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Chemical profiles of the essential oil of wild and in vitro regenerated Zataria multiflora Boiss. (Lamiaceae)

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Zataria multiflora Boiss. is an aromatic shrub belonging to the family Lamiaceae and its aerial parts are used in traditional medicine, pharmaceutical and food industries. In the present study, the content and chemical composition of the essential oils from regenerated plants grown '*in vitro*' were assessed by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) and compared with those of wild plants. In total, 29 and 16 compounds were identified and quantified in wild and *in vitro* regenerated plants, representing 99.6% and 99.4% of the total oil, respectively. The major identified components in the oil from wild and *in vitro* regenerated plants were carvacrol (35.0% and 49.2%), thymol (9.6% and 11.8%), *p*-cymene (11.7% and 5.8%), carvacrol methyl ether (7.5% and11.3%) and γ-terpinene (4.7% and 9.4%). The oils were dominated by oxygenated monoterpenes followed by monoterpene hydrocarbons. Our results indicate that *in vitro* propagated plants produce oil more rich in oxygenated sesquiterpenes than wild plant. Quantitative and qualitative variations were observed both in wild and in micropropagated plants in relation environmental factors. Thus, micropropagation provides plants suitable for the industrial exploitation of this species.

KEYWORDS: *Zataria multiflora* Boiss., Lamiaceae, essential oil, *in vitro* culture, thymol

INTRODUCTION

The family of Lamiaceae is one of the largest and most distinctive families of flowering plants, with *ca.* 258 genera and 6970 species worldwide. Lamiaceae with 46 genera and *ca*. 420 species and subspecies have a great diversity and distribution in the flora Iran [1-3]. Altogether 124 species and subspecies (30%) of this family are endemic to Iran [4]. Lamiaceae plants are well known for the essential oils and many biologically active oils have been isolated from various members of this family [5–7]. Some are one of the major sources of culinary, food flavoring, vegetable, and medicinal plants all over the world. A wide range of compounds such as terpenoids, phenolic compounds, and flavonoids have been reported from the members of the family [8–10].

Zataria multifora Boiss. as a suffruticose perennial shrub with 40-80 cm height is a member of this family and is known by the common Persian name of "Avishan Shirazi". The plant grows wild on the rocky and gravelly slopes from south to central parts of Iran in the Saharo-Sindian and Irano-Turanian regions and also in Pakistan and Afghanistan. *Z. multifora* has thyme-like fragrance and its generic name is derived from the arabic word "za'tar", meaning thyme [11]. *Z. multifora* is extensively used in traditional medicine as a condiment, antiseptic, analgesic (pain-relieving) and carminative (anti-flatulence and intestine-soothing). Biological activities of the plant have been also attributed to essential oil containing mainly phenolic compounds, thymol and carvacrol [12-15].

In recent years the monograph of *Z. multifora* has been introduced and presented in Iranian herbal pharmacopoeia [16]. According to this monograph, main chemical constituent of the plant has been described as carvacrol (61.0 %) and thymol (25.0 %) and also its herbal drug should contain at least 0.6 % oil. The huge amounts of herbal drugs of this species are harvested from the wild every year and are sold in the inner markets or exported. In recent years several pharmaceutical and cosmetic products have been introduced to the market by pharmaceutical companies [16].

Owing to over-exploitation of wild plants for commercial purposes and a low propagation rate in nature, *Z. multifora* is now almost extinct and is listed as an extremely vulnerable species in Iran [17]. *In vitro* culture offers a viable approach to propagate this species since it can also be used as

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a complimentary strategy for conservation and utilization of genetic resources. Further, *in vitro* plant regeneration through axillary bud culture is not only an easy and economic way of obtaining a large number of consistently uniform and true-to-type plants within a short span of time, but also offer an alternative to field agriculture to manufacture economically important secondary metabolites such as flavors, dyes, and pharmaceuticals within controlled laboratory environments [18,19]. Recently developed *in vitro* propagation techniques offer high-rate multiplication alternatives for plants of horticultural, economical and medicinal importance [20], as well as medium- to long-term conservation of valuable germplasm by means of slow growth storage and cryopreservation [21]. Although the essential oil of *Z. multifora* has been studied previously [22-24], but to the best of our knowledge, there is no data reporting aroma composition of the essential oil obtained from *in vitro* regenerated plants. Further, we speculate that *in vitro* plantlet culture may offer a means to procure essential oil compounds *in vitro* at the commercial level. In the present work, the chemical composition of the *in vitro* regenerated *Z. multifora* oil isolated by hydrodistillation was studied and compared with those of wild plants for the first time. These results can be considered for further strategies like *in vitro* shoot cultures for enhanced production of valuable phenolic terpenoids as thymol and carvacrol on a large scale.

EXPRIMENTAL

Plant material

The aerial parts of *Z. multiflora* were collected at vegetative stage from Jiroft (28º 41′ N, 57º 42′ E at an altitude of 710 m), Kerman Province, Iran. A Voucher specimen has been deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran.

In vitro regeneration

In vitro shoot proliferation of *Z. multiflora* were performed by culturing of internodal segments (3 cm) of wild growing stock plant on Murashige and Skoog (MS) medium [25] fortified with 1.5 mg/L BAP plus 1.0 mg/L IBA. Rooting of proliferated shoots was also performed on B5 medium [26] supplemented with 1.5 mg/L IBA. The cultures were incubated at 25 ± 2°C under a 16-h photoperiod, with light provided by cool daylight fluorescent lamps (40 $\mu mol^{-1}\ m^{-2}\ s^{-1}$), and were proliferated by monthly subcultures to fresh medium of the same type.

Essential oil isolation

The essential oil of air-dried samples (30 g) was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus, recommended by the British Pharmacopeia [27]. The essential oil was dried over anhydrous sodium sulfate (Na_2SO_4) and kept at 4ºC in dark vial until analyzed and tested.

GC-FID analysis

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was carried out on fused silica capillary DB-5 column (30 m × 0.25 mm i.d.; film thickness 0.25 µm). The injector and detector temperatures were kept at 250 ºC and 300 ºC, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.1 ml/min; oven temperature program was 60–250 ºC at the rate of 4 ºC /min and finally held isothermally for 10 min; split ratio was 1:50.

GC-MS analysis

GC-MS analysis was carried out by use of Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (60 m × 0.25 mm i.d.; film thickness 0.25µm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 200 ºC and 250 ºC, respectively. Mass range was from 35 to 456 amu. Oven temperature program was the same given above for the GC.

Identification and quantification of the oil components

The constituents of essential oils were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C_6–C_{24}) and the oil on a DB-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (Adams and Wiley 7.0) or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature [28]. For quantification purposes, relative area percentages obtained by FID were used without the use of correction

factors.

RESULTS AND DISCUSSION

The hydrodistillation of the air-dried aerial parts of wild and *in vitro* regenerated *Z. multiflora* gave yellow pale oils in 2.6 and 0.9 (w/w%, based on dry weight) yield, respectively. The chemical composition of the oil samples was mainly investigated using both GC-FID and GC-MS techniques. Qualitative and quantitative analytical results are listed in Table in the order of their elution on the DB-5 column.

GC-FID chromatograms of the essential oils from wild and *in vitro* regenerated plants with identified major compounds are shown in the Figure 1.

Table. Essential oil composition of wild and in vitro regenerated plants of Zataria multiflora

No.	RIa	Compounds	%		Identification methods
			Wild plant	In vitro regenerated plant	
1	929	α-Thujene	1.2	0.4	RI, MSb
2	939	α-Pinene	5.0	0.6	RI, MS, CoIc
3	954	Camphene	0.4	-	RI, MS
4	983	β-Pinene	1.9	0.2	RI, MS, CoI
5	988	Myrcene	2.0	1.2	RI, MS, CoI
6	1008	α-Phellandrene	0.3	0.1	RI, MS
7	1020	α-Terpinene	1.8	1.5	RI, MS
8	1028	p-Cymene	11.7	5.8	RI, MS
9	1032	Limonene	0.9	-	RI, MS, CoI
10	1035	1,8-Cineol	1.6	-	RI, MS, CoI
11	1060	γ-Terpinene	4.7	9.4	RI, MS
12	1069	cis-Sabinene hydrate	0.6	1.0	RI, MS
13	1091	Terpinolene	0.3	-	RI, MS
14	1097	Linalool	2.6	-	RI, MS, CoI
15	1101	trans-Sabinene hydrate	0.3	-	RI, MS
16	1182	Terpinen-4-ol	1.6	0.2	RI, MS
17	1196	α-Terpineol	1.5	-	RI, MS
18	1202	cis-Dihydro carvone	0.2	-	RI, MS
19	1233	Thymol methyl ether	1.1	-	RI, MS
20	1244	Carvacrol methyl ether	7.5	11.3	RI, MS
21	1288	Thymol	9.6	11.8	RI, MS, CoI
22	1305	Carvacrol	35.0	49.2	RI, MS, CoI
23	1352	Thymol acetate	0.5	-	RI, MS
24	1371	Carvacrol acetate	2.8	0.5	RI, MS
25	1435	(E)-Caryophyllene	2.3	4.8	RI, MS
26	1454	Aromadendrene	0.4	-	RI, MS
27	1508	Viridiflorene	0.2	1.4	RI, MS
28	1593	Spathulenol	0.4	-	RI, MS
29	1600	Caryophyllene oxide	1.2	-	RI, MS
		Monoterpene hydrocarbons	31.1	20.2	
		Oxygenated monoterpenes	64.0	73.0	
		Sesquiterpene hydrocarbons	2.9	6.2	
		Oxygenated Sesquiterpenes	1.6	-	
		Total identified	99.6	99.4	

[a]Retention indices relative to C6–C24 n-alkanes on a DB-5 column; [b]mass spectrometery; [c]co-injection with authentic

compounds

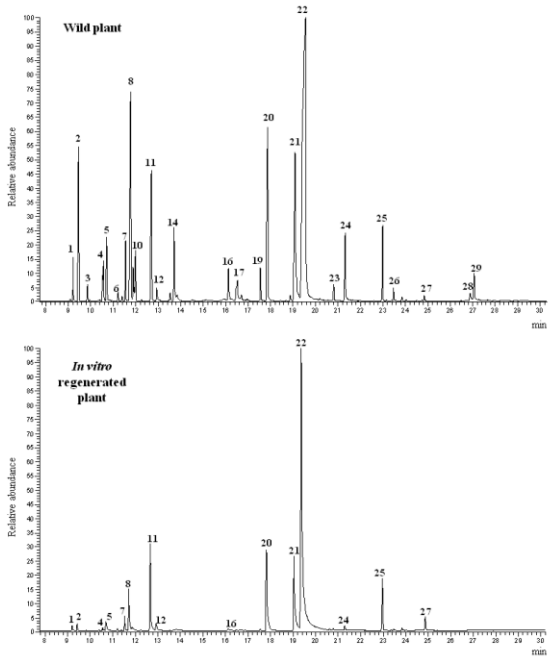

Fig. 1. Gas chromatography–flame ionization detector (GC–FID) chromatograms of the essential oil from wild and *in vitro* regenerated *Zataria multiflora* Boiss.

In total, 29 and 16 compounds were identified and quantified in wild and *in vitro* regenerated plants, representing 99.6% and 99.4% of the total oil, respectively. The major identified components in the oil from wild and *in vitro* regenerated plants were carvacrol (35.0% and 49.2%), thymol (9.6% and 11.8%), *p*-cymene (11.7% and 5.8%), carvacrol methyl ether (7.5% and 11.3%) and γ-terpinene (4.7% and 9.4%).

Chemical structure of the major identified components is shown in the figure 2.

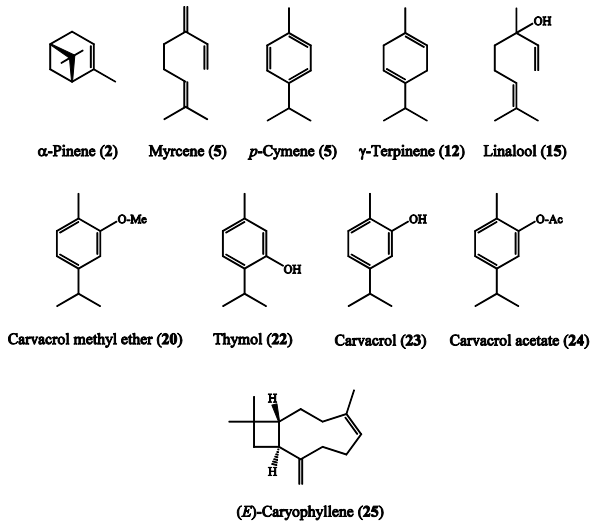

Fig. 2. Chemical structure of the major identified compounds in the essential oil of wild and *in vitro* regenerated *Zataria multiflora* Boiss.

The classification of the identified compounds, based on functional groups is summarized at the end of Table. The oils were dominated by oxygenated monoterpenes (64.0 and 73.0% in the oils from wild and *in vitro* plants, respectively) followed by monoterpene hydrocarbons (20.2 and 31.1% in the oils from *in vitro* and wild plants, respectively). It was found that the chemical profile of the essential oil from *in vitro* regenerated plant is qualitatively different than wild plant oil. The essential oils of wild and *in vitro* regenerated plants comprised 13 and 9 monoterpene hydrocarbons, 11 and 5 oxygenated monoterpenes, 3 and 2 sesquiterpene hydrocarbons, respectively (Table). Monoterpene hydrocarbons camphene, limonene, terpinolene and *trans*-sabinene hydrate were absent in the essential oil profile of *in vitro* regenerated plants. 1,8 Cineol, linalool, α-terpineol, *cis*-dihydro carvone, thymol methyl ether and thymol acetate as oxygenetaed monoterpenes were not detected in the essential oil obtained from regenerated plants grown '*in vitro*'. Oxygenated sesquiterpenes spathulenol and caryophyllene oxide were only detected in the essential oil of wild plant. Our results showed that both wild and clonally propagated plants contained high levels of monoterpene hydrocarbons *p*-cymene and γ-terpinene (Table). However, the relative concentration of *p*-cymene was higher and γ-terpinene was lower in the wild plant; as well, α-pinene was also higher. A major difference between the oils from *in vitro* and *in vivo Z. multiflora* plants was observed in the content of oxygenated monoterpenes; in the oil from micropropagated plants the amount of the oxygenated monoterpenes carvacrol, thymol and carvacrol methyl ether was higher than that found in the oil from *in vivo* plants. Similarity in the chemical composition of essential oils from *in vitro* and *in vivo* plants has been reported by some other authors [29-31]. For example, Kuźma et al. (2009) [29] have reported that a chemical profile of the essential oil from *Salvia sclarea in vitro* plants was similar to that of the control mother plants, with linalool as the main compound. It has been also reported that a chemical profile of the essential oil from *in vitro* grown *Origanum vulgare* L. ssp. *hirtum* was comparable to that *in vivo* plants [31]. On the other hand, comparative studies on the essential oils from *in vitro* and *in vivo* plants of *Salvia przewalskii* showed numerous differences between the two oil profiles

[32]. It has been reported that micro-shoots, which are the normal sites for secondary metabolism in nature, readily manifest commercially desired secondary metabolites *in vitro* [19,33]. For example, in the Lamiaceae family, essential oil synthesis occurs primarily in the leaf epidermal cells and storage primarily in glandular leaf trichomes [34]. Examination of the leaf surfaces of foliage from tissue culture plantlets also reveals the occurrence of these leaf trichomes and they also readily produce volatile essential oils [33]. Our results revealed that *Z. multiflora* plantlets grown *in vitro* are quantitatively potent in the production of the major essential oil components as well as the wild plant. It can be conclude that *in vitro* regeneration of *Z. multiflora* would be of great interest for the cloning of valuable genotypes, for example, the plants that contain high level of phenolic terpenoids as carvacrol and thymol. The rapid cloning may represent a way to exploit the natural variability of this species. This technique would also be useful to mass produce this plant, relieving wild populations from the pressure produced by intensive collection. Further, these results strongly suggest that using *in vitro* plantlets as a means to produce secondary metabolites is possible for future commercial applications.

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ХИМИЧЕСКИ ПРОФИЛИ НА ЕТЕРИЧНО МАСЛО НА ДИВОРАСТЯЩИ И ИН-ВИТРО РЕГЕНЕРИРАНИ *Zataria multiflora* BOISS. (*LAMIACEAE*)

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(Резюме)

Zataria multiflora Boiss. е ароматен храст, принадлежащ на семейство Устноцветни (Lamiaceae), чиито надземни части се използват в традиционната медицина, фармацевтичната и хранително-вкусовата промишленост. В текущото проучване са изследвани съдържанието и химическият състав на етеричните масла, получени от регенерирани растения, отгледани „ин витро" чрез газова хроматография – пламъков йонизационен детектор (GC-FID) и газова хроматография – мас спектрометрия (GC-MS) и сравнени с тези на диворастящи растения. Общо 29 и 16 съединения са идентифицирани и определени количествено съответно в диворастящи и ин витро отгледани растения, представляващи 99, 6% и 99, 4% от общото количество на маслата. Основните определени съединения в маслото от дивите и инвитро регенерираните растения са карвакрол (35,0% и 49,2%), тимол (9,6% и 11,8%), п-цимен (11,7% и 5.8%) метилов етер на карвакрола (7,5% и 11,3%) и γ-терпипен (4,7% и 9,4%). Маслата съдържат основно окислени монотерпени, последвани от монотерпенови въглеводороди. Нашите резултати показват, че ин витро размножените растения произвеждат масла, по-богати на окислени сескитерпени в сравнение с дивите. Качествени и количествени различия се наблюдават както при диворастящите, така и в микроразмножените растения в зависимост от условията на околната среда. Това показва, че микроразмножаването осигурява растения, подходящи за промишленото използване на този вид.