow rate of 1.1 mL.min^{-1}, the split ratio was 1:10, the ionization energy was 70 eV, and the scan time was 0.4 seconds. The components of the EO were determined by comparing their mass spectra to those of a GC/MS system equipped with a DB-5 fused silica computer library or standard compounds, and their retention indices were confirmed by comparing them to those of standard compounds or data published in the literature. The retention index of all volatile elements was determined using a series of n-alkanes homologs (32).

Evaluation of anti-inflammatory activity by inhibition of protein denaturation

The protein denaturation inhibition method was used to calculate the in vitro anti-inflammatory efficacy. It was modified from the original Mizushima and Kobayashi (1968) technique (33). Various EO sample solution concentrations and reference medicine (diclofenac sodium) were created (100, 300, 500, 700, and 900 µg.mL^{-1}). The test control solution was made up of 0.5 mL of 3 % (w/v) aqueous human serum albumin, HSA (0.45 mL), and 0.05 mL distilled water. Similarly, the 0.5 mL product control solution contained distilled water (0.05 mL), and the test solution (0.45 mL). HSA (0.45 mL) and test solution were also included in the 0.5 mL test solution (0.05 mL). Diclofenac sodium (0.05 mL) and HSA were included in the 0.5 mL standard solution (0.45 mL). The pH of the aforementioned solutions was adjusted to 6.4 using HCl (1 N). The solutions were then heated at 51°C for 60 min after being incubated at 37°C for 20 min. After chilling, the preceding combinations were added with phosphate buffer (2.5 mL). HSA denaturation was evaluated using the absorbance at 416 nm using a UV-Visible spectrophotometer, and the inhibitory percentage of was calculated as follows:

$$Percentage\ Inhibition = 100 - [\frac{A\ of\ test\ solution\ - A\ of\ product\ control}{A\ of\ test\ control} \times 100] \quad (1)$$

Results and discussion

Statistical analysis in the UAE process

To optimize the extraction parameters, a subset of RSM called the Box-Behnken design was used (34). The combination of independent variables listed in Table 2 was used to determine the EO yield. There are a total of 17 sets of trials in the BBD design, including 5 midpoints. With a low coefficient of variation, the midpoint tests were sufficiently repeatable and reliable (Table 2). Consequently, the connection between the input variables and the measured response could be described by the second-order polynomial model in Table 3.

The ANOVA results of the proposed quadratic model are shown in Table 4. The p-value is a statistical significance index that is used to determine each coefficient's statistical significance. The model is statistically significant if the F-value is high (F = 27.22) and the p-value is low (p<0.0001). Furthermore, the coefficient of determination (R^2) was used to assess the model's quality. The R^2 value was 0.97, indicating that the experimental and expected values are well correlated. The lack of a fit index is not significant compared to the pure error, indicating that the model fits well, and the independent variables have a significant impact on the response. In conclusion, the proposed model is able to plot response surface curves and predict ideal extraction settings to optimize EO values.

Table 2. Box–Behnken design matrix in coded values, experimental and predicted results.

Run	Factors			Response		
	Time (min) X_1	Temperature (ºC) X_2	F/S ($g.L^{-1}$) X_3	Experimental essential oil ($mg_{EO}/g_{\ of\ plant}$)	Predicted essential oil ($mg_{EO}/g_{\ of\ plant}$)	|Error|* (%)
1	30	25.00	11.25	8.21	8.63	4.87
2	60	42.50	11.25	10.11	10.40	2.79
3	90	42.50	7.50	6.47	6.78	4.57
4	30	42.50	7.50	8.01	7.72	3.76
5	60	42.50	11.25	10.58	10.40	1.73
6	90	60.00	11.25	6.08	5.66	7.42
7	60	42.50	11.25	10.82	10.40	4.04
8	60	60.00	7.50	6.27	6.38	1.72
9	60	42.50	11.25	10.11	10.40	2.79
10	60	42.50	11.25	10.55	10.40	1.44
11	30	60.00	11.25	7.26	7.44	2.42
12	90	42.50	15.00	8.20	8.49	3.41
13	30	42.50	15.00	8.91	8.60	3.60
14	60	60.00	15.00	6.60	6.72	1.78
15	90	25.00	11.25	9.54	9.36	1.92
16	60	25.00	15.00	10.23	10.10	1.29
17	60	25.00	7.50	7.98	7.86	1.53

*Percentage of Error calculated as [100 × (EO $_{Predicted}$ - EO $_{Experimental}$)/ EO $_{Predicted}$], where EO is the essential oil values.

Table 3. Final equation for the essential oil value of *T. daenensis* in terms of coded or actual factors.

Response	Types	Equations
Essential oil value	Actual	$-18.05313 + 0.19023 \times time + 0.47140 \times temp + 2.40506 \times \frac{F}{S} - 1.19524E-003 \times time \times temp + 1.84444E-003 \times time \times \frac{F}{S} - 7.31429E-003 \times temp \times \frac{F}{S} - 1.40778E-003 \times time^2 - 4.55347E-003 \times temp^2 - 0.09276 \times \left(\frac{F}{S}\right)^2$
	Coded	$+10.43 - 0.26\,X_1 - 1.22\,X_2 + 0.65\,X_3 - 0.63\,X_1X_2 + 0.21\,X_1X_3 - 0.48\,X_2X_3 - 1.27\,{X_1}^2 - 1.39\,{X_2}^2 - 1.27\,{X_3}^2$

Table 4. The ANOVA analysis for the response surface quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	F-value	*p-value* Prob > F
Model	42.78	9	4.75	27.22	0.0001 *significant.*
X_1	0.55	1	0.55	3.16	0.1188
X_2	11.88	1	11.88	68.06	<0.0001
X_3	3.39	1	3.39	19.43	0.0031
$X_1.X_2$	1.58	1	1.58	9.02	0.0198
$X_1.X_3$	0.17	1	0.17	0.99	0.3537
$X_2.X_3$	0.92	1	0.92	5.28	0.0552
X_1^2	6.76	1	6.76	38.71	0.0004
X_2^2	8.19	1	8.19	46.90	0.0002
X_3^2	6.79	1	6.79	438.87	0.0004
Residual	1.22	7	0.17		
Lack of fit	0.83	3	0.28	2.81	0.1722 *not significant.*
Pure error	0.39	4	0.098		
Cor total	44.00	16			
R^2	0.9722				
Adjusted R^2	0.9365				

Fig. 2A shows a plot of actual response versus expected response values. It helps in identifying a value or collection of values that the model function cannot readily predict (35). The Fig. 2A also demonstrates that the prediction is relatively close to the practical data using the suggested correlation. Fig. 2B also presents the residuals versus the fitted response. The assumption of constant variance is challenged. The plot should behave like a random scatter, with a constant range of residuals distributed across the graph (35). These findings support the validity and dependability of the proposed correlation. The variance between the predicted response and the actual data is also shown in Fig. 2C as a normal probability plot of the residuals. The normal probability plot shows whether or not the residuals have a normal distribution. The points in this scenario are connected by a straight line (Fig. 2C). As shown in Fig. 2C, the points in this plot follow a normal distribution around a straight line.

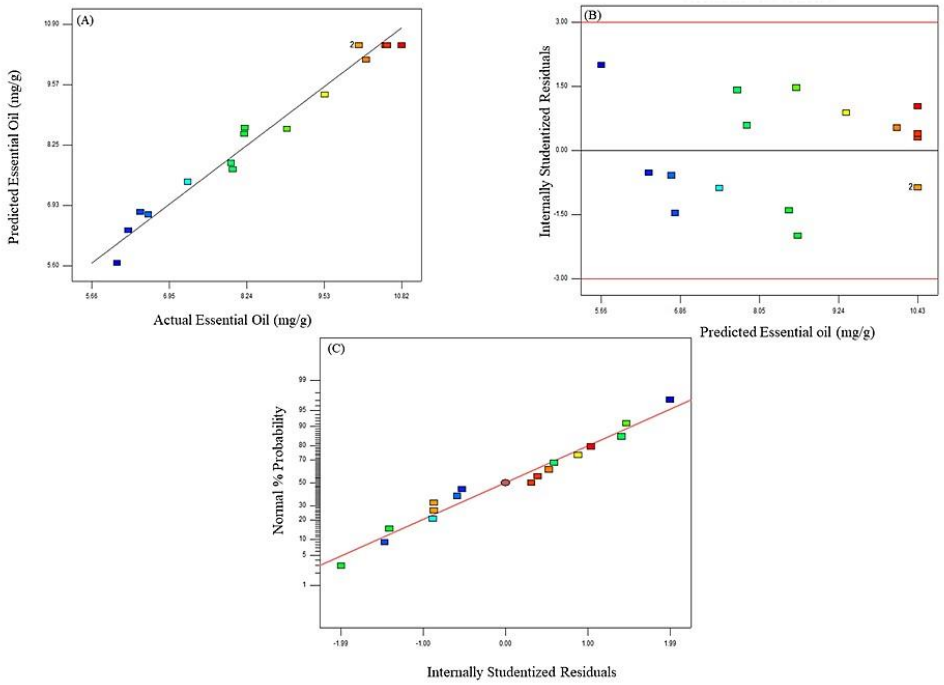

Fig. 2. Evaluation of RSM proposed correlation between predicted and experimental essential oil values from *T. daenensis* aerial parts; A) Predicted versus actual values plot, B) Residual versus predicted plot, C) Normal plot of residuals.

Common effect of extraction factors

Since the operating parameters can show considerable influence on the extraction processes, the extraction conditions of EO from *T. daenensis* were evaluated. Fig. 3 demonstrates the relationship between the independent and interactive dependent variables is depicted as a three-dimensional response level diagram in Fig. 3. It is noted that the high-frequency vibration, shear stress, and radiation pressure are all mechanical effects of ultrasound wave propagation. They have the potential to damage the tissues and cells of *T. daenensis*. It enables the solvent to completely penetrate plant tissue and cells allowing therapeutic agents to be extracted from the cells of *T. daenensis*. In addition, the ultrasonic waves cause cavitation, which breaks down cell walls and membranes, making it easier to release the therapeutic substances (24, 36). The UAE time is acknowledged as a significant aspect of the EO extraction process since

it's crucial to establish the extraction time in order to prevent wasting too much energy. The F/S ratio is yet another crucial factor that has been studied to increase extraction effectiveness. To maximize extraction yield, it is also important to take into account the ratios of sample to solvent and particle size since these variables affect cavitation phenomena and the final extraction concentration. (37).

Increasing the solvent-to-feed ratio within a certain ratio can dissolve the target compounds more efficiently, leading to a higher extraction yield (38). Fig. 3A depicts the impact of UAE duration and F/S ratio on the volume of extracted EO. A considerable curvature can be shown, indicating that two parameters have an interaction effect on the response. It was found that the best region for extracting the most EO is in the middle of UAE time and at a fairly high level of the F/S ratio (see also Table 2). However, the quantity of extracted EO was slightly reduced at the high UAE time and high F/S ratio. Fig. 3B illustrates the impact of UAE time and temperature affect the quantity of EO extracted. According to Fig. 3B, simultaneous increase in the UAE time and temperature variables (movement on the dimeter axis) lead to increase the amount of EO then decreases the extracted EO. It was revealed that the most favorable condition for achievement of the high yield is using low temperature (35 - 45°C) and low UAE time (45 - 60 min) during extraction.

Based on the slope of the curve in Fig. 3B, the temperature has a significant impact on the extraction process for each UAE time value. Lower temperatures lead to greater extraction of 9.75 $mg.g^{-1}$. This might be explained by the EO compounds' characteristics. Despite having boiling points of 200°C or higher, EO compounds only volatilize at about 100°C when vapor or boiling water is present and atmospheric pressure is present (39). Since heat is probably contributing toward the formation of free radicals, it is crucial that autoxidation and hydroperoxide degradation speed up as the temperature increases. (40). It's ought to be mentioned that thermolabile flavors or aromatic metabolites, which are susceptible to degradation by heat, play an important role in altering the properties of EO (41). Higher temperatures actually led to yield reduction of EO due to undesirable processes such as decomposition and hydrolysis. Another theory suggests that the problem is caused by the higher temperatures and the long UAE time associated with a process known as "supercavitation" (42). Increasing the vapor pressure reduces the ultrasonic power transmitted to the medium by increasing the production of bubbles around the ultrasonic horn tip. The interaction of temperature and F/S ratio on the amount of extracted EO is depicted in Fig. 3C. In Fig. 3C, the surface reaches the plateau in the F/S ratio ranges of 13.0 - 15.0 $g.L^{-1,}$ temperature of 35 - 45ºC, and, with the highest potential amount of EO extracted 9.5 to 10.0 mg. g^{-1} (movement on the diameter axis).

Optimizing the extraction conditions

The regression equation was employed to determine the best values for variable leading to the optimum extraction conditions. A number of optimal points proposed by the Design-Expert software is shown in Table 5, indicating that the UAE model accurately matched the experimental data and thus optimized the *T. daenensis* EO method. To validate these findings, all tests were run in triplicate under proposed conditions and compared to the standard procedure. Temperatures of 30 - 40°C, F/S ratios of 14.00 - 15.00 $g.L^{-1}$, and UAE times of 45 - 55 minutes were found to be optimal extraction conditions. In the conventional approach, the maximum EO yield was 8.49 $mg.g^{-1}$ of the plant (F/S ratio of 14.52 $g.L^{-1}$), whereas the extraction yield in the UAE system (53.96 min UAE duration, F/S ratio of 14.52 $g.L^{-1}$, and 30.06°C UAE temperature) was 10.51 $mg.g^{-1}$ of EO. Compared to the conventional settings, these data show an almost 23.8% increase in EO extraction.

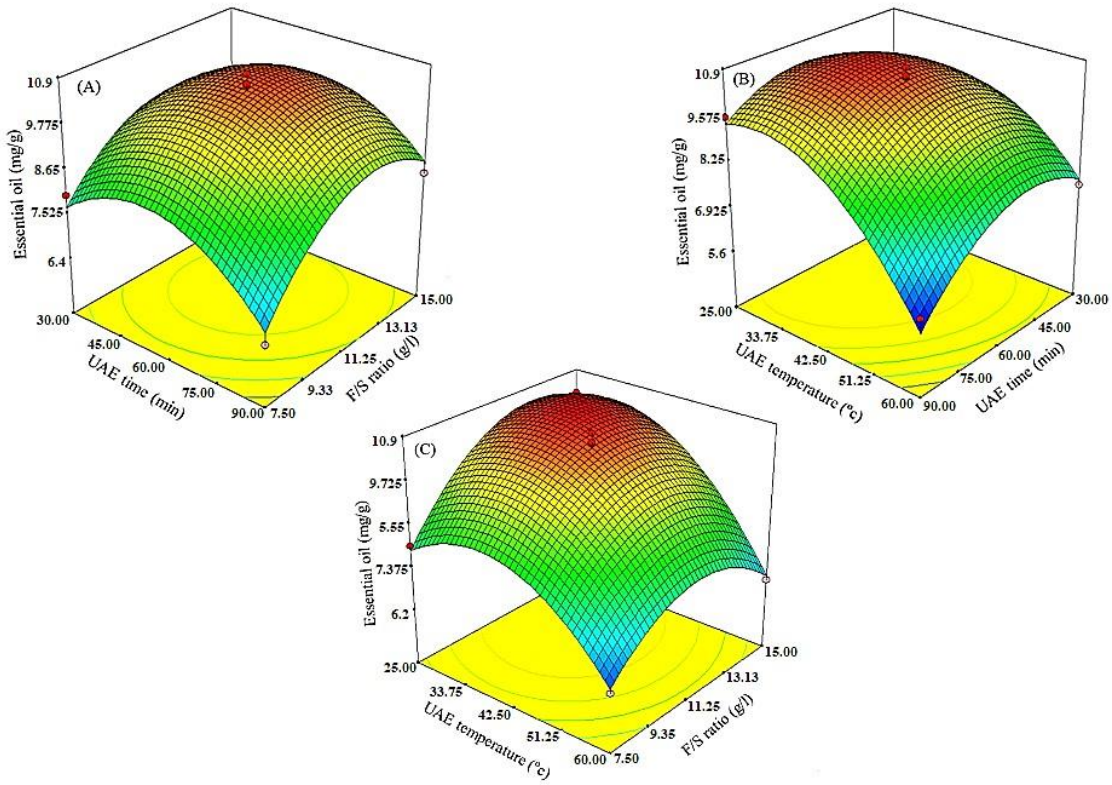

Fig. 3. Response surface plots for the effect of (A) F/S ratio and UAE time; (B) UAE temperature and UAE time; (C) F/S ratio and UAE temperature on the value of the essential oil. (UAE = Ultrasound-assisted extraction. F/S ratio = feed to solvent ratio).

Table 5. The number of optimal points employed in UAE system

No.	Time (min) X_1	Temperature (°C) X_2	F/S($g.L^{-1}$) X_3	Predicted essential oil value (mg $_{EO}$/g $_{of\ plant}$)	Reported essential oil value (mg $_{EO}$/g $_{of\ plant}$)	Desirability
1	49.03	35.00	14.99	10.04	10.28	1.000
2	48.89	36.11	14.20	10.33	10.46	1.000
3	53.96	30.06	14.52	10.37	10.51	1.000

Microscopic analysis

The UAE method was used to detect morphological changes in the surface structure of *T. daenensis* leaf samples, and the results were compared using a SEM test to better demonstrate the efficiency of the UAE method. Fig. 4 (A-D) shows the microstructure of *T. daenensis* leaf samples after treatment using conventional and UAE methods at the recommended optimal conditions (UAE time 53.96 min, F/S ratio 14.56 $g.L^{-1}$, and temperature 30.06°C). Figures 4A and 4B show that the conventionally handled sample still has a significant quantity of intact, undamaged, and blocked hole tissues. On the other hand, Fig. 4C and 4D demonstrate how most of the trichomes' "hair" structures are severed and broken after ultrasonic treatment. The cells adjacent to the structures also seem to have undergone significant ablation. Additionally, because of cracks, bigger holes, and voids on the sample surfaces brought on by the ultrasonic waves, the surface structures of the UAE-treated sample were more disrupted than those of the

conventionally extracted sample. The ultrasonic waves can enhance the process of extracting plant compounds by introducing porosity and holes in the cell membrane as well as by facilitating and accelerating mass transfer. (43, 44). This covers both the extension of the tissue to make space for the solvent and the leakage of chemicals into the solvent from the tissue. Over the contraction cycle, the cavitation bubbles close to the plant material surface collapses, enabling the microjet to penetrate the surface. (45-47). As a result, acoustic cavitation homogenized and dissolved the large particles, and improved the contact area of the *T. daenensis* leaf tissue with the solvent. The cell walls of the *T. daenensis* leaf tissue were damaged by ultrasonic sonication, allowing more water solvent to penetrate inside the cells and release the active ingredients. UAE disturbed the *T. daenensis* leaf tissue more than conventional extraction, which could improve mass transfer and lead to stronger and faster release of the active ingredients, resulting in higher yield, shorter extraction time, and enhanced quality of extracted EO.

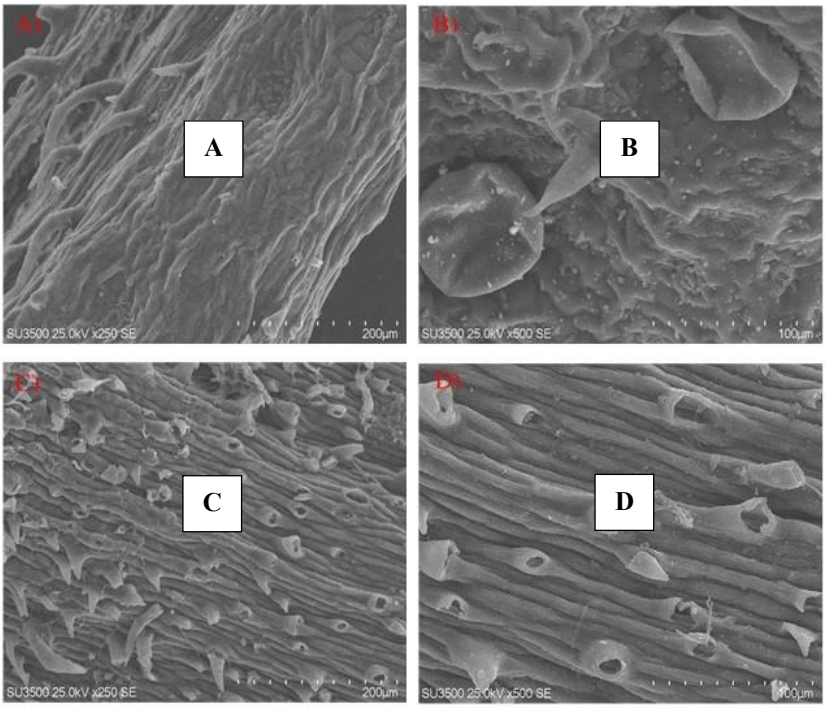

Fig. 4. Effect of power ultrasound on *Thymus daenensis* aerial parts: SEM microscopic observation of plant surface using conventional extraction method, magnification 200 µm (A), 100 µm (B), respectively. SEM microscopic observation of plant surface after UAE, at optimized conditions, magnification 200 µm (C), 100 µm (D), respectively.

GC-MS analysis of essential oil

Table 6 depicts the chemical content of EO extracted from *T. daenensis* using conventional HD and UAE techniques evaluated qualitatively and quantitatively using GC and GC-MS. GC-MS analyses demonstrated that the main components of *T. daenensis* EOs are thymol and carvacrol. (Chromatogram in Fig. 5). Table 6 shows that a total of nine components were distinguished as accounting for 100 percent of the total oil. The major compositions of EO of *T. daenensis* were found to be carvacrol, thymol, *ρ*-cymene, γ-terpinene, caryophyllene, linalool, α-terpinene, and borneol. The oils had a high percentage of carvacrol in both the UAE and conventional methods, with 57.82 % and 48.47 %, respectively.

Also, thymol was the next component with a high percentage of 22.20 % and 18.88 %, respectively in the UAE and conventional methods, which have increased by a total of 12.67 % in UAE extraction.

The high percentage of monocyclic monoterpene (carvacrol, thymol, α-terpinene, *p*-cymene, etc.) in *T. daenensis* EO extracted under UAE compared to the conventional method shows an increase in the amount of the major components recovered by the UAE method. This could be due to the more efficient liberation of certain components from the secretory structures of *T. daenensis* leaves or to transformations of unstable chemical compounds after ultrasonic irradiation. For example, *p*-cymene could be oxidatively hydroxylated at a position similar to the hydroxyl group position in *α*-terpineol. The hydroxylation of *p*-cymene also results in the biosynthesis of thymol, a completely different monoterpene. (48). UAE extraction method reduced *p*-cymene and γ-terpinene contents while, the contents of carvacrol, thymol EO of *T. daenensis* were increased significantly. As shown in Table 6, in the conventional extraction method the rate of *p*-cymene was 9.59 %, whereas in the UAE method was reduced to 2.28 %, and this might be a reason for the increase of thymol and carvacrol in EO extracted by the UAE. The aforementioned findings showed that ultrasound irradiation would enhance the content of effective components rather than alter the primary active ingredients in *T. daenensis* EOs.

Table 6. Chemical composition of the *Thymus daenensis* EO in conventional and ultrasonic methods at the optimized condition.

No.	Compounds	Percent (%)		RI*
		Conventional method without ultrasonic	Ultrasonic method	
1	α-terpinene	0.890	0.172	1012
2	*p*-cymene	9.590	2.278	1022
3	γ-terpinene	8.723	2.025	1054
4	linalool	3.914	4.125	1098
5	borneol	4.653	5.744	1175
6	thymol	18.88	22.20	1297
7	carvacrol	48.47	57.82	1313
8	*trans*-caryophyllene	3.441	3.891	1419
9	caryophyllene oxide	1.439	1.745	1584

*RI: Retention indices relative to C9-C22 *n*-alkanes on the DB-5 column.

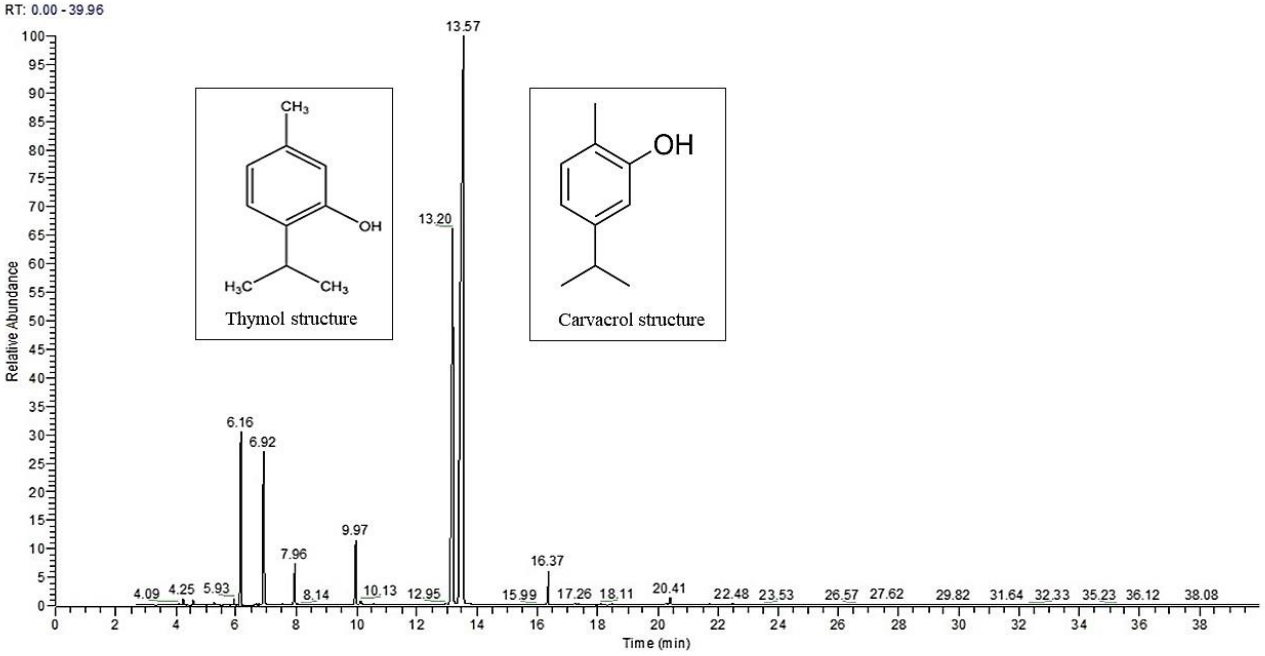

Fig. 5. GC-MS chromatography profile of *Thymus daenensis* essential oil.

Effect of anti-inflammatory activity of the EO

Nonsteroidal anti-inflammatory drugs (NSAIDs), steroidal anti-inflammatory drugs (SAIDs), and immunosuppressants are used to treat inflammatory diseases. However, their use has been restricted owing to the possible adverse consequences (49, 50). The importance of EOs as a rich source of active chemicals in the search for novel bioactive natural products that reduce inflammation is becoming more widely acknowledged. For example, many monocyclic monoterpenes, such as carvacrol, thymol, *α*-terpineol, *p*-cymene, and other chemicals, have been derived from different plants. They have been found to have a variety of biological and therapeutic benefits, including anti-inflammatory, antioxidant antipruritic, and analgesic effects (51, 52). Phenolic terpenes, including carvacrol and thymol, are the major components in the *Thymus* genus (8, 53).

As a result, monoterpenes have been identified as potential compounds that can be used to treat a wide range of diseases. Monoterpenes can be considered as the key molecules for the development of effective anti-inflammatory drugs, as they have shown numerous modes of action on different targets in *in vitro* and *in vivo* studies (54). It is well known that inflammation is caused by the denaturation of protein molecules (55). The approach of Mizushima et al. is one of the most popular in vitro techniques for assessing anti-inflammatory effects by reducing protein denaturation. (33, 56). Proteins lose their tertiary structure and convert to secondary structure as a result of denaturation, which is often cited as the cause of inflammation.

Albumin protein denaturates at physiological pH when phosphate-buffered saline sodium is present (57). The anti-inflammatory properties of carvacrol (58), thymol (59), *p*-cymene (60) have been already reported. However, in this work, perceptible concentration of monocyclic monoterpenes in the extracted EO in suppressing the denaturation of albumin protein is highlighted in this work. Using the

human serum denaturation technique, Fig. 6 illustrates the *in vitro* anti-inflammatory activity of EOs and diclofenac sodium as an NSAID at various doses. According to Boukhatem, Darwish, et al. (2020) the rich fraction of *Thymus vulgaris* EO presented a dose-dependent maximum inhibition of denaturation of protein albumin of 96.35% at 0.5 µl.mL^{-1} (IC50 value of 6.843 ± 0.830 µl.mL^{-1}) whereas sodium diclofenac revealed maximum inhibition of 96.89% at the concentration of 1 mg.mL^{-1} (61).

As can be seen in Fig. 6, EO showed 79, 81, 82.75, 84, and 86 percent inhibition of denaturation of HSA at different concentrations (100, 300, 500, 700, and 900 µg.mL^{-1}), whereas standard diclofenac sodium showed 86, 88, 91, 93, and 96.5 percent at the same concentration ranges. In general, as concentrations rise from 100 to 900 µg.mL^{-1}, the impact of inhibition by diclofenac sodium is generally greater than that of EO. Even though EO and diclofenac sodium were used in various concentrations, there was no discernible change in the percent inhibition. This could be attributed to the high concentration of monoterpenes in EO.

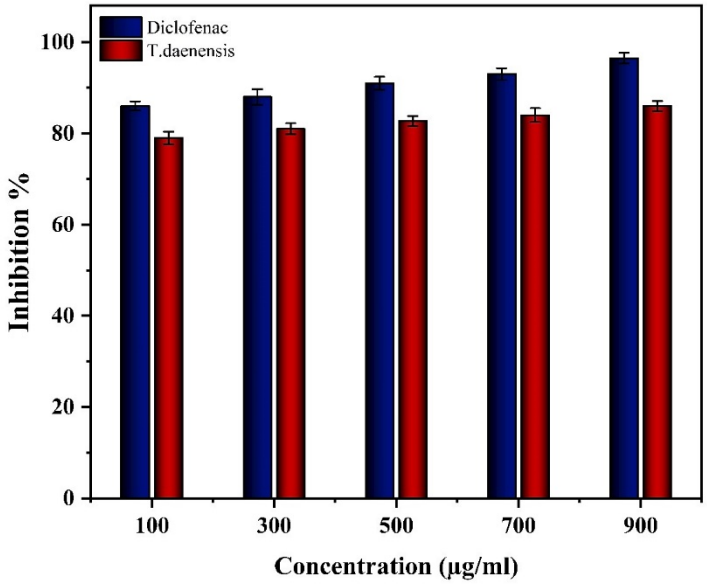

Fig. 6. Comparison of the effect of essential oil extracted from *Thymus daenensis* and diclofenac sodium on inhibition of albumin denaturation.

Conclusion

To improve the extraction of EOs from *T. daenensis* leaves, an effective continuous flow batch procedure supported by an ultrasonic horn coupled with a Clevenger device was designed in this research. The effects of operating factors on EO extraction were thoroughly studied and compared with the conventional method. In comparison to the traditional method, the efficient UAE system resulted in increasing the number of monocyclic monoterpenes, carvacrol, and thymol in the extracted *T. daenensis* EO. The differences in the results could be explained by the effective contact area between the solid phase and the solvent, as well as acoustic cavitation processes, and a high-volume circulating sonication system. The plant matrix's cell walls may be broken, releasing the contents, by the high local pressure and temperature used in the UAE method as well as the micro-mixing in the active zone. The yield of T. daenensis EO, on the other hand, was negatively impacted by the prolonged sonication period and

elevated operating temperature. In addition, the anti-inflammatory activity of the components of *T. daenensis* EO at various dosages revealed promising results that were roughly comparable to those of standard diclofenac sodium. According to the results, the UAE system can be developed as an effective, reliable, and beneficial method for the extraction of natural products and/or the development of industrial applications.

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Authors' Contributions

Asghar Hadi Darabad: Conceptualization, Investigation, Formal analysis, Writing-original draft. **Masoud Rahimi:** Methodology, Data Curation, Formal analysis, Visualization, Writing-review and editing. **Hasan Rafati:** Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Formal analysis, Writing-review and editing.

Declaration of Interest

The authors of this article declared no conflict of interest.

Ethical Considerations

All ethical principles were adheried in conducting and writing this article.

Transparency of Data

In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

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