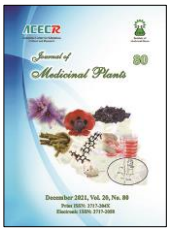
J. Med. Plants 2021; 20(80): 47-59

Institute of Medicinal Plants

Journal of Medicinal Plants

Journal homepage: www.jmp.ir



Research Article

Simultaneous ultrasound-assisted hydrodistillation of essential oil from aerial parts of the *Satureja khuzistanica* Jamzad and its antibacterial activity

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ARTICLE INFO

Keywords:
Stureja khuzistanica
Lamiaceae
Carvacrol
Ultrasound-assisted hydrodistillation
Distillate
Antibacterial

ABSTRACT

Background: Ultrasonic assisted extraction (UAE) is a robust and efficient method as a desirable alternative to conventional strategies for extracting active ingredients from different parts of plants. Coupling this technique with hydrodistillation is an effective method for producing essential oil from plant material. **Objective**: In this study, we successfully combined ultrasonic technology with hydrodistillation (HD) and optimized the system to reduce the time of the isolation and increase the yield of the essential oil obtained from *Stureja khuzistanica* Jamzad (SK). **Methods**: In the next step, isolation time, yield, and quality of essential oils obtained by conventional and ultrasonic-assisted methods were compared. Ethyl acetate (EA) was used to recover the remaining essential oil in the distillate. **Results:** All oils and distillates were analyzed qualitatively and quantitatively via the GC-FID and GC-MS techniques. The minimum inhibitory concentration (MIC) of SK essential oil against *Escherichia coli* (0.5 mg/ml to 8.0 mg/ml) and *Staphylococcus aureus* (0.25 mg/ml to 8.0 mg/ml) were calculated. **Conclusion:** Our results show that while the obtained essential oils chemical profile and biological properties are comparable, this method can enhance essential oil isolation yield efficiency by up to 40 %.

1. Introduction

As a result of industrialization during the past and the present century, the mass production and consumption of unhealthy food products, especially in developing countries, is increased [1]. One of the best alternatives for many harmful chemicals, food additives, and pharmaceutical products is natural essential oils [2]. Many essential oils were recently identified as green alternatives as a natural antioxidant and natural antimicrobial for replacing hazardous chemical and synthetic products in the food, cosmetic and pharmaceutical industries [3]. With the growth of these industries, the demand for natural essential oils has dramatically increased. Many procedures have been used to isolate the essential oils from the desired plants, all of which are based on distillation and extraction techniques [4]. Water

Abbreviations: UAE, Ultrasonic Assisted Extraction; HD, Hydrodistillation; SK, *Satureja khuzistanica* Jamzad; EA, Ethyl Acetate; MIC, Minimum Inhibitory Concentration

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doi: 10.52547/jmp.20.80.47

Received 18 September 2021; Received in revised form 28 November 2021; Accepted 30 November 2021

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[DOI: 10.52547/jmp.20.80.47]

and steam distillation are the main techniques applied to isolate essential oils from plant materials [5, 6]. These techniques are time-consuming and need many infrastructures, triggering many researchers to look for more reasonable procedures to isolate desired essential oils. Microwave-assisted extraction (MAE) [7], liquid-phase microextraction (LPME) [8], membrane-based extraction techniques [9], and supercritical fluid extraction (SFE) [10] are the most utilized techniques for the isolation and extraction of essential oils from plant sources.

Recently, a novel method has been developed to isolate essential oil using a combination of hydrodistillation and ultrasonic techniques [11 - 13]. Ultrasound-assisted extraction (UAE) is a robust and effective method as a desirable alternative for the conventional strategies used to extract active ingredients from different parts of plants [14]. During a typical UAE procedure, an ultrasonic wave passes through the target sample tissue, causing the solvent to penetrate the sample and extract the target molecules effectively. The ultrasonic wave's primary goal is to facilitate the mass transfer between the sample and the extraction solvent [15]. Applying the ultrasound wave is usually carried out via two different strategies: ultrasonic bath and ultrasonic probes. In both systems, the piezoelectric transducers are adopted as vibration sources [16]. The only difference between these two strategies is how the ultrasonic energy is transferred, wherein in an ultrasonic bath, the power is homogenously dispersed, whereas, in a probe-based system, the ultrasound energy is focused due to the use of a conical horn [17-19].

Also, different methodologies such as ultrasound pretreatment and direct Sono-hydrodistillation were applied. The data revealed the maximum yield at the minimum time that could be obtained when the direct application of ultrasound (sono-clevenger) was used [20]. Pingret *et al*. developed a new process employing ultrasound technology to improve the hydrodistillation extraction (sono-clevenger) of fresh orange peel essential oil [11]. However, the data do not show significant differences in final yields but improve over the conventional clevenger technique. Some reports approved using ultrasound probes in continuous and pulsed pretreatments combined with the traditional hydrodistillation process resulted in shorter extraction time and higher extraction yields compared to the non-sonicated leaves [21]. The available studies on the ultrasonic pretreatment of plant materials for essential oil isolation suggest that low frequencies ranged from 20 to 50kW and sonication time ranged from 20 to 40 min. Thereupon a time reduction to near 70 % concerning the conventional hydrodistillation was obtained [20]. This may be accompanied by a growth in the extraction of bioactive compounds and consequently improving the biological activities of essential oils [20, 21]. Furthermore, ultrasound-assisted extraction (UAE) combined with other extraction technologies such as supercritical extraction allowed reduced time and solvent volume [20, 22-27].

The genus *Satureja*, commonly known as savory, is an aromatic plant endemic to the Middle-East, South-Eastern Europe, North Africa, and Central Asia and belongs to the Lamiaceae family [28]. These plants were widely prescribed in traditional medicine as a remedy for different types of diseases. The plants of this genus have been used for curing crucial diseases such as muscular pains [29], stomach, bowel disorders [30], nausea [31], indigestion [32], and diarrhea [33, 34]. In Iran, seventeen *Satureja* species grow indigenously, and some cultivate food and pharmaceutical applications. *S. khuzistanica* Jamzad (SK) is an endemic

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species to Iran [35]. SK, was used as a flavoring agent in the food industry due to its aromatic and antiseptic properties[36]. Moreover, SK essential oil was shown antibacterial activities because of the high level of carvacrol in the essential oil in a study conducted by Deans *et al.* [37]. Therefore the efficient isolation of high purity SK essential oil is valuable.

A new method was used for ultrasonic-assisted hydrodistillation with in-line distillate extraction to isolate SK essential oil efficiently. Both methods were successfully coupled via an innovative approach. This was followed by an online liquid-liquid extraction strategy for the chemical profiling of corresponding distillates. The isolated essential oils were qualitatively and quantitatively analyzed by gas chromatography equipped with a mass spectrometry detector. Two main components of essential oil were successfully identified and determined using the proposed procedure as an applicable method to isolate the essential oils from plant samples. Hence, their antibacterial activities were tested against gram-positive and gram-negative bacteria.

2. Materials and Methods

2.1. Materials and apparatus

GC-grade ethyl acetate (EA) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). The ultrapure water used in the experiments was obtained from an AquaMax ultra-pure water purification system from Younglin (Seoul, South Korea). All the glass tools in this method, comprising distillation and condenser systems, were made manually in the laboratory.

2.2. *Plant material*

The aerial part *S. khuzistanica* was provided by Vasha Darou Pars Herbal Pharmaceutical Co., Qom, Iran, in 2017. The plant materials were identified by Dr. Javad Hadian and a voucher specimen (MPH-1414) at the herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

2.3. Essential oil isolation

The essential oil of each air-dried plant part (50 g) was isolated by hydrodistillation three-time using a clevenger-type apparatus recommended by the British Pharmacopoeia. The essential oil content of plant materials was determined by repeating experiments three times. The essential oils were dried over anhydrous sodium sulfate and kept at 4 ºC in dark vials until analyzed and tested.

2.4. UAE followed by hydrodistillation

The hyphenation of ultrasonic-assisted ultrasonic homogenizer (UP200S; Hielscher, Teltow, Germany) equipped with a titanium probe of 14 mm in diameter was employed. The sono-clevenger has been hyphenated with an ultrasound device according to the schematic representation in Fig. 1. The sonication power (20, 30, and 50 W at 24 kHz) was used to isolate essential oil in the sono-clevenger method with 30 seconds on/off interval. The distilled water passed through the EA (20 ml) as an extraction solvent used as an online liquid-liquid extraction system. The flow rate of distilled water passing the solvent was 1.5 ml/min. Eventually, the collected EA was evaporated under reduced pressure by a Heidolph rotary vacuum evaporator. The residual essential oil was reserved for further analysis.

2.5. Analysis, identification, and quantification of the oil components GC–FID and GC-MS analysis

The analysis was carried out on a fused silica capillary DB-5 column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The injector and

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detector temperatures were kept at 250 and 300 ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.1 ml/minute; the oven temperature program was 60-250 ºC (4 ºC /min) and finally held isothermally for 10 min; the split ratio was 1:50. Gas chromatography/mass spectrometry (GC-MS) analysis was conducted using Thermoquest–Finnigan gas chromatograph equipped with a fused silica capillary DB-5 column (60 m × 0.25 mm i.d.; film thickness 0.25 µm) coupled with a TRACE mass spectrometer (Manchester, UK). Helium was used as a carrier gas with an ionization voltage of 70 eV. Ion source and interface temperatures were 200 and 250 ºC, respectively. The mass range was 35 - 456 amu. The oven temperature program was the same as above for the GC-FID. The essential oil was diluted in EA by the proportion of 1:10 and 1 µl injected from this solution for analysis. For GC-MS analysis, the solvent delay mode has been applied.

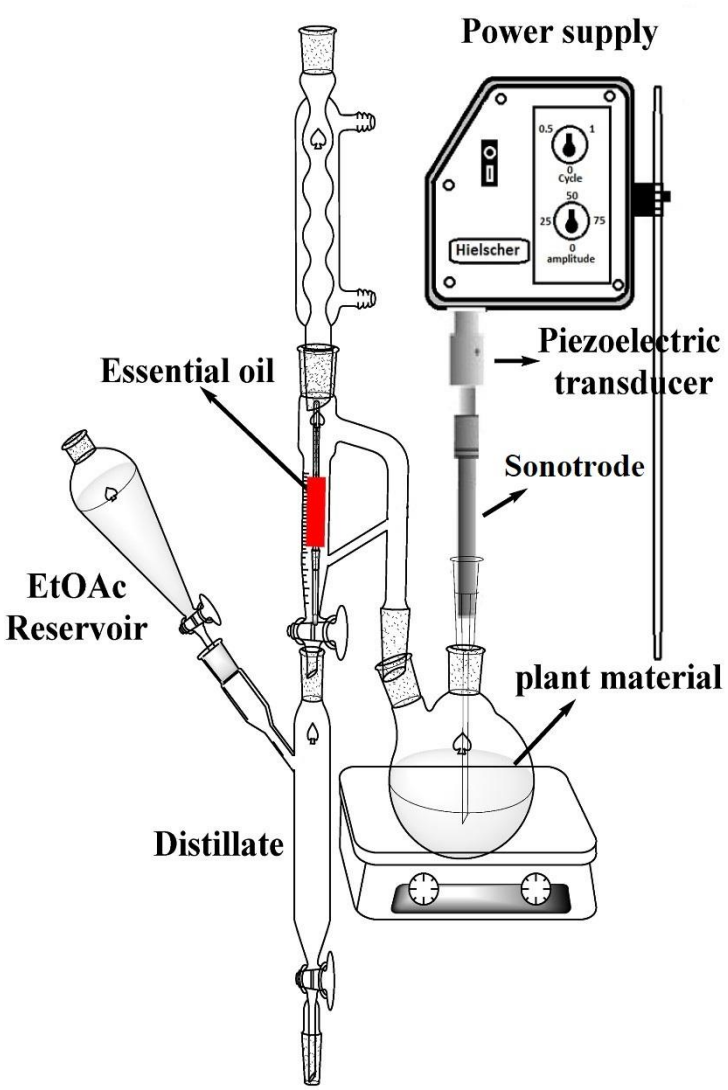


Fig. 1. Instrumental design for simultaneous ultrasonic-assisted isolation of *S. khuzistanica* essential oil and its corresponding distillate**.**

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The constituents of essential oils were identified by calculating their retention indices under temperature-programmed conditions for n-alkanes ($C_6 – C_{24}$) and the oil on a DB-5 column under the same chromatographic conditions. Each compound was identified by comparing their mass spectra with those of the internal reference mass spectra library (Adams and Wiley 7.0) or with authentic compounds and confirmed by comparing their retention indices with authentic compounds or those reported in the literature [38]. For quantification purposes, relative area percentages obtained by FID were used without the use of correction factors.

2.6. Scanning electron microscopy (SEM)

SEM data of dried *Satureja* leaves were obtained for both samples undergone hydrodistillation (for 4 hours) and sono-clevenger (for 60 min). The leaves were fixed on the specimen holder with aluminum tape and then sputtered with gold in a sputter coater. Finally, the plant cell photos were taken at the central laboratory of Shahid Beheshti University by using the SEM, model 3500, manufactured by Hitachi (Japan).

2.7. Biological assays

The bacteriostatic activity of the volatile constituents against the Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), the Gram-negative bacteria: *Escherichia coli* (ATCC 25922) were determined using the dilution technique [39]. The oils were prepared at 10 mg/ml using sterile distilled water containing 10 % Tween 80. Serial dilutions of the stock solutions in broth medium (100 ml of Muller Hinton broth) were prepared in a microtiter plate (96 wells). Following this, 1 ml of the microbial suspension (in sterile distilled water) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked, and the plates were incubated as described above. The minimum inhibitory concentrations (MIC) were determined as the lowest concentrations preventing visible growth. Standard antibiotics (Netilmicin and amoxicillin with clavulanic acid) were used to control the sensitivity of the bacteria tested.

3. Results

The essential oil of air-dried plant material of SK (20 g) was isolated by hydrodistillation for three h using a clevenger-type apparatus recommended by the British Pharmacopoeia. Then, the equipment is designed to isolate the plant's essential oil and remain in distillate by online extraction with an organic solvent (Fig. 1).

The sonication power was set at three different energy levels of 20, 30, and 50 W, based on the literature data [11 - 13]. Considering essential oil quantity reached a constant value, EO isolation time was chosen at 60 minutes for all experiments. Table 1 presents the experimental details of the experiments designed. Consequently, the overall yield of conventional clevenger-type apparatus was 2.5 ml/ 100 g DW for 4 hours. On the other hand, the isolation of essential oil with sono-clevenger system yield was 3.0, 3.3, and 3.5 ml/100 g DW at 20, 30, and 50 W, respectively, after 60 minutes.

Furthermore, comparing the kinetic of essential oil yield by hydrodistillation and sono-clevenger method was shown in Fig. 2. The kinetic of the extraction process can be tracked by determining the amount of essential oil yield as a function of time (or solvent consumption), providing an overall extraction curve. In brief, isolation yield maximum with the sono-clevenger technique started at a much earlier time than that with HD (25 min vs. 90 min, respectively). In the

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hydrodistillation, the isolation process of essential oil is slow, and in 90 minutes, the maximum level of 2.5 % will reach, wherein sono-clevenger, this process is much faster in 30 minutes, the highest level achieved. The results show that using the ultrasound reduced the time required to achieve this yield by a factor of 3. The major effects of ultrasound in a liquid medium are attributed to the cavitation phenomena, which comes from the physical processes that create, enlarge, and implode micro bubbles of gases dissolved in the liquid. The temperature and the pressure inside of these bubbles have been estimated to be up to 5000 K and 5000 atm. This phenomena dramatically accelerates the chemical reactivity of the medium by which the mass transfer from plant cell will increase.

Table 1. The sample codes and experiments

Sample code	Description	EO (in ml) from 100 g of SK*(Yield)
Exp. 1	Blank sample only EA	-
Exp. 2	EO obtained by hydrodistillation	2.5
Exp. 3	EO obtained with sonication power 50 W	3.5
Exp. 4	EO obtained by sonication power 30 W	3.3
Exp. 5	EO obtained by sonication power 20 W	3.0
Exp. 6	EO obtained from distillate in 50W	0.1
Exp. 7	EO obtained from distillate in 30W	0.1
Exp. 8	EO obtained from distillate in 20W	0.1

*SK: *Stureja khuzistanica Jamzad*; EA: ethyl acetate; EO: essential oil

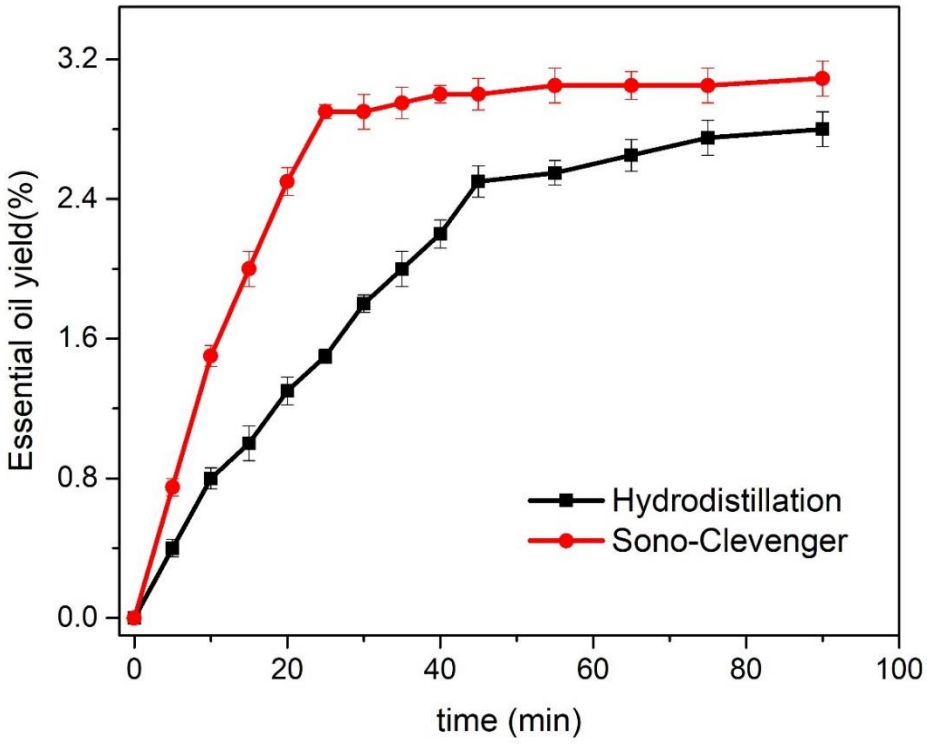


Fig. 2. The comparison of extraction yield as a function of time for hydrodistillation and the sono-clevenger method

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In another word, as shown in Fig. 3, it seems that the plant cell walls of plant secretory structures can be easily destroyed by sonic waves, thereby facilitating the release of metabolites in the extraction solvent [40]. It means sonic waves facilitate solvent to flow into the SK plant cells and enhance EO isolation from the secretory glands. Comparing SEM data related to SK leaves treated by HD (Fig. 3A) and ultrasound-assisted HD (Fig. 3B) reveals more efficient disruption of plant cell walls by the sonic waves. As a result, an enhancement in EO extraction by about 40 % compared to conventional hydrodistillation methodology was observed.

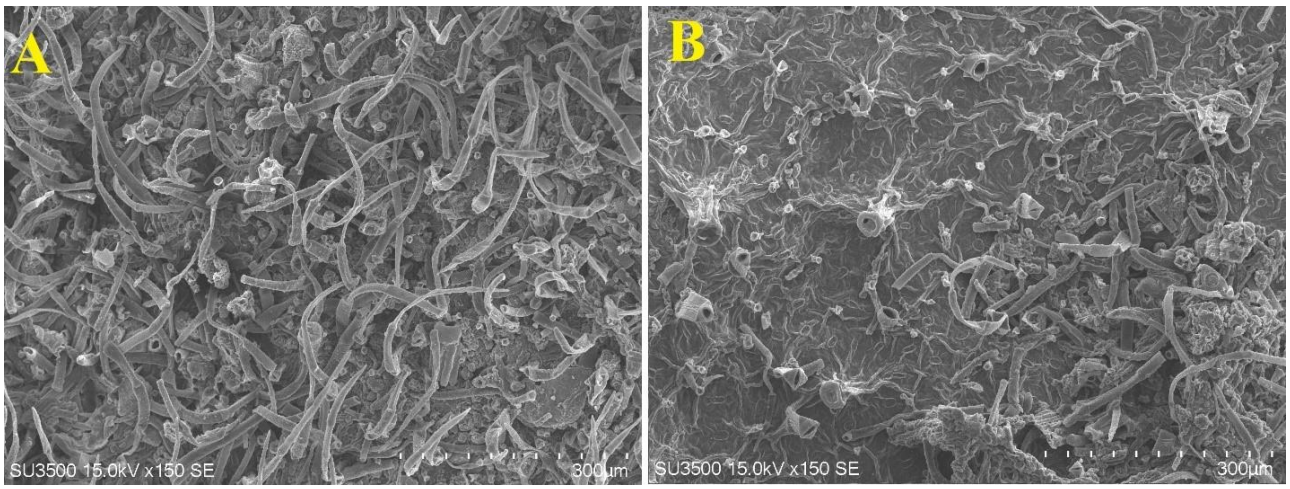


Fig. 3. Scanning electron micrographs (SEM) related to *Satureja* leaves: (A) after hydrodistillation for 4 hours, and (B) after sono-clevenger treatment for 60 min.

4. Discussion

The GC-MS profiles for essential oils produced by conventional hydrodistillation and the sono-clevenger at three different energies were compared. The resultant hydrosols were extracted following the essential oil isolation using a liquid-liquid extraction strategy based on EA as the acceptor phase. Table 2 represents the identification of the essential oils and corresponding distillates by GC-MS and their quantification by GC-FID obtained under different conditions. Carvacrol is the most abundant phytochemical found in all studying essential oils, and distillates ranged from 96.7-98.7 %. The other minor compounds presented in studying oils and distillates could be considered as follows: α-pinene ranged from 0.1-0.2 %, *p*-cymene (tr-1.2 %), 4-terpineol (0.2-0.6 %) and γ-terpinene (tr-0.3 %). The highest amount of carvacrol (98.7 %) was obtained at the sonication power of 50 W. The lowest amount of carvacrol was found in the hydrodistillation. The only trace of thuja-2,4(10)-diene was detected in EO obtained by conventional hydrodistillation and UAE at a power of 50W. The *p*-cymene quantity ranged from 0.3 % to 1.1 %, which was at its highest amount in UAE hydrodistillation at the power of 50W. The range of γ-terpinene detected from 0.1 to 0.3 % in the UAE power of 20W, whereas only the trace of *p*-cymene in all oils obtained by UAE, while in conventional oil and all distillates ranged from 0.1to 0.2 %. A trace amount of α-terpineol (0.03-0.2 %) was detected in all studying oils and distillates. In the conventional hydrodistillation method, 4-

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terpineol quantity was ranged from 0.2 to 0.6 % in the resulting oil at a power of 30 W. Thymol almost detected at 0.2 % in all oils and distillates. The trace amount of carvacrol acetate, *α*-humulene, *trans*-caryophyllene, and *β*-bisabolene are detected in studying oils and distillates. Except for some small differences, shortening the process of extracting essential oils by applying the ultrasonic technique did not result in any significant loss in the quality of EO when compared to the conventional method. There are many reports [42] in which the ultrasonic pretreatment before the hydrodistillation step has been investigated, improving both the quality and quantity of the essential oil and decreasing the time and energy consumption compared to the conventional techniques. The effects on the chemical composition were dependent on the evaluated conditions. Table 2 shows small changes such as *α*-terpineol production in sono-clevenger. This may relate to the ease of releasing the essential oils from secretory glands or to transformations of unstable chemical compounds during the ultrasound application. On the other hand, an increase in the essential oil quality can be attributed to the low level of degradation of thermal compounds.

Table 2. Essential oil composition obtained from different strategies (Exp.2-Exp. 8).

No	Compounds	Rt	RI * Lit.	RI Exp.	Exp. 2
1	*α*-Pinene	3.92	932	933	0.1
2	Thuja-2,4(10)-diene	5.40	953	954	tr
3	1,3,6-Octatriene	4.88	1032	1030	tr
4	*γ*-Terpinene	5.56	1054	1053	0.1
5	*p*-Cymene	6.27	1089	1080	1.1
6	*p*-Cymenene	6.51	1089	1080	0.2
7	*α*-Terpineol	7.25	1179	1175	tr
8	Terpinen-4-ol	9.18	1174	1180	0.6
9	Thymol	10.81	1289	1270	0.2
10	**Carvacrol**	**12.81**	**1298**	**1282**	**96.8**
11	Carvacrol acetate	14.14	1370	1329	0.1
12	*α*-Humulene	15.22	1452	1427	0.1
13	*trans*-Caryophyllene	17.37	1417	1425	0.1
14	*β*-Bisabolene	13.14	1505	1501	0.3
	Total identification				99.73
	Monoterpene hydrocarbons (MH)				1.5
	Oxygenated monoterpenes (MO)				97.7
	Sesquiterpene hydrocarbons (SH)				0.5

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Table 2. Essential oil composition obtained from different strategies (Exp.2-Exp. 8). (Continued)

No	Compounds	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
1	*α*-Pinene	0.2	0.1	-	0.1	tr	tr
2	Thuja-2,4(10)-diene	-	-	tr	-	-	-
3	1,3,6-Octatriene	-	-	0.1	-	-	-
4	*γ*-Terpinene	0.3	tr	0.1	tr	0.1	0.1
5	*p*-Cymene	0.9	0.3	-	1.2	1.0	1.3
6	*p*-Cymenene	tr	tr	tr	0.1	0.2	0.1
7	*α*-Terpineol	0.1	0.1	0.2	0.1	-	-
8	Terpinen-4-ol	0.3	0.2	0.4	0.5	0.5	0.4
9	Thymol	0.1	0.2	0.2	0.2	0.2	0.2
10	**Carvacrol**	**97.5**	**98.4**	**98.7**	**96.8**	**96.7**	**96.3**
11	Carvacrol acetate	0.1	0.1	0.1	0.1	0.1	0.1
12	*α*-Humulene	tr	0.1	-	0.1	0.1	0.1
13	*trans*-Caryophyllene	0.2	0.2	0.13	0.3	0.3	0.3
14	*β*-Bisabolene	0.2	tr	-	0.3	0.3	0.3
	Total identification	99.9	99.7	99.93	99.8	99.5	99.2
	Monoterpene hydrocarbons (MH)	1.4	0.4	0.2	1.4	1.3	1.5
	Oxygenated monoterpenes (MO)	98.1	99	99.6	97.7	97.5	97
	Sesquiterpene hydrocarbons (SH)	0.4	0.3	0.13	0.7	0.7	0.7

Components are listed in order of elution from a DB-5MS column. RI: Retention indices calculated against C8-C24 n-alkanes on the DB-5MS column; tr: traces <0.1, *: Retention indices based on Adams (2017); MH: Sr. No 1-6; MO: Sr. No 7-11; SH: Sr. No 12-14

The antibacterial activity (MIC) of studying oils and distillates against *Escherichia coli* and *Staphylococcus aureus* were compared. Except for the blank sample, which contains no plant materials, all EOs obtained by hydrodistillation, sono-clevenger, and distillate products showed significant antibacterial activity with MIC: 1.0 mg/ml *E. coli* and 0.5 mg/ml *S. aureus*. The antibacterial properties of essential oil from nine *Satureja* species (*S. bachtiarica*, *S. mutica*, *S. sahandica*, *S. macrantha*, *S. atropatana*, *S. edmondi*, *S. spicigera*, *S. isophylla* and *S. intermedia*) were assessed [41]. On average, minimum inhibitory concentration (MIC) ranged from 4 mg/ml to 16 mg/ml against *E. coli* and 1 mg/ml to 2 mg/ml against *S. aureus*, respectively. To conclude, our results are based on the previously reported data [42] and confirming somehow there are no significant differences among their chemistry-related biological effects.

5. Conclusion

In summary, the results indicate while the overall yield of conventional clevenger-type apparatus was 2.5 ml/ 100 g DW for 4 hours, using the sono-clevenger system, the overall

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yield increased up to 40 % at a reduced time by a factor of 3. Furthermore, the kinetic study revealed an extraction yield with the sono-clevenger technique started at a much earlier time than that with HD (25 min vs. 90 min, respectively). Thus, the antibacterial effect of two essential oils and their comparison of chemical profiles confirmed no significant change in the chemical composition of the essential oils. The results suggest it as an applicable method for industrial scale.

Author contributions

SRR: Experimental part, writing the manuscript, HR and SNE: Supervision, experimental validation in phytochemical part, developing the draft of the paper.

Conflict of interest

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

Acknowledgement

The authors gratefully acknowledge the financial support of Shahid Beheshti University, Tehran, Iran. The plant material provided by Vasha Darou Pars Herbal Pharmaceutical Co and supported this research.

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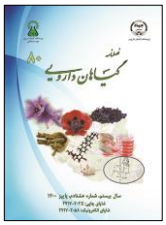
How to cite this article: Rasouli SR, Nejad Ebrahimi S, Rezadoost H. Simultaneous ultrasound-assisted hydrodistillation of essential oil from aerial parts of the *Satureja khuzistanica* Jamzad and its antibacterial activity. ***Journal of Medicinal Plants*** 2021; 20(80): 47-59.
doi: 10.52547/jmp.20.80.47

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مقاله تحقیقاتی

بکارگیری همزمان امواج فراصوت و تقطیر با آب جهت استخراج اسانس اندام هوایی مرزه خوزستانی و بررسی خواص ضدباکتریایی آن

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اطلاعات مقاله

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نعناعیان
کارواکرول
امواج فراصوت
عرقیات
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چکیده

مقدمه: استفاده از استخراج با کمک امواج فراصوت (UAE) یک روش قوی و موثر به عنوان جایگزینی مطلوب برای استراتژی‌های متداول جهت استخراج مواد فعال از قسمت‌های مختلف گیاهان است. کوپل کردن این تکنیک با روش تقطیر با آب یک روش کارآمد برای تولید اسانس از مواد گیاهی است. **هدف:** در این تحقیق روش کوپل شده همزمان با استفاده از امواج مافوق صوت به منظور کاهش زمان اسانس‌گیری و افزایش بازده آن به منظور استحصال اسانس از گیاه مرزه خوزستانی مورد بررسی قرارگرفت. **روش بررسی:** در مرحله بعد، زمان جداسازی، عملکرد و کیفیت اسانس‌های بدست آمده با استفاده از روش‌های متداول و امواج فراصوت مقایسه شد. اتیل استات برای بازیابی باقیمانده اسانس موجود در عرق استفاده شد. **نتایج:** همه اسانس‌ها و عرق‌ها با استفاده از تکنیک‌های GC-FID و GC-MS از نظر کیفی و کمی تجزیه و تحلیل شد. در این کار تحقیقاتی خاصیت ضدباکتریایی اسانس گرفته شده یا به عبارت دیگر حداقل غلظت بازدارندگی در برابر باکتری گرم منفی اشریشیاکلی برابر با ۰/۵ تا ۸/۰ میلی‌گرم در میلی‌لیتر و برای باکتری گرم مثبت استافیلوکوکوس اورئوس برابر با ۰/۲۵ تا ۸/۰ میلی‌گرم در میلی‌لیتر بود. **نتیجه گیری:** روش بکار گرفته شده در این کار تحقیقاتی نشان داد که با حفظ ترکیبات شیمیایی اسانس‌ها و خواص بیولوژیکی آن‌ها می‌تواند کارایی بازده جداسازی اسانس را تا ۴۰ درصد افزایش دهد.

مخففها: UAE، استخراج به کمک امواج فراصوت؛ HD، تقطیر با آب؛ SK، *Stureja khuzistan*ica Jamzad؛ EA، اتیل استات؛ MIC، حداقل غلظت بازدارندگی

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تاریخ دریافت: ۲۷ شهریور ۱۴۰۰؛ **تاریخ دریافت اصلاحات:** ۷ آذر ۱۴۰۰؛ **تاریخ پذیرش:** ۹ آذر ۱۴۰۰

doi: 10.52547/jmp.20.80.47

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