

Investigation of Composition and Antimicrobial Properties of *Lavandula stoechas* Essential Oil Using Disk Diffusion and Broth Microdilution

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ABSTRACT

Background and Objective: *Lavandula stoechas* is a species of native and permanent plants in Golestan province that belongs to the family *Lamiaceae*. *L. stoechas* has been used in traditional medicine for treatment of various diseases. The aim of this study was to extract essential oil using steam distillation method from the flowers of *L. stoechas* collected from Jahan-nama region in the Golestan province, and evaluate its antibacterial activity.

Methods: Steam distillation (Clevenger) and GC-MS system were used to separate volatile oils and identify the essential oil components, respectively. Two methods of disk diffusion and broth micro dilution were used to evaluate the antimicrobial effect of *L. stoechas* essential oil. Six bacterial species including *Staphylococcus aureus*, *Bacillus sp.*, *Enterococcus faecalis*, *Salmonella enteritidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were tested.

Results: The essential oil yield was 0.28%. The main components were camphor (71.86%), 1, 8-cineole (4.08%), linalool (3.77%) and borneol (3.19%). The essential oil showed no inhibitory effect on *P. aeruginosa* and *E. faecalis*, while it had different inhibitory effects on other bacteria. *S. aureus* and *Bacillus sp.* showed the highest sensitivity with inhibition zone diameter of 32 and 29 mm, respectively.

Conclusion: The results of this study showed that the essential oil of *L. stoechas* has high inhibitory and antimicrobial activity particularly against Gram-positive bacteria, which may be due to the presence of 71.86% camphor in its composition.

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

INTRODUCTION

Nature has been a rich source of medicinal compounds, some of which are stored in plants. Among these important compounds are the extracts or essential oils that have numerous biological effects. Favorable, rational and efficient use of such resources is simpler and less expensive than chemical pharmaceutical industry. It can also provide a major part of society's healthcare needs and prevent the withdrawal of large amounts of money from the country. *L. stoechas* is a species of plant in the family *Lamiaceae* (1). *L. stoechas* grows in most parts of the world and is abundant in the south of France, the Mediterranean region and Toronto. It appears in different types depending on the soil and environmental conditions (2). In Iran, *L. stoechas* can be found in three provinces, Golestan, Khorasan and Mazandaran. Its flowers and flowering branches can be used. The components of essential oil of *L. stoechas* flowers include: α -Pinene, Camphene, Verbenene, β -Pinene, β -Trans-Ocimene, Cymene, 1,8-Cineole, Linalool Oxide, O-Methylphenylol, α -Thujone, Linalool, Camphor, Borneol, Citronellal, Cryptone, Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde, 6,6-dimethyl, Myrtenol, Eucarvone, Carveol, Propanal, 2-methyl-3-phenyl, 1-Carvone, Phellandral, Anethole, Cuminol, Neryl alcohol, β -Selinene, α -Terpinene, Caryophyllene oxide, Delta-Cadinene, β -Eudesmol and α -Bisabolol. According to traditional medicine, *L. stoechas* has a warm and dry nature with carminative, antidepressant, antiseptic, stomach tonic and antibacterial properties. It can also relieve muscle spasms, soothe the nerves, relieve insomnia, enhance the memory, eliminate forgetfulness, stimulate blood flow, relieve asthma and control growth, dizziness and convulsions. The diluted *L. stoechas* is also used in dressing wounds and injuries (3). Vakilian et al. showed that inhalation of *L.*

stoechas essential oil has a direct impact on reducing labor pain. The result of their study indicated that aromatherapy could be a suitable alternative for other alternative pain relief therapies such as massage, acupuncture, electrical stimulation of the skin, etc. (4). Traditional methods include distillation, pressure, abrasion, scarification, organic solvent extraction and extraction by hydrolytic enzymes. New methods include supercritical extraction using carbon dioxide, ultrasound-assisted extraction, accelerated solvent extraction, extraction using hot water and headspace extraction. Choosing the suitable method for the extraction of essential oils is performed based on the type and part of plant used for the extraction. Essential oils are generally extracted by distillation of aromatic plants (5). Essential oils eliminate microorganisms by destroying cell wall and proteins, causing dysfunction in membrane enzymes, affecting DNA and RNA production, etc. (6). The indicator bacteria used in this study include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Enterococcus faecalis* and *Salmonella enteritidis*. *S. aureus* causes a wide range of infections from simple skin infections such as pimples and boils to life-threatening diseases such as pneumonia and meningitis. *P. aeruginosa* causes soft tissue infections, bacteremia and bone and joint infections. *E. coli* causes food poisoning and diarrhea. *E. faecalis* causes endocarditis and urinary tract infections. *S. enteritidis* causes paratyphoid disease. These bacteria were tested as environmental pathogens. Given the medicinal, antimicrobial and antibacterial importance of *L. stoechas*, this study aimed at extracting essential oil from the plant (collected from the mountains of Jahan-nama in the Golestan province) and investigating its antimicrobial effects.

Figure 1- *L. stoechas* in Jahan-nama region



MATERIAL AND METHODS

The plant's flowers were collected in May 2013 from the Jahan-nama Mountains (at a distance of 85 km from Gorgan in the Golestan province) and then shade dried. The steam distillation method (Clevenger) was used for extracting the essential oil. First, 150 grams of dried and ground flowers were placed in a 1-liter flask along with 700 ml of distilled water. Essential oil extraction continued for 5 hours (7). After the extraction, 0.431 grams of essential oil were obtained from 150 grams of dried plant. The resulting essential oil yield was 0.28%. The essential oil was then poured into dark vials and stored at 4 °C until the time of analysis.

Essential oil yield = weight of the resulting essential oil / weight of dried plant in grams × 100 The resulted essential oil was injected into gas chromatograph with temperature programmed for complete separation of the essential oil constituents. The retention index of constituents and their percentage were calculated. A GC instrument with flame detector (manufactured by Agilent Co., model 7890A) with HP-5 column (30m × 320µm × 25µm) and mobile phase nitrogen were used to analyze and identify terpenes. GC-MS system used was with gas chromatography [Manufactured by Agilent Co. Model 7890N] connected to spectrometer Model 5975 CEI, which has split inputs, mobile phase Helium and HP-5MS column (30m × 250µm × 25µm) similar to the columns used in the gas chromatograph. Temperature program for the column was as follows: initial temperature of the column was maintained at 60 °C for 4 minutes, then increased to 100 °C at rate of 3 °C per minute and remained at this temperature for 2 minutes, and then became constant at 225 °C with rate of 4 °C per minute. Both injection and detection were carried out at 260 °C. Column's carrier gas flow was at linear velocity of 1 ml/min. Identification of essential oil components was done using mass spectroscopy and their comparison with mass spectra of standard compositions and information available at GC-MS system's library (8). Disk diffusion method: this method was done according to CLSI standard. The method is briefly described below : Essential oil dilution: Since the essential oil is water insoluble, gum arabic

solution was used, which does not affect bioassays. In addition, 0.25 grams per hundred gum arabic solution was used at ratio of 1:1 to dissolve essential oil. This solution was diluted with gum arabic to prepare 1/2 to 1/512 serial dilutions. Preparation of discs containing essential oil: 40 µl of each concentration of the essential oil were separately added to each 6mm sterile paper disk (Padtan Teb Co.), and then kept at 37 °C for 30 minutes to be absorbed. Preparations of microbial suspension: The studied microorganisms included three Gram-positive bacteria: *E. faecalis* (PTCC 1393), *B. cereus* (ATCC 1247) and *S. aureus* (Standard col) and three Gram-negative bacteria: *S. enteritidis* (PTCC 1639), *P. aeruginosa* (PTCC 1214) and *E. coli* (PTCC 1399). The bacteria were cultured and after ensuring about the purity, bacterial suspension equivalent to 0.5 McFarland standards (1.5×10^8 cfu/ml) was prepared. Investigating the antibacterial effect: Using a sterile swab, 100 ml of the prepared suspension was spread on Mueller-Hinton agar and cultured uniformly. The discs impregnated with different concentrations of essential oil were placed on the surface of agar medium. The plates were incubated for 24 hours at 37 °C, and the diameter of inhibition zone around the discs was measured in millimeters. All stages of the experiments for each bacterial strain were repeated three times and the average of the triplicates was calculated. Gentamicin and gum arabic solution disks were used as positive and negative control, respectively. Determination of minimum inhibitory concentration and minimum bactericidal concentration (MIC, MBC): For this purpose, bacterial suspension with dilution of 10^5 cfu/mL was used. Then, 100 µl of the intended dilution of the essential oil and 100 µl of the bacterial suspension were added to each ELISA well. ELISA plates were incubated for 24 hours at 37 °C. The lowest concentration at which no turbidity was observed was determined as the MIC. In order to determine the MBC, culture was performed on nutrient agar medium from dilutions at which no turbidity was observed. The plates were incubated at 37° C for 24 hours. MBC was defined as the concentration at which colony count was zero.

RESULTS

L. stoechas essential oil yield was 0.28% and 31 compounds were identified that accounted for 98.14% of the total essential oil. Table 1 shows the components of *L. stoechas* essential oil along with retention time (RT) and percentage of each compound. The main components of the essential oil included camphor (71.86%), 1, 8-cineole (4.08%), linalool (3.77%) and borneol (3.19%). The effect of different concentrations of the essential oil in the disk diffusion method on *S. enteritidis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* are shown in Table 3.

The inhibitory effect of each concentration against each bacterium was evaluated by measuring the diameter of inhibition zones. The results showed that dilutions of 1/2, 1/4, 1/8 and 1/16 of the essential oil had the strongest inhibitory effect against *S. aureus* followed by *Bacillus SP.*, *E. coli* and *S. enteritidis*. The essential oil at dilutions of 1/32 and 1/64 had inhibitory effect only against *S. aureus*. *P. aeruginosa* (Gram-positive) and *E. faecalis* (Gram-negative) were resistant to all dilutions of the essential oil and no inhibition zone was observed in their cases.

Table 1- The chemical composition of *L. stoechas* essential oil extracted by Hydrodistillation

R.T (min)	Yield (%)	compounds	Entry
6/671	1/17	α -Pinene	1
7/185	1/21	Camphene	2
7/38	0/38	Verbenene	3
8/217	1/06	β -Pinene	4
8/797	0/18	β -Trans-Ocimene	5
9/341	0/3	Cymene	6
10/44	4/08	1,8-Cineole	7
12/263	0/85	Linalool Oxide	8
12/695	0/14	O-Methylphenylol	9
12/916	0/72	α -Thujone	10
13/876	3/77	Linalool	11
15/935	71/86	Camphor	12
16/659	3/19	Borneol	13
17/127	0/33	Citronellal	14
17/501	0/6	Cryptone	15
17/933	0/4	Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde, 6,6-dimethyl-	16
18/128	0/16	Myrtenol	17
18/58	0/18	Eucarvone	18
19/381	0/44	Carveol	19
20/259	0/69	Propanal, 2-methyl-3-phenyl-	20
20/475	0/39	l-Carvone	21
22	0/77	Phellandral	22
22/601	0/19	Anethole	23
23/315	0/37	Cuminol	24
25/939	0/15	Neryl alcohol	25
30/432	1/35	β -Selinene	26
31/315	0/34	Alpha.-Terpinene	27
33/544	1/05	Caryophyllene oxide	28
35/383	0/16	Delta.-Cadinene	29
35/66	1/23	β -Eudesmol	30
36/61	0/43	α -Bisabolol	31

RT (seconds): the time between sample injection and washing of each component.

Table 2- Antimicrobial activity of *L. stoechas* essential oil determined by disk diffusion method

Amount of essential oil (µL)	Test microorganism					
	<i>S. enteritidis</i> (PTCC 1639)	<i>B. cereus</i> (ATCC 1247)	<i>E. coli</i> (PTCC 1399)	<i>S. aureus</i> (col)	<i>P. aeruginosa</i> (PTCC 1214)	<i>E. faecalis</i> (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	-	-

Table 3- MIC and MBC of *L. stoechas* essential oil against the selected bacteria and yeast by broth microdilution method

Pathogen	MIC	MBC
<i>S. aureus</i>	1.51	1.26
<i>E. coli</i>	1.32	1.32
<i>B. cereus</i>	1.64	1.32
<i>S. enteritidis</i>	1.32	1.16

Unit: µg/mL

DISCUSSION

Skoula et al. reported α -pinene, 1, 8-cineole, fenchone, camphor and myrtenyl acetate as the main components of *L. stoechas* flowers' essential oil (9). Zuzarte et al. extracted the essential oil of *L. stoechas* flowers by steam distillation and reported fenchone (37%) and camphor (27.3%) as the main components (10). Kirmizbekmez et al. also used the same method for essential oil extraction and reported alpha-fenchone, myrtenyl acetate, alpha-pinene, camphor and 1, 8-cineole as the components (11). Rasouli et al. reported linalol, 1, 8-cineole, camphor and terpinene-4-ol as the components of *L. stoechas* essential oil. They also evaluated the antimicrobial activity of the essential oil and reported that its antibacterial activity is due to presence of monoterpenoids (12). Bouzoutia et al. extracted essential oil from dried leaves of *L. stoechas* in Tunisia and reported fenchone (68.2%) and camphor (11.2%) as its components. Moreover, evaluation of the essential oil's antibacterial activity against six bacteria showed that *S. aureus* is more sensitive than the others (13). Akgun et al. extracted the essential oil of *L. stoechas* flowers in Turkey using carbon dioxide (supercritical fluid) and reported that it is mainly consisted of camphor and fenchone (70%) (14). Nasrolahi omran et al. extracted the essential oil of *L. stoechas* by steam

distillation (using Clevenger apparatus), and evaluated the antibacterial activity of the plant by the diffusion method (15). Dob et al. reported the *L. stoechas* essential oil yield of 1.1% in Algeria (16). Fadaei et al. evaluated the antibacterial activity and chemical composition of *L. angustifolia* and reported linalol, 1, 8-cineole, camphor and terpinene-4-ol as the components of this essential oil. The results of their study showed that this essential oil has equal antimicrobial activity against *E. coli* and *B. subtilis* (17). In the present study, the essential oil yield was 0.28, while in the report by Dob et al. it was 1.1%. This difference could be due to the plant's habitat conditions (16). The main component of the essential oil of *L. stoechas* was identified as camphor, which is consistent with the result of Akgun et al. (14). In Kirmibekmez et al. report, fenchone was reported as the main component of *L. stoechas* essential oil. This difference may be related to differences in habitat conditions at sample collection location (11). Bouzoutia et al. reported that camphor (11.2%) and fenchon (68.2%) are the main components of *L. stoechas* essential oil. This difference can be attributed to differences in habitat conditions and plant parts that were used for essential oil extraction (leaves were used in this study) (13). Rasouli et al. reported linalool (36.9%) as the main component of *L.*

angustifolia essential oil. This difference may be attributed to differences in the species and antibacterial properties of this plant due to presence of monoterpenoids (12). The results of evaluating the antibacterial property of *L. stoechas* essential oil in the present study are similar to previous studies (10, 11). Based on the results, it can be stated that *L. stoechas* essential oil has higher antimicrobial effects on *S. aureus*.

CONCLUSION

It seems that the antimicrobial properties of *L. stoechas* essential oil can be attributed to

the presence of 71.86% camphor in its composition. The results of this study showed that this plant has strong antibacterial property with the greatest effect against *S. aureus*.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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