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The composition and antibacterial activity of the essential oil of *Levisticum officinale* Koch flowers and fruits at different developmental stages

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Abstract: The composition and antibacterial activity of the essential oil of *Levisticum officinale* Koch at different developmental stages (flower, immature fruit, green mature fruit and ripened fruit) is reported. The essential oils were obtained by hydrodistillation of air-dried samples and their antibacterial activities were tested against seven bacteria. The yield of oil (w/w %) in different stages was in the order: immature fruit (1.5 %) > green mature fruit (1.0 %) > > ripened fruit (0.6 %) > flower (0.1 %). The essential oils were analyzed by GC and GC–MS. In total, 27, 31, 28 and 26 constituents were identified and quantified in the mentioned samples, respectively. Monoterpene hydrocarbons were the main group of compounds in the green mature fruit (79.2 %), immature fruit (78.4 %), ripened fruit (75.2 %) and flower (44.0 %). The antibacterial activity of the oils was evaluated by the disk diffusion method using Müller–Hinton agar and determination of inhibition zones. The results of the bioassays showed some variations between the three tested oils in their inhibitory activity against the tested bacteria at a 10 µl disc^{-1} concentration. The oils from mature and ripened fruit exhibited potent antibacterial activity against *Bacillus subtilis*, with minimum inhibitory concentration (*MIC*) values of 0.90 mg ml^{-1} in mature and ripened fruits.

Keywords: Levisticum officinale Koch; Apiaceae; essential oil; antibacterial activity; reproductive stage.

INTRODUCTION

Lovage (*Levisticum officinale* Koch) is a perennial herbaceous plant from the Apiaceae family with origins in Iran and Afghanistan; it can now be found throughout the world.[1–4] The plant has been alternatively classified as *Ligus-*

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ticum levisticum L., *Hipposelinum levisticum* Britt. and *Angelica levisticum* Baillon.[5] The essential oil of roots, seeds and leaves of lovage are used in a wide variety of applications including food flavoring, medicinal preparations, aromatherapy, perfumery and industrial fragrances.[2,6,7] Moreover, the plant is used in Iranian folk medicine for the treatment of several gastrointestinal, nervous and rheumatic disorders.[2,8] The essential oil composition of the plant was previously studied in different countries and more than 190 compounds were reported in its root, seed or leaf oil.[9] It was found that the chemical compositions of the essential oils distilled from separate botanical parts of this plant are rather different.[10–12] The chemical constituents of lovage root oil are mainly phthalides including *n*-butylidene phthalide and *n*-butyl-phthalide, sedanonic anhydride, terpenoids such as α-terpineol, carvacrol, phenylpropanoids such as eugenol and volatile acids.[5,13,14] Polyacetylenes as antimycobacterial compounds have also been reported from the plant.[15] The effect of harvesting time, plant age, cutting frequency and the method of plantation establishment on the essential oil yield and components in different parts of *L. officinale* was investigated previously.[7,11,16,17] It was found that the flowers and seeds produced the highest yields of the oil with β-phellandrene (40.8 and 61.5 %, respectively) as the main constituent, while α-terpinyl acetate (≈70.0 %) was reported as the principal constituent of the leaves and stems oils.[7] In another study, the oil of lovage fruits contained β-phellandrene (69.3 %), α-terpinenyl acetate (4.2 %) and α-terpineol (2.1 %) as the major components.[12] It was reported that the essential oil content was similar in roots, stems, petioles, leaves and inflorescence, while the highest content was found in seeds (1.9 %).[18] Seasonal variations in the composition of headspace volatiles were also determined,[10] of which, β-phellandrene was the most abundant component in all plant parts except for root. Samiee *et al*. reported terpinyl acetate (40.5 %) and β-phellandrene (16.7 %) as the main constituents in the essential oil and β-phellandrene (23.0 %), naphthalene (20.6 %) and γ-terpinene (12.1 %) as the major components in the methanol extract of the plant from Iran.[19] Recently, (*Z*)-falcarinol, *n*-octanal, palmitic acid, (*Z*)-ligustilide, (*Z*)--3-butylidenephthalide, *trans*-β-farnesene have been reported as the main compounds of the essential oil of hairy root cultures of *L. officinale*.[20–22] Variations in the essential oil composition of roots and leaves of *L. officinale* from different European countries have also been studied. Ten compounds, including *trans*-*p*--mentha-2,8-dien-1-ol, *iso*-thujyl alcohol, *p*-mentha-1,5-dien-8-ol, bicyclo[3.2.0]-heptan-3-ol, 2-methylene-6,6-dimethyl, *trans*-carveol, perillaldehyde, sabinyl acetate, perillyl alcohol, the methyl ester of methylpentadecanoic acid and methylhexadecadienoic acid, were introduced for the first time.[23] To the best of our knowledge, there is no previous report on the essential oil analysis and antibacterial activity of *L. officinale* at different developmental stages. Thus, in this pa-

per, the composition and antibacterial activity of the essential oils of this plant at different stages of its development are reported.

EXPERIMENTAL

Plant material

These experiments were conducted during 2007–2009 at the field of the Medicinal Plants and Drugs Research Institute of Shahid Beheshti University, located in Evin (35°48' N, 51°23' E at an altitude of 1785 m) in the north of Tehran, Iran. The plant seeds were obtained from the seed bank of the Medicinal Plants and Natural Products Research Institute, Iranian Academic Center for Education, Culture and Research (ACECR) and were sown in a greenhouse in the last week of February, 2007. Nine-week-old seedlings were transplanted at 50 cm row-to-row and 30 cm plant-to-plant spacing in the experimental field in May, 2007. The sampling was realized from a 2-year-old cultivated population by the random collection of 10 individuals for each developmental stage. For the collection of the flowers, all of them on the inflorescence were opened. The samples at the fruiting stage were collected at three different times of fruit maturation, *i.e*. immature (infructescence with young fruits 15 days after flowering), mature (infructescence with solid and dark green colored fruit) and ripened (infructescence with yellowish fruits just in the deciduous time). Voucher specimens (No. 200364-7) representative of each sample were deposited at the Medicinal Plants and Drugs Research Institute Herbarium (MPH), Shahid Beheshti University of Tehran.

Essential oil isolation procedure

The essential oil of air-dried samples (100 g) of each stage was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (1993).[24] The isolated oils were dried over anhydrous sodium sulfate and stored in dark tightly closed vials at 4 °C until analysis.

Essential oil analysis procedure

GC analysis was conducted using a Varian CP-3800 instrument equipped with a DB-1 fused silica capillary column (25 m×0.25 mm i.d., film thickness 0.25 μm). Nitrogen was used as the carrier gas at a constant flow rate of 1.1 ml min^{-1}. The oven temperature was held at 60 °C for 1 min, then programmed to 250 °C at a rate of 4 °C min^{-1}, and then held for 10 min. The injector and detector (FID) temperatures were kept at 250 and 280 °C, respectively. GC/MS analysis was realized on a Thermoquest-Finnigan Trace GC/MS instrument equipped with a DB-1 fused silica column (60 m×0.25 mm i.d., film thickness 0.25 μm). The oven temperature was raised from 60 to 250 °C at a rate of 5 °C min^{-1} and then held at 250 °C for 10 min; the transfer line temperature was 250 °C. Helium was used as the carrier gas at a flow rate of 1.1 ml min^{-1}; the split ratio was 1/50. The quadrupole mass spectrometer was scanned over the 45–465 amu range with an ionizing voltage of 70 eV and an ionization current of 150 μA.

Identification and quantification of the oil components

The constituents of the essential oils were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C6–C24) and the oil on a DB-1 column under the same chromatographic condition. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds (purchased from Sigma-Aldrich and Merck) or with those reported in the literature.[25] For quantification purpose, the relative area percentages obtained by FID were used without the use of correction factors.

Antibacterial activity

The antibacterial activity of the oils was evaluated by the disk diffusion method using Müller-Hinton agar[26] and determination of the inhibition zones. The essential oils were tested at a concentration of 10 μl per disk. The microorganisms used were as follows: *Bacillus subtilis* ATCC 9372, *Enterococcus faecalis* ATCC 15753, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27852, and *Klebsiella pneumoniae* ATCC 3583. For the determination of the minimum inhibitory concentration (*MIC*), a microdilution broth susceptibility assay was used, as recommended by NCCLS.[27] The technical data were described previously.[28] Ampicillin was used as the standard reference for the positive control.

RESULTS AND DISCUSSION

Essential oil analysis

The essential oils had a light yellow color with distinct sharp odor. The yield of the essential oils (w/w %) of the plant at different developmental stages were in the order: immature fruit (1.5 %) > green mature fruit (1.0 %) > ripened fruit (0.6 %) > flower (0.1 %). The qualitative and quantitative analytical results are listed in Table I together with the retention indices of the identified compounds, where all the constituents are arranged in order of their elution on the DB-1 column. In total 27, 31, 28 and 26 constituents, respectively, were identified and quantified in the studied samples representing 95.9, 99.9, 98.7 and 92.7 % of the total oil, respectively. A comparison among the composition of the essential oils revealed both quantitative and qualitative differences. The GC and GC–MS analyses showed that the distribution of saturated hydrocarbons of the oil from flower was remarkably different from that of the oils at the fruiting stage. The results revealed that the saturated hydrocarbons from the flower (12.3 %) were present in higher amount than in the other samples. Heneicosane (6.0 %) and tricosane (3.0 %) were found only in the oil of the flower. The major constituent of the oil of the flower was *β*-pinene (17.7 %), but it was found that this compound decreased gradually in subsequent developmental stages. The *β*-pinene and *α*-pinene contents were highest in the essential oil of the first harvest and decreased with progressive maturation of the fruit. On the contrary, *β*-phellandrene was found as the principal component of the oil after fruit initiation, *i.e.*, it constituted 11.7 % of the oil of the flower but increased remarkably in the fruit oils, constituting 62.4, 60.5 and 56.4 % of the green mature, immature and ripened fruit oils, respectively. *β*-Phellandrene has already been reported as the main constituent in the essential oil from the flowers and fruits in previous reports.[7,10,12] *β*-Gurjunene (2.8 %), globulol (0.7 %) and geranyl acetate (3.3 %) were found only in the oil of the flower. *α*-Phellandrene, *δ*-elemene and germacrene-D were absent completely in the oil of flower but were present in trace or low amounts in the other samples. The essential oil obtained from immature fruit contained the highest contents of sabinene (2.3 %), isomenthol (5.6 %), *cis*-dihydrocarvon (0.6

%), germacrene-D (0.6 %), elemol (0.5 %) and *trans*-nerolidol (1.6 %) compared with the other samples.

TABLE I. Composition of the essential oil of *Levisticum officinale* at different developmental stages

RI[a]	Compound	From flowers %	In fruiting stages, %			Identification methods
			Immature	Mature	Ripened	
0935	*α*-Pinene	5.3	4.6	4.3	2.9	RI, MS[b], CoI[c]
0969	Sabinene	1.7	2.3	1.5	1.1	RI, MS
0976	*β*-Pinene	17.7	11.5	4.1	2.9	RI, MS, CoI
0982	Myrcene	1.3	t[d]	t	t	RI, MS
1002	*α*-Phellandrene	–	t	t	t	RI, MS
1010	*δ*-3-Carene	3.0	2.8	5.7	6.8	RI, MS
1017	*ortho*-Cymene	1.4	t	t	t	RI, MS
1026	*β*-Phellandrene	11.7	56.4	62.4	60.5	RI, MS
1038	*cis*-Ocimene	1.9	0.8	0.8	0.8	RI, MS
1083	Terpinolene	–	–	0.4	0.2	RI, MS, CoI
1111	*cis*-*p*-Menth-2-en-1-ol	–	0.3	–	0.2	RI, MS
1162	Isomenthol	1.8	5.6	3.9	2.1	RI, MS
1166	4-Terpineol	0.5	0.6	–	–	RI, MS
1168	*cis*-Dihydrocarvone	–	0.6	–	–	RI, MS
1217	Cuminyl aldehyde	–	0.5	t	–	RI, MS
1265	*p*-Cymene-7-ol	–	0.4	t	–	RI, MS
1339	*δ*-Elemene	–	0.4	0.3	0.3	RI, MS
1358	Geranyl acetate	3.3	–	–	–	RI, MS, CoI
1385	*α*-Copaene	1.3	0.3	0.3	0.2	RI, MS
1392	*β*-Elemene	1.0	1.2	1.3	1.3	RI, MS
1433	*α*-Humulene	0.9	0.4	0.3	0.2	RI, MS
1436	*β*-Gurjunene	2.8	–	–	–	RI, MS
1446	(*Z*)-*β*-Farnesene	4.3	0.2	0.2	0.2	RI, MS
1473	*γ*-Curcumene	8.0	0.2	0.2	0.3	RI, MS
1484	Germacrene-D	–	0.6	0.3	t	RI, MS
1489	Zingiberene	0.8	0.5	0.8	t	RI, MS
1500	Germacrene-B	0.8	1.6	t	1.2	RI, MS
1503	*β*-Bisabolene	1.6	0.8	1.8	–	RI, MS
1518	*β*-Sesquiphellandrene	2.1	1.2	2.4	5.1	RI, MS
1541	Elemol	–	0.5	–	–	RI, MS
1548	*trans*-Nerolidol	0.8	1.6	–	–	RI, MS
1562	*γ*-Elemene	–	0.8	0.8	1.2	RI, MS
1574	Spathulenol	8.9	1.1	1.9	2.7	RI, MS
1593	Globulol	0.7	–	–	–	RI, MS
1601	Hexadecane	3.3	2.1	2.5	2.2	RI, MS
1691	3-*iso*-Thujopsanone	–	–	2.5	0.3	RI, MS
2097	Heneicosane	6.0	–	–	–	RI, MS
2301	Tricosane	3.0	–	–	–	RI, MS
–	Monoterpene hydrocarbons	44.0	78.4	79.2	75.3	–

TABLE I. Continued

RI[a]	Compound	From flowers %	In fruiting stages, %			Identification methods
			Immature	Mature	Ripened	
–	Oxygenated monoterpenes	8.9	8.0	3.5	2.3	
–	Sesquiterpene hydrocarbons	20.3	9.0	9.1	10.7	
–	Oxygenated sesquiterpenes	10.4	2.4	4.4	2.2	
–	Other	12.3	2.1	2.5	2.2	
Total identified		95.9	99.9	98.7	92.7	

[a]Retention indices relative to C_6–C_{24} *n*-alkanes on a DB-1 column; [b]mass spectroscopy; [c]co-injection with authentic compounds; [d]trace, less than 0.1 %

The classification of the identified compounds based on functional groups is summarized at the end of Table I and shows that monoterpene hydrocarbons were the main group of compounds in all samples. The monoterpene hydrocarbons content was the lowest in the flower and increased with subsequent harvesting times to reach maximum in the mature fruit and then decreased in the ripened fruit. In this study, the oil from green mature fruit contained β-phellandrene in higher amount (62.4 %) than the oils from ripened fruit (60.5 %), immature fruit (56.4 %) and flower (11.7 %). The other major monoterpene hydrocarbons which were found in all samples were β-pinene, α-pinene and δ-3-carene, while in another study, α-terpinenyl acetate, α-terpineol, limonene and myrcene were reported as the major monoterpenes.[12] Monoterpene hydrocarbons identified in the oil of flower were present in lower amount than in the oil of other stages. On the other hand, in the essential oil of flower, sesquiterpenes were one of the dominant fraction with spathulenol (8.9 %) as the major compound and their percentage decreased with progressive fruit maturation.

Antibacterial activity

The disk diffusion method, used in preliminary screening of the antibacterial activity, showed that the oils from the three different fruiting stages of *L. officinale* were active against all the tested bacteria. Moreover, the oils proved to be highly active against the tested Gram-positive bacteria, especially *B. subtilis* that was more sensitive than others, and the Gram-negative bacterium, *E. coli* (Table II). The oils were moderately active against *K. pneumoniae* and *P. aeruginosa*. The antibacterial activities of the oils were also determined using the microtiter 96-well dilution method, by measuring the minimal inhibitory concentration (*MIC*) against the tested bacteria (Table II). The essential oils of mature and ripened fruits exhibited the highest activity against *B. subtilis* with an *MIC* value of 0.90 mg ml^{-1}. In addition, the highest activity of the oil of mature fruit was observed against *S. epidermidis*, with an *MIC* value of 0.90 mg ml^{-1}. The oils showed the

lowest activity against *K. pneumoniae* and *P. aeruginosa*, with the *MIC* values ranging from 14.4 to 15.2 mg ml^{-1}.

TABLE II. Antibacterial activity of *L. officinale* essential oil at different fruiting stages

Microorganism	Essential oil[a]						Ampicillin[b]
	Immature		Mature		Ripened		
	DD[c]	*MIC*[d]	*DD*	*MIC*	*DD*	*MIC*	*DD*
B. subtilis	25.0±0.9	3.8	36.0±0.5	0.9	35.0±0.4	0.9	14.0±0.7
E. faecalis	19.0±0.7	15.2	17.0±0.4	7.5	13.0±0.4	7.2	11.0±0.4
S. aureus	17.0±0.5	3.8	21.0±0.5	3.7	16.0±0.5	3.6	13.0±0.6
S. epidermidis	23.0±0.7	1.9	26.0±0.5	0.9	25.0±0.6	1.8	19.0±0.5
E. coli	19.0±0.6	15.2	18.0±0.4	7.2	15.0±0.7	7.2	12.0±0.5
K. pneumoniae	10.0±0.8	>15.2	10.0±0.7	>14.4	9.0±0.4	>14.4	–
P. aeruginosa	11.0±0.7	>15.2	8.0±0.6	>14.4	9.0±0.5	>14.4	9.7±0.7

[a]Tested at a concentration of 10 µl disc^{-1}; [b]tested at a concentration of 10 µg disc^{-1}; [c]diameter of inhibition zone (mm) including disk diameter of 6 mm: inactive (–), moderately active (7–14) and highly active (>14); [d]minimum inhibitory concentration, values as mg ml oil^{-1}

CONCLUSIONS

The study of a plant as a source of aromatic and flavoring compounds requires the analysis of not only the whole plant but also its individual parts at their different developmental stages. While the best harvesting time of lovage to obtain sharp odorant compounds such as α- and β-pinene is the flowering stage, β-phellandrene as the main compound of the plant with a peppery-minty and slightly citrusy odor was achieved at the fruiting stage. Chemical characterization and antibacterial screening studies on the plant-based essential oils could also lead to the discovery of new natural antibacterial agents. In addition to perfume and tobacco products, the essential oil of lovage is used as a flavor agent in major food products, such as beverages, frozen dairy desserts, candy, gelatins and pudding, and meat and its products. Although the antimycobacterial activity of polyacetylenes, such as falcarinol and falcarindiol, from the plant has recently been studied,[15] the present study is the first report on the antibacterial activity of the essential oil from fruits of *L. officinale* at different developmental stages. The oils showed promising antibacterial activity against, *B. subtilis* and *S. epidermidis*. The present results revealed that the essential oils tested represent an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent the growth of bacteria and extend the shelf life of processed food.

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ИЗВОД

САСТАВ И АНТИБАКТЕРИЈСКА АКТИВНОСТ ЕТАРСКОГ УЉА ЦВЕТА И ПЛОДА БИЉКЕ *Levisticum officinale* Koch У РАЗЛИЧИТИМ РАЗВОЈНИМ ФАЗАМА

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Одређиван је састав и антибактеријска активност етарског уља биљке *Levisticum officinale* Koch у различитим развојним фазама: цвет, незрео плод, зелени зрео плод и потпуно зрео плод. Етарско уље је добијено из сувих узорака дестилацијом воденом паром, а антибактеријска активност је одређивана спрам седам врста бактерија. Принос уља (масени %) у различитим фазама је био следећи: незрео плод (1,5 %) > зелени зрео плод (1,0 %) > потпуно зрео плод (0,6 %) > цвет (0,1 %). Састав етарског уља је одређиван методама GC и GC–MS. У ова четири узорка је идентификовано и квантификовано редом 27, 31, 28 и 26 састојака. Монотерпенски угљоводоници су чинили главну групу једињења: 79,2 % у зеленом зрелом плоду, 78,4 % у незрелом плоду, 75,2 % у потпуно зрелом плоду и 44,0 % у цвету. Антибактеријска активност уља је одређивана методом прстенасте дифузије у Милер-Хинтоновом агару мерећи зону инфибиције. Коришћено је 10×10 µl уља за инхибицију и резултати су били различити за уља добијена из биљке у различитим развојним фазама. Најјача антибактеријска активност је испољена спрам *Bacillus subtilis*. *MIC* вредност је била 0,90 mg ml^{-1} са уљем потпуно зрелог плода.

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