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Effect of density and year of cultivation on essential oil content and chemical compounds of *Achillea millefolium* subsp. *elbursensis*

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To domesticate and cultivate *Achillea millefolium* subsp. *Elbursensis* Hub.-Mor., its seeds were collected from natural habitat in the Dizin region and were cultivated in the research field in March 2017. Effects of cultivation density (5, 8, 12 shrubs per m^2 and cultivation year (2017 and 2018) on essential oil percentage and main chemical compounds were investigated. Essential oil extraction of flowering shoots was carried out by the hydrodistillation method. Determination of chemical compounds was done by (GC) and (GC-MS). Chamazulene was determined as the main compound in all treatments. Effect of cultivation year and density on the essential oil content and main compounds was significant. The highest essential oil percentage was found in 12 shrubs per m^2 density during two examined years. The highest essential oil yield (0.42%) was observed at 12 shrubs per m^2 in 2018. But the lowest essential oil percentage (0.14%) was found at 5 shrubs per m^2 in 2017. The maximum content of camphor (8.6%) and borneol (10.30%) was obtained at 5 shrubs per m^2 density in 2018. The highest chamazulene (75.63%) was found at 12 shrubs per m^2 density in 2018.

Keywords: *Achillea millefolium* subsp. *elbursensis* Hub.-Mor., Borneol, Camphor, Chamazulene, Domestication, Essential oil percentage.

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Introduction

Asteraceae family has various flowering plants and contains about 600 genera and 23000 species. Genus *Achillea* includes 115 species that are distributed in the North hemisphere and 19 herbaceous and perennial aromatic plants in Iran[1]. *Achillea millefolium* subsp. *elbursensis* Hub.-Mor., commonly known as yarrow, is a permanent shrub containing stem with a height of 20 to 40 cm and leaves with delicate hairs almost broad and dense. The leaves of the middle part of the stem are more or less rectangular, and their length is up to 1.5 mm. The flowers are white and purple. The flowering time is summer and distributed in Mazandaran and Tehran province[2]. In conventional medicine, yarrow is used as a tonic, relaxation, rheumatic problem, nervous pain relief, diuretic, cold treatment, digestive system, gastric diseases and haemorrhoids improvement[3-4]. In a recent study, the apoptosis effect of essential oil from *A. millefolium* against HeLa cells was proven[5]. One of the main compounds of essential oil from *Achillea* species is chamazulene that is derived from matricine with anti-inflammatory property. Therefore, it is recommended for use in cosmetic and hygienic products to cure skin inflammation diseases[6].

Effect of cultivation date and density has been investigated in various medicinal plants such as *Matricaria chamomilla*[7], *Achillea millefolium*[8], *Thymus* species[9-10], *Catharanthus roseous*[11], *Dracocephalum moldavica*[12], *Coriandrum sativum*[13], and *Foeniculum vulgare*[14].

Essential oil compositions of various *Achillea* species have been studied by several researchers[5,15-21]. Jaim and *et al.*[22] studied essential oil percentage of *A. millefolium* subsp. *elbursensis* and chemical constituents by using hydrodistillation and steam distillation methods. Askari *et al.*[23] compared essential oil of leaf and flower of *A. millefolium* subsp. *Elbursensis* under habitat and cultivated conditions.

In this study, the effect of cultivation year and density on essential oil percentage and main chemical compounds of *A. millefolium* subsp. *elbursensis* was investigated.

Materials and Methods

Seeds of *A. millefolium* subsp. *elbursensis* were collected from natural habitat located in the Dizin

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region (2800 m altitude, the highest altitude of the ski slope) of Tehran province. Seeds were planted in cultivation try containing peat moss under greenhouse conditions in March 2017. The voucher specimen (No. 104120 TARI) was deposited in the Research Institute of Forests and Rangelands (RIFR), TARI herbarium. Then seedling was transplanted to the field condition with different density (5, 8, and 12 shrubs/m^2) in April 2017 (Table 1). The experiment was conducted in a completely randomized block design with three replications in the Research field of medicinal plants located in the Alborz Research Center, RIFR. The soil sample was collected randomly from each Crete and then mixed together. Physicochemical analysis of soil sample was determined in Soil Lab of RIFR (Table 2).

Essential oil extraction

Flowering shoots were collected from three replications of each treatment. All samples were dried under lab temperature and milled into small pieces. Essential oil extraction was done by hydrodistillation method with Clevenger apparatus[24,25]. Essential oil percentage was calculated through a proven protocol. The essential oils were poured into a small container and were stored in a refrigerator at 4 ºC. The quality and quantity of essential oils were analyzed with Gas Chromatography (GC) and Gas Chromatography equipped Mass Spectrometry (GC-MS) instruments.

Table 1 — Map of plant cultivation in the research field of Alborz research station, Research Institute of Forests and Rangelands.

Cultivation condition	5 plants/m^2	8 plants/m^2	12 plants/m^2
Plot length (m)	6	6	6
Plot width (m)	3	3	3
Plot area (m^2)	18	18	18
Rows distance (cm)	50	40	30
Plants distance (cm)	40	30	30
Numbers of rows	6	7	10
Number of plants in each row	15	20	20
Total number of plants	90	140	200
Area occupied with each plant	0.09	0.13	0.2

Gas chromatography

GC analysis was done by Shimadzu GC-9A Gas chromatograph equipped with a DB5 fused silica column (60 m x 0.25 mm; film thickness 0.25 µm). The oven temperature was kept at 50 °C for 5 minutes and then was programmed to 270 °C at a rate of 3 °C/min, injector and detector (FID) temperature were 280 °C. Helium was used as carrier gas at a linear velocity of 32 cm/s. The relative amounts of individual components were 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 analysis was achieved on a Varian 3400 GC-MS system equipped with a DB-5 fused silica column (60 m x 0.25 mm, film thickness 0.25 µm) and was interfaced with a Varian ion trap detector. Essential oil was diluted with dichloromethane 1:100 ratio and 1 µL of the diluted sample was injected into the GC-MS. The oven temperature was programmed for 50-270 °C at a rate of 3 °C/min, injector, and transfer line temperature was 280 and 290 °C, respectively. Helium was employed as carrier gas with a linear velocity of 31.5 cm/s, split ratio 1/60, ionization energy 70 ev, scan time 1s and mass range 40-400 amu.

Identification of chemical components

The oil components were identified by comparing their mass spectra with those of the computer library NIST library and Willey[26-30] or with authentic compounds. The accuracy of chemical compounds was confirmed by comparison of their retention index with those of authentic compounds or with published data in the literature.

Result

In this study, the effect of cultivation density and year of cultivation on essential oil percentage and main chemical compounds were investigated (Table 3). The effect of cultivation density on essential oil percentage was significant (P <0.05). The highest essential oil content (0.4%) was obtained in 12 shrubs per m^2 and the lowest essential oil content (0.19%) was recorded in 5 shrubs per m^2.

Table 2 — Physicochemical characteristics of experimental field soil

Depth of sampling (cm)	Electrical conductivity (ds/m)	Organic carbon (%)	Nitrogen (%)	Potassium (mg/kg)	Phosphorus (mg/kg)	pH	Sand (%)	Silt (%)	Soil texture
0-30	0.1413	8.5	0.095	238	8.95	7.64	70.4	30.27	Sandy-loam

Table 3 — Effect of density on essential oil content and main chemical compounds of *Achillea millefolium* subsp. *elbursensis*

Density (plants/m^2)	Essential oil content	Camphor	Borneol	E-anethole	E-caryophyllene	E-nerolidol	Caryophyllene oxide	Chamazulene
5	0.19^{b}	7.40^{a}	8.66^{a}	4.80^{a}	2.80^{a}	2.88^{a}	3.05^{a}	48.27^{c}
8	0.23^{ab}	0.76^{b}	0.2^{b}	2.21^{a}	2.93^{a}	1.90^{a}	5.30^{a}	62.30^{b}
12	0.4^{a}	1.2^{b}	0.33^{b}	0.58^{a}	2.46^{a}	1.75^{a}	3.01^{a}	71.92^{a}

Means followed by the same letters are not significantly different at 5% probability level

Table 4 — Effect of density on essential oil content and main chemical compound of *Achillea millefolium* subsp. *elbursensis*

Treatment Year	Density (plants/m^2)	Essential oil content	Camphor	Borneol	E-anethole	E-caryophyllene	E-nerolidol	Caryophyllene oxide	Chamazulene
2017	5	0.14^{b}	6.2^{a}	7.3^{a}	1.63^{a}	2.83^{a}	4.63^{a}	3.6^{a}	53.37^{c}
	8	0.22^{ab}	1.3^{b}	0.3^{b}	1.46^{a}	2.83^{a}	1.73^{a}	6.30^{a}	62.87^{b}
	12	0.39^{a}	1.1^{b}	0.3^{b}	0.63^{a}	2.46^{a}	1.86^{a}	3.96^{a}	68.20^{b}
2018	5	0.25^{ab}	8.6^{a}	10.3^{a}	7.30^{a}	2.76^{a}	1.13^{a}	2.5^{a}	43.17^{d}
	8	0.25^{ab}	0.23^{b}	0.3^{b}	2.96^{a}	3.03^{a}	2.06^{a}	4.26^{a}	61.73^{b}
	12	0.42^{a}	1.3^{b}	0.3^{b}	0.53^{a}	2.46^{a}	1.63^{a}	2.06^{a}	75.63^{a}

Means followed by the same letters are not significantly different at 5% probability level

The effect of cultivation density on main chemical compounds including camphor, borneol, and chamazulene was significant at $P < 0.05$ (Table 3). However, cultivation density didn't have a significant effect on E-anethole, E-caryophyllene, and E-nerolidol as well as caryophyllene oxide.

The interactive effects of year and density on essential oil percentage and camphor, borneol as well as chamazulene were significant at $P < 0.01$ (Table 4). The highest essential oil percentage (0.42%) was observed in 2018 at 12 shrubs per m^2. The lowest essential oil percentage (0.14%) was seen in 2017 at 5 shrubs per m^2. The highest camphor content (8.6%) and the minimum camphor percentage (0.23%) were obtained in 2018 at 5 shrubs per m^2 and 8 shrubs per m^2, respectively. The highest borneol (10.30%) was found in 2018 at 5 shrubs per m^2 and the lowest borneol (0.3%) was seen in 2018 at 8 and 12 shrubs per m^2. The highest chamazulene (75.63%) was observed in 2018 at 12 shrubs per m^2 and the lowest chamazulene content (43.17%) was seen in 2018 at 12 and 5 shrubs per m^2. The list of all identified chemical compounds is given in Table 5.

Discussion

Essential oil percentage fluctuated between 0.19 to 0.4% in different cultivation density. The study on the interactive effect of cultivation year and cultivation density showed that essential oil percentage varied between 0.14 to 0.42%. The essential oil percentage of leaf and inflorescence of this subspecies collected from natural habitat were 0.11 and 0.53%, respectively. Also, the essential oil percentage collected from the experimental field in two consecutive years was 0.28 and 0.26% for leaf sample and 0.5 and 1.3% for inflorescence[23]. Jaim and *et al*.[22] mentioned that essential oil percentage of inflorescence and leaf of *A. millefolium* L. subsp. *elbursensis* collected from natural habitat was 0.2 to 0.5% and 0.1%, respectively. In this study, the enhancement of essential oil percentage in the second year of cultivation was evident. These phenomena related to better establishment and adaptation of plants in field condition during the second year in comparison to the first year of cultivation[31]. Essential oil percentages of *A. millefolium* from India was 0.7%[(ref 21)] from Estonia was 0.9-9.5%[(ref 32)], and flowering shoots from Shiraz habitat was 1.2%[(ref 33)].

The main identified chemical constituents were chamazulene, camphor, borneol, E-anethole, E-caryophyllene, E-nerolidol, and caryophyllene oxide. Chamazulene has been reported as the main compound in different investigations[18,22-23,34] and some reports introduced camphor as one of the main compounds in Yarrow[5,17,19,35].

Effect of cultivation year on essential oil percentage was significant at $P < 0.05$. Essential oil content increased in the second year in all density

Table 5 — Chemical compositions mean of *Achillea millefolium* subsp. *elbursensis* identified by GC-MS

Chemical compounds			2017			2018		
	RI*	RI**	5 plants/m^2	8 plants/m^2	12 plants/m^2	5 plants/m^2	8 plants/m^2	12 plants/m^2
ρ-cymene	1055	1026	-	0.1	0.6	0.7	0.1	-
1,8-cineole	1065	1033	0.3	1.1	4.1	1.9	1.1	0.2
Camphor	1181	1143	6.2	1.3	1.1	8.6	0.23	1.3
Borneol	1222	1165	7.3	0.3	0.3	10.3	0.3	0.3
α-terpineol	1236	1189	0.3	-	0.4	0.6	0.6	-
Methyl chavicol	1297	1196	0.3	0.2	0.3	0.4	0.4	-
Geranial	1320	1253	0.5	0.4	0.5	5.9	0.7	-
E-anethole	1330	1285	1.6	1.5	0.6	7.3	3.0	0.5
Carvacrol	1370	1298	0.1	-	0.3	0.3	0.2	0.1
Geranyl acetate	1377	1381	0.5	0.6		0.7	0.3	0.3
Trans-myrtanol acetate	1379	1387	0.2	-	0.3	0.7	2.2	0.5
α-cedrene	1458	1409	0.4	0.2	0.2	0.1	0.6	-
E-caryophyllene	1473	1418	2.8	2.8	2.5	2.8	3.0	2.5
β-chamigrene	1511	1475	0.7	0.6	0.2		1.4	-
γ-gurjunene	1520	1477	0.5	0.5	0.9	1.9	1.4	2.1
Germacrene D	1537	1480	1.4	1.7	1.4	1.4	1	0.7
Bicyclogermacrene	1548	1494	0.6	1.6	0.7	0.7	0.4	0.1
E-E-α-farnesene	1562	1506	0.1	0.1	-	0.4	0.6	0.4
E-nerolidol	1575	1564	4.6	1.7	1.9	1.1	2.1	1.6
Spathulenol	1652	1576	-	0.9	-	1	2.4	0.6
Caryophyllene oxide	1662	1581	3.6	6.3	4.0	2.5	4.3	2.1
Humulene epoxide II	1693	1608	0.5	0.7	0.3	0.2	0.3	0.8
β-eudesmol	1736	1651	1.4	2.8	2.2	1.3	2.9	2.4
(Z,E)-farnesyl acetate	1822	1701	1.2	1.8	1.2	0.9	1.9	2.7
Chamazulene	1837	1732	53.4	62.9	68.2	43.2	61.7	75.6
Oplopanone	1859	1740	0.6	0.7	0.6	0.4	0.5	0.6
Khusimol	1867	1743	1.6	2.4	1.6	0.5	0.9	0.6
α-sinensal	1892	1757	1.2	1.8	1.4	0.7	1.2	1.6
Total			91.9	95.0	95.7	96.5	95.7	97.6
Number of chemical compounds			26	25	25	27	28	22

RI* library retention index

RI**literature retention index

treatments. Chamazulene as the main compound increased in 12 shrubs per m^2 in two consecutive years. Dadkhah *et al.*[7] showed that the cultivation season had a significant effect on chamomile essential oil under field condition and increasingly affected essential oil yield. Ghani and Azizi[8] found that cultivation date changes essential oil yield and percentage in *A. millefolium* subsp. *millefolium*. Lebaschy *et al.*[10] reported that the highest essential oil yield and thymol content of thymus species were in 8 and 6 shrubs per m^2, respectively. In conclusion, high shrub density increases essential oil yield because of higher competition among plants to obtain mineral from soil and utilization of environmental factors such as light and moisture.

Conclusion

In this study, the effect of cultivation density and year of cultivation on essential oil percentage and chemical composition of the plant were studied. The highest essential oil percentage and chamazulene content were observed during the second year of cultivation at 12 shrubs per m^2 density. Consequently, harvesting of flowering shoots in the second year and cultivation of plants at 12 shrubs per m^2 density are recommended to obtain maximum essential oil percentage and chamazulene content. This plant can be introduced in the cosmetics industry due to its valuable amounts of chamazulene or as an ornamental plant in horticultural practices.

Declaration of interests

The authors declare that there are no conflicts of interest.

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