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A Comparative Study of Extraction Methods and Components Identification of Teucrium Chamaedrys and Investigation of Their Antibacterial Effect

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KEYWORDS

Extraction
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Microwave-assisted
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HIGHLIGHTS

- We attempted to extract the essential oils from a species of herbs in Iran, called Teucrium Chamaedrys.
- Using hydrodistillation as a usual method, microwave-assisted, and headspace solid-phase microextraction as two new methods.
- Chromatography/mass spectrometry (GC/MS) was used to investigate the estimated compounds.
- The antibacterial effect against gram-positive bacteria of the essential oil was proved.

ABSTRACT

Ethnopharmacological relevance: Teucrium Chamaedryswas historically used as a medicinal herb for the treatment of gout and sometimes as a component of Venice treacle.

Aim of the study: Our aim was identification and extraction of components of Teucrium Chamaedrys using hydrodistillation as a usual method, microwave-assisted, and headspace solid-phase microextraction as two new methods and comparing their results. Chromatography/mass spectrometry (GC/MS) was used to investigate the estimated compounds. Finally, we study their antibacterial effect.

Results: 17 compounds were identified accounting for a total of 85.35% of the total essential oil obtained from the hydrodistillation method. In addition, 15 compounds were found accounting for 79.26% of the total essential oil, obtained from the solid phase microextraction. In the microwave method, the essential oil obtained did not have the required quantity and quality to identify.

Conclusion: By comparing the above results, it is concluded that more compounds can be isolated and identified through hydrodistillation.The results showed that the main composition of this plant is caryophyllene in different ways and a total of different forms of this composition in both methods showed that a higher percentage of these compounds is obtained in the steam distillation process, and factors such as plant drying and extraction method will have a significant effect on the extraction percentage. The antibacterial effect against gram-positive bacteria of the essential oil extracted by hydrodistillation and solid-phase microextraction methods was proved.

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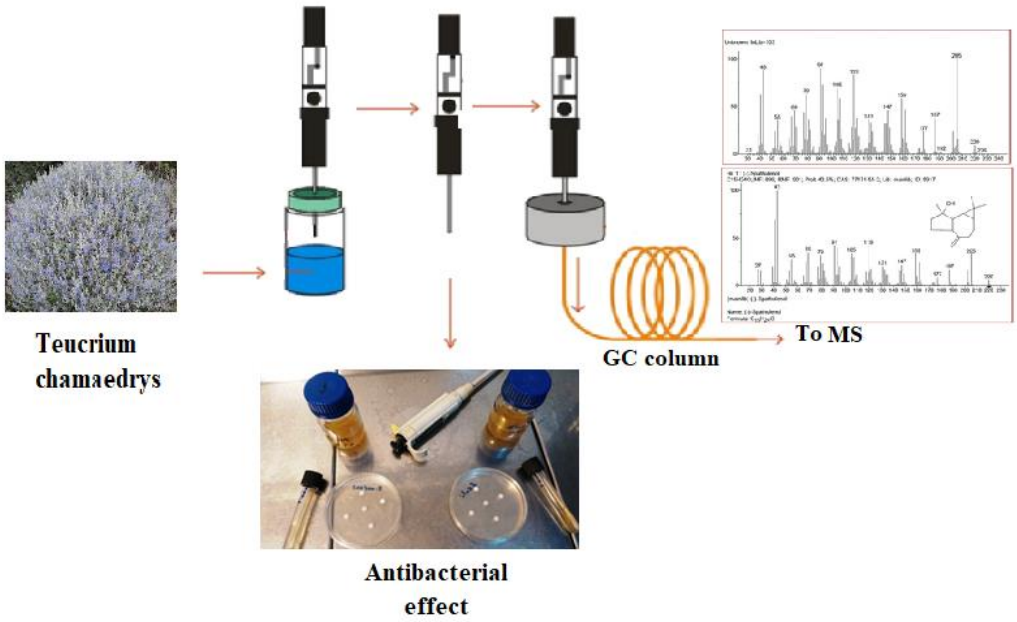
GRAPHICAL ABSTRACT



INTRODUCTION

Natural compounds found in the plant's cells, which play a basic role in the biological activities of plants,are known as primary metabolites, such as hormones and proteins, and secondary metabolites are the compounds that are found in plants under tense stress conditions, to cope with such conditions and continue to survive (like essential oils) [1].

Essential oils (essences) are a complex mixture of volatile organic chemicals with fat and heavy metal levels. They produce the smell or taste of the plant, the proper use of which requires good knowledge.Since the use of plants as sources of medicinal and food compounds requires the knowledge of their essential oils, these compounds must first be extracted from the plant. Different extraction methods are used to achieve the best results for each plant and each combination. A proper extraction method should lead to the maximum number and maximum amount of extraction. After extraction, the effective compounds in the extracted essential oil should be identified.The extraction of a chemical species of natural or laboratory samples for analysis or applications in food and pharmaceutical industries requires the removal of other chemical species existing in the sample. In other words, it is always necessary to purify the required chemical species for the purposes mentioned above. All physical or chemical processes used in this regard are referred to as isolation methods.

Since essential oils are widely used in the pharmaceutical, food, and healthcare industries, isolation methods have been evaluated to extract these active compounds from plant sources in a shorter time with lower solvent consumption. The shorter time required for extraction and separation reduces costs and the more stable the extracted compounds are. In addition to the extraction time, the amount of solvent used at this stage is also very important. Consumption of less solvent reduces costs and less damage to the environment. Extraction by microwaves (MAE) [2] and solid phase microextraction (SPME) [3]are very effective and efficient as novel methods for extracting active compositions from plant tissues. Each of these methods has its advantages and disadvantages. In this study, by using them and comparing the results, we

examined the efficiency of each to extract the essential oil of the Teucrium Chamaedrys plant.Considering the fact that many plant species in Iran contain essential oils, it is necessary to study further. Therefore, the essential oil of a type of herbs, Teucrium Chamaedrys, wasobtained by hydrodistillation (HD) as a traditional method, and microwave-assisted, headspace solid-phase microextraction as modern methods. The essential oils obtained were studied and identified by the gas chromatography-mass spectrometry (GC-MS) method.

The hydrodistillation extraction method is used as a basic method in extracting essential oils from plants due to its simplicity and available solvent. This method, despite the stated advantage, has a small extraction percentage. In contrast, modern methods have a high extraction percentage, lower solvent consumption, and a desirable extraction time, but are costly[4-8].

Essential oils are aromatic compounds widely used in the perfume, pharmaceutical, and food industries. Essences are a mixture of more than 200 different combinations. Essential oils are generally produced by aromatic plants. The most important plants producing essential oils belong to the Labiatae, composite, Umbelliferae, pine, and citrus [4].

Essential oils are produced and stored in various plant organs, depending on plant families in different organs, such as glandular trichomes (Labiatae) and parenchyma cells (Umbelliferae). Essential oils play an important role in plant protection as antimicrobial, antiviral, antimicrobial [5], and antifungal [6] agents, and also as insecticides and in-plant antiherbivore defense [7]. In addition, these compounds play a key role in attracting insect pollinators and the reproduction of plants. For example, bees are sensitive to natural smells, and the same applies to insects such as moths that are active at night [8].One of the properties of some essential oils is their antioxidant activity [9]. Studies have shown that the use of such oils can have a more effective biological effect, such as preventing cardiovascular disease, stop the cells from aging, and prevent cancer cells from growing and developing by removing free radicals [10].

Experimental section

Materials and instruments

The plant sample was prepared with the help of the Research Institute of Forests, and Rangelands and Rangeland Research Institute, Tehran, Iran. A complete sample with roots, stems, leaves, and flowers (generally, the main organ) was dried and sent to the Iranian Research Institute of Plant Protection, the Department of Plant Sciences, to determine the scientific name. Then, it was stored for 72 hours in the shade and at room temperature to dry. To extract the essential oil of the plant, the Clevenger apparatus was used for hydrodistillation (Fig. S1).

In the first method, the essence was extracted by a microwave apparatus (Ethos Plus, a milestone model with a power of 1000W and a frequency of 2450 Hz (Fig S2).

In the other method, the essential oil was separated by Suplco (SPME Fiver Assembly Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), 65 mμ (Fig. S3). To identify the essential oil obtained from the Hewlett Packard GC/MS system with the following specifications (Table S1), the following instruments and devices were used to perform microbial tests:AMSCO E599-383 autoclave from the USA, Lab-line 702 incubator made in the USA, FARPAJOUH Microbiological Safety Cabinetclass 2 made in Iran, Memert-UNB500 300 °C oven and Eppendorf research plus 1000 micropipette.

Chemicals and Reagents

Normal hexane solvent (Merck) was prepared to inject into the GC/MS system without any purification. Mueller-Hinton Agar was prepared for microbial testing as the culture medium, as well as two bacterial strains for the Iranian Research Organization for Science and

Technology with the following specifications:Müller Hinton culture medium (Merck), Microbial strain was prepared from the Industrial Research Organization of Iran with the following numbers; Escherichia Coli (ATCC: 10536, PTCC: 1338) and Staphylococcus aureus (ATCC: 6538, PTCC: 1112), Dimethyl sulfoxide Culture medium (Merck), Sodium without water (Merck).

HD Preparation

400 g of the plant's aerial organ was extracted from Clevenger in 5 hours. Dehydration was performed on organic oils obtained with water-free, sodium sulfate (Na_2SO_4).

MAE Preparation

To extract the essential oil of the plant, 250 g of the plant's dried aerial organ was grounded in and placed in the microwave apparatus for 20 minutes in MAE for extracting the essence. Dehydration was performed from the essential oils with water-free, sodium sulfate (Na_2SO_4).

SPME Preparation

To extract the essential oil of the plant understudy, in the SPME method (Fig. S3), 200 g of the plan's aerial organ, not completely dried, was placed in the Circulator and exposed to 50 °C for 30 minutes. After that, the SPME syringe (Suplco, PDMS-DVB-poly dimethylsiloxane-vinyl benzene fiber, 65 µm) was placed in the headspace for extraction of essential oil and absorbing compounds by the fiber.After dehydration, the oil extracted was diluted (rate: 1 to 10) with the normal hexane solvent for qualitative and quantitative analysis. The samples prepared were injected by chromatography-coupled mass spectrometry.

Antibacterial properties

In the current study, the agar diffusion (disk diffusion) method was used to study the antimicrobial effect of the extracted essential oils according to Neels M62-A7 protocol. In the agar diffusion method, the bacterium was uniformly cultured on the surface of the Mueller-Hinton agar medium, then placed on the disk surface at a distance of 2-4 cm from the material under study with a specific concentration, and then incubated enough and examined. Therefore, the material was then absorbed into the medium after a specific time and an inhibition zone (halo) would form around the disk proportional to the strength of the antibiotic in inhibiting the growth of bacteria if the bacterium was sensitive to the material [11].

Bacterial culture medium preparation

To prepare disks, Whatman Filter Paper No.1 was used. The disks are about 6 mm in diameter. After punching the paper filter, the disks were placed on a glass plate and completely sterilized for 2 hours at 170 °C. Then, the dilution of 1 to 3 of the essential oils was done in the Dimethyl sulfoxide (DMSO) solvent and the disks were impregnated with 15 µl of the essential oil diluted by micropipette.

First, we poured a certain amount of Merck agar powder into an Arlen disk containing the appropriate amount of distilled water and shook it well to completely dissolve the powder. The powder was dissolvedin distilled water to make it completely transparent by heating. After closing the container, it was placed in an autoclave to sterilize (at 121°C for 15 minutes). After autoclave and cooling (about 40-50 °C) under the hood, we transferred 25 to 30 ml of the medium into a 10 ml plate and continue so that the depth of the environment reached up to approximately 4 cm. These media were kept at room temperature to cool down and completely become stiff. The refrigerator can be used if the media are to be kept longer.

Escherichia Coli was used as a Gram-negative bacterium and Staphylococcus aureus as a Gram-positive bacterium (Table S2). The lyophilized strains were opened under sterile conditions and the hood class 2 and injected 2 ml of Tryptic Soy Broth medium into a lyophilized ampoule, and after creating a suspension, 50 ml of fluid and

lyophilizedbacterial cells were injected into the Tryptic Soy Broth. We then incubated it at 35 °C for 24 hours. After the complete growth of the bacteria in the liquid medium, we cultivated it on the Tryptic Soy agar solid medium by using a microbiological loop. After 24 hours, 3 to 5 colonies were separated from the plate surface and prepared in 4 to 5 ml of sterile suspension with 0.5% MacFarland turbidity (0.9% NaCl). According to the McFarland turbidity standard, 0.5% MacFarland is equivalent to 1 to 2 CFU (Colony Forming Unit) per milliliter of Escherichia coli. To prepare 0.5 McFarland, 1.175% of barium chloride ($BaCl_2.2H_2O$) was poured into a 100 ml volumetric flask with 1% sulfuric acid. The solution is stable at room temperature and in the dark for 6 months.

Diffusion

We inserted a sterile swab into a bacterial suspension and, after removing the extra solution, cultivated it in different directions on the culture medium with an angle of approximately 60 degrees so that no blank space would remain on the medium and the bacterium was cultivated uniformly on the medium. After drying the surface of the medium, which should not last more than a quarter, we placed the disks at a distance from the medium with sterile pins, and after 18 hours at 35 °C, and measured the zones of (growth) inhibition with a caliper (Fig. 1).

Results and Discussion

The essential oils obtained from different extraction methods were placed into a gas chromatography machine with a mass spectrometer, and the chromatograms were obtained.

Fig 1. The culture medium and prepared discs.

The constituents of each essential oil are various alkenes or terpene groups or complex and organic compounds that are separated in a gas chromatogram according to the temperature equilibrium and detected by a mass spectrometer device under the library of the device, which includes the standard range of thousands of compounds.In the essential oil obtained using Hydrodistillation, 17 compounds totaled 85.35% of the total essential oil were identified. The main constituents of essential oil in this method are listed in Table 1.

The mass spectrum obtained from the GC/MS system and the standard range of most of the compounds in the essence of Teucrium Chamaedrys obtained through the Hydrodistillation method are shown in Figure 2 as an example and Figures S4 to S9.

In the essential oil obtained through SPME, 15 compounds totaled 72.263% of the total essential oil were identified. The main constituents of essential oil in this method are listed in Table 2.

The mass spectrum obtained from the GC/MS system and the standard range of most of the compounds in the essence of Teucrium Chamaedrys obtained through the SPME method is illustratedin Figure 3 as examples and Figures S10 to 15.

Table 1. Composition of the percentage of substances contained in the plant oil's organic base oil Teucrium Chamaedrys from Hydrodistillation.

Compound	**R.T**	**%**
γ-Terpinene	10.25	0.57
Thymoquinonp	10.50	1.71
Undecane	11.78	3.23
α-Caryophyllene	12.18	13.81
Spathulenol	13.60	6.01
Naphthalenepropanol	13.87	0.79
Dodecane	14.48	6.80
Caryophyllene oxide	16.52	5.01
Hexahydrofamesly Acetone	17.78	10.90
Spathulenol	18.77	0.35
Caryophyllene	19.12	20.71
Hexadecanoic acid	19.74	0.63
Bicyclo (3.1.1) dimethyl	20.26	0.67
Docosane	20.44	5.19
Bicyclogermacrene	21.78	2.83
Germacrene-D	23.09	4.44
2-Pentadecanone, 6,10,14-trimethyl	24.86	2.00
Total	**17Compounds**	**85.35%**

Table 2. Combination of the percentage of substances contained in the plant oil's organic base oil Teucrium Chamaedrys derived from the SPME method.

Compounds	**R.T**	**%**
Undecane	4.490	7.056
Dodecane	8.501	5.910
Cyclobuta[1,2:3,4] Dicyclopentene	11.264	4.540
Caryophyllene	11.756	16.233
Bicyclo[3.1.1] dimethyl	11.854	6.575
1,6,10-Dodecatriene	12.034	3.963
α-Caryophyllene	12.184	7.025
1H-Cyclopenta[1,3]cyclopropa [1,2]benzene	12.510	2.244
Bicyclogermacrene	12.729	4.871
Spathulenol	13.730	5.171
Caryophyllene oxide	13.808	9.389
Oxabicyclo [9.1.0]dodeca	14.099	2.410
tau.-Cadinol	14.415	2.995
2-Pentadecanone, 6,10,14-trimethyl	16.464	1.642
Naphthalenepropanol	19.514	1.805
Total	**15Compunds**	**72.263%**

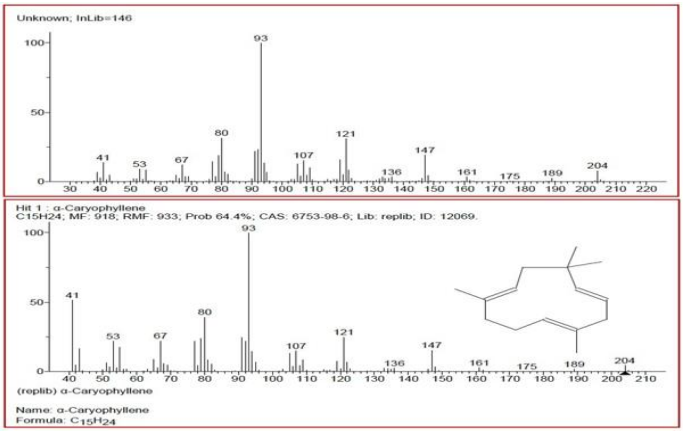


Fig 2. The mass spectrum obtained from the GC/MS device and the standard α-Caryophyllene spectrum after extraction from the upper space

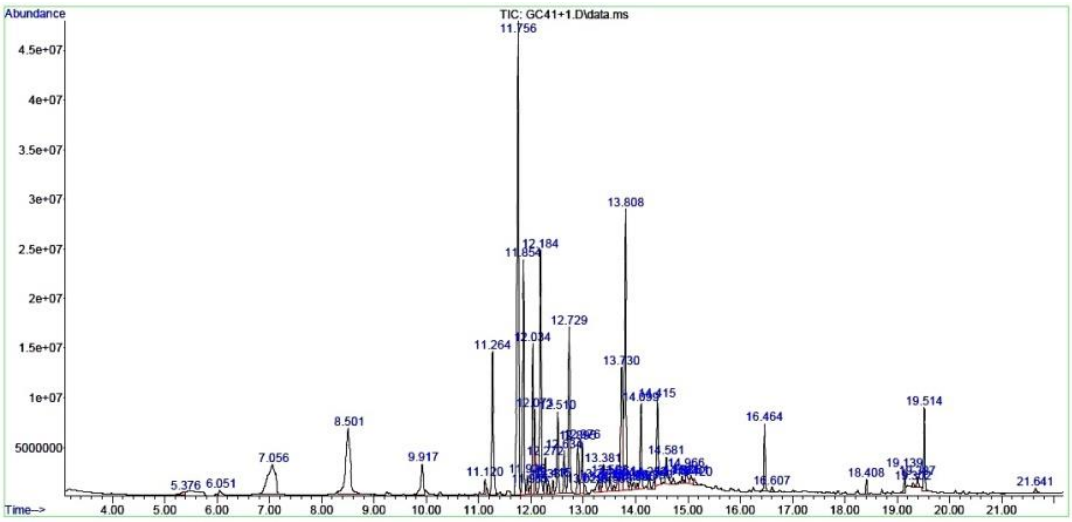


Fig 3.GC-MS Chromatogram of Teucrium Chamaedryscomponents after extraction from machine GC:HP-6890 and MASS:HP-5973. SPME-fiberP-5MS (5% phenyl dimethylsiloxane) where AP isaminopropyl and SPME is solidphase microextraction. Experimental conditions: sample weight, 200 g; extraction time, 3 min; microwave power, 70 W

Examining effective parameters

To obtain the best and most effective extract, it is necessary to pay attention to some of the properties of the herbal substance, the proper solvent selection, and the accuracy of the extraction steps, and it should be kept in mind that the high yield in the extract does not mean high yield of the composition in the extract. Medicinal herbs contain many secondary compounds that often have important biological activity and since plant extracts are widely used in the pharmaceutical, food, and healthcare industries, extraction technologies are evaluated to better extract these active compounds from plant sources. Traditional methods such as soxhlet extraction that has been used for many years is very time-consuming and consumes a lot of solvents[12]. Therefore, there is a high demand for new extraction methods that provide shorter time, lower solvent consumption, and environmental protection. New extraction methods are more common today, such as extraction with microwave waves, [13] extraction with ultrasound [14], and solid phase microextraction [15].

Anti-bacterial Test

The results of the experiment on the effect of essential oil of the culture medium on the gram-positive and gram-negative bacteria are in Figure 4 and Table 3.

Effect of essential oil on two different methods of extraction of Teucrium Chamaedrys on gram-positive and gram-positive grams of bacteria, which was carried out by disc diffusion method, showed that the growth hole of both essential oils on the gram-positive bacteria has a larger diameter, and this result showed the effect of different combinations of caryophyllene that has antibacterial properties in the face of bacteria and has been confirmed in studies of this kind.

The results of the test of the effect of essential oil on the culture medium on the gram-positive and gram-negative bacteria are as follows: Essential oil No. 1 wasobtained from the Hydrodistillation method and Essential oil No. 2 was obtained from the solid phase microextraction.

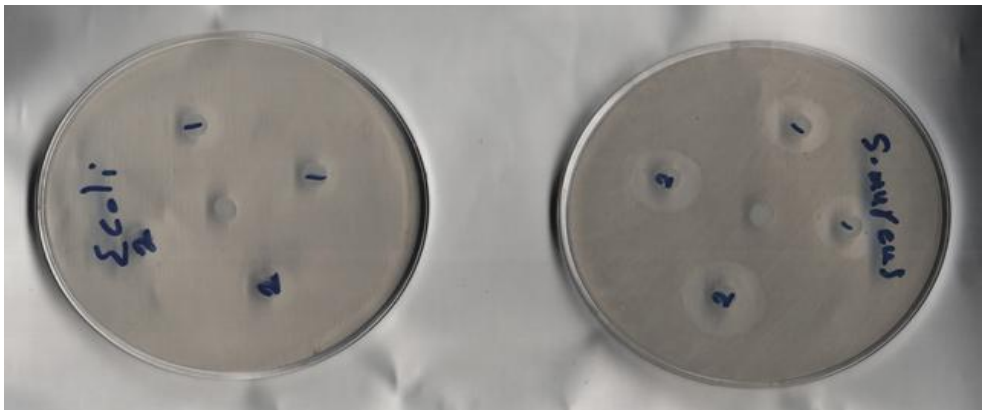


Fig 4. Non-growth halo in two culture media. The left side of the gram-negative bacterium and the right side of the gram-positive bacterium.

Table 3. Results of the diameter of the growth holes in two replicates.

Diameter of growth DMSO (mm)	Diameter of growth 2 (mm)		Diameter of growth 1(mm)		ATCC	Bacteria name	row
0	7.10	9.30	7.44	8.14	10536	Escherichia Coli	1*
0	15.31	15.80	12.05	12.36	6538	Staphylococcus Aureus	2**

1 * = Extraction essential oil from Hydrodistillation

2 ** = Extracting essential oil from the solid phase micropropagation method

In this research, in addition to performing traditional extraction methods, new methods of extracting essential oils from the plant in question are examined simultaneously, as well as differences. It has some advantages over other researches.The table below shows the differences between this study and several similar studies.

Table 4: Comparative table of results of several extraction methods

Research	Subspecies matching	Number of extraction methods	Microbial tests
[16]	×	✓	×
[17]	✓	✓	×
[18]	×	×	✓
[19]	✓	✓	✓

Conclusion

Different extraction and separation techniques are used to extract essential oils in plants, and for each plant and each condition, one method extracts and identifies the best and most compounds. In addition, many native plants of Iran have not been studied for essential oil extraction. To find the best way to extract the most essential oils of the plant Teucrium chamaedrys, we used different extraction methods. In addition, to identify the extracted compounds, we used the GC/Ms device, which has the highest quality and sensitivity in identifying the structure of the compounds.

In this study, the volatile compounds in Teucrium chamaedrys were studied and analyzed by a Clevenger apparatus and a microwave system based on solid phase microextraction. In the essential oil obtained through hydrodistillation, 17 compounds were identified, which together accounted for 85.35% of the total essential oil. In addition, in the essential oil obtained through solid phase microextraction, 15 compounds were identified totaled 79.26% of the total of the essential oils with the frequencies listed in the tables. In the microwave method, the essential oil did not have the required quantity and quality for identification.

By comparing the above results, it is concluded that more compounds can be isolated and identified using steam distillation. The result showed that the main composition of this plant is caryophyllene in various methods. The various forms of this composition in both methods indicated that a greater percentage of these compositions are obtained in the process of steam distillation, and factors such as complete drying of the plant, the type of solvent, and the solid phase in different methods of extraction will have a significant effect on the extraction percentage.

The diameter of the inhibition halo revealed that although the essence obtained by SPME was less than that of the Hydrodistillation method, the quality of the caryophyllene compositions strengthened the anti-bacterial effect. In other words, the solid-phase micro-extraction method, as a new method of extraction, fulfills the qualitative requirement of the essential oil.

The study of the effect of essential oil obtained through the two methods on the Gram-negative and gram-positive bacteria carried out by disk diffusion method showed a larger diameter of the inhibition hale of both essential oils on the gram-positive bacteria that is indicative of the effect of different caryophyllene compositions, which shows antibacterial properties facing bacteria. The effect has been confirmed in similar studies.

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