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Original Article

Comparison of Essential Oils Composition Between in-vitro Plantlets and Greenhouse Plants from Various Populations of *Dracocephalum kotschyi* Boiss.

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ABSTRACT

Dracocephalum kotschyi Boiss. belongs to the family Lamiaceae, a perennial herbaceous medicinal plant that is native to Iran and is considered an endangered species. In-vitro plantlets (seven populations) were raised in MS medium supplemented with 0.1 mg/l BAP and 0.01 mg/l NAA and the rooted plantlets were acclimatized successfully under greenhouse conditions. In- vivo plants (eight populations) were propagated under greenhouse condition. The essential oils were isolated by hydro distillation and identification of chemical compounds was done by a combination of capillary GC and GC-MS instruments. Twenty-five and forty compounds were identified in the different populations of in-vitro plantlet and in-vivo plant constituting 85.8%-99.68% and 85.1%-95.06% of essential oils, respectively. The major components of in-vitro plantlet on different populations were Verbenone (2.5%-82.47%), Geranyl acetate (28.35%-62.07%), Methyl geranate (0.98%-79.06%), Neral (0.64%-3.13%), Geranial (4.93%-14.39%), Limonene (0.37%-10.36%) and E-Anethole (7.62%-20.71%). The composition of essential oil from greenhouse plant populations were dominated by Neral (11.24%-74.80%), Limonene (0.17%-25.26%), Geraniol (0.54%-40.81%), Geranial (0.04%-9.15%), Methyl geranate (0.16%-28.48%), E-Anethole (0 %-0.1%) and Verbenone (0.39%-23.96%). The highest values of Neral, Limonene and Geraniol percentage were obtained from greenhouse conditions. In contrast, the maximum values of Verbenone, Geranyl acetate, Methyl geranate, Geranial, and E-Anethole were observed in the essential oils of in-vitro plantlets. This study demonstrated difference of chemical composition between in-vitro plantlets and greenhouse plants of different populations on *D. kotschyi* species. Also, new chemotypes of *D. kotschyi* has been introduced for further research.

INTRODUCTION

Dracocephalum genus, belonging to the Lamiaceae family, includes 190 species in the world [1]. This genus has about eight fragrant annual and perennial herbaceous species in Iran. *D. kotschyi* is an important aromatic species due to synthesis and production of essential oils. This species is one of the exclusive species in Iran that grows in the mountainous regions of the north, west, and central parts of Iran [2,3]. The Persian name of this species is Zarringiah or Badranjboyeh denaei [4]. Many studies approved the antibacterial activity [5]; anti hyperlipidemic [6]; Immune system modulatory [7]; anti nociceptive [8] and cytotoxic effects [9,10] of this species. According to previous studies, various compounds such as limonene, geranial, geranyl acetate, methyl geranate were found in the essential oils of this species as well as other compounds such as citral, β-caryophyllene, terpinyl acetate, myrthenol, α-pinene, β-ocimene, carvacrol and γ-

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terpinene were identified [11-20]. Reproduction of this species is through seeds. However, due to seeds dormancy and low germination rate, their propagation and development are limited [21]. *D. kotschyi* species is in danger of extinction for some reasons such as incorrect exploitation and harvest as well as unsuitable natural conditions for its growth.

In-vitro propagation techniques are an efficient, rapid, and economic method for propagation of aromatic and medicinal plant species [22]. However, investigation on quality and quantity of essential oils of propagated plants is necessary. In-vitro propagation of aromatic and medicinal plants and analysis of essential oils have been conducted in various plants such as *Thymus vulgaris* [20]; *Ocimum basilicum* [23]; *Melissa officinalis* [24]; *Lavandula angustifolia* [25]*; Teucrium scorodonia* [26]; *Caryopteris species* [27]; *T. caespititius* [28]; *Varronia curassavica* [29]; *Teucrium polium* [30]; *Lessertia frutescens* L. [31]; *Salvia fruticaosa* [32]; *Lavandula viridis* [1]; *Minthostachys mollis* [33]; *Lippia dulcis* [34]. The aim of this study was to comparison of essential oils composition among in-vitro plantlets and greenhouse plants on different populations of *D. kotschyi* species.

MATERIAL AND METHODS

Plant Material

The seeds of different populations of *D. kotschyi* species were obtained from the Gene Bank Natural Resources, Research Institute of Forests and Rangelands (RIFR) [Table 1]. In order to seed germination, the surface sterilization of seeds carried out with 50% commercial bleach solution (sodium hypochlorite with 5% active chlorine) for five minutes and then immersed in 70% ethanol for five seconds then rinsing three times with sterile distilled water. Finally, the seeds were put in glass tubes, containing 20 ml half-strength MS medium.

In-vitro Plantlet Production

In order to *in-vitro* plantlet production, the shoot tips of seedlings were excised and then cultured in a glass jar with 50 ml MS culture medium [35] supplemented with 0.1 mg/l BAP, 0.01 mg/l NAA, 30 g/l sucrose and gelled with 6 g/l agar. The pH was adjusted to 5.8 with KOH before the addition of agar and autoclaved for 20 min at 121 °C. Sub culturing was done every four weeks and all samples were maintained at growth room with a photoperiod of 16h light provided by cool white fluorescent lamps (12000 lux) and 8h darkness under 25 °C temperature.

Table 1 characteristics of different populations of *D. kotschyi* Boiss. used in this study

Sample code	Gene Bank code	Origin	Method of culture
1	39385	Qazvin 4 (Takestan)	Seedling
2	12938	Qazvin 2	Seedling
3	12938	Qazvin 2	tissue culture
4	221-2	Darbandsar 1	Seedling
5	221-2	Darbandsar 1	tissue culture
6	180	Chalus	Seedling
7	180	Chalus	tissue culture
8	221-5	Darbandsar 3	Seedling
9	221-5	Darbandsar 3	tissue culture
10	221-3	Darbandsar 2	Seedling
11	221-3	Darbandsar 2	tissue culture
12	798	Qazvin 1	Seedling
13	798	Qazvin 1	tissue culture
14	29652	Qazvin 3	Seedling
221	29652	Qazvin 3	tissue culture

After shoot proliferation, all shoots with suitable length were transferred to MS medium supplemented with ½ Nitrate compounds (NH_4NO_3 and KNO_3) without growth regulators or 0.5 mg/l IBA and 0.5 mg/l NAA. In the next step, the rooted plantlets were cultured in a small pot containing a mixture of peat moss and perlite (3:1) and kept in an incubator with 20 ± 1 °C temperature and a

photoperiod of 16h light and 8h darkness and covered with polythene bags for one month. The plantlets were irrigated and sprayed daily with Hoagland's nutrition solution. After two months, plantlets were transferred to greenhouse condition and cultivated in pots containing silt, soil and manure (2:1:1). Irrigation was conducted every day and liquid fertilizer was added to each pot weekly.

Cultivation of greenhouse plants

In order to cultivation plants under greenhouse condition, after germination of seeds in optimal condition and producing seedling of different populations of *D. kotschyi* species, they were transferred in a small pot containing a mixture of peat moss and perlite (3:1) and adapted to greenhouse condition as well as in-vitro plantlets.

Isolation procedure

Air-dried parts of the plants were subjected to hydro distillation method for 3h using Clevenger-type apparatus. Essential oils were dried over anhydrous sodium sulfate and stored in sealed vials at low temperature (4 °C) before analysis.

Gas Chromatography (GC)

GC analysis was performed using a Shimadzu GC-9A Gas chromatograph equipped with a DB5 fused silica column (60 m x 0.25 mm; film thickness 0.25 micron). The Oven temperature was held at 50 ºC for 5 min and then was programmed to 270 ºC at a rate of 3 ºC/min, injector and detector (FID) temperature were 280 ºC, helium was used as carrier gas at a linear velocity of 32 cm/s. The relative amounts of individual components are based on electronic integration of peak area without the use of an internal standard or FID response factor correction.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was carried out on a Varian 3400 GC-MS system equipped with a DB-5 fused silica column (60 m x 0.25mm, film thickness 0.25 micron) and interfaced with a Varian ion trap detector. The Oven temperature was 50-270 ºC at a rate of 3 ºC/min, injector and transfer line temperature were 280 ºC and 290 ºC, carrier gas, helium with a linear velocity of 31.5 cm/s, split ratio 1/60, ionization energy 70 ev; scan time 1 s; mass range 40-400 amu.

Identification of components

The oils component was identified by comparing their mass spectra with those of computer library [36-38] or with authentic compounds. This was confirmed by comparison of their retention indices with those of authentic compounds or with data published in the literature.

Statistical analysis

In order to determine the variation between individuals and localities on different culture methods, Factorial experiment based on CRD design was performed on normal data and means were compared by Duncan's test using SAS (1996), ver.6.12 software.

RESULTS

The percentage of identified essential oils compound is listed in table (2 and 3). In order to their elution on the DB-5 column, 25 and 40 compounds were identified in different populations of *D. kotschyi* propagated under in-vitro conditions and greenhouse plants respectively. The main compounds among seven populations of *in-vitro* plantlets were limonene, verbenone, geranial, E-anethole, methyl geranate and geranyl acetate. The main compounds among eight populations of greenhouse plants dominated in limonene, neral, verbenone, geraniol, geranial, methyl geranate and geranyl acetate. Different in-vitro plantlets of *D. kotschyi* populations exhibited a chemical variability, their composition being dominated either by oxygenated monoterpene or by ester (Table 2). The main compound of essential oils in population of Qazvin1 was limonene (10.02%) and verbenone (68.79%). Also the major chemical compounds of Qazvin2 were limonene (10.36%) and verbenone (82.47%). The main compound identified in the essential oils of population of Qazvin3 was verbenone (11.39%) and methyl geranat (79.06%). In population of Darbandsar1, geranyl acetate (50.87%), E-anethol (15.73%), geranial (11.03%) and methyl geranate (5.93%) were identified as main compounds. The main compound of essential oils in population of Darbandsar2 was geranyl acetate (62.07%), methyl geranate (8.30%), E-anethol (7.62%) and geranial (4.93%). In population

of Darbandsar3, geranyl acetate (35.51%), methyl geranate (21.33%), geranial (5.88 %) and E-anethol (9.8%) were formed as main compound of essential oils. In Chalus population, geranyl acetate (28.35%) E-anethol (20.71%), geranial (14.39%) and methyl geranate (11.98%) were identified as main compound of essential oils.

A tremendous chemical variability was also observed among different populations of greenhouse plants. The major component in most populations was oxygenated monoterpene and monoterpene (Table 3).

The main compound of essential oils in population of Qazvin1 was neral (61.04%) and limonene (25.26%), also the major compounds in populations of Qazvin2 and Qazvin3 were neral (74.80% and 69.24%) and limonene (9.05% and 16.62%). In population of Darbandsar1, neral (47.23%), verbenone (23.96%) and limonene (13.53%) were identified as main compound of essential oils. The main compound of essential oils in population of Darbandsar2 was neral (34.10%), verbenone (19.46%), spathulenol (8.91%), and caryophyllene oxid (3.60%). In population of Darbandsar3, neral (11.24%) methyl geranate (28.48%) and geraniol (12.27%) were identified as main compound of essential oils. In Chalus population, the main compound of essential oils included geraniol (40.81%), methyl geranate (14.43%), neral (19.24%) and α-campholenal (4.21%). In population of Qazvin4 (Takestan) the main compound of essential oils were dominated by neral (74%) and limonene (3.63%).

Statistical analysis according to Factorial test based on completely randomized design demonstrated that there are significant differences among populations just for six combinations of the measured essential oils ($P< 0.01$). Also there are significant difference between method of cultures and interaction between (populations and method of cultures) for some combinations of the measured essential oils ($P<0.01$ and 0.05) (Table 4). Comparison mean of essential oils composition obtained from in-vitro propagations and greenhouse plants of *D. kotschyi* species in different populations is shown in Table 5. In in-vitro propagation method the mean value of geranial, E-anethole, verbenone and geranyl acetate compounds were significantly different mainly from greenhouse culture method. Also, the mean value of production of compounds such as limonene and neral were higher in greenhouse culture and were significantly different from in-vitro culture. The mean value of limonene was varied from 0.36% in Darbandsar2 region to 14.16% in Qazvin1 region. The mean value of neral was varied from 3.44% in chalus region to 37.67% in Qazvin2 region. The mean value of verbenone was varied from 0.26% in Darbandsar3 region to 41.23% in Qazvin2 region. The mean value of methyl geranate was varied from 0.71% in Qazvin2 region to 18.15% in Darbandsar3 region. The mean value of geranyl acetate was varied from 0.15% in Qazvin3 region to 31.58% in Darbandsar2 region (Table5).

Using principal components analysis (PCA) the first five independent components accounted for about 83.03% of total variation. The first component emphasized linalool, neral, E-anethole, geranyl acetate and germacrene -D percentage values which had the highest coefficients of Eigen vectors and were important essential oils for classification of populations with about 27.22% of total variation. The second component emphasized p-cymene, 1,8-cineole, carvone, allo-aromadendrene, δ-cadinene and spathulenol values which had the highest coefficients of Eigen vectors and were important essential oils for classification of populations with about 20.13% of total variation.

The third component emphasized limonene, carvone, methyl geranate and α-copaene values which had the highest coefficients of Eigen vectors and were important essential oils for classification of populations with about 13.30% of total variation. The fourth component emphasized α-pinene and geranial values which had the highest coefficients of Eigen vectors and were important essential oils for classification of populations with about 12.25% of total variation. The fifth component emphasized γ-terpinene and α-terpineol values which had the highest coefficients of Eigen vectors and were important essential oils for classification of populations with about 10.13% of total variation (Table 6).

Grouping of studied populations was based on their essential oils compound (Fig. 1). The results showed that different populations of *D. kotschyi* contain of different culture methods have been grouped in separate cluster. By cutting the dendrogram resulting from cluster analysis by Ward's method with cophenetic correlation coefficient (r= 0.95) with a metric distance of 4.08, the populations were

classified into six groups. Darbandsar1 and Darbandsar2 populations obtained from in-vitro culture together are classified in a separate group due to high level of geranyl acetate compound. Qazvin1 and Qazvin2 populations obtained from greenhouse culture together are classified in a separate group duo to high level of neral and limonene compounds.

Table 2 Comparison percentage of essential oils composition among different populations of *in vitro* plantlet in *D. kotschyi* Boiss.

N0	Chemical compound	RI	Qazvin 1	Qazvin 2	Qazvin 3	Darbandsar 1	Darbandsar 2	Darbandsar 3	Chalus
1	α-pinene	986	-	0.24	-	-	-	-	-
2	p-cymene	1028	-	0.24	0.59	0.13	-	-	-
3	Limonene	1032	10.02	10.36	1.88	1.54	0.63	1.16	0.37
4	1,8-cineole	1035	0.19	0.12	-	0.23	0.19	-	0.38
5	γ-terpinene	1065	0.08	-	0.22	0.34	0.08	-	0.83
6	Terpinolene	1091	0.32	-	-	0.33	0.35	0.89	1.95
7	Linalool	1100	0.67	0.14	-	0.22	0.14	0.46	0.26
8	α-terpineol	1190	-	0	-	-	-	-	1.13
9	Verbenone	1206	68.79	82.47	11.39	4.25	2.86	-	2.5
10	*Trans* carveol	1220	1.32	0.76	0.2	-	-	0.51	0.86
11	Neral	1242	-	-	-	0.64	3.13	1.15	1.39
12	Geranial	1270	-	-	-	11.03	4.93	5.88	14.39
13	Bornyl acetate	1284	1.36	0.53	-	-	-	-	-
14	E-Anethole	1285	-	-	-	15.73	7.62	9.8	20.71
15	methyl geranate	1324	2.88	0.98	79.06	5.93	8.30	21.33	11.98
16	Neryl acetate	1359	-	-	-	1.52	1.11	0.85	3.32
17	Geranyl acetate	1379	-	-	-	50.87	62.07	35.51	28.35
18	β-cubebene	1387	0.48	0.16	-	0.38	0.34	-	0.24
19	longifolene	1404	-	0.92	1.55	0.07	-	-	0.84
20	α-gurjunene	1409	1.62	0.79	1.2	0.28	0.20	0.91	-
21	α-cedrene	1410	0.72	0.48	-	0.33	0.48	0.43	0.55
22	Allo-aromadendrene	1463	-	0.15	0.3	0.1	0.26	0.74	-
23	Germacrene D	1480	3.23	0.38	0.65	0.97	1.65	2.69	0.98
24	δ-cadinene	1526	-	0.55	-	-	-	-	-
25	Spathulenol	1578	2.39	0.41	-	1.15	0.68	3.49	-
Monoterpene			10.42	10.84	2.69	2.34	1.06	2.05	3.15
Oxygenated monoterpene			70.97	83.49	11.59	32.74	18.87	17.8	41.62
Ester			2.88	0.98	79.06	58.32	71.48	57.69	43.65
Sesquiterpene			6.05	3.43	3.7	2.13	2.93	4.77	2.61
Oxygenated sesquiterpene			2.39	0.41	-	1.15	0.68	3.49	-
Total			94.07	99.68	97.04	96.04	95.02	85.8	90.66

-: trace< 0.05 RI:retention indices in elution order from DB-5 column

Table 3 Comparison percentage of essential oils composition among different populations of greenhouse plant in *D. kotschyi* Boiss.

No	Chemical compound	RI	Qazvin 1	Qazvin 2	Qazvin 3	Darbandsar 1	Darbandsar 2	Darbandsar 3	Chalus	Qazvin 4 (Takestan)
1	α-pinene	986	1.46	0.88	0.55	-	-	2.62	0.59	0.16
2	p-cymene	1028	0.72	0.54	0.42	0.16	-	0.09	0.46	0.12
3	Limonene	1032	25.26	9.05	16.62	13.53	0.17	2.46	2.75	3.63
4	1,8-cineole	1035	0.46	0.08	0.31	0.05	-	0.02	-	-
5	Z-β-ocimene	1042	-	-	-	0.03	-	-	-	-
6	E-β-ocimene	1050	-	-	-	-	-	0.1	0.7	-
7	γ-terpinene	1065	-	0.15	-	-	-	-	-	-
8	Linalool	1100	-	-	-	0.06	-	0.19	0.49	0.51
9	α-campholenal	1127	0.31	0.53	-	0.58	-	2.41	4.21	0.63
10	*Trans* limonene	1141	-	-	-	0.14	-	-	-	-
11	α-terpineol	1190	-	-	-	0.06	0.4	0.11	-	-
12	Verbenon	1206	-	-	-	23.96	19.46	0.39	-	-
13	Trans-carveol	1220	-	-	-	-	0.34	-	-	-
14	Neral	1242	61.04	74.80	69.24	47.23	34.10	11.24	19.24	74
15	Carvone	1244	-	-	-	0.54	1.17	0.44	-	-
16	Geraniol	1256	1.57	0.65	0.54	0.90	0.57	12.27	40.81	1.50
17	Linalool acetate	1260	-	-	-	-	-	0.15	-	-
18	Geranial	1270	-	0.038	0.15	-	0.11	9.15	0.52	0.47
19	bornyl acetate	1284	-	-	-	-	-	0.11	-	-
20	E-Anethole	1285	-	-	-	0.1	-	-	-	-
21	Thymol	1293	-	-	-	-	1.91	0.14	-	-
22	Carvacrol	1300	-	-	-	-	1.4	-	-	-
23	Methyl geranate	1324	1.28	-	-	0.16	0.99	28.48	14.43	0.48
24	Limonene aldehyde	1326	-	-	-	1.05	2.52	-	-	-
25	Piperiteneoxid	1365	-	0.26	-	0.13	-	0.22	-	-
26	α-copaene	1378	-	-	-	0.26	-	0.15	-	-
27	Geranyl acetate	1379	0.65	1.88	0.68	1.29	1.65	7.48	0.3	1.13
28	β-bourbonene	1384	0.07	0.27	-	0.15	0.22	0.09	0.62	1.21
29	β-cubebene	1387	0.13	0.51	0.42	0.35	0.73	0.33	0.71	1.94
30	iso-longifolene	1389	0.83	1.96	1.09	1.80	3.81	2.88	0.86	-
31	β-elemene	1394	-	-	-	-	0.45	0.14	0.44	2.04
32	Cyperene	1398	-	0.27	-	-	-	-	-	2.06
33	β-longipinene	1399	-	-	-	-	-	-	-	1.73
34	β-humulene	1440	-	-	-	-	0.77	0.26	-	-
35	Allo-aromadendrene	1463	-	-	-	0.53	-	-	-	0.76
36	Germacrene D	1480	-	-	0.31	0.13	0.21	0.32	0.31	0.33
37	β-selinene	1486	-	-	-	-	-	0.69	-	-
38	δ- cadinene	1526	-	-	-	1.09	0.91	0.54	-	-
39	Spathulenol	1578	-	-	-	0.78	8.91	1.20	-	-
40	Caryophylleneoxid	1584	-	-	-	0.19	3.60	0.43	-	-
Monoterpene			27.44	10.62	17.59	13.72	0.17	5.27	4.5	3.91
Oxygenated monoterpene			62.91	76.09	70.24	74.48	61.98	36.36	65.27	77.1
Ester			1.93	2.14	0.68	1.58	2.64	36.44	14.73	1.61
Sesquiterpene			1.03	3.01	1.82	4.31	6.33	5.14	2.49	10.07
Oxygenated sesquiterpene			-	-	-	0.97	13.28	1.89	-	-
Total			93.31	91.86	90.33	95.06	85.09	85.1	87.44	92.53

-: trace <0.05, RI:retention indices in elution order from DB-5 column

Table 4 The results of factorial experiments for essential oils data based on CRD design

S.O.V.	df	M.S (Mean Square)										
Compounds		α-pinene	p-cymene	limonene	1,8-cineole	γ-terpinene	linalool	α-terpineol	neral	geranial	E-anethol	verbenone
Population (P)	6	0.062 **	0.015 **	0.84 **	0.004 ^{ns}	0.004 ^{ns}	0.001 ^{ns}	0.002 ^{ns}	0.56 ^{ns}	0.254 ^{ns}	0.309 **	1.807 **
Method of Culture (MC)	1	0.24 **	0.027 **	1.08 **	0.0004 ^{ns}	0.005 ^{ns}	0.011 ^{ns}	0.0005 ^{ns}	11.79 **	0.544 *	1.48 **	0.816 **
P × MC	6	0.052 *	0.012 **	0.28 *	0.008 **	0.005 ^{ns}	0.002 ^{ns}	0.004 ^{ns}	0.833 *	0.303 *	0.282 **	1.056 **
Error	28	0.016	0.003	0.094	0.002	0.003	0.003	0.003	0.338	0.119	0.076	0.229
C.V%		14.01	6.02	20.4	5.28	5.89	6.72	6.50	28.32	27.14	23.30	28.24

^{ns}: non-significant, **: significant at 1% , *: significant at 5%

Continued Table 4

S.O.V.	df	M.S (Mean Square)							
Compounds		carvone	methyl geranate	geranyl acetate	α-copaene	allo-aromadendrene	germacrene D	δ -cadinene	spathulenol
Population (P)	6	0.007 ^{ns}	0.418 ^{ns}	1.23 **	0.006 ^{ns}	0.0097 ^{ns}	0.0186 ^{ns}	0.018 ^{ns}	0.084 ^{ns}
Method of Culture (MC)	1	0.004 ^{ns}	0.306 ^{ns}	1.649 **	0.044 **	0.005 ^{ns}	0.1737 *	0.011 ^{ns}	0.005 ^{ns}
P × MC	6	0.0227 ^{ns}	0.603 *	1.26 **	0.006 ^{ns}	0.004 ^{ns}	0.0189 ^{ns}	0.022 ^{ns}	0.025 ^{ns}
Error	28	0.012	0.232	0.128	0.004	0.008	0.024	0.013	0.061
C.V%		12.23	29.10	23.35	7.36	10.72	16.23	13.07	25.52

^{ns}: non-significant, **: significant at 1% , *: significant at 5%

Table 5 Comparison mean of essential oil composition from *in vitro* propagations and green house plants of *D. kotschyi*

R	Chemical compounds	Culture methods		populations						
		Green house	Tissue culture	Chalus	Darbandsar3	Darbandsar2	Darbandsar1	Qazvin1	Qazvin2	Qazvin 3
1	α-pinene	0.91 a	0.03 b	0.09 bc	1.74 a	0 c	0 c	0.67 abc	0.79 ab	0 c
2	p-cymene	0.26 a	0.08 b	0.07 c	0.06 c	0 c	0.14 bc	0.37 ab	0.43 a	0.09 c
3	Limonene	7.92 a	2.42 b	0.52 b	1.28 b	0.36 b	7.53 a	14.16 a	10.64 a	1.78 b
4	1,8-cineole	0.10 a	0.09 a	0.06 ab	0.01 b	0.09 ab	0.14 ab	0.25 a	0.13 ab	0 b
5	γ-terpinene	0.04 a	0.11 a	0.13 ab	0 b	0.04 ab	0.20 a	0 b	0.12 ab	0.04 ab
6	Linalool	0.04 a	0.13 a	0.12 a	0.14 a	0.07 a	0.11 a	0.11 a	0.07 a	0 a
7	α-terpineol	0.06 a	0.05 a	0.18 a	0.07 a	0.13 a	0.03 a	0 a	0 a	0 a
8	Neral	35.67	0.66 b	3.44 b	5.78 ab	12.93 ab	23.94 ab	31.83 ab	37.67 a	11.60 ab
9	Geranial	1.78 b	3.24 a	2.48 ab	5.51 ab	2.50 ab	7.01 a	0.05 b	0.03 b	0 b
10	E-anethole	0.01 b	4.78 a	3.45 bc	1.63 bc	3.81 ab	7.91 a	0 c	0 c	0 c
11	Verbenone	5.36 b	16.59	0.41 b	0.26 b	7.47 b	14.10 ab	11.46 b	41.23 a	1.89 b
12	Carvone	0.27 a	0.14 a	0 a	0.29 a	0.39 a	0.27 a	0.23 a	0.26 a	0 a
13	Methyl geranate	5.16 a	7.66 a	4.40 ab	18.15 a	4.48 ab	3.04 ab	0.90 b	0.71 b	13.17 ab
14	Geranyl acetate	2.13 b	19.17	4.77 b	10.90 a	31.58 a	26.08 a	0.28 b	0.79 b	0.15 b
15	α-copaene	0.06 b	0.26 a	0.09 ab	0.17 ab	0.24 ab	0.29 a	0.12 ab	0.24 ab	0 b
16	Allo- aromadendrene	0.14 a	0.13 a	0 a	0.12 a	0.41 a	0.31 a	0 a	0.07 a	0.05 a
17	Germacrene D	0.18 b	0.78 a	0.21 a	0.81 a	0.89 a	0.55 a	0.53 a	0.19 a	0.21 a
18	δ-cadinene	0.34 a	0.08 a	0 a	0.36 a	0.30 a	0.54 a	0 a	0.27 a	0 a
19	Spathulenol	1.19 a	0.59 a	0 a	1.38 a	3.31 a	0.96 a	0.39 a	0.20 a	0 a

D. kotschyi Boiss. in different populations

In each column the same letters are not significantly different at P≤0.05

Table 6 Eigenvectors from the first five principal components for 19 essential oils to classify 14 populations of *D. kotschyi* Boiss.

Chemical compounds	First component	Second component	Third component	Fourth component	Fifth component
α-pinene	-0.109	-0.057	-0.235	0.477	-0.306
p-cymene	-0.249	-0.329	0.136	0.209	-0.071
Limonene	-0.278	-0.183	0.362	0.170	-0.089
1,8-cineole	0.022	-0.318	0.285	0.266	0.071
γ-terpinene	0.179	-0.236	0.029	0.208	0.337
Linalool	0.331	0.047	0.062	-0.031	-0.305
α-terpineol	0.003	0.197	-0.218	0.202	0.352
Neral	-0.309	-0.166	0.111	0.184	0.232
Geranial	0.282	-0.008	-0.166	0.450	-0.071
E-anethole	0.372	-0.117	0.099	0.145	0.237
Verbenone	-0.059	0.215	0.406	-0.027	-0.291
carvone	-0.130	0.426	0.092	0.214	-0.087
Methyl geranate	0.088	0.042	-0.398	0.219	-0.294
Geranyl acetate	0.368	-0.069	0.139	0.165	0.120
α-copaene	0.261	0.131	0.383	0.153	-0.213
Allo-aromadendrene	-0.021	0.331	0.242	0.039	0.325
Germacrene D	0.365	0.039	0.183	-0.118	-0.051
δ-cadinene	-0.161	0.348	0.132	0.324	-0.036
Spathulenol	-0.0197	0.362	-0.055	0.137	0.305
Eigen Value	5.17	3.82	2.53	2.33	1.92
Percentage of	27.22	20.13	13.30	12.25	10.13
Cum Percentage of	27.22	47.35	60.65	72.90	83.03

Hierarchical Clustering, Method =Ward Clustering History

	Distance		Leader	Joiner
Number of Clusters	13	1.572592107	29652G	180G
	12	1.9953019197	798T	2215T
	11	2.4136712157	29652T	29652G
	10	2.6830670144	12938G	798G
	9	2.7894877802	2212T	2213T
	8	3.2481561203	29652T	798T
	7	3.3943390793	12938T	2212G
	6	**4.0874678027**	**29652T**	**180T**
	5	4.5542882425	12938T	2213G
	4	5.4248414293	29652T	2215G
	3	6.1481021583	29652T	12938T
	2	6.5620005083	29652T	12938G
	1	7.0661813692	29652T	2212T

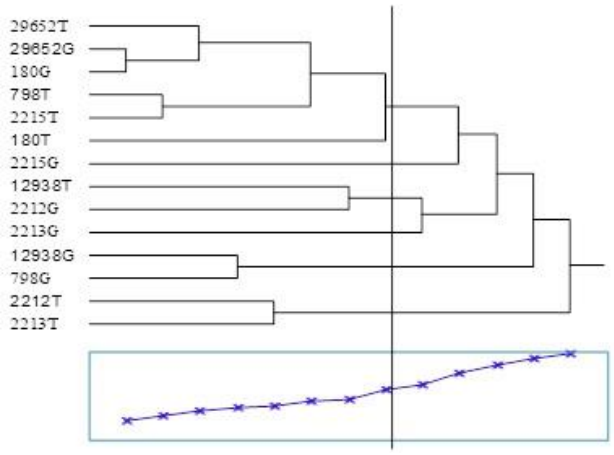

Fig. 1 Dendrogram of 14 populations of *D. kotschyi* Boiss. by analyzing 19 essential oils compound using Ward's cluster analysis method. Cophenetic correlation r = 0.95. (G: greenhouse; T: *in vitro*)

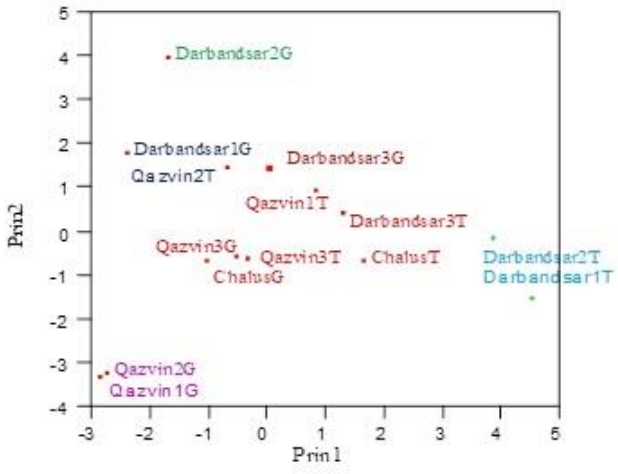

Fig. 2 Scatter plot of 1populations for the first two principal components. (G: greenhouse; T: *in vitro*)

Darbandsar 2 and Darbandsar 3 populations obtained from greenhouse culture, each one is classified as a separate group due to high levels of neral, verbenone and spathulenol and high levels of methyl geranate and geranial compounds in Darbandsar2 and Darbansar 3 separately. The Population of Qazvin2 obtained from in-vitro culture and population of Darbansar 1 obtained from greenhouse together are classified in a separate group due to high levels of verbenone and limonene compounds. Other populations of *D. kotschyi* are classified as a separate group due to more and less similar compounds. The diagram of populations' dispersion, based on the first two components, showed that the populations separated into six groups, which completely fits with results obtained through the grouping analysis by Ward's method (Fig. 2).

This study confirms that the essential oils of plants such as *D. kotschyi* species in different methods of cultures (in-vitro or greenhouse) and various individuals are different. Depending on what kind of essential oils compound, we expect to produce by the plant; different methods of reproduction should be used. For example, for the production of verbenone and methyl geranate, it is better to collect this plant from a suitable area and propagate it by in-vitro culture. This study also introduced new chemotypes o f *D. kotschyi* with high levels of verbenone (Qazvin1 and Qazvin2), methyl geranate (Qazvin3) and geranyl acetate (Darbandsar1 and Darbansar2) compounds, all of which were propagated by in-vitro method. In greenhouse condition we introduced new chemotypes of *D. kotschyi* with a high level of neral (Qazvin1, Qazvin2, Qazvin3 and Qazvin4) compound.

DISCUSSION

The study of secondary metabolites in plants is of major interest in the areas of plant biotechnology and phytochemistry [22]. Comparison of essential oils composition between field plants and in-vitro-cultured plants provided variable results [28]. In some studies, such as *Salvia fruticosa* [32] and *Agastache rugosa* [39] variation in chemical composition of essential oils was observed. According to our result, the main compound of essential oils differed between in-vitro plantlets and greenhouse plants. In addition, the percentage of chemical compounds varied between in-vitro plantlets and greenhouse plants. The amount of verbenone, methyl geranate and geranyl acetate was increased in in-vitro plantlets. However, a high level of neral and limonene were found under greenhouse

conditions. The variation in chemical compound of essential oils between in-vitro plantlets and greenhouse plants is related to different environment of in-vitro and greenhouse culture condition. Under in-vitro conditions, the relative humidity was high. In addition, auxin and cytokinine hormones stimulate secondary metabolite accumulation [40] and rejuvenation of shoots causes accumulation of volatile compounds [30]. However, in greenhouse conditions, the relative humidity was low, light intensity was high, and non-sterile condition caused monoterpene accumulation [41]. In contrast to the results of this study, the chemical composition of essential oils between in-vitro culture and field condition was reported to be similar in plants of *Lavandula viridis* [42], *Lavandula pedunculata* [43], *Minostachys mollis* [33], *Mentha spicata* [44], *T. mastichina* [45], *Varronia curassavica* [29].

According to Andrys and Kulpa (2017) the chemical constituent of borneol was dominant in both in-vitro plantlets and in-vivo plants of *lavandula angustifolia.* However, the significant difference was found between in-vitro and field conditions. The essential oils composition of intact plant and in-vitro shoots and adventitious roots of *Caryopteris* species varied significantly which is in agreement with our results [27]. Similar results mentioned in relation to chemical composition of *Teucrium scorodonia* ssp. Scorodonia [26]

Limonene, geranial and geranyl acetate were reported as main compounds of *D. kotschyi* in the past studies [15,18]. Najafpour Navaei and Mirza (2007) compared the essential oils composition of *D. kotschyi* between habitat and field condition. They reported that limonene (29%), methyl geranate (17.7%) and geranial (15.8%) were identified in the plant samples collected from natural habitats and myrtenol (30.8%), limonene (23.6%) and geranial (14.3%) were identified in the plants cultivated under field condition.

Saeidnia et al. (2007) reported that geranial (35.8%) and limonene (15.8%) were the main compounds of *D. kotschyi* species. In another study, α-pinene (7.32%), caryophyllene oxide (6.6%), 4- terpineole (4.1%), and germacrene D (4%) were reported to be the main compounds in the plant samples collected from Isfahan population [17]. According to Karamkhani (2003) [46], verbenone was identified in *D. kotschyi* plant samples collected from Bojnoord town. The amount of this compound in water distillation and steam distillation method was 21.37% and 19.89%, respectively. According to this study, verbenone was the major compound of essential oils in in-vitro plantlets. Verbenone is a natural chemical compound that is used against *Dendroctonus ponderosae* of pine trees [47]. Therefore, in-vitro plantlet production of *D. kotschyi* is important for commercial production of verbenone chemotype. Variation in chemical composition was observed among populations of this species. This difference may be due to the genetic variation among populations of this species [48].

According to cluster analysis, the essential oils compound diversity displayed by in-vitro plantlets and greenhouse plants can be attributed to the geographical origin of populations and is affected by culture conditions. It should be mentioned that the chemical composition and percentage are affected by genotype, culture conditions, environmental factors, and their interaction.

CONCLUSION

In this study, chemical compounds and their percentage were compared between in-vitro plantlets and greenhouse plants of *D. kotschyi* populations. The main compounds identified in various populations were limonene, neral, verbenone, methyl geranate, geranyl acetate, E-Anethol and geranial. The conducted protocol of this experiment would be applied to an investigation of volatile chemical compounds of medicinal plants especially Lamiaceae family.

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