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Antimicrobial activity of the essential oils of *Lavandula stoechas* flowers extracted by microwave

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Abstract
Lavandula stoechas genus is an important member of family Lamiaceae, are widely distributed in Golestan province of IRAN. *Lavandula* has been used in traditional medicine to treat various diseases. The chemical compounds of essence were identified by GC-MS analysis. In order to evaluate the antimicrobial effect of essential oil, Broth microdilution (MIC determine the minimum inhibitory concentration) methods was used. The antimicrobial activity was tested against on Gram Positive and Negative Bacteria (*staphylococcus aureus, bacillus cereus, enterococcus faecalis, salmonella enteritidis, escherichia coli* and *pseudomonas aeruginosa)*. The yield of essential oil by microwave under optimum condition was obtained 0.112%. 28 components with 98.93% yields have been identified. The main compounds of essential oil obtained camphor, 1,8-Cineole, Linalool and borneol. The essential oil of *L. stoechas p.* showed antibacterial activity against most of the tested standard bacteria strains expects *pseudomonas aeruginosa* and *enterococcus faecalis*. *Staphylococcus aureus* and *bacillus cereus* with 29 and 32 mm respectively diameter was showed the highest sensitivity. MIC and MBC methods also show that all bacteria have the same minimum inhibitory and fatality concentrations except Enterococcus facials with minimum inhibitory concentration of 1.32 and minimum concentration fatality of 1.16. The results showed that the essential oil of *Lavandula stoechas* was revealed highly inhibitory antimicrobial activity especially on gram positive bacteria and can be used instead of chemical drugs to treat bacterial infections.

Keywords: *L. stoechas* ssp, essential oil, camphor, antimicrobial effect.

1. Introduction
Plants are important in human life because of their roles in the treatment of different illnesses. Nowadays, due to side effects of chemical drugs attention to pharmaceutical plants has increased. *Lavandula stoechas* belong to Lamiaceae family [1], which are distributed through the world, especially Mediterranean regions, south of Franch and Torento [2]. In Iran are found in Golestan, Khorasan and Mazandaran provinces. *Lavandula stoechas* is an important economic plant source of essential oil is used as natural antimicrobials. Antimicrobials activity of oil related to the monoterpenoid compounds and Linalool, 1,8-Cineole, Camphor, Terpinene-4-ol were the major component in the oil [3-4]. Previous studies on the essential oil of this genus which was collected from Tonesia and Turkey mainly contained Fenchone and Camphor [5-7]. α-pinene, 1,8-cineole, Fenchone, Camphor and Myrtenyl acetate were the major component in the essential oil of *Lavandula stoechas P.* flower [8]. There is some essential oil studies on Lavandula species which grow in Turkey, the recent ones were performed by supercritical fluid extraction [8-9]. The essential oil of the Greek *Lavandula stoechas* was reported by Kokkalou [10]. The study on *Lavandula stoechas* ssp. *stoechas* was its about nonvolatile compounds**,** afforded tri terpenoids [11]. Microwave-assisted extraction (MAHD) is an important alternative method in the extraction of different compounds such as essential oil and other organic compounds from plants. Because of its advantages which mainly are reduced extraction time and solvents, selectivity, lowering of energy costs and high yields have attracted much interest particularly from the viewpoints of green chemistry. In this work, much effort has been focused on the extraction of essential oils from the flower of *Lavandula stoechas P.* which is harvested in Jahan-Nama Mountain in Gorgan city (Golestan province, Iran) by using MAHD method. In the present study, the essential oil of *Lavandula stoechas* obtained by MAHD, investigated by GC-MS analysis and evaluated for its antibacterial activities. To the best of our knowledge it is the first report on the extraction of volatile oils from the flower of *Lavandula stoechas P.* by MAHD and bioactive constituents of Iranian *Lavandula stoechas*.

2. Material and Methods

2.1 Sample Collection: The flower of *Lavandula stoechas* P. were collected from the Jahan-Nama Mountain, 85 km from Gorgan (Golestan province) on April 2014. A voucher specimen was deposited in the Herbarium of the Faculty Sciences, Golestan University, Gorgan, Iran. The harvested materials from the flower were air- dried in shaded place at ambient temperature (25 °C) and air circulation. The air-dried material was mixed and grounded into a homogeneous fine grade powder by disintegrator. The average particle size was 0.7 mm**.**

2.2 Sample Extraction: Extraction of the essential oil was performed using multi-mode microwave (Microsynth. 1000 W, Milestone), which was equipped with Clevenger-type apparatus, temperature controller and time controller. 80 g of sample were placed in a 1000 mL flat bottomed flask and added 600 mL distilled water. The flask containing the sample was set to allow heating by microwave oven for 35 minutes and power 400 W. The yield of the oil was obtained 0.112%. At the end, the extracted was dried and placed into the test tube under refrigeration until its analysis.

2.3 Essential Oils Chemical Composition: Chemical composition of essential oil was determined by gas chromatography (Agilent technology model 7890 N) coupled to mass spectrometry detector (5975 CEI) (GC-MS). A capillary column HB-5 (30 m long, 0.25 mm diameter and 0.25 µm film thicknesses) was employed, using Helium carrier gas at constant flow of 4 mL/min. The injector and detector temperature were 260 °C and 300 °C respectively. The column temperature was set at 60 °C for 4 min, 100 °C at a rate of 3 °C/min for 2 min and then programmed until 225 °C at a rate of 4 °C/min isothermal at this temperature for 10 min. The components of the essential oil were identified by comparing their mass spectra with those in the mass spectra libraries and comparison of individual relative retention times by comparing their relative retention index to series of n-alkanes (C8-C26). For quantification purpose, the percentage composition of the oils was computed by the normalization method from FID areas calculated as mean values of three injections of oil. The individual identified components with their relative percentages by MAHD are given in Table 1.

2.4 Antimicrobial activity: Antimicrobial activity of *Lavandula stoechas* P. oil was determined by using well diffusion [12] and broth micro dilution (MIC determine the minimum inhibitory concentration) methods. Standard bacteria strains *Enterococcus faecalis (PTCC 1393), bacillus cereus (ATCC 1247) and Staphylococcus aureus* (*col*) as gram-positive and *Pseudomonas aeruginosa* (*PTCC 1214*), *Escherichia coli* (*PTCC 1399*) and *salmonella enteritidis (PTCC 1639)* as gram-negative bacteria were used [13]. For antimicrobial susceptibility methods described below, the bacterial suspensions were prepared and the turbidity was adjusted equivalent to a 0.5 McFarland standard.

Because of insolubility of oil in water, Arabic gum was used as solvent. 250 mg Arabic gum dissolved in a 100mL water then 1500 µL oil add to 1500 µL of solution and the mixture was vigorously shacked. Prepared solution was used as Stock solution (1/2 dilution) and accordingly diluted to 1/4-1/64 of the oil. For the disk diffusion method, 100 µl of the 0.5 McFarland suspension prepared above was inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate (pH 5.9) with a sterile cotton-tipped swab to form an even lawn. Sterile papers disks (6 mm in diameter) impregnated with 40 µl diluted solution were placed on the surface of each MHA plate using a sterile pair of forceps. The plates were incubated aerobically at 37 °C for 24 h. The diameter of inhibition zone was measured after 24 h incubation using a ruler or caliper.

In Broth micro dilution method, 125 mg Arabic gum dissolved in a small amount of distilled water and 500 µL oil add to 500 µL of solution and the mixture was vigorously shacked. Prepared solution was used as Stock solution (1/2 dilution) and accordingly diluted to 1/4-1/512 of the oil. Susceptibility panel in 96-well microtiter plates were prepared by dispensing 100 µl of oils solutions with the highest concentrations into the column wells.

In MIC, MBC method, 10^5 cfu/mL dilutions of suspension bacteria were used. 100 µL of essence and 100 µL suspension bacteria was added in Elisa well the Gram positive and negative bacteria were cultivated on Mueller-Hinton agar. Using a sterile Pasteur's pipit, 100 µL diameter wells with defined distances were made on Mueller-Hinton agar media. Then, by a sampler 100µL of the dilution of 1/2-1/64 were poured in wells. Plates were incubated in 37 °C for 24 hours and then antimicrobial activity was evaluated by measuring the zone of inhibition around the disc against the test microorganism. The Arabic gum solution (2.5 g/L) as negative control and gentamicin antibiotic (10 μgr) as positive control was used and different dilutions of essential oil of *Lavandula stoechas P.* were treatments. To ensure, each concentration of essential oils and antibiotics of experiment was repeated three times for each bacterial strain.

3. Results and discussion

3.1 Chemical composition of essential oil

The hydrodistillation of the leaves of *Lavandula stoechas P.* gave yellowish oil with a yield of 0.08 (w/w). Oils were subsequently analyzed by GC and GC/MS and the individual identified chemical composition, their retention indices and relative percentages are listed in table 1. In the oil 29 components were characterized representing 98.93% of the total oil. As shown in table 1 the main constituents of the essential oil was comphor (60.53%) 1,8-cineole (11.48%), linalool (4.22%) and borneol (4.10%).

Table 1: Compared the Chemical composition the essential oil from *Lavandula stoechas* P. extracted by MAHD

No.	Compounds	Yield (%) MAHD	Retention Index (RI)	Time (min)
1	Pinene***α-***	0.67	936	6.7
2	Camphene	0.81	952	7.2
3	Verbenene	-	963	7.4
4	Pinene***β-***	0.66	978	8.2
5	Trans-Ocimene***β-***	0.08	1055	8.8
6	Cymene	0.2	1025	9.4
7	1,8-Cineole	11.48	1033	10.5
8	Linalool Oxide	1.35	1082	12.3
9	O-Methylphenylol	0.49	1086	12.7
10	Thujone***α-***	1.12	1103	12.9
11	Linalool	4.22	1101	13.9

12	Camphor	60.53	1145	15.9
13	Borneol	4.10	1168	16.7
14	Citronellal	0.74	1175	17.1
15	Cryptone	1.4	1182	17.5
16	Bicyclo [3.1.1] hept-2-ene-2-carboxaldehyde, 6,6-dimethyl	0.7	1189	17.9
17	Myrtenol	0.56	1193	18.1
18	Eucarvone	0.58	1207	18.6
19	Carveol	0.86	1226	19.4
20	Propanal, 2-methyl-3-phenyl	1.02	1234	20.3
21	l-Carvone	0.78	1240	20.5
22	Phellandral	0.6	1276	22
23	Anethole	0.32	1284	22.6
24	Cuminol	0.67	1301	23.3
25	Neryl alcohol	0.55	1437	25.9
26	β-Selinene	0.85	1487	30.4
27	Caryophyllene oxide	1.35	1584	33.5
28	Delta-Cadinene	0.46	1520	35.4
29	β-Eudesmol	1.78	1652	35.6

Grouped compounds	MAHD (%)
Monoterpens hydrocarbon	2.22
Oxygenated monoterpenoid	88.17
Sesquiterpens hydrocarbon	1.31
Oxygenated sesquiterpenes	3.13
Aromatic and phenolic compounds	2.7
Ketones	1.4
Total	98.93

The constituents of *Lavandula stoechas* P. was identified as hydrocarbon monoterpenes (2.22%), oxygenated monoterpenes (88.17%), hydrocarbon sesquiterpenes (1.31%), oxygenated sesquiterpenes (3.13%), phenolic and aromatic compounds (2.7%) and ketones (1.4%). Compare to the other genus of *Lavandula* it has been obtained rich proportion of oxygenated monoterpenes in oil.

The GC chromatogram of essential oil from the flower of *Lavandula stoechas* P. by MAHD is shown in Fig. 1 and which revealed the presence of 29 compounds (98.93% of total oil) in table 1. The major compounds identified in the oils were comphor (60.53%), 1,8-cineole(11.48%), linalool(4.22%) and borneol (4.10%).

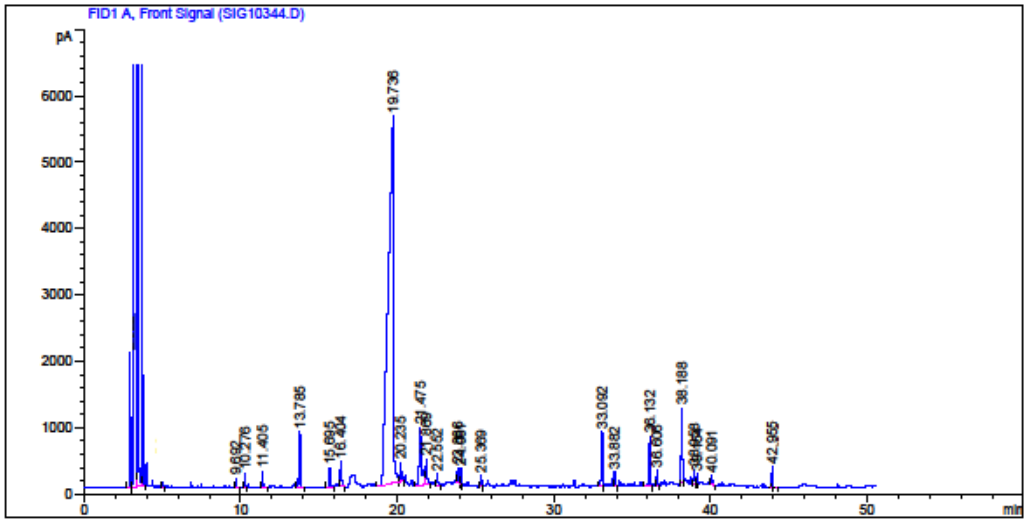

Fig 1: GC chromatogram of the essential oil from *Lavandula stoechas* P. oil extracted by MAHD

3.2 Antibacterial activity

Antimicrobial Activity of *Lavandula stoechas* oil Determined by Disk Diffusion and the results are given in mm as zone diameter by disc diffusion method. The essential oil was tested against standard bacterial strains, and showed mild activity mainly against the pathogens Salmonella enteritidis and Escherichia coli, among which Staphylococcus aureus was the most affected. Staphylococcus aureus was the sensitive bacteria to the dilution of, 1/32 and 1/64. Antibacterial activity against most of the tested standard bacterial strains except, Pseudomonas aeruginosa, and Enterococcus faecalis (Table 2). The result showed that Pseudomonas aeruginosa and Enterococcus faecalis were resistant to tested essential oils. It has been demonstrated that Gram-positive bacteria (S. aureus and B. cereus) are more susceptible to essential oils than Gram-negative ones. The fact that Gram-negative organisms are less susceptible to the action of antibacterials has been ascribed to the presence of a hydrophilic outer membrane surrounding the cell wall membrane which blocks penetration of hydrophobic essential oils into target cell membrane [14]

Table 2: Antimicrobial Activity of *Lavandula stoechas* oil Determined by Disk Diffusion

	Test microorganism					
Amount of essential oil (µL)	***Salmonella enteritidis* (PTCC 1639)**	***Bacillus cereus* (ATCC 1247)**	***Escherichia coli* (PTCC 1399)**	***Staphylococcus aureus* (col)**	***Pseudomonas aeruginosa* (PTCC 1214)**	***Enterococcus faecalis* (PTCC 1393)**
½	14	29	17	32	-	-
¼	11	21	10	25	-	-
1/8	10	19	10	20	-	-
1/16	9	12	9	14	-	-
1/32	***0***	***0***	***0***	7	-	-
1/64	***0***	***0***	***0***	7	-	-

Inhibitions are expressed in mm and include the diameter of the paper disc

Table 3 reports the Minimum Inhibitory Concentration (MIC) and the Minimum Bacterial concentration MBC values of the essential oils against 4 Gram positive and Gram negative bacterial strains. *Lavandula stoechas P. oil* exhibited potent antibacterial effect against contaminating microorganisms, *in vitro*.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Lavandula stoechas* essential oil against selected bacteria and the yeast by broth microdilution method

Pathogen	MIC	MBC
Staphylococcus aureus	1.51	1.26
Escherichia coli	1.32	1.32
Bacillus cereus	1.64	1.32
Salmonella enteritidis	1.32	1.16

The MIC of the essential oil against the tested microorganisms (except for Pseudomonas aeruginosa and Enterococcus faecalis in which the oil was ineffective in tested concentrations) was 1.32- 1.64 µL/mL in microdilution methods. The lowest concentration in which more than 99.9% reduction in microbial counts was observed for all microorganisms (except for Pseudomonas aeruginosa and Enterococcus faecalis) was 1.16 – 1.32 µL/mL of the essential oil, which was recorded as MBC. Biological activity of essential oils depends on their chemical composition, which is determined by the genotype and influenced by environmental and agronomic conditions. The activity of oils could be ascribed to the higher content of Camphor, in agreement with the reported relationship between activity and the presence of some components, especially 1, 8-Cineole, Linalool and Borneol [15, 16]. 1, 8-Cineole, borneol, camphor, and α-/β-thujone extracted from Salvia officinalis L. chiefly contribute to the anti-inflammatory activity of sage infusion in human gingival fibroblasts [17]. These active compounds such as Linalool being considered by a number of studies as possessing a strong antimicrobial effect [16-18] and key odorant molecule with valuable biological properties [20]. Many essential oils high in linalool and beta-pinene are well known for their ability to reduce sad and anxious feelings [21]. Borneol is a natural product with strong inhibitory effects on proinflammatory TRPA1 noxious cold-sensor ion channels [22]. Essential oils represent complex mixtures of chemical compounds with different antimicrobial properties, and for these reasons it is very difficult to reduce their antimicrobial effect to one or several active principles [5]. Our results demonstrated that the use of of *Lavandula stoechas P*. flower essential oil in concentrations higher than MIC values (i.e. >1.5 µL/mL) can reduce the risk of food poisoning due to consumption of contaminated products. This finding confirms the interest of these plants for pharmaceutical and nutraceutical applications.

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5. References

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