ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
https://www.icf.ro/rrch/

Rev. Roum. Chim.,
2024, *69*(1–2), 41–48
DOI: 10.33224/rrch.2024.69.1-2.05

A COMPARATIVE STUDY OF MICROWAVE-ASSISTED AND CONVENTIONAL HYDRO DISTILLATION METHODS FOR EXTRACTING ESSENTIAL OILS AND EVALUATING THEIR ANTIMICROBIAL ACTIVITY FROM *CLEOME KHORASSANICA*, NOVEL RESEARCH

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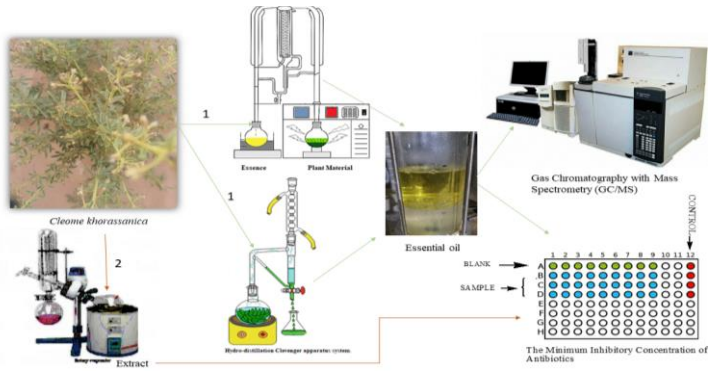
Received June 14, 2023

This study reports on the phytochemical analysis and antimicrobial activity evaluation of essential oils extracted from the aerial parts of *Cleome khorassanica* using both microwave-assisted water distillation (MAHD) and classical hydro distillation (HD). Gas chromatography coupled with mass spectrometry was used to analyze the chemical composition of the essential oils. The main components identified in the MAHD-extracted oil were γ-Cadinene (34.29%) and δ-Cadinene (19.52%), while o-Isopropenyltoluene (46.7%) and Duodecyclic acid (10.96%) were the major components in the HD-extracted oil. The MAHD technique showed higher extraction efficiency in a shorter time than the conventional HD method. The antimicrobial activity of the essential oils was evaluated using the minimum inhibitory concentration (MIC) method. The results showed that the essential oil and methanolic extract of *Cleome khorassanica* exhibited significant inhibitory activity against *Staphylococcus aureus*, *Aspergillus niger*, *Aspergillus alternata* and *Fusarium solani*. Gram-positive bacteria and fungi were more susceptible to the essential oil than Gram-negative bacteria. Furthermore, the samples extracted by MAHD demonstrated better antimicrobial activity compared to the HD-extracted oil.



INTRODUCTION

Cleome khorassanica (*C. khorassanica*) is a plant belonging to the Cleomaceae family that has been studied for its pharmacological properties. It is native to Iran and Afghanistan and has been used in traditional medicine for centuries. Recent studies have shown that Cleome plants contain a

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variety of bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenolic acids that exhibit significant antimicrobial properties. Moreover, previous studies on different species of Cleome have demonstrated promising biological activities, including anti-tumor, anti-hyperlipidemia, anti-hyperglycemic, anthelmintic, antibacterial, and anti-inflammatory properties.[1,2]

The antimicrobial activity of Cleome extracts has been attributed to their ability to disrupt the cell membrane integrity of microorganisms or inhibit their enzymatic activities.[3] In traditional medicine, *C. khorassanica* has been used for various purposes. The plant has been used as an antispasmodic, diuretic, and laxative. It has also been used to treat respiratory problems such as asthma and bronchitis. In addition, *C. khorassanica* has been used to treat skin conditions such as eczema and psoriasis.

Efficiency and time of essential oil extraction

The extraction of essential oils from medicinal plants is a crucial step in the production of many pharmaceutical and cosmetic products. Hydro distillation (HD) is a conventional method for preparing essential oils from medicinal plants.[4] However, this method has some limitations such as long extraction time, high energy consumption, low efficiency, and high solvent consumption. Exposure to high temperatures can also cause the decomposition of some essential oil compounds and the loss of volatile compounds.[5,6] To overcome these limitations, there has been growing interest in exploring new and efficient methods for essential oil extraction. Non-conventional methods such as supercritical carbon dioxide extraction, microwave extraction, and ultrasound extraction have been studied. However, these methods cannot fully replace the HD method. Tadić *et al*. (2012) reported that supercritical carbon dioxide extraction cannot replace conventional HD due to significant differences in the chemical composition of essential oils.[7]

The aim of this study is to evaluate the efficiency and time of essential oil extraction from *C. khorassanica* using two different methods: water distillation and microwave-assisted water distillation. The use of microwaves allowed for a more rapid and homogeneous transfer of heat, resulting in shorter extraction times.[8] Moreover, the microwave method consumed less energy than the conventional Clevenger method. Both microwave and water distillation methods used water as a solvent, which is an environmentally friendly option.[9,10]

In addition to higher extraction efficiency and energy-saving, the microwave method also offers selectivity in the extraction of specific compounds from plant materials. This selective extraction is a significant advantage over the Clevenger method. Microwave energy can improve the release of volatile compounds from the plant matrix, leading to an increase in distillation efficiency. Furthermore, microwave-assisted extraction causes the walls of the plant tubers to break quickly, resulting in higher extraction efficiency and shorter extraction times.[11]

Overall, the results of this study suggest that microwave-assisted extraction is an efficient and environmentally friendly method for essential oil extraction from *C. khorassanica*. This method offers several advantages over conventional water distillation and Clevenger methods and could be used as an excellent alternative for the production of essential oils from plant materials.[12]

METHODOLOGY

The entire plant organ of wild *C. khorassanica* was collected from the North Khorasan province of Iran in May 2020 at the full growth stage. The plant material was taxonomically identified by Mahmood Zakaee, a plant taxonomist at the Department of Basic Science College, Ferdowsi University, Mashhad, Iran. The voucher specimen (20995) was deposited in the herbarium center at Ferdowsi University, Mashhad, Iran. The aerial part of the plant was air-dried in the shade and subsequently ground into medium-sized particles. To avoid increasing the oxidation rate of the essential oil compounds or conversion rate to resin materials, the plant material was ground to a medium degree. It should be noted that crushing the plant material into very small parts is not recommended.[13]

Essential oil by hydro distillation

For essential oil extraction, 100 grams of the chopped plant material were placed in a one-litre

flask, and two-thirds of it was filled with distilled water. The essential oil was extracted from the plant material using a Clevenger apparatus for 210 minutes. The weight of the essential oil obtained from the hydrodistillation method was determined to be 0.65gr of the dry weight of the plant, as indicated by the formula mentioned below(formula 1). The efficiency of essential oil production was found to be 0.65% of the dry weight of the plant. The dehydrated essential oil was collected in a closed, dark container using anhydrous sodium sulfate and stored at 4°C until further analysis.

Formula 1:

$$\text{Yield\%} = \frac{\text{Weight of essential oil}}{\text{Weight of raw material}} \text{x } 100$$

Microwave-Assisted Hydro Distillation (MAHD)

Microwave-assisted hydro distillation (MAHD) was used for the extraction of essential oil from *C. khorassanica*. This method is a combination of microwave heating and distillation, using distilled water as the solvent. The apparatus used for this purpose consisted of a microwave oven, a condenser in the upper part of the oven to remove and cool the vapors, and a magnetic stirrer.

To extract the essential oil, 100 grams of the aerial part of the plant were mixed with distilled water and irradiated with a frequency of 2450 MHz and a power of 800 watts for 35 minutes. The contents inside the balloon were mixed with the help of a magnetic stirrer during irradiation. After the end of irradiation, the extracted essential oil was collected. To hydrate the outer layer of the plant, before using the steam distillation method, we placed 100 grams of the aerial parts of the plant in 650 milliliters of distilled water at room temperature (25 degrees Celsius) for 70 minutes. According to the formula mentioned above (formula1), the weight of the essential oil obtained from the microwave-assisted extraction method was found to be 0.9 grams per gram of the dry weight of the plant, and the efficiency of essential oil production was found to be 0.9% of the dry weight of the plant. This method of hydration was adapted from Arabshahi-Delouee and Urooj (2007).[14]

Minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of the samples, the MIC dilution method was employed. The MIC assay is a useful tool for evaluating the antimicrobial activity of natural products and synthetic compounds. It provides a quantitative measure of the potency of the test sample against a specific microorganism and can be used to screen large numbers of samples in a relatively short time. However, it should be noted that the MIC value is influenced by various factors, such as the type and concentration of the microorganism, the growth medium, the incubation time and temperature, and the presence of other compounds in the test sample. Therefore, it is important to carefully control these variables and perform appropriate statistical analyses to obtain reliable and reproducible results. In brief, a sterile 96-well plate was used, and the micro dilution technique was applied. In each of the first to eighth rows of the plate, 95 microliters of Hinton Broth molar culture medium (Merck, Germany) and 5 microliters of microorganism were added. The first well of each row was treated with 100 microliters of the sample, while the eighth well was treated with 95 microliters of successive dilutions of the extract. The ninth well was used as a blank control, and the tenth well contained 95 microliters of culture medium and 5 microliters of microorganism without any sample. The eleventh well was treated with an antibiotic control. The MIC can be determined visually by comparing the growth in the wells with the growth in the control wells containing no test sample. After incubation for 24 hours at 37°C, the presence of turbidity was observed and compared to the control row. The absence of bacterial growth was indicated by transparency, while the presence of turbidity indicated bacterial growth. The MIC is defined as the lowest concentration of the test sample that completely inhibits visible growth of the microorganism. This method has been widely used in previous studies to determine the MIC of essential oils against various microorganisms.[15,16]

Characteristics of gas chromatography/mass spectrometer

Gas chromatography/mass spectrometry (GC/MS) has been widely used for the analysis of chemical compounds in various fields, including pharmaceuticals and food industry. In this study, GC/MS was performed using an Agilent Technologies model 7890A gas chromatography device and an Agilent Technologies model 5975C mass spectrometer. The sample injection part was maintained at a temperature of 280°C, and the carrier gas used was helium (99.99%) with a flow rate of 1 milliliter per minute. The system was set in split mode with a split ratio of 100:1. The column used was a model 5MS-HP capillary column with a length of 30 meters, an inner diameter of 0.25 mm, and a film thickness of 0.25 micrometers. The column temperature was increased from 60°C to 210°C at a rate of 3°C per minute, followed by an increase to 240°C at a rate of 20°C per minute and maintained at this temperature for 8.5 minutes, resulting in a total operating time of 60 minutes.

The solvent delay time in the mass spectrometer was set to 3 minutes, and the mass range was 45-550 amu. The mass spectrum source temperature was set to 230°C, the mass spectrum quad temperature was 150°C, and the transfer line temperature was 280°C. After performing the injections and obtaining the chromatograms, the identification and quantification of each compound were done using their mass spectrum. The identification of the compounds was based on Wiley Registry 10th Edition/NIST 2012 Mass Spectral Library data, DVD-ROM – May 13, 2013. The retention index of the compounds was calculated using a standard sample of normal alkanes injected into the MS/GC machine under the same temperature program and column. The retention index of compounds in *C. khorassanica* leaf essential oil was determined using the normal retention time of injected alkanes.

Methanolic extract preparation method

In this study, the methanolic extract of the plant was prepared using the following method: 100 grams of the powdered plant material were weighed using a digital scale and placed in an Erlenmeyer flask. Merck methanol solvent was added to each sample, enough to completely cover the powder. The Erlenmeyer flasks were then covered with aluminum foil and placed on a shaker machine at 90 rpm for 48-72 hours to ensure homogenization of the solvent and the plant. The resulting mixture was filtered using filter paper. The extract was then separated from the solvent using a rotary evaporator (Heidolph, Germany).

RESULTS

In this study, essential oils were extracted from the aerial parts of *C. khorassanica* using both conventional water distillation (HD) and microwave-assisted water distillation (MAHD) methods. The essential oils were analyzed using GC/MS, and 34 compounds were identified in the essential oil of the *C. khorassanica* plant by HD method, while 18 compounds were identified by the MAHD method (Table 1, Fig.1). The number and percentage of compounds in the two methods were significantly different. Among the most important similar compounds, in the water distillation method o-Isopropenyltoluene, Duodecyclic acid, α-Cadinol and δ-Cadinene are 46.7, 10.96, 1.14, 1.98% respectively, but in the microwave method, these compounds are 0.46% respectively, 0.33, 16.63 and 19.52 percent. The percentage of extracted compounds in the MAHD method (99.36%) was higher than in the HD method (95.44%), although a higher number of compounds were extracted using the HD method (Table2). The compounds were characterized by comparing their mass spectra and retention indices with those in the NIST library. The main compounds in both methods with the highest percentage are listed in Table 2. The extraction time for the hydro distillation method was 210 minutes, while the microwave method reduced the extraction time to 35 minutes. Essential oil extraction was conducted in three stages, with the first stage involving injection of the extracted oil into a GC-MASS device for chemical compound identification. In the two subsequent stages, the extracted oils were subjected to antimicrobial testing for further analysis. The use of three different stages was to ensure the reported efficiency of the process.

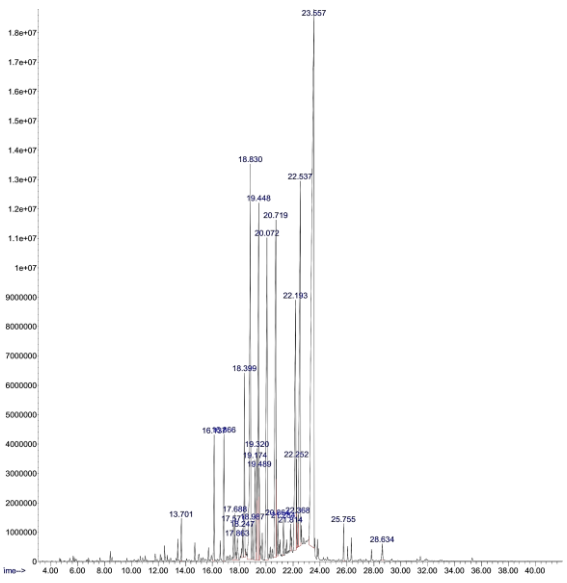


Fig1: Chromatogram of the chemical compounds identified via GC-MS of *Cleome khorassanica* by microwave assisted

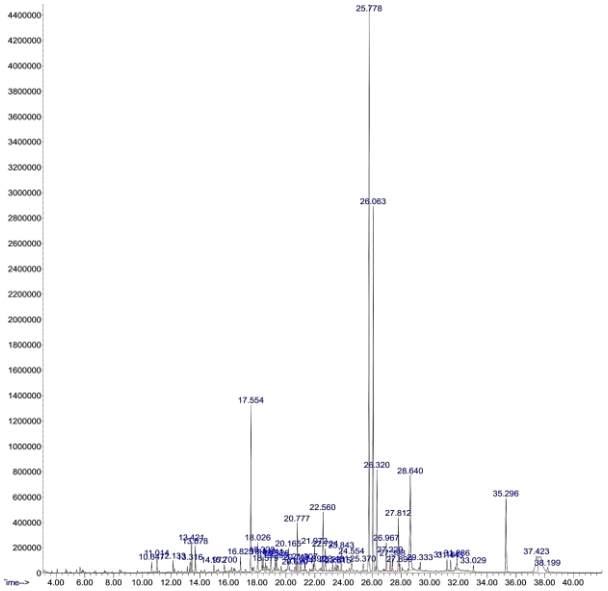


Fig2: Chromatogram of the chemical compounds identified via GC-MS of *Cleome khorassanica* by hydrodistillation assisted

Table 1

Chemical composition of coriander seeds essential oil of *C. khorassanica* in HD and MAHD

	Compound	**MAHD**	**HD**	**RI[a]**	**Formola**
1	Azulene	…	0.47	1186	$C_{10}H_8$
2	Esdragol	…	0.59	1200	$C_{10}H_8$
3	Cuminal	…	0.49	1242	$C_{10}H_{12}O$
4	Anethol	…	0.46	1287	$C_{10}H_{12}O$
5	Thymol	0.49	2.22	1291	$C_{10}H_{14}O$
6	β-elemene	1.69	0.37	1351	$C_{10}H_{16}$
7	β-Bisabolene	0.34	0.80	1358	$C_{15}H_{24}$
8	β-Caryophyllene	1.71	1.09	1380	$C_{15}H_{24}$
9	α -Cubebene	1.06	…	1380	$C_{15}H_{24}$
10	trans-Caryophyllene	…	1.41	1425	$C_{15}H_{24}$
11	Geranyl acetone	0.53	6.59	1455	$C_{13}H_{22}O$
12	α-Decene	…	1.24	1473	$C_{10}H_{20}$
13	β -Ionene	…	0.54	1489	$C_{13}H_{20}O$
14	bicyclogermacrene	9.61	…	1506	$C_{15}H_{24}$
15	δ-Cadinene	19.52	1.98	1528	$C_{15}H_{24}$
16	α -Farnesene	0.81	0.4	1534	$C_{15}H_{24}$
17	Elemol	8.36	…	1559	$C_{15}H_{26}O$
18	Duodecyclic acid	0.33	10.96	1563	$C_{15}H_{24}O$
19	Caryophyllene oxide	2.17	2.22	1589	$C_{15}H_{24}$
20	Viridiflorol	0.51	2.28	1593	$C_{15}H_{26}O$
22	Tetradecanal	…	0.37	1599	$C_{14}H_{28}O$
22	Tetradecanal	…	0.37	1599	$C_{14}H_{28}O$
23	Ledol	0.47	…	1612	$C_{15}H_{26}O$
24	β -turmerone	…	2.48	1642	$C_{15}H_{22}O$
25	α -Eudesmol	0.38	…	1660	$C_{15}H_{26}O$
26	α -Cadinol	16.63	1.14	1668	$C_{15}H_{26}O$
27	Cetylic alcohol	…	1.13	1669	$C_{16}H_{34}O$
28	Lauryl ethoxylate	…	0.32	1676	$C_{14}H_{30}O_2$
29	γ -Cadinene	34.29	0.33	1714	$C_{15}H_{24}$
30	Myristic acid	…	1.01	1727	$C_{14}H_{28}O_2$
31	o-Isopropenyltoluene	0.46	46.7	1761	$C_{10}H_{12}$
32	Eicosane	…	0.41	1999	$C_{20}H_{42}$
33	Phytol	…	0.56	2113	$C_{20}H_{40}O$
34	3,8-Dimethyldecane	…	2.88	2372	$C_{12}H_{26}$
		99.36%	95.44%		

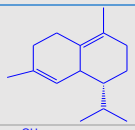
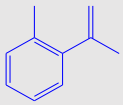
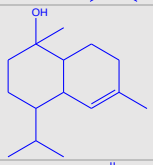
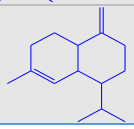
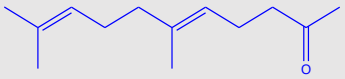
Note: [a]Retention indices using a 5MS-HP column

In a similar study on the hydraulic distillation of rosemary essential oil using microwaves, it was reported that distillation with microwave water was superior in terms of saving energy and extraction time. In this study, the quality of essential oil improved due to the identification of oxygenated compounds in the MAHD method Moradi S. *et al*., 2018.[17] The extraction efficiency was significantly dependent on the extraction technologies used, as shown in a phytochemical study on *Cleome brachycarpa*, where microwave-assisted extraction resulted in 37% yield compared to ultrasound-assisted extraction and Soxhlet extraction.[18]

The concentration of monoterpene hydrocarbons in the MAHD method was lower compared to HD, while the concentration of oxygenated compounds and sesquiterpenes increased. This trend was observed in other studies, including those by Lucchesi *et al.*[19] Okoh *et al.*, Bendahou *et al.*[2,0] Okoh *et al.*[21] and Ferhat *et al.*[9] They also showed that the content of oxygenated compounds in the essential oil obtained from the microwave is higher than the essential oil obtained from hydro distillation. However, a different report by Wang et al showed that the content of oxygenated compounds in the essential oil obtained from HD was higher.[11] The reason for this discrepancy may be related to the type of plants, and the content of oxygenated compounds in the oil is probably not related to the extraction method.[17] Table 1 shows the similar and non-similar compounds of the microwave method and extraction with a Clevenger. As can be seen, the percentage composition of some components increased and some decreased.

Table 2

Main compound in essential oil of HD and MAHD

Compound	**MAHD**	Structure	**Compound**	**HD**	**Structure**
δ-Cadinene	19.52%		o-Isopropenyltoluene	46.7%	
α -Cadinol	16.63%		Duodecyclic acid	10.96%	
γ -Cadinene	34.29%		Geranyl acetone	6.59%	

Antimicrobial properties of essential oil extracted by MAHD and HD methods and Methanolic extract of *C. khorassanica*

Table 3 shows the minimum inhibitory concentration of essential oils against Gram-positive and Gram-negative bacteria. The study's most notable results indicate that the HD-extracted essential oil exhibited inhibitory effects on the growth of, *S. aureus* at 32 mg/mL, *A. niger* at 32 mg/mL, and *F. solani* at 32 mg/mL. On the other hand, the MAHD-extracted essential oil had inhibitory effects on the growth of S. aureus, *A. niger*, and *F. solani* with minimum inhibitory concentrations of 32, 8, and 16 mg/mL, respectively. Additionally, the methanolic extract of *C. khorassanica* showed inhibitory effects on the growth of *F. solani*, *A. alternata*, *A. niger*, *S. aureus*, and *S. epidermis* with concentrations of 16, 32, 16, 32, and 32 mg/mL, respectively. The best inhibitory effects were observed against gram-positive bacteria and fungi, while all three tested samples had the lowest inhibition in gram-negative bacteria.

In a previous study conducted in 2022, the flavonoid salvigenin was extracted from *Cleome turkmena*, and its effect on inhibiting the growth of several bacteria and fungi was investigated using the minimum inhibitory concentration (MIC) and disc diffusion (DD) methods.[22] The best results were observed against the Gram-positive Bacteria *Bacillus pumilus* with a MIC value of 64 µg/ml, which was consistent with our results on *C. khorassanica*.

Additionally, previous studies on different ethanol, chloroform, ethyl acetate, and N-hexane extracts of the *C. turkmena* plant showed the best microbial inhibition against Gram-positive bacteria. The best result was reported in that study on the inhibition of extracts with polar solvents against *Staphylococcus epidermidis* and *Staphylococcus aureus.*[23] In another study conducted in 2021, the best inhibitory effect of the essential oil and ethanol extract of *Cleome brachycarpa* and *Cleome quinquenervia* was reported against *A. niger* and *F. solani* fungi.[15] It can be predicted that the genus Cleome has the best inhibition against gram-positive bacteria and fungi. In traditional medicine and local use, these plants are also used for their antifungal properties. The findings of the present study mostly support the green technology for the efficient extraction of *C. khorassanica* essential oil, the observations showed that the antimicrobial capacity in the MAHD method improves performance and inhibits the growth of microbes.

Table 3

Antimicrobial activity (MIC mg/ml) of various essential oil and extracts of *C. khorassanica*

Bacterial strain	Methanolic extract	Microwave essence	Hydrodistillation essence	ampicillin	gentamicin	fluconazole
Gram-negative bacteria						
E. coli	128 mg/ml	128mg/ml	256mg/ml	16 mg/ml	...	...
K. pneumonia	256 mg/ml	512mg/ml	512mg/ml	8 mg/ml	...	...
E. albertii	128 mg/ml	128mg/ml	256mg/ml	8 mg/ml	...	...
Gram-positive bacteria						
C. glutamicum	64 mg/ml	64mg/ml	128mg/ml	...	8 mg/ml	...
S.aureus	32 mg/ml	32 mg/ml	32mg/ml	...	16 mg/ml	...
S. epidermis	32 mg/ml	64mg/ml	64mg/ml	...	16 mg/ml	...
Fungi						
F. solani	16 mg/ml	16mg/ml	32mg/ml	...	...	8 mg/ml
A. alternata	32 mg/ml	8mg/ml	32mg/ml	...	...	8 mg/ml
A. niger	16 mg/ml	16mg/ml	32mg/ml	...	...	8 mg/ml

CONCLUSIONS

In conclusion, the present study provides valuable insights into the potential of Microwave-assisted hydrodistillation (MAHD) and Hydrodistillation (HD) methods for the efficient extraction of *C. khorassanica* essential oil. The results showed that the essential oil extracted by both methods had a strong inhibitory effect on the growth of various Gram-positive bacteria and fungi, making it a promising natural source of antimicrobial agents. The findings of this study support the use of green technology for the extraction of essential oils from *C. khorassanica*, which offers many benefits, including higher yield, shorter extraction time, and better quality of the extracted oil. The present study provides scientific evidence to support the traditional use of *C. khorassanica* as a natural source of antimicrobial agents. The results of this study could be useful for researchers, pharmaceutical companies, and food industries looking for natural and sustainable alternatives to synthetic antimicrobial agents. Overall, the findings of this study provide a strong case for the use of *C. khorassanica* as a natural source of antimicrobial agents, and the suggested methods of MAHD and HD for the extraction of essential oil from the plant could provide a valuable alternative to conventional methods.

Acknowledgements. We would like to express our sincere gratitude to the Sciences and Research Branch of the Islamic Azad University (IAU), Tehran, Iran, for their financial support.

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