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In vitro Propagation and Volatile Compound Characterization of *Lavandula stoechas* L. subsp. *stoechas*- An Economically Important Source of Essential Oil

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Abstract: The objective in this study is to isolate and characterize volatile compounds of *Lavandula stoechas* L. subsp. *stoechas* rooted with micropropagation techniques and grown under suitable *in vitro* conditions. Microdistillation procedure was applied on aerial parts of this plant. The volatile compounds were characterized by GC-FID and GC-MS systems, simultaneously. Based on results, twenty-two compounds were identified. Major volatile compounds included camphor (38.5%), bornyl acetate (10.6%), α-fenchone (8.9%), 1,8-cineole (4.3%), α-pinene (4.0%), linalool (3.5%), viridiflorol (3.8%) myrtenal (2.7%), geranyl acetate (2.1%). These results were positively related with data published regarding the plant growing wild.

Keywords: *Lavandula stoechas* subsp. s*toechas*; tissue culture; microdistillation; volatile compounds.

1. Introduction

Natural products can be found everywhere and are still gaining popularity, and the use of plant products such as extracts, essential oils, fixed oils, etc. in industrial applications has been rising. In addition, people are paying more attention to natural products for preventing and/or treating of various diseases or promoting of well-being. Medicinal and aromatic plants-based natural products are very popular. Being one of the most attractive families, Lamiaceae includes many well-known herbs, shrubs and rarely tree or vines, of horticultural and economic significance [1]. Lamiaceae family contains about 240 genera and around 7200 species around the world. Many members of Lamiaceae are cultivated in dry, mild and cold districts of Asia, Europe and North Africa due to their aromatic qualities and ease of cultivation [2,3]. This family contains thousands of flowering species widely used in landscapes and butterfly gardens [2]. Most species of this family produce essential oils secreted by glandular hairs on aerial vegetative and some reproductive organs [4].

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Essential oils, obtained as secondary metabolites, are highly complex mixtures of volatile compounds. Essential oils constitute a major group of agro-based industrial products, and they find applications in several types of industries including food products, drinks, perfumes, pharmaceuticals and cosmetics [5]. Essential oils exert many benefits, such as a pleasant aroma, especially in perfumes and to impart shine or conditioning in a hair care products, and for emolliency or improving the elasticity of the skin [6].

Typical secondary metabolites of Lamiaceae include various terpenoids, especially mono-, sesqui-, di- and tri-terpenes. A typical and most characteristic feature of most Nepetoideae is the production and accumulation of comparably large amounts of volatile monoterpenes, which are usually sequestered in specialized glands and trichomes. Few genera produce sesquiterpenes, furthermore, biologically active diterpenes have been found in some members of the Nepetoideae. Diterpenes are the interest in the isolation of these compounds is due to their biological activity, ecological and taxonomic function and use as templates for synthesis [1].

Essential oils distilled from members of the genus *Lavandula* have been used for cosmetics and therapeutic purposes for centuries. It is widely employed in all types of soaps, lotions and perfumes, with the most commonly used species being *Lavandula angustifolia*, *L. latifolia*, *L. stoechas* and *L*. x *intermedia* [6]. *Lavandula stoechas* L. known as French lavender grows perennially and is widely found in countries of Mediterranean region. It widely grows in Marmara, Aegean and South East Anatolian regions of Turkey as well [7,8]. The leaves of *L. stoechas* are small with petioles, hairy and long, have white greyish green appearance. The plant grows from root with few branches that are very similar rosemary. The fruits of the plant are light brown in color, flowers are the economic parts of the plant [9]. Since the plant has antibacterial characteristics, it is widely used in Turkish traditional medicinal system for treatment of problems related to throat and urinary tract [10,11].

Plants are known to be used for different purposes all over the world from ancient times. Many studies have reported medicinal properties of plant products based on different research groups. Therefore, plants are becoming more and more popular among people. Besides, the chemical synthesis of most pharmaceutically important secondary metabolites is not economically convenient, these compounds are isolated from wild growing or cultivated plants. However, because of extensive and non-regulated collections, many plant species have become threatened and endangered. It is obviously clear that all plants are not cultivated, species present in wild habitats are facing threat due to the overexploitation for their byproducts [12,13]. During the last few years, the interest in using micropropagation techniques for rapid and large scale propagation of medicinal and aromatic plants has been significantly increased [12]. Micropropagation is rapid multiplication of any selected plants using *in-vitro* culture techniques. In vegetative propagation, use of micropropagation has become very popular because of its application in agriculture, forestry, and horticulture. It has been possible to propagate forest trees, crop plants, vegetables, ornamentals, medicinal plants, and other commercial useful plants via this technique [14]. In the field literature, there are some reports on species belonging to the Lamiaceae family that are economically important: *Origanum* [15-17], *Salvia* [18-20], *Sideritis* [21], *Micromeria* [13], *Thymus* [22], *Ajuga* [23], *Lavandula* [24-26]. *Lavandula* species are produced as both vegetative and generative. Since the poor germination of seeds, the plant mostly reproduced vegetatively with cuttings. Hence, micropropagation techniques of *Lavandula* are deeply focused on *in vitro* methods. *In vitro* micropropagation of the varieties such as *L. dentata*, *L. viridis*, *L. latifolia*, *L. angustifolia* and *L* x *intermedia* were carried out successfully [24-28].

Studies on micropropagation of *Lavandula stoechas* are very limited. In 1996, Nobre reported that *in-vitro* clonal propagation of *L. stoechas* had been achieved from mature field-grown plants [29]. But, there is still need for biotechnological methods in addition to classic techniques of breeding for improvement of the plant. So, this study was the first report on micropropagation techniques of seeds of *L. stoechas* cultivated in Turkey. The volatile compounds of samples were determined by GC-FID and GC-MS systems, simultaneously.

2. Materials and Methods

The experimental design was given in supporting information in Figure S1.

2.1. Plant Material

The plant material was selected as micropropagated and acclimatized plant transferred to the soil under field conditions. Our research group previously published the data for the acclimatization *in vitro* regenerated *Lavandula stoechas* subsp. *stoechas* under field conditions [30-31]. After optimization of parameters for the acclimatized plant, the plant started flourishing and growing well under Mediterranean continental type field conditions. Seeds of this plant were collected from Prof. Dr. Neşet Arslan, Section Medicinal and Aromatic Plants, Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey.

2.2. Culture Conditions for Lavandula stoechas subsp. *stoechas*

Surface sterilization conditions for 50 seeds of the plant were determined. These seeds were surface sterilized with 20% commercial bleach (Ace, 5% NaOCl) for 8 min followed by 3 x 5 min rinsing with sterilized bidistilled water. The seeds were germinated on Murashige and Skoog (MS) [32] medium supplemented with 30 g/L sucrose 0.65% agar (Sigma type A). The pH of all culture media was adjusted to 5.7 ± 0.1 before autoclaving at 120ºC for 20 min. about 35 mL of autoclaved medium was poured into each of the petri dish or culture vessels. All cultures were incubated at 24 ± 1ºC under 16 h light photoperiod provided by cool white, fluorescent light (4000 lux). The cotyledon node explants were taken from 10 days old seedlings. Cotyledon nodes of germinating seeds were used as explants that were cultured on MS medium containing 0.25 mg/L BAP (6-benzylaminopurine) for eight weeks. Well-developed micropropagated shoots were rooted on MS medium that contained 1 mg/L IBA (indol-3-butyric acid). Thereafter, rooted plants were transferred to tubes containing peat moss covered with black polyethylene bags at room temperature. Peat moss was previously sterilized at 160ºC for 2 h.

2.3. Isolation and Analyzes of the Volatiles

2.3.1. Microdistillation

Volatiles were obtained by microdistillation of the aerial parts (500 mg) through an Eppendorf MicroDistiller® with 10 mL distilled water per sample vial. The micro-hydrodistillation programme was automatically set as below: the sample vial was heated to 108°C at a rate of 20°C/min and kept at this temperature for 90 min, then heated to 112°C at a rate of 20°C/min and kept at this temperature for 30 min. The sample was subjected to a final post-run for 2 min under same conditions. The collecting vial, which contained a solution of NaCl (2.5 g) and water (0.5 mL) plus *n*-hexane (350 µL) to trap volatile components, was cooled to –5°C during distillation. After distillation was completed, the organic layer (*n*-hexane) in the collection vial was separated and analyzed by GC-FID and GC-MS systems, simultaneously.

2.3.2. GC-MS (Gas Chromatography-Mass Spectrometry) Analysis

GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 mm film thickness) was employed with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min kept constant at 220°C for 10 min and followed by elevating the temperature to 240°C at a rate of 1°C/min. Injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was *m/z* 35 to 450.

2.3.3. GC-FID (Gas Chromatography-Flame Ionization Detector) Analysis

GC analysis was carried out using an Agilent 6890N GC system using FID detector temperature of 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column at the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.3.4. Identification of Volatile Compounds

Identification of the volatile compounds was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, MassFinder 3 Library) [33,34] and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils. Additionally, MS literature data [35,36] was also used for the identification.

3. Results and Discussion

Twenty-two volatile compounds of aerial parts of micpropagated *L. stoechas* subsp. *stoechas* were identified representing 95.9% of the sample by GC and GC-MS systems, simultaneously. Major compounds included camphor (38.5%), bornyl acetate (10.6%), α-fenchone (8.9%). Detailed information is given in Table 1.

There's only one study about micropropagation of *L. stoechas* in Turkey. One published study on a possible tissue culture method for *L. stoechas* [37]. It was reported that the essential oil of *Lavandula stoechas* flowers grown wild in Adana, Turkey was characterized with fenchone (32%), camphor (14.7%), myrtenyl acetate (11.7%) and 1,8-cineole (7.7%) [38].

The essential oil of the leaves of *L. stoechas* collected from Kairouan, Tunisia was reported [39] and fenchone (68.2%) and camphor (11.2%) were found to be major compounds of the twenty-eight identified volatiles. Three samples of *L. stoechas* obtained from organic farming fields in Spain were subjected to steam distillation. The essential oils were characterized with fenchone (33-37%), camphor (16-24%) and eucalyptol (syn:1,8-cineole) (17-18%) by GC-MS system [40]. Compositions of the essential oils of *L. stoechas* collected from various locations in Algeria were analyzed by GC and GC-MS systems. Major essential oil components were fenchone (11.27-37.48%), camphor (1.94-21.8%), 1,8-cineole (0.16-8.71%) and viridiflorol (2.89-7.38%) [41].

In Sardinia, Italy, essential oils obtained by hydrodistillation from the leaves and flower of *L. stoechas* subsp. *stoechas*. According to, GC and GC-MS results, fenchone (52.6, 66.2%) and camphor (13.13, 27.08%) were identified as the major compounds [42].

Essential oil compositions of *L. stoechas* and *L. stoechas* subsp. *stoechas* were summarized at below. In another study on the effects of hormones concentrations and *in vitro* conditions changes on tissue culture of *L. stoechas* subsp. s*toechas* [43]. In that study, rooted plants were adapted in wild conditions, essential oil yields were calculated but the essential oil compositions were not published.

It was reported that oxygenated monoterpenes constituted the highest proportion of the oil from *L. stoechas* subsp. *stoechas* growing wild in Tunisia, among which fenchone (34.3%), camphor (27.4%) and lavandulyl acetate (5.6%) were predominated [44]. Essential oils from leaves and inflorescens of the Greek samples of *L. stoechas* subsp. *stoechas* were reported with fenchone (39.9, 21.0%) and camphor (24.2, 26.3%) as major compounds [7].

Table 1. The composition of volatile compounds of aerial parts of micropropagated *L. stoechas* subsp. *stoechas**

RRI[a]	RRI[b]	Compound	%[c]	IM[d]
1025[e]	1032	α-Pinene	4.0	tR, MS
1077[f]	1076	Camphene	1.4	tR, MS
1212 [f]	1203	Limonene	1.4	tR, MS
1213[g]	1213	1,8-Cineole	4.3	tR, MS
1399[h]	1406	α-Fenchone	8.9	tR, MS
1488[f]	1497	α-Copaene	1.9	MS
1515[h]	1532	Camphor	38.5	tR, MS
1543[h]	1553	Linalool	3.5	tR, MS
1579[h]	1590	Bornyl acetate	10.6	tR, MS
1631[h]	1648	Myrtenal	2.7	MS
1680[h]	1684	*trans*-Verbenol	1.2	tR, MS
1691[h]	1704	Myrtenyl acetate	0.7	MS
1694[h]	1706	α-Terpineol	1.6	tR, MS
1699[h]	1719	Borneol	1.2	tR, MS
1751[h]	1765	Geranyl acetate	2.1	tR, MS
1755[h]	1773	δ-Cadinene	2.0	MS
1900[h]	1900	*epi*-Cubebol	1.8	MS
1918[h]	1957	Cubebol	1.0	MS
2089[h]	2104	Viridiflorol	3.8	tR, MS
2215[e]	2209	T-Muurolol	0.2	MS
	2210	Copaborneol	1.6	MS
	2289	4-*oxo*-α-Ylangene	1.5	MS
		TOTAL	**95.9**	

*RRI[a] from literature, e) [48], f) [49], g) [50], h) [51]. RRI[b]: Relative retention indices calculated against *n*-alkanes; %[c]: calculated from FID data; IM[d]: Identification Method; tR, identification based on the retention times (tR) of genuine standard compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data. The results were given for micropagated and acclimatized plant material on peat moss.

Several studies were reported on the essential oil of *L. stoechas* subsp. *stoechas* from Turkey, and the major compounds were determined as fenchone (31.8-56%), camphor (5.9-45.8%), 1,8-cineole (3.8-15.6%) and myrtenyl acetate (6.3%) [45] and camphor (45.8%), α-fenchone (31.8%), bornyl acetate (4.2%) and followed the other compounds such as camphene, viridiflorol, myrtenyl acetate and α-pinene. This study also showed similar patterns of major compounds especially for the ratio of fenchone and camphor which are the isomer of each. Regarding to biosynthetic pathway, the change of ratios of those compounds could be from the harvesting period of plant species and climate factors as expected usually. However we can clearly claim that those compounds are the main chemotaxonomic marker of this species.

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Supporting Information

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