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Extraction of *Ziziphora tenuior* essential oil using supercritical CO_2

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ABSTRACT

Ziziphora tenuior is an edible medicinal plant which belongs to Labiatae family. It is often used as a treatment for some diseases such as edema, insomnia, and hypertension in Turkey, Iran and China. In this study, the essential oil of Ziziphora tenuior was extracted by using a technique called 'supercritical fluid extraction'. In addition, the components of the essential oil were identified by chromatography analysis. The main components of the essential oil were 53.977% of p-mentha-3-en-8-ol and 38.481% of pulegone. It must be noted that CO_2 *was used as the supercritical fluid because it has a moderate critical temperature and pressure. In this study, the effects of some extraction parameter variations on the extraction yield were examined. Amongst the said parameters were pressure, temperature and mean particle size.*

Keywords: Carbon dioxide; Extraction; Supercritical Fluid; Separation; Labiatae; *Ziziphora tenuior*
Abbreviations: *CVD: Cardiovascular diseases-Z: Ziziphora-SFE: Supercritical fluid extraction-SC-*CO_2*: Supercritical Carbon dioxide*

INTRODUCTION

Herbal medicine has been used as a common way to treat diseases since ancient times. Herbal medicines are prepared in different forms and products, such as raw, brewed or boiled medicine, extracted oil, etc. The low price of herbal medicines and their negligible side effects are their main advantages as compared to chemical drugs. During the last century, using medicinal plant as a common treatment has been resumed. Some plants are used to prepare medicines in the form of oils.

An essential oil or essence, which is present in different parts of the plant, is a hydrophobic liquid. It consists of volatile and aromatic compounds from plants [1, 2]. Essential oils are used to cure some diseases.

Cardiovascular diseases (CVD) are the principal cause of death in all developed countries accounting for 50% of all deaths [3]. Arterial hypertension is the most common cardiovascular disease [4]. In view of the spread of this disease, researchers are looking for new treatments. Herbal medicine is a good choice for this purpose. *Ziziphora* is an edible medicinal plant used to cure arterial hypertension. Moreover, its essential oils are used for treating some diseases such as edema, insomnia, lung abscess, tracheitis, hemorrhoids and hypertension [5,6]. The antimicrobial activity of the essential oil on *Salmonella* typhi Vi+ makes it useful in the treatment of typhoid fever, too[7]. This plant is also used as an appetitive, carminative, antiseptic and wound-healing material[8]. Iranian, Turkish and Chinese people have been using this plant to treat the said diseases since ancient times[9, 5].

Ziziphora belongs to the Labiatae family and consists of four species, namely: *Z. clinopodioides* Lam., *Z. capitata* L., *Z. persica* BUNGE. and *Z. Tenuior*. This plant grows wildly in some parts of Iran, Turkey, Afghanistan, Iraq and Azerbaijan[10]. *Ziziphora* is used as an additive to yogurt to improve its aroma and flavor[9, 11]. It is also used to make some kind of cheese which is named 'herby cheese' [12]. Essential oil of *Ziziphora* is prepared by distillation or brewing. Supercritical fluid extraction (SFE) is a new technique for this purpose.

The vegetal oil extraction with supercritical fluids has been studied since the 1980s [13]. In SFE, unlike the liquid-liquid extraction technique, the solvent can be easily recycled by depressurizing. This will decrease the operating costs. The researchers used supercritical CO_2(SC-CO_2) as a solvent. Contrary to organic solvents, CO_2 as a supercritical fluid is a non-toxic, non-flammable and environmental-friendly solvent. Supercritical fluids have properties between those of in gas and liquid. The solvation strength and the density of supercritical fluids are close to those of liquids, while its viscosity and diffusivity are closer to those of gases. Low viscosity and high diffusivity of supercritical fluids cause a better mass transfer as compared to organic solvents. Moreover, very low surface tension enables supercritical fluids to penetrate easily into the porous structure of the solid matrix.SFE technology is also suitable to decrease volatility and thermal degradation components during the extraction. Because of these reasons, researchers have paid a special attention to SFE as an advanced technique of separation [14, 15, 16, 17].

Godze Elgin Meral et al. reported chemical compositions of *Z. taurica* Subsp. *Cleonioides* essential oil and investigated its antibacterial activity[9]. Sezai Ercisli and Suzan Ozturk extracted the essential oil of *Z. Persica BUNGE* by methanol extract technique. They also examined the chemical compositions of the essential oil. The essential oilin vitro antimicrobial activities were studied, too[18]. In another study, Sezai Ercisli and Suzan Ozturk reported chemical compositions of the essential oil obtained from methanol extract of aerial parts of *Z. clinopodioides.* Moreover they investigated its antimicrobial activity against 52 Gram-positive and Gram-negative bacteria [11]. François Senejoux et al. used the organ bath technique to explore the vasodilating effects of hexane, dichloromethane and aqueous fraction of a crude hydroalcoholic extract of the whole plant of *Z. Clinopodioides* on isolated rat thoracic aortas and investigated the underlying mechanisms of the most potent extract[6]. Steam distillation of *Ziziphora* has already been used, but no report has yet appeared on the SFE of this plant. However SFE of some other plants from this family has been studied. For example, Paul Barton et al. extracted the essential oils of spearmint and peppermint with SFE and reported their chemical compositions [19]. O. Bensebia et al. extracted the essential oil of rosemary by means of SFE and modeled the process mathematically[20].

In the present study, the essential oil of *Z. Tenuior* is extracted by using SFE, and its chemical compositions are reported. Then the effects of three parameters on the extraction yields are investigated. Amongst the said parameters were the pressure, temperature and mean particle size.

MATERIALS AND METHODS

Materials

Plant Material

Z. Tenuior was collected from the northern part of Shiraz, Iran, in October 2012. Then it was dried in a dark place and at the room temperature (around 25°C). The sample was ground in a Panasonic blender (Model MX-J225G) to produce powder. The averages of particles size on the basis of ASTM E11 were 0.212, 0.300 and 0.420 mm.

Reagent

Carbon dioxide with the purity of 99.95% was obtained from Abu-Qaddareh Company (Shiraz). Dichloromethane with the purity of 99.99%, purchased from Merk Kga A, was used as a solute recovery.

Methods

Hydrodistillation

The oil of the plant (50gr material) was obtained by hydrodistillation for 3.5 hours on a Clevenger-type apparatus. The extraction yield on the basis of dry weight was 1.3%.

Design of Experiments

In SFE, due to the high number of tests and high pressure, we like to reduce the number of experiments. To do this, design of experiments (DOE) is needed. Using DOE, we can gain the information in the shortest possible time and with lowest costs. There are three strategies to design experiments: a) One-at-a-time; b) Full factorial; c) Fractional factorial. Taguchi is a kind of fractional factorial design.

Taguchi method reduces the number of experiments, costs and time. Because of these advantages and facility to use, the researchers used this method to design the experiment. The results coming from this method are so reliable. Beside these, we can repeat the experiments in different arrays and conditions to get better results[21, 22, 23].

No researches have been done on *Z. Tenuior* essential oil SFE, but in most of the researches on some species of the family Labiatae and other families of herbs, essential oil SFE has been done based on between two to four parameters. Therefore, it was decided to use three parameters which are the most important. The pressure and temperature of the extraction as well as the mean particle size of samples are the most important parameters which affect the extraction yield of herbal essence.

According to the laboratorial pilot and error tests in different conditions, parameter ranges have been determined as summarized in Table 1[2, 24, 25, 26].

Table 1- Variable Parameter Ranges

No.	Parameters	Unit	Variable range
1	Pressure(P)	bar	$160<P<190$
2	Temperature(T)	°C	$40<T<50$
3	Mean particle size (di.)	µm	212<di. <420

As Table 2 shows, in this study, the pressure and temperature of the extraction tanks and the mean particle size of the feed powder have been chosen as the efficient factors on total extraction yield in three different levels. L.9 array has been prepared based on this table. For this purpose, Minitab 16 software was used.

Table 2-Experimental Levels of the Factors Used in the Taguchi Method Supercritical Fluid Apparatus

No.	Pressure (bar)	Temperature (˚C)	Mean particle size (µm)
1	160	40	212
2	175	45	300
3	190	50	420

As it is shown in Fig. 1, the SFE apparatus consisted of a CO_2 cylinder, a refrigerator to change CO_2 phase from gas to liquid, a handle reciprocating pump, a double-wall tank to load CO_2, a barometer and an extraction tank with the internal diameter of 12.5 mm and length of 40 cm. The extraction tank had two parts. In the inner part, the sample was placed; while in the outer part, water was circulating to increase the temperature of the flow. The temperatures of input and output water flow were controlled and at the end, a Joule-Thomson valve had been embedded in the outgo of the line. After the extraction, the essential oil+CO_2mixturewasexpanded to atmospheric pressure. The essential oil was solved in dichloromethane, and $CO_2$was released into the air (before it was released to the air, its flow was measured by a volumetric flow meter).

Supercritical Fluid Extraction Procedure

The aerial parts of the Ziziphora plant were dried out in the room temperature for 72 hours. Then it was grounded by Panasonic grounder (model MX-J225G), and it was sieved with different sizes of 212, 300 and 420 µm on the basis of ASTM E11. Eight grams of the sample was measured by RADWAG balance (model WAS 220/C/2) and was placed into the extraction tank. CO_2 gas emitted from the cylinder was changed to liquid while passing through the refrigerator. Then it was compressed with the handle pump, and its pressure was increased to the desired pressure. The supercritical CO_2 was in contact with the sample for 30 minutes and during this time, it solved the essential oil of the sample. This situation is called “static time”. Then the exterior needle valve of extractor was opened for 45 minutes, which is named “dynamic time”. Essential oil+CO_2 passed from a U-shape tube in which dichloromethane was at 0°C. The essential oil was solved in dichloromethane, and CO_2 was released into the atmosphere. In the end, the essential oil+dichloromethane mixture was collected as a sample, which was used in GC analysis.

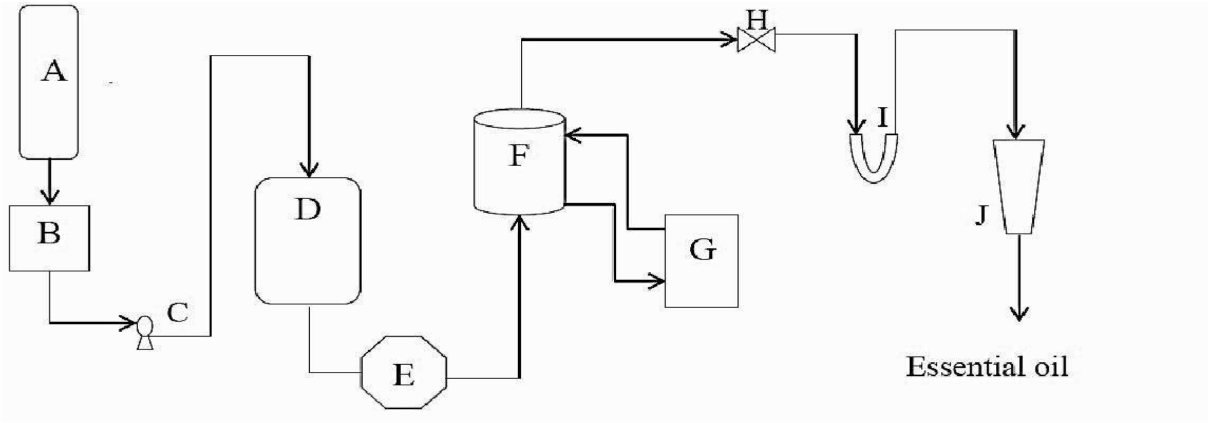

Fig. 1-Experimental Apparatus for the Extraction of Z. Tenuior

The Experimental Apparatus for the Extraction of Z. Tenuior: A- Cylinder of CO_2 *,B- Refrigerator, C- Handle Reciprocating Pump, D- A Tank for* CO_2 *Loading, E- Barometer, F- Extraction Tank, G- Heater for Heating Water, H- Joule-Thomson Valve, I- Dichloromethane in U-Shape Tube at 0°C , J- Flow meter*

GC and GC-MS analysis

The GC-FID analysis was performed using Agilent GC-6890N gas chromatograph, which is made in the U.S., with nitrogen as the carrier gas with a velocity of 1.4mlit/s on HP-5 (Dimethylsiloxane, 5% phenyl) column (30×0.25 mm id, Film thickness 0.25µm). The SFE samples (1µl) were injected (without any further dilution) using the split mode with a split ratio of 1/10. The oven temperature program was 60°C, which was then increased to 230°C at a rate of 3°C/min, and it remained at 230°C for 5 minutes. The injector and detector temperature were held at 240 and 260°C, respectively. The percentages of components were calculated by the area normalization method, without considering response factors.

The GC-MS analysis was performed using a Variane 3400 equipped with a DB-5 column (30×25 mm internal diameter, film thickness 0.25 µm, and with helium as a carrier gas). The SFE samples (0.2µl) were injected (without any further dilution) using split mode with a split ratio of 1/60 and with split flow of 7.166ml/min. The oven temperature program at first was 60°C for 3 minutes, and then it was increased to 230°C at a rate of 3°C/min, and it remained at 230°C for 5minutes. The transfer line temperature was 280°C. The ionization energy was 69.922eV with a scan time of 1 second and mass range of 34-500amu. The injector and detector temperatures were held at 300 and 270°C, respectively.

RESULTS AND DISCUSSION

In this study, the effects of three important factors on the extraction yield were investigated. These factors included the pressure and temperature of the extractor and the mean particle size of feed powder.

This experiment was designed based on Taguchi method and according to L.9 orthogonal array with Minitab 16 software. Table 3 shows the experimental conditions of SFE for *Z. Tenuior* essential oil. Moreover, the components of the *Z. Tenuior* essential oil obtained from the SC-CO_2 extraction was identified and quantitated in different experimental conditions.

The Effect of Particle Size

As it was expected from previous studies [20, 21, 27, 28, 29, 30], the extraction yield rose with the particle size of raw materials decrease. This fact is shown in Fig. 2.

The extraction yield rose when the particle size was decreased. This is due to the increase in the interfacial area. Milling the material before the extraction process increases the interfacial area. Moreover, grinding the particles helps release oil from the cells. Some cells are broken in the grinding process, and the trapped oil will be released from the broken cells. This amount of released oil is more accessible by SC-CO_2, and it is solved so quickly in the said solvent. Beside these reasons, the intraparticle resistance to mass transfer is decreased by milling the particles. As a result, the diffusion path in solid becomes shorter. Thus, the solute will be transported more easily, and it causes extraction yield to rise. The above reasons can explain why the rise in the extraction yield occurs with the particle size decrease.

Table 3-Three Factors, Three Levels (L.9) Orthogonal Array Design for SFE of Z. Tenuior

Run	Pressure (bar)	Temperature (˚C)	Mean Particle Size (µm)	Yield (%)	Static Time (min)	Dynamic Time (min)	Flow Rate (cm^3/min)
1	160	40	212	1.20	30	45	400
2	160	45	300	1.18	30	45	400
3	160	50	420	0.89	30	45	400
4	175	40	300	2.80	30	45	400
5	175	45	420	2.80	30	45	400
6	175	50	212	2.90	30	45	400
7	190	40	420	1.30	30	45	400
8	190	45	212	1.90	30	45	400
9	190	50	300	1.59	30	45	400

The Effect of Pressure

Table 4 shows the analysis of variance (ANOVA) results. This Table indicates that the pressure of supercritical fluid is the most important factor in SFE of *Z. Tenuior.*

As it is shown in Fig. 2, the pressure had two different effects on the extraction yield in this study. At first, when the pressure was increased from 160 to 175bar, the extraction yield rose. However, we see a reduction in the amount of extraction yield when the pressure is increased from 175 to 190bar.

Table 4 -ANOVA of the Experiments (at 90% Confidence)

S.V. [a]	S.S [b]	DF [c]	MS [d]	F Value
Pressure	4.8	2	2.4	58.62
Temperature	0.066	2	0.033	0.04
Mean Particle Size	0.171	2	0.086	0.0902

[a:] *Source of Variance*
[b:] *Sum of Square*
[c:] *Degree of Freedom*
[d:] *Mean Square*

In the first stage, the extraction yield rose with pressure increase. As Table 3 shows, the pressure increased from 160 to 175 bar resulted in 146% rise in the extraction yield. The pressure increase causes the fluid density to rise. As a consequence, the solvent power for solving substances will increase. Moreover, because of the solvent viscosity, the mass transfer coefficient in the fluid phase decreases with pressure increase [20]. In this stage, the positive effects are stronger than the negative ones.

In the second stage, the pressure had a negative effect on the extraction yield. Increasing the pressure from 175 to 190 bar caused a 45-percent reduction in the extraction yield. This unexpected reduction in the extraction yield with the pressure increase can be attributed to a decrease in the diffusion rates of the extracted essence from the plant matrix to the supercritical fluid. The diffusivity causes a decrease in the interaction between the supercritical fluid and the solute contained within the matrix. Furthermore, the mass transfer coefficient in the solid increases with the pressure increase, and it results in mass transfer resistance reduction.

The Effect of Temperature

Temperature has two different effects on the extraction yield, positive effect and negative effect. For each condition, there is a competition between the positive and negative effects.

The increase in temperature causes the transport properties increase. Amongst the said properties are vapor pressure of essential oil, binary diffusion coefficient and volatility of essential oil in supercritical CO_2. In other words, we can say the vapor pressure and diffusion coefficient of essential oil increase as the temperature increases. On the other hand, the density and solvent power of SC-CO_2 increases as the temperature decreases. As a consequence, driving force in the fluid phase increases [27, 30, 31].

For a volatile solute, there is a competition between the essential oil solubility in CO_2 (which decreases as the temperature increases) and its volatility (which rises with increase in temperature) [20].

As we see in Fig. 2, at first, the extraction yield rose as the temperature was increased. On the other hand, there was an extraction yield reduction as the temperature was increased in the second stage. When the temperature was increased from 40 to 45 °C, the positive effects such as volatility of essential oil became stronger than negative effects like its solubility in CO_2. Thus, the extraction yield rose in the first stage. However, when the temperature was increased from 45 to 50 °C, we saw opposite behaviors. At this stage, the negative effects are greater than the positive ones, and the extraction yield reduced with the temperature increase.

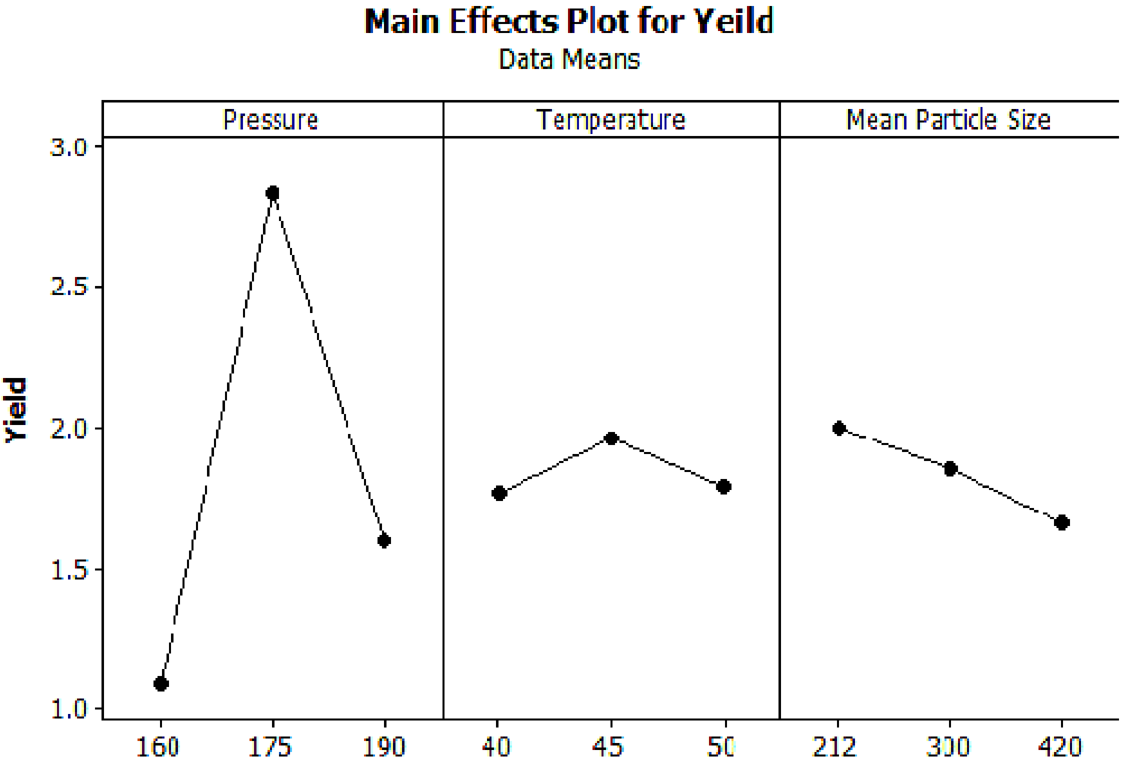

Fig. 2-The Effects of Temperature and Pressure and Mean Particles Size on the Extraction Yield

Analysis Results

The hydrodistillation of *Z. Tenuior* areal parts gave the essential oil in 1.3% (w/w) yield, based on the dry weight of the plant. However, the essential oil could be achieved in 0.89% to 2.9% (w/w) in different runs in SFE. Thus, by optimizing the conditions, there can be better yields in SFE than in hydrodistillation. Moreover, the essential oil of SFE had a deeper color and aroma in comparison to the essential oil obtained from hydrodistillation.

The main components of *Z. Tenuior*, which are identified by GC/MS analysis of the extracts, are presented in Table 5. The identification of the components was done through the electronic library WILEY7 and through the standard mass chromatogram of the components. As it can be seen in Table 5, the components present in higher quantities are 53.977% of p-mentha-3-en-8-ol, 38.481% of pulegone, and 1.651% of p-mentha-3,8-diene. The essential oil also contained smaller percentages of β-pinene; 4aα, 7α, 7aα-nepetalactone; α-thujene; caryophyllene oxide; limonene; E-caryophyllene and terpinolene. P-mentha-3-en-8-ol and pulegone are the main components of *Z. Tenuior*, and they cause the antimicrobial activities of the essential oil. Also, a typical chromatogram of the SFE is given in Fig. 3. To show the selectivity of SFE, two runs (8, 9) were chosen randomly, and the components of their essential oil were reported. These components are identified by GC analysis. The results are presented in Table 6.

Table 5-Compositions of Z. Tenuior Essential Oil Obtained by SFE

Components	R.T. [a]	K.I. [b]	% in Oil	Molecular Formula	Molar Mass(gr/mol)
Tricyclene	-	926	t [c]	$C_{10}H_{16}$	136.23
α-thujene	3.950	930	0.484	$C_{10}H_{16}$	136.23
α-pinene	4.207	939	0.122	$C_{10}H_{16}$	136.23
Sabinene	-	975	t	$C_{10}H_{16}$	136.23
β-pinene	4.898	979	0.891	$C_{10}H_{16}$	136.23
Myrcene	5.204	990	0.093	$C_{10}H_{16}$	136.23
P-mentha-1(7),8-diene	5.686	1004	0.042	$C_{10}H_{16}$	136.23
Limonene	6.243	1029	0.317	$C_{10}H_{16}$	136.23
P-mentha-3,8-diene	7.605	1072	1.651	$C_{10}H_{16}$	136.23
Terpinolene	8.039	1088	0.194	$C_{10}H_{16}$	136.23
P-mentha-3-en-8-ol	11.678	1150	53.977	$C_{11}H_{20}O$	168.28
Cis-pulegol	13.275	1229	0.190	$C_{10}H_{18}O$	154.25
Pulegone	14.942	1237	38.481	$C_{10}H_{16}O$	152.23
Neoiso-isopulegyl Acetate	17.139	1313	0.044	$C_{12}H_{20}O_2$	196.29
4aα,7α,7aα-nepetalactone	18.538	1360	0.500	$C_{10}H_{14}O$	166.22
α-copaene	19.293	1376	0.122	$C_{15}H_{24}$	204.36
β-bourbonene	19.631	1388	0.134	$C_{15}H_{24}$	204.36
E-caryophyllene	20.976	1419	0.221	$C_{15}H_{24}$	204.36
Germacrene D	23.415	1485	0.137	$C_{15}H_{24}$	204.36
γ-cadinene	25.103	1513	0.053	$C_{15}H_{24}$	204.36
Caryophyllene Oxide	27.348	1583	0.324	$C_{15}H_{24}O$	220.36
Hexadecanoic Acid	41.749	1984	0.136	$C_{16}H_{32}O_2$	256.42
Total		-	**98.113**		

[a] *R.T.: Retention Time*
[b] *K.I.: Kovats Index*
[c] *t: Trace (<0.05%)*

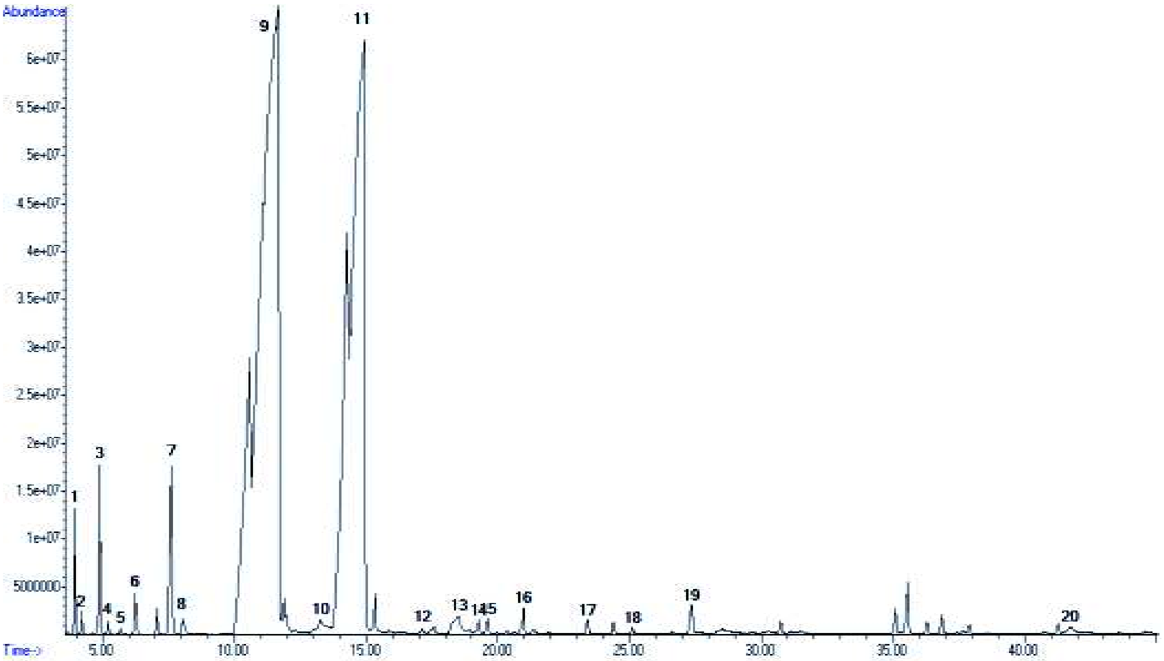

Fig. 3-Typical Gas Chromatogram of the Essential Oil of Z. Tenuior

The different compounds are shown with numerals; (1) α-Thujene; (2) α-Pinene; (3) β-Pinene; (4) Myrcene; (5) p-Mentha-1(7),8-diene; (6) Limonene ; (7) p-Mentha-3,8-diene; (8) Terpinolene; (9) p-Mentha-3-en-8-ol; (10) cis-Pulegol; (11) Pulegone; (12) neoiso-Isopulegyl acetate; (13) 4aα,7α,7aα-Nepetalacetone; (14) α-Copaen; (15) β-Borbonene; (16) (E)- Caryophyllene; (17) Germacrene D; (18) γ-Cadinen; (19) Caryophyllene oxide; (20) Hexadecanoic acid.

Table 6 -GC Analysis for Z. Tenuior Essential Oil in Randomized Runs

Components	Run 8 Essential Oil Components (%)	Run 9 Essential Oil Components (%)
Tricyclene	-	-
α-thujene	-	0.01
α-pinene	-	-
Sabinene	-	-
β-pinene	-	-
Myrcene	-	-
p-mentha-1(7),8-diene	-	-
Limonene	-	-
P-mentha-3,8-diene	-	-
Terpinolene	-	-
P-mentha-3-en-8-ol	0.09	0.10
Cis-pulegol	0.22	0.19
Pulegone	0.25	0.12
Neoiso-isopulegyl Acetate	1.42	0.06
4aα,7α,7aα-nepetalactone	-	0.03
α-copaene	-	0.11
β-bourbonene	0.19	0.08
E-caryophyllene	0.07	0.03
Germacrene D	0.10	0.05
γ-cadinene	-	0.02
Caryophyllene Oxide	0.09	18.02
Hexadecanoic Acid	-	-

CONCLUSION

This study was conducted on the extraction of the essential oil of *Z. Tenuior* by using SC-CO_2 in different conditions. Moreover, its components were identified. The effects of some process parameters on the extraction yield were investigated. Amongst the said parameters were the pressure and temperature of the extractor and the mean particle size of the raw materials. It was shown that the pressure is the most important factor in SFE. The extraction yield rose as the pressure was increased from 160 to 175bar. This is due to an increase in the fluid density, which results in a rise in the power of solvent. Moreover, the pressure increase affects the solvent viscosity. On the other hand, when the pressure was increased from 175 to 190bar, the extraction yield reduced. This is associated with the decrease in diffusion rates of the extracted essential oil from the plant matrix to the supercritical fluid. The extraction yield rose with an increase in the temperature at first. This is due to the increase in the diffusion coefficient, volatility of SC-CO_2 and vapor pressure of essential oil. On the contrary, the extraction yield reduced as the temperature was increased. This opposite behavior is due to the decrease in the density and solvent power of SC-CO_2. The extraction yield reduced as the particle size was increased. This behavior is because of an increase in the interfacial area resulting from grinding. Beside this, it is attributed to releasing the trapped oil, which is more accessible to SC-$CO_{2,}$ from the plant cells.

In comparison to hydrodistillation, a higher extraction yield can be achieved in SFE. Moreover, deeply-colored essential oil can be obtained in SFE. Last but not least, in SFE, the operating costs are lower than hydrodistillation. The chromatography results (GC/MS) showed that pulegone and p-mentha-3-en-8-ol were the main components of the *Z. Tenuior* essential oil. The maximum yield for SFE was 2.9%. This extraction yield was obtained at the pressure of 175bar, temperature of 50°C and mean particle size of 212µm. In other words, by means of SFE, more than 2 kg of valuable and costly essential oil can be extracted from each 100 kg of dried aerial parts of *Z. Tenuior* plant.

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