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Effect of altitude on essential oil composition and on glandular trichome density in three *Nepeta* species (*N. sessilifolia*, *N. heliotropifolia* and *N. fissa*)

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Abstract. In the current study, the trichome density and the chemical variation of the hydrodistillated essential oils were determined along the altitudinal gradient in *Nepeta sessilifolia*, *N. heliotropifolia*, and *N. fissa*. The capitate glandular trichomes were predominant in all populations studied. Denser leaves indumentum was observed in high altitude populations of *N. heliotropifolia*, and in low altitude populations of *N. sessilifolia* and *N. fissa*. Moreover, the inter-population chemical polymorphism was detected. In the lower population of *N. sessilifolia,* spathulenol (14.2%) was the major compound of essential oils. This oil had great amount of oxygenated sesquiterpene (35.3%), while the oil of high altitude population had great amount of diterpenes, namely phytol (32.8%). In *N. fissa,* the main compound in lower population was β-caryophyllene (33.1%), whereas in the higher population it was caryophyllene oxide (21.5%). In *N. heliotropifolia*, 1,8-cineole (20.1%) was as the principal oil compound in low altitude population and β-caryophyllene (18.8%) in the high altitude population. We suggested that the amount of different types of monoterpenes decreases along the altitudinal gradient and the amount of oxygenated compounds increases with the elevation.
Keywords: *Nepeta fissa*; *Nepeta heliotropifolia*; *Nepeta sessilifolia*; chemical polymorphism; indumentum; altitudinal gradient; Iran.

Efectos de la altitud sobre la composición química de los aceites esenciales y la densidad de los tricomas glandulares en tres especies del género *Nepeta* (*N. sessilifolia*, *N. heliotropifolia* and *N. fissa*)

Resumen. En el presente trabajo se ha estudiado el efecto del gradiente altitudinal sobre la densidad de los tricomas y la variabilidad química de los aceites esenciales hidrodestilados en *Nepeta sessilifolia*, *N. heliotropifolia* y *N. fissa*. Se han observado los tricomas glandulares capitados como el tipo predominante en todas las poblaciones estudiadas. Mayor densidad del indumento foliar fue observado en las poblaciones situadas a mayor altitud en *N. heliotropifolia* y en las de menor altitud en *N. sessilifolia* y *N. fissa*. Además se han detectado diferencias en la composición química entre las poblaciones de la misma especie. En la población de *N. sessilifolia* situada a menor altitud se ha detectado el espatulenol como el mayor componente del aceite (14.2%). Este aceite presentaba mayor cantidad de los sesquiterpenos oxigenados (35.3%), mientras el aceite obtenido de la población situada a más altitud presentaba una gran cantidad de los diterpenos, especialmente fitol (32.8%). En *N. fissa*, la población situada a menor altitud tenía como el componente principal de los aceites esenciales el β-cariofileno (33.1%) en comparación con la de mayor altitud, cuyo componente principal fueron los óxidos cariofilenos (21.5%). En *N. heliotropifolia*, hemos observado que el componente principal a menor altitud fue el 1,8-cineol (20.1%), que presenta mayor cantidad de los monoterpenos oxigenados. En la población de mayor altitud, se ha observado gran cantidad de los sesquiterpenos (43.1%) con β-cariofileno (18.8%) como el componente principal. Finalmente, podemos concluir que existe una relación positiva entre el gradiente altitudinal y los niveles de los compuestos oxigenados y una relación negativa con los niveles de diferentes monoteprenos.
Palabras clave: *Nepeta fissa*; *Nepeta heliotropifolia*; *Nepeta sessilifolia*; variablidad química; indumento; gradiente altitudinal; Irán.

Introduction

Variations in essential oil compositions confirm the possibility of chemical polymorphism among different populations of the same plant species (Yarmohammadi *et al.,* 2017). Therefore, it is very important to characterize and identify the existence of chemotypes of the plant material used in chemical, agronomic, and pharmacological investigations to produce the herbal medicines. The pharmacological activitiy of the same species could vary due to differences in composition of essential oil (Lima *et al.,* 2003; Potzernheim *et al.,* 2006; Paula *et al.,* 2011).

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Kofidis & Bosabalidis (2008) proposed that altitude is one of the most important ecological factors. Different environmental factors like light intensity, wind exposure, ozone concentration, and partial CO_2 pressure may also vary among different altitudes.

Gianoli & González-Teuber (2005) showed that the trichomes are typically long and dense in morphological traits. Several investigations have focused on examining trichomes response to soil water deficit to determine whether the trichomes increase it resistance ability towards drought stress in plants (Gianoli & González-Teuber, 2005; Meng *et al.,* 2014). Indumentum is rather plastic adaptive pattern towards the drought as barrier against the influence of CO_2 and H_2O exchange, which decreases excessive transpiration and photoinhibition (Pallioti *et al.,* 1994; Gianoli & González-Teuber, 2005). Moreover, trichomes can decline the plant solar radiation absorption and deduct its temperature by increasing the leaf surface boundary layer (Schreuder *et al.,* 2001).

Furthermore, the essential oil, in Labiatae species, is accumulated and secreted by glandular trichomes, and each type of glandular hair stores special compounds. Therefore, all changes and variability of trichome type and number alter the amount and chemical composition of the essential oils (Askary *et al.,* 2016a, b).

Nepeta is one of most important genera of Labiatae family in Iran. Seventy-nine species of this genus grow widely in different parts of Iran (Jamzad, 2012) and sixteen of them were used for treatment of different diseases, as culinary or industrial plants (Naghibi *et al.,* 2005). Three of them have been selected for this study: *Nepeta sessilifolia* Bunge., *N. heliotropifolia* Lam. and *N. fissa* C.A. Mey. Iran is one of the main distribution centers of *N. fissa* (Jamzad, 2012), perennial glabrous to densely puberulous plant. Leaves are usually triangular, rarely rhomboid. Inflorescence is usually lax and flowers congested or loose. Calyx is tubular and corolla is blue or lilac to purple, exserted from calyx teeth. This species grows in volcanic and serpentine rocks and its flowering time is July. *Nepeta heliotropifolia* and *N. sessilifolia* are endemic species of Iran (Sajjadi & Khatamsaz, 2001; Jamzad, 2012). *Nepeta heliotropifolia* is perennial plant with several stems. Leaves are oblong, ovate or elliptic. Inflorescence is widely branched thyrse. Calyx is long with triangular-lanceolate teeth. Corolla is pale lilac to blue. This aromatic herb grows in steppe, fallow or cultivated fields and slope of mountain. Its flowers were seen from June to July. *Nepeta sessilifolia* is distinctly varied from the other *Nepeta* species with its sessile cauline leaves. Moreover, this species has flowers with long exserted stamens. It grows in rocky slope of mountain in west and central parts of Iran and its flowers appear between June and July (Jamzad, 2012).

Formisano *et al.* (2011) have stated that nepetalactones, iridoids and their glucosides, diterpenes, triterpenes, and flavonoids are major constituents of *Nepeta* taxa. Depending upon the composition of the major compounds in the essential oils, *Nepeta* species have been divided into two groups: one that contained different isomers of nepetalactone and the second that had compounds other than isomers of nepetalactone like 1,8-cineole, β-caryophyllene, caryophyllene oxide, β-farnesene, α-citral and β-citronellol as their major constituents (Sajjadi, 2005). Medicinal properties of *Nepeta* species are related to terpenoids and flavonoids. Compounds such as 1,8-cineole are very common in *Nepeta* and have expectorant, antiseptic and anthelmintic activities (Jamzad, 2001).

In this study, we examined the effect of altitude on the variations in essential oil compositions of three *Nepeta* species: *N. sessilifolia*, *N. heliotropifolia*, and *N. fissa.* Almost all previous studies on the essential oils of these species (Baser *et al.*, 2000; Sajjadi & Khatamsaz, 2001: Jamzad *et al.*, 2008; Batooli & Safaei-Ghomi, 2012; Yarmohammadi *et al.*, 2017; Talebi *et al.*, 2017) were based on one mixed collective plant sample from one or two populations. By this way, we obtain a general picture of essential oil composition of these plants, and we lack the information about the chemical variability of its populations along the altitude gradient. It is well-known fact that biosynthesis of terpene is influenced by various ecological factors, therefore, the aims of our study were: a) to characterize the essential oils of these species at population level; b) to compare the essential oil composition between the populations of the same species; c) to study the effect of altitude on essential oil compositions and d) to investigate the glandular trichome intra-population variations.

Material and methods

Plant material. We selected two populations from each species at two different altitudes (with at least 300m differences) (Table 1). We identified the plants according to Flora of Iran (Jamzad, 2012). Aerial parts of the studied populations were harvested at the flowering stage of plant development (June) during 2017. We selected forty-five plant samples from each population with a minimum distance of 80m, mixed them for homogenization, and made three replications for essential oil extractions. The voucher specimens were lodged in the Herbarium of Arak University (AUH).

Isolation and analysis of essential oil. The aerial parts of the plant material were dried at room temperature. Essential oils were achieved by hydro-distillation method in a Clevenger-type apparatus (Anon. 2011) and its yields were calculated based on dried weight using the equation: $EO\% = (M / B_m) \times 100$ where M is the mass of the extracted oil (g) and B_m is the initial plant biomass (g) (Da Costa *et al.,* 2014). The volatile compounds were analyzed by Gas Chromatography (GC) and Mass Spectrometry (MS). An Agilent 6890 N GC system was used, equipped with a 5975 MSD and an FID, using an HP-5 MS column (30m × 0.25mm, 0.25mm film thickness). The volume of injection was 2μL and the temperature of injector 200°C with a 10:1 split ratio. The carrier gas was Helium, and the rate of gas flow was 1.0ml/min (constant flow mode). The temperature of the column was linearly programmed in the range of 60-280°C with step of 3°C /min and held at 280°C for 5 min.

The transfer line was heated at 250°C. The retention indices of essential oil compounds were determined in two series of n-alkanes (C8-C20 and C21-C40) under equal chromatographic conditions. Identification of components was based on the comparison of experimental data (their retention indices and mass spectra) with those obtained from authentic samples, the NIST AMDIS software, Wiley libraries, the Adams database, and available literature (Adams, 1995). The parameters of MS scan included an electron impact ionization voltage of 70 eV, a mass range of 40–400 $m.z^{-1}$ and a scan interval of 0.5s. Relative percentages of the identified components were computed from the peak area of GC.

Table 1. Harvesting collection locations of the studied *Nepeta* species.

Coding	Populations	Habitat	Latitude	longitude	Voucher number
NHLA	*N. heliotropifolia* Lam.	Markazi province, Kharaghan, Vidar, 2100 m a.s.l.	35° 19′ 39.89” N	050° 06′ 50. 40” E	2001-AUH
NHHA	*N. heliotropifolia* Lam.	Markazi province, Kharaghan, Vidar, 2410 m a.s.l.	35° 20′ 20.50” N	050° 06′ 49. 31” E	2002-AUH
NSLA	*N. sessilifolia* Bunge	Markazi province, Arak, Sefidkhani mountain 1920 m a.s.l.	33° 59′ 30.7” N	049° 34′ 12. 09” E	2003-AUH
NSHA	*N. sessilifolia* Bunge	Markazi province, Arak, Sefidkhani mountain 2290 m a.s.l.	33° 59′ 25.2” N	049° 34′ 18. 01” E	2004-AUH
NFLA	*N. fissa* C.A. Mey.	Markazi province, Nobaran, Sangak, 1930 m a.s.l.	35° 15′ 01.2” N	049° 45′ 32. 7” E	2005-AUH
NFHA	*N. fissa* C.A. Mey.	Markazi province, Nobaran, Sangak, 2280 m a.s.l.	35° 27′ 39.6” N	049° 50′ 24. 10” E	2006-AUH

Light microscopy (LM). Six mature leaves were selected from six plant samples of each population. The leaves were fixed in the FAA (formaldehyde 90%, ethanol 5% and acetic acid 5%) solution. The plant samples were dehydrated in an ethanol series (Johansen, 1940). The anatomical analysis of the hairs was based on the semi-thin sections made from transects obtained from the central part of the leaf blade. The leaves slices were stained with a 1% aqueous methylene blue and carmine solutions. The observations were made and microphotographs were shot using a Cannon Power shot adapted to an Olympus CH_2 optic light microscope.

Scanning electron microscopy (SEM). Small parts (6mm×10mm) of each selected leaf were fixed in a 5% glutaraldehyde solution in 0.1M phosphate buffer (pH 7.0) for 10h at 24°C. Then, the plant samples were washed in the same buffer three times at 15min intervals and after that were dehydrated in different series of ethanol (30, 50, 70, 90, and 95%) followed by the double usage of absolute alcohol. Dehydrated leaf slices were transferred to acetone. In this time, plant slices dried at a critical point in liquid CO_2 and coated with gold using the Polaron SC 7640 sputter coater (Robards, 1978). The images of glandular trichomes were captured with SU 3500 scanning electron microscope, at 5–10 kv accelerating voltage.

Statistical analyses. The mean and standard deviation of the recorded hairs number were calculated. Data were standardized (mean=0, variance=1) for Correspondence Analysis (CA), Principal Coordinate Ordination (PCO) and Unweighted Paired Group Using Average method (UPGMA) (Higgs, 1991; Podani, 2000). One-way analysis of variance (ANOVA) was used to compare the trichomes numbers among the studied species. We used SPSS 9.0 (1998) and MVSP 2.0 (1998). Trichomes densities of four slices per leaf were counted with light microscope. Digital image processing has been used for leaf area measurement by Image Tool 2.0. Trichomes density was calculated by dividing the hair number per mm^2 of leaf area.

Results

Essential oil analyses

All the studied populations yielded essential oil from 0.10 to 0.25% based on dry weight (Table 2). The obtained essential oils were analyzed by GC/MS.

Essential oil compositions of *Nepeta sessilifolia* populations. Forty-three compounds were identified from the essential oil composition of *N. sessilifolia.* It is worth nothing that the main group of compounds varies between both localities studied. While the low altitude population (NSLA) showed sesquiterpenes (56.8%) as predominant ones, the high altitude populations (NSHA) was characterized by other compounds (52.5%). Other difference was the amount of monoterpenes, in NSLA they sum 31.9% while in NSHA were not detected. In both cases, the oxygenated sesquiterpenes were more abundant with 35.3% and 35.5% in NSLA and NSHA, respectively. The biggest differences between both localities were the absence of monoterpenes and the big amount of other compounds in NSHA in comparison with NSLA (Table 2). The principal compounds of NSLA were identified as spathulenol (14.2%), *epi*-cedrol (8.8%), germacrene D (6.9%), bicylcogermacrene (5.0%), α-terpinyl acetate (4.8%) and β-caryophyllene (4.3%). n-hexadecanoic acid (0.9%) and phytol (1.3%) were recorded in the low content. In NSHA population the main constituents were: phytol (32.8%), n-hexadecanoic acid (13.4%), caryophyllene oxide (11.9%), *epi*-cedrol (8.0%), spathulenol (7.8%), (*Z,Z,Z*)-9,12,15-octadecaterien-1-ol (5.2%) and 14-hydroxy-9-*epi*-β- caryophyllene (4.3%). β-caryophyllene (1.3%) and germacrene D (1.2%) were registered in small amount. α-terpinyl acetate and bicylcogermacrene were not detected.

Table 2. Chemical constituents identified in the essential oils of the studied populations of *Nepeta* species. (KI: Kovats' Index).

No	Compounds	NSLA (%)	NSHA (%)	NFLA (%)	NFHA (%)	NHLA (%)	NHHA (%)	KI
	Essential oil yield (%)	0.1	0.15	0.2	0.2	0.2	0.25	
Monoterpene hydrocarbons								
1	Santene	–	–	–	–	0.3	–	883
2	Thujene	–	–	–	–	0.3	–	928
3	α-Pinene	2.7	–	10.5	–	2.1	–	935
4	Sabinene	1.23	–	–	–	5.2	1.0	976
5	β-Pinene	0.4	–	0.8	–	12.4	2.7	982
6	Myrcene	0.4	–	–	–	0.2	–	993
7	δ-2-Carene	1.3	–	–	–	–	–	1013
8	α-Terpinene	4.1	–	–	–	–	–	1022
9	*(p)*-Cymene	4.1	–	–	–	0.5	–	1032
10	Limonene	0.6	–	0.5	–	0.4	–	1034
11	*(Z)*-β-Ocimene	0.4	–	–	–	0.3	–	1041
12	δ-Terpinene	1.5	–	–	–	0.4	–	1064
13	Terpinolene	0.5	–	–	–	–	–	1090
14	Geijerene	0.9	–	–	–	–	–	1147
Oxygenated monoterpenes								
15	1,8-Cineole	3.5	–	–	–	20.1	4.3	1038
16	Linalool	0.7	–	–	–	–	–	1107
17	*trans*-pinocarveol	–	–	–	–	2.9	1.4	1151
18	*cis*-Verbenol	0.5	0	1.7	–	–	–	1152
19	*tran*-Verbenol	0.7	–	–	1.39	0.6	0.4	1156
20	Pinocarvone	–	–	–	–	1.8	–	1176
21	δ-Terpineol	–	–	–	–	0.5	–	1182
22	Terpinen-4-ol	1.8	–	–	–	0.6	0.5	1191
23	α-Terpineol	1.1	–	–	–	–	–	1207
24	Myrtenol	–	–	–	–	2.7	3.2	1207
25	Myrtenal	–	–	–	–	3.0	–	1209
26	Geraniol	–	–	–	–	–	1.3	1259
27	*iso*-Verbenol acetate	0.7	–	–	–	–	–	1302
28	α-Terpinyl acetate	4.8	–	–	–	–	0.7	1355
29	Geranyl acetate	–	–	–	–	–	1.0	1385
Sesquiterpene hydrocarbons								
30	α-Cubebene	0.5	–	–	–	–	–	1351
31	α-Copaene	–	–	1.5	1.1	0.7	–	1381
32	β-Bourbonene	–	–	0.5	–	1.9	–	1390
33	β-Elemene	–	–	–	–	0.6	–	1395
34	β-Caryophyllene	4.3	1.3	33.1	13.8	4.5	18.8	1427
35	*cis*-Thujopsene	–	–	0.6	–	–	–	1444
36	*α*–Humulene	0.68	–	1.3	–	0.8	1.6	1464
37	*allo*-Aromadendrene	–	–	–	–	0.2	1.1	1469
38	*cis*-Muurola-4(14),5-diene	1.82	–	–	–	–	–	1471
39	Germacrene D	6.91	1.2	9.6	4.8	11.6	3.3	1490
40	δ-Selinene	0.84	–	–	–	–	–	1499
41	Bicyclogermacrene	5.01	–	4.0	1.6	2.5	11.5	1505
42	*(E,E)*-α-Farnesene	0.26	–	1.4	1.0	0.9	6.7	1457
43	β-Bisabolene	–	–	–	1.1	–	–	1514
44	δ-Cadinene	1.2	–	1.5	–	0.6	–	1514
45	β-Sesquiphellandrene	–	–	0.7	–	–	–	1526

Oxygenated sesquiterpenes								
46	Dictamnol	0.3	–	–	–	–	–	1452
47	Elemol	2.1	–	–	–	–	5.5	1560
48	Spathulenol	14.2	7.8	0.8	4.0	4.1	7.1	1591
49	Caryophyllene oxide	4.2	11.9	11.9	21.5	8.3	8.8	1596
50	Humulene epoxide II	–	–	–	–	0.8	–	1626
51	*epi*-Cedrol	8.8	8.0	–	–	–	–	1628
52	Caryophylla-4(14), (15)- dien-5- α-ol	–	0.6	–	–	–	–	1654
53	α-Cadinol	2.3	0.6	–	–	0.0	–	1657
54	Eudesmol	3.4	2.6	–	–	–	1.7	1672
55	14 -hydroxyl-9-*epi -(E)*- caryophyllene	–	4.3	–	2.9	–	–	1687
Others								
56	*(E)*-2-Hexanal	–	–	–	–	0.3	–	861
57	α–Campholenal	0.4	–	0.6	–	0.4	–	1137
58	6,6–Dimethyl–2–methylene bicycle [2.2.1] heptan–3–one	–	–	–	–	–	0.6	1175
59	Cumin aldehyde	–	–	–	–	0.3	–	1258
60	Tetradecanoic acid	–	1.1	–	–	–	–	1771
61	*n*-Hexadecanoic acid	0.9	13.4	1.0	3.5	–	1.8	1972
62	Phytol	1.3	32.8	1.0	1.3	2.8	5.5	2058
63	*(Z,Z,Z)*-9,12,15-Octadecaterien-1-ol	–	5.2	–	–	–	–	2076
64	Incensole	–	–	–	1.8	–	–	2083
65	Incensole acetate	–	–	–	2.4	–	–	2089
66	Tetracosane	–	–	–	1.1	–	–	2400
67	Eicosane	–	–	2.7	–	–	–	2461
68	Pentacosane	–	–	–	–	–	–	2462
69	Hexacosane	–	–	–	–	–	–	2600
70	Heptacosane	–	–	–	7.2	–	–	2652
71	Octacosane	–	–	2.3	5.4	–	–	2850
72	Nonacosane	–	–	3.0	6.8	–	–	2856
73	Treiaconate	–	–	–	3.9	–	–	2918
	Monoterpene Hydrocarbons	18.1	–	11.8	–	22.1	3.7	
	Oxygenated Monoterpene	13.8	–	1.7	1.40	32.2	12.8	
	Sesquiterpene Hydrocarbons	21.5	2.5	54.3	23.4	24.3	43.0	
	Oxygenated Sesquiterpene	35.3	35.5	12.7	28.4	13.2	23.1	
	Other	2.6	52.5	10.6	33.4	3.8	7.9	

Essential oil compositions of *Nepeta fissa* populations. Thirty-two compounds were identified from the essential oil of *N. fissa* populations. In both populations, the predominant compounds were sesquiterpenes with 67.0 % and 51.8 %, at low altitude (NFLA) and high altitude (NFHA), respectively. Moreover, in these populations, the amounts of oxygenated sesquiterpenes were low and nearly equal with 1.7% and 1.4 % in both localities. The biggest differences between these populations were the presence of monoterpene hydrocarbons and the big amount of sesquiterpene hydrocarbons in NFLA in comparison with NFHA. Other difference was the amount of other compounds, in NFLA they sum 10.6% while in NFHA were 33.4 %. The principal compounds of NFLA were identified β-caryophyllene (33.1%), caryophyllene oxide (11.8%), α-pinene (10.5%), germacrene D (9.6%) and bicylcogermacrene (4.0%). Heptacosane, hexacosane, pentacosane and treiaconate were not registered. In NFHA population, caryophyllene oxide (21.5%), β-caryophyllene (13.8%), heptacosane (7.2%), nonacosane (6.8%), octacosane (5.4), germacrene D (4.4%) and spathulenol (4.0%) were the major constituents, while α–pinene and eicosane were not detected.

Essential oil compositions of *Nepeta heliotropifolia* populations. Forty-five components were identified from the essential oil composition of *N. heliotropifolia.* It is valuable nothing that the main group of compounds differs between the localities studied. While the low altitude population (NHLA) showed monoterpenes (54.3%) as predominant ones, the high altitude population (NSHA) was characterized by sesquiterpenes (66.1%). Other difference was the amount of other compounds in NHHA, they sum 7.9% while in NHLA were 3.8%. The biggest differences between these populations were the big amount of monoterpene hydrocarbons and oxygenated monoterpene in NHHA in comparison with NHLA. In addition, in NHHA amounts of sesquiterpene hydrocarbons and oxygenated sesquiterpenes were nearly two times more than NHLA. The principal compounds of NHLA were identified 1,8-cineole (20.1%), β-pinene (12.4%), germacrene D (11.6%), caryophyllene oxide (8.3%), sabinene (5.2%), β-caryophyllene (4.5%), spathulenol (4.1%) and myrtenal (3.0%). (*E, E*)-α-Farnesene, elemol and *epi*-cedrol were not detected. In NHHA population, β-caryophyllene (18.8%), bicylcogermacrene (11.5 %), caryophyllene oxide (8.8 %), spathulenol (7.1%), *(E, E)*-

α-farnesene (6.7 %), elemol (5.5 %), and phytol (5.5%) were major compounds while the β-pinene, α-pinene and sabinene were registered in small amounts.

The studied populations were presented in UPGMA tree (Figure 2). The tree has two branches. The small one represents NSHA and NFHA populations, while the rest of populations is plotted in the bigger one. It is divided into two groups: NSLA and NHLA populations clustered together either the NHHA and NFLA samples. These results showed that the similarity of the chemical compositions between (1) the low altitude populations of *N. sessilifolia* and *N. heliotropifolia* and (2) the high altitude populations of *N. sessilifolia* and *N. fissa*. Moreover, the PCO plot demonstrates similar outputs (Fig.2). Two distinct groups were found in this plot. NSHA and NFHA populations made a small group and placed far from the others. NSLA and NHLA samples were grouped together either the NHHA and NFLA populations. CA-joined plot showed that every examined population was characterized by specific compounds, which can be used for identification. For example, the percentages of phytol and n-hexadecanoic acid were specific traits for NSHA population, and terpinen-4-ol, terpinyl acetate, *(Z-O)*-cymene and myrcene for NSLA population (Fig. 3).

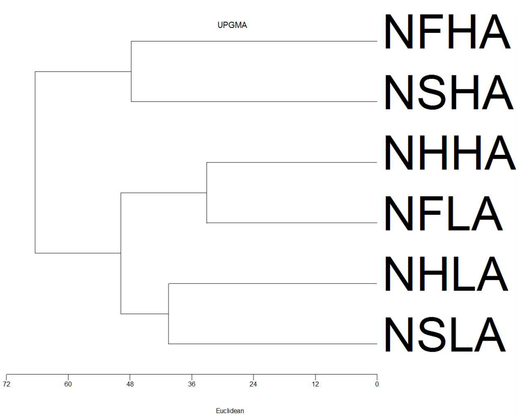

Figure 1. UPGMA tree of essential oil chemical compositions of the *Nepeta* populations.

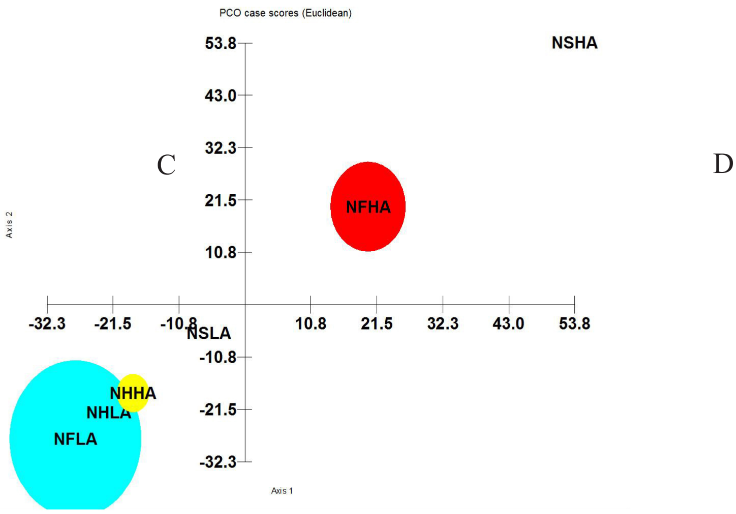

Figure 2. PCO plot of the studied *Nepeta* populations based on the oil chemical compounds.

Table 3. Mean value ± standard deviation of the observed glandular trichomes among the studied populations.

Populations	Capitate type II	Capitate type I	peltate
NSHA	73.5 ± 2.89	42.5 ± 0.99	24.0 ± 2.02
NSLA	118 ± 3.97	181.3 ± 1.02	1.0 ± 0.21
NFHA	0	13.1 ± 1.34	2.0 ± 0.52
NFLA	1.5 ± 0.3	25 ± 2.11	4.0 ± 0.69
NHHA	1.0 ± 0.2	22.33 ± 1.12	4.66 ±0.93
NHLA	0	11.0 ± 0.54	2.0 ± 0.41

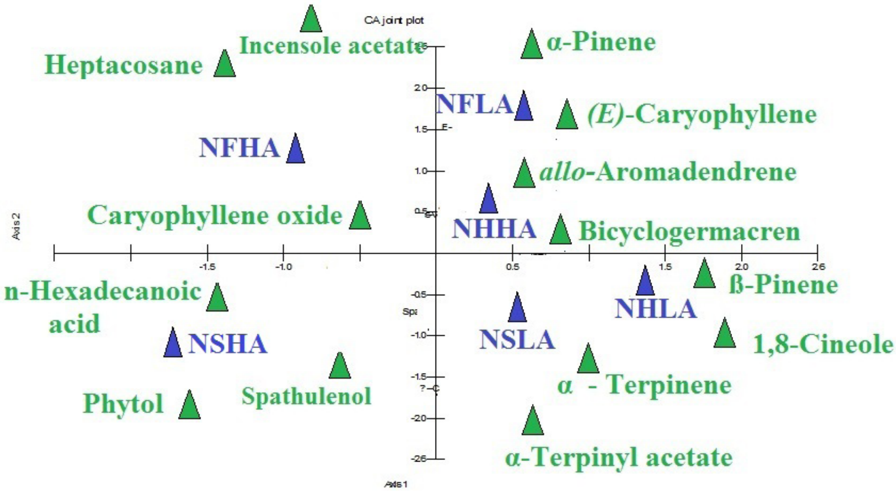

Figure 3. CA-Joined plot of the studied populations (blue symbols) with the chemical compounds of essential oil (green symbols)

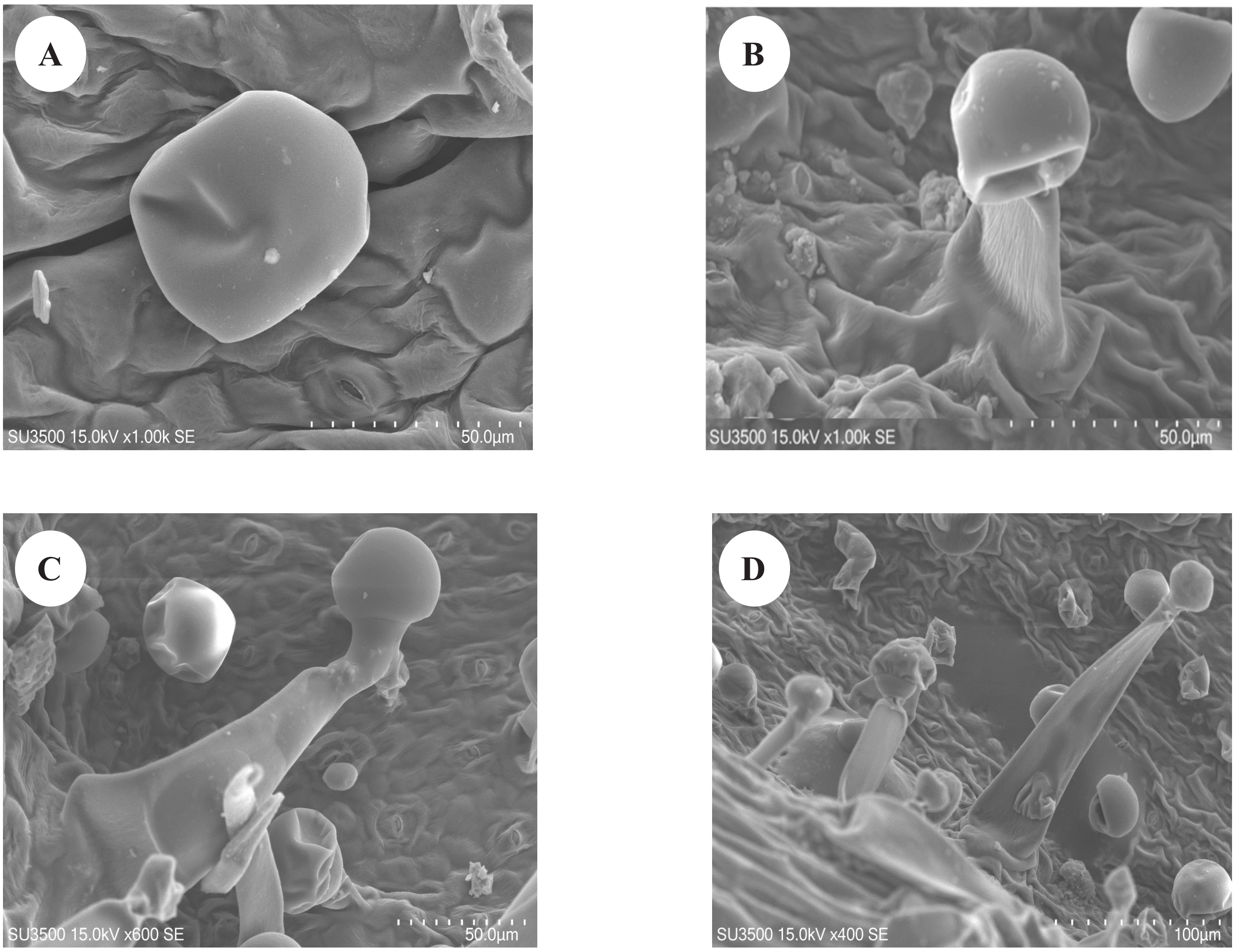

Figure 4. Electronic micrographs of glandular trichomes on the leaves of the studied *Nepeta* taxa. A) Peltate trichomes, B; capitate type I, C; capitate type II, D; comparison of capitate type I and II.

Trichomes variability. In our research we studied the type and density of glandular trichomes on both leaves surfaces. We registered three types of glandular trichomes, namely: peltate, capitate type I and type II. The peltate trichomes had a short one-celled stalk that was followed by a disc-shaped head that was consisted of four secretory cells (Figure 4A). Capitate trichomes were of two types based on the stalk length. Capitate type I had a small basal cell, a short uni-celled stalk and a head with one globoid cell (Figure 4B). In capitate type II there was one short uni-celled base, a stalk with 2 to 3 cells, a small neck that was followed by one globoid head cell

(Figure 4C). Numbers of observed glandular trichome in the studied populations are presented in Table 3. We found that the indumentum had bigger density in low altitude population of *N. sessilifolia* compared to high altitude population. The number of capitate trichomes type I and II were bigger by 2 and 4 times in NSLA population than in NSHA population, while peltate trichomes were more frequent in NSHA population. Anyway, the most abundant trichomes in NSHA and NSLA populations were capitate trichomes types I and II, respectively. However, ANOVA test did not showed significant variations ($p \leq 0.05$) in the number of observed glandular trichomes between both populations of *N. sessilifolia* (Table 4). We also registered that in *N. fissa*, leaf indumentum was denser in NFLA population than NFHA population. The number of peltate and capitate hairs was decreased by two times in NFHA population compared to NFLA population. We registered that the capitate type I was the most abundant trichome in both populations. Results of ANOVA test proved significant variation ($p \leq 0.05$) in the number of peltate trichome between both populations of *N. fissa*. In *N. heliotropifolia*, NHHA population had denser indumentum than NHLA population. We also detected that the numbers of peltate and capitate type I were higher by two times in NHHA population than in NHLA population and the capitate type I was the most abundant glandular hair in both populations. ANOVA test showed significant differences ($p \leq 0.05$) in the number of capitate type II between both populations of this species.

Table 4. ANOVA results on the glandular trichomes type between both populations of the studied *Nepeta* species.

trichomes		Sum of Squares	df	Mean Square	F	Sig.
N. sessilifolia population						
Capitate type II	Between Groups	3408.167	1	3408.167	0.688	0.453
	Within Groups	19809.333	10	4952.333		
	Total	23217.500	11			
Capitate type I	Between Groups	1568.167	1	1568.167	0.269	0.631
	Within Groups	23299.333	10	5824.833		
	Total	24867.500	11			
Peltate	Between Groups	96.000	1	96.000	1.000	0.374
	Within Groups	384.000	10	96.000		
	Total	480.000	5			
N. fissa populations						
Capitate type II	Between Groups	1.500	1	1.500	3.000	0.158
	Within Groups	2.000	10	0.500		
	Total	3.500	11			
Capitate type I	Between Groups	192.667	1	192.667	4.516	0.101
	Within Groups	170.667	10	42.667		
	Total	363.333	11			
Peltate	Between Groups	8.167	1	8.167	12.250	0.025
	Within Groups	2.667	10	0.667		
	Total	10.833	11			
N. heliotropifolia populations						
Capitate type II	Between Groups	5340.167	1	5340.167	865.973	0.000
	Within Groups	24.667	10	6.167		
	Total	5364.833	11			
Capitate type I	Between Groups	170.667	1	170.667	3.425	0.138
	Within Groups	199.333	10	49.833		
	Total	370.000	11			
Peltate	Between Groups	0.667	1	0.667	0.143	0.725
	Within Groups	18.667	10	4.667		
	Total	19.333	11			

Discussion

In the current study, we have investigated altitudinal changes in essential oil composition and glandular trichome density in three species of *Nepeta* from Iran; widespread *N. fissa* and endemic *N. heliotropifolia* and *N. sessilifolia*. Essential oil has several roles in plant life and its adaptation with various environmental factors (Uphof & Hummel, 1962; Corsi & Bottega, 1999). Zouari (2013) has stated that each essential oil is characterized by some major compounds which can reach high levels, as compared to other compounds exist in trace amounts. However, for a given species, natural factors may be at the origin of the variability of the chemical composition of these essential oils, and a subsequent variability in their quality. These factors may be intrinsic, related to the plant, or extrinsic such as the plant environment that causes the occurrence of several chemical races or chemotypes within the same species.

The composition and profile of essential oil highly varied between both populations of *N. sessilifolia* and it seems that these populations can be definite as chemotypes. The altitude caused the decreasing of the amount of total monoterpenes and sesquiterpenes, while increased the percentages of oxygenated diterpenes and fatty acid. The percentage of sesquiterpene hydrocarbon decreased with an increase in altitude, while the amount of oxygenated sesquiterpene did not differ. We can state that the amount of oxygenated compounds increased in the essential oil of high altitude plants. High doses of different types of UV radiations leading to development of oxidative stresses in plant and this condition made higher amounts of oxygenated compounds in NSHA compared with NSLA populations. Furthermore, terpenoids were easily oxidized or hydrolyzed and transformed to other compounds. Bilger *et al.* (2007) have stated that plants at higher altitudes face higher UV-B radiations that have pleiotropic effects on contents of secondary metabolites. Our results agreed with previous findings. Concentration of terpenes differs in essential oil compositions along the altitude gradient. The majority of altitudinal differences in terpene chemistry were among the sesquiterpene with most of the sesquiterpenes decreasing as altitude increased (Lockhart, 1990). We detected highest amounts of monoterpene in NSLA while NSHA had zero amount of monoterpenes. Plant uses essential oil for attracting specific pollinator insect. It seems that low altitude population of *N. sessilifolia* had highest amount of monoterpene for attracting the pollinators. The air temperature decreases with an increase in altitude, therefore in higher temperature monoterpenes emit more easily than lower temperature. Holopainen *et al.* (2013) have suggested that monoterpenes are high volatile terpenoids emitted with warmer temperature than sesquiterpenes. However, Lockhart (1990) has stated that the putatively susceptible zone at high altitudes could be caused by relative increase of monoterpenes and oxygenated monoterpenes. The main compound of low altitude, spathulenol, decreased about one-half in high altitude population; in addition, other main compounds of NSLA, β-caryophyllene, germacrene D, α-terpinyl acetate and bicylcogermacrene were registered in zero or small amounts in NSHL. It may be possible that low amount of spathulenol in NSHA is due to absence of bicyclogermacrene in this population. Toyota *et al.* (1996) have stated that bicyclogermacrene is converted to spathulenol by autooxidation. Authors have also supposed that in NSHL population, sesquiterpene hydrocarbons such as β-caryophyllene, were influenced by UV radiation and turned into oxygenated ones such as caryophyllene oxide. It is important know that caryophyllene oxide amount amplified nearly 3 times in NSHA compared with NSLA. Although, the roles of other factors such as genetic drift, introgression of traits through hybridization and phenotypic plasticity should not be ignored (Eckert *et al.,* 2008; Raguso, 2008). Moreover, the major compound, phytol, from NSHA was recorded in the low content in NSLA. It seems that one of possible reasons for high amount of phytol in NSHA population is hydrolysis of chlorophyll to phytol by enhanced UV radiation. Salama *et al.* (2011) proposed that the contents of chlorophyll were affected by enhanced UV radiation. Different types of chlorophyll (a, b) and total amounts were decreased compared with the control plants and reduced with the enhanced UV radiation. In addition, chlorophyllase catalyzes the hydrolysis of chlorophyll to chlorophyllide and phytol (Matile *et al.,* 1999). These authors have imagine that plants of high altitude population of N. sessilifolia use phytol for chemical defense. Studies have showed that some insects use phytol and its various metabolites as deterrents against predation (Vencl & Morton, 1998). Jamzad *et al.* (2008) examined the essential oil composition of another population (Ghamshelo area-Isfahan Province) of this species and registered that linalool acetate (14.7%) and linalool (14.2%) were the main components of the essential oil. But linalool and linalool acetate had zero or trace amounts in NSHA and NSLA populations. Batooli & Safaei-Ghomi (2012) have recently investigated the essential oil of *N. sessilifolia* collected from Kashan. They reported that spathulenol (25.8%) and lavandulyl acetate (16.7%) were the major compounds. These proved that the percentages of spathulenol from Kashan population amplified more than two and three times in comparison to NSLA and NSHA populations, respectively. However, we did not detect lavandulyl acetate in our studied populations. NSHA was plotted far from NSLA in UPGMA tree and also PCO plot. Each of these populations was characterized by special type/amount of compounds. High altitude population of *N. sessilifolia* was clustered together high altitude population of *N. fissa.* Therefore, the essential oil composition of these populations are the most similar. Though these populations belong to various species and their habitats were far from each other. These authors revealed the influence of altitude on the major essential oil compounds in low and high populations of *N. sessilifolia*. They also suggested that variation in the essential oil compositions of *N. sessilifolia* may result from interactions between environmental or micro-environmental factors. Chauhan *et al.* (2016) believed that variation in the essential oil compositions between different altitudes may results of adaptation to particular habitats. Furthermore, plants' age and genetic variability can show some effect on the composition of the essential oil. Our collected plant samples of both altitudes were morphologically very similar, in the same phenologic period and a like in age. Moreover, the edaphic conditions were similar between these populations. Therefore, it seems that these differences in compositions of oil related to adaptation to various climatic conditions that was seen in different altitudes.

Study of trichomes confirmed that the type of prominent trichomes differed between low and high altitude populations, likewise the number of peltate hairs highly differed between populations. The leaves of low altitude plants had more trichomes and denser indumentum. The soil moisture and air humidity decline with altitude decreasing, therefore plants utilize special strategies for water loss prevention. The change in indumentum density is one of them, which was registered in different species. For instance, Pérez-Estrada *et al.* (2000) studied trichomes density among populations of *Wigandia urens* and observed that the leaves trichomes density of populations growing in drier habitats was higher than in wetter plots. They believed that glandular hairs can help to minimize water loss and to maximize the sunlight reflection. Therefore, leaf surface in NSLA population had denser indumentum for water loss prevention.

The essential oil compositions and profile highly varied between both populations of *N. fissa.* We suggested that the amount of both types of monoterpene and sesquiterpene changed along the altitudinal gradient. Highest amounts of monoterpene were recorded in low altitude population while high altitude population had very low amount of monoterpenes. It seems that in NFHA monoterpenes rapidly degrade under influence of high solar radiation. Moreover, the percentage of sesquiterpene hydrocarbons decreased with an increase in altitude, while the amount of oxygenated sesquiterpenes increased. Authors also detected that the amount of other compound in NFHA were 3 folds more than NFLA. In NFHA population, other compounds were characterized by high amounts of long-chained hydrocarbons. It seems that these compounds are polymerized into epidermal waxes. They prevent plant aerial parts from high UV radiation, wind blowing and other stresses that present in high altitude habitats. Bernard *et al.* (2012) showed that these alkanes are major parts of cuticular waxes which cover aerial parts of plant and play a critical role in plant protection from various biotic and abiotic agents such as UV radiation and cold temperature. Lockhart (1990) stated that there were increasing and decreasing trends in concentration of terpenes in essential oil compositions along the altitude gradient. The majority of altitudinal differences in terpene chemistry were among the sesquiterpenes with most of the sesquiterpenes decreasing as altitude increased. However, the putatively susceptible zone at high altitudes could be caused by relative increase of monoterpenes and oxygenated monoterpenes. The low and high populations of this species clustered separately in UPGMA tree and PCO plot. Furthermore, each of these populations was characterized by special type/amount of compounds. In our research we registered approximately twice as much variation in the amounts of two main compounds of essential oil composition, β-caryophyllene and caryophyllene oxide, between populations of *N. fissa*. In low altitude plant, β-caryophyllene amount was 33.1%, while it decreased to 13.8% in high altitude plants. Reverse pattern was detected for caryophyllene oxide and its amount varied from 11.9% in low altitude population to 21.5% in high altitude population. These authors have supposed that in NFHA, UV-induced oxidative stress converted β-caryophyllene into caryophyllene oxide, because caryophyllene oxide is an oxidation derivative of β-caryophyllene (Fidyt *et al.,* 2016). Although, we think that genetic factors may be associated with environmental factors in this area. Our findings agreed with previous investigations on the essential oil compositions from this species. For example, Talebi *et al.* (2017) examined essential oil compositions of two populations (Polor and Dizin) of this species. In Polor samples, the main constituents of oil were: phytol (20.0%), caryophyllene oxide (8.3%) and β-caryophyllene (7.8%). In Dizin population, the amounts of caryophyllene oxide (1.2%) as well as β-caryophyllene (0.9%) were very low. Authors also detected that there was 7-folds difference in the amount of caryophyllene oxide and β-caryophyllene in the essential oil compositions from these populations. Likewise, similar results were also found in older studies (Baser *et al.* 2000; Sefidkon *et al.,* 2002) on oil compositions from *N. fissa*. Baser *et al.* (2000) identified caryophyllene oxide (24%) and β-caryophyllene (8.3%), as the main compounds of oil compositions in Turkish population. Volatile oil from the aerial parts of *N. fissa* from Tehran Province (Iran) was analyzed by Sefidkon *et al.* (2002). In this study, β-caryophyllene (17.4%) and caryophyllene oxide (12.3%) were registered as the main compounds. These proved that the percentages of β-caryophyllene in Tehran population (Iran) amplified more than two times, but in oil of Tehran samples caryophyllene oxide percentage was about one-half. These investigations confirmed that caryophyllene oxide and β-caryophyllene are two main compounds of *N. fissa* essential oil, while their percentages highly vary infra-specifically. It shows that ecological factors have strong effect on percentages of these compounds in this species. Populations living at high altitude are adapted to the ecological conditions of these habitats, including cold, dryness, high levels of ultraviolet radiation, and difficulty of reproduction. Moreover, various studies (Graves & Taylor, 1988; Kao *et al.*, 1998) showed that climate and vegetation significantly varied along the altitudinal gradient of these habitats. Low-altitude plants need to adapt to adverse conditions of high temperatures, excessive radiation and minimal precipitation (Fahn & Cutler, 1992). To the contrary, high altitude plants do not suffer from drought, but they have to face the unfavorable conditions like low temperatures and high irradiance (Angelopoulos *et al.*, 1996).

Trichomes investigation proved that the type of prominent trichomes varied between low and high altitude populations of *N. fissa*, moreover the number of peltate trichomes differed significantly between populations. The leaves of low altitude plants had more trichomes and denser indumentum. Because, in drier environment (low altitude) glandular trichomes density is higher than in wetter habitat (high altitude) and these trichomes play a defensive role against water loss. Difference in trichomes density along the altitudinal variations were reported in several taxa. For example, Sheue *et al.* (2003) studied the influence of altitude on the abundance of glandular trichomes. They showed a higher density of internal glands in population of *Pinus taiwanensis* in middle altitudes, in comparison with samples growing at higher altitudes. Beside, Horgan *et al.* (2009) have stated that the density of glandular trichome decreased with increasing altitude of origin in *Solanum berthaultii* populations.

The main group of compounds varied between both populations of *N. heliotropifolia*. In NHLA, amount of total monoterpenes was three times bigger than NHHA population, while in high altitude samples, amount of both types of sesquiterpenes and other compounds were amplified twice more than low altitude plants. These authors have believed that different stresses such as low temperature, wind blowing and UV radiation at higher altitude degrade monoterpenes. Besides, the roles of genetical parameters are very important. For example, Dieckmann & Palamand (1974) reported that oxidation, disproportionation, polymerization and cyclization lead to the monoterpenes degradation. Moreover, several authors (Eckert *et al.,* 2008; Raguso, 2008) have stated that the infra-specific difference in secondary metabolite production in plants has been explained by genetic drift, relaxed selective pressure, introgression of traits through hybridization, gene pleiotropic effects, and phenotypic plasticity. For instance, qualitatively and quantitatively variations in secondary metabolites have been recorded among populations of the same species (Peracino *et al.,* 1994; Muñoz-Bertomeu *et al*., 2007). Three main compounds of essential oil, 1,8-cineole (20.1%), β-pinene (12.4%), germacrene D (11.6%), from NHLA decreased more than four folds compared to NHHA population. Moreover, in NHHA population the main compounds, β-caryophyllene, bicylcogermacrene and spathulenol amplified more than four times compared to NHLA population. We can stated that high amount of spathulenol in NHHA is due to presence of high presentage of bicyclogermacrene. Because, autooxidation converts bicyclogermacrene to spathulenol (Toyota *et al.* 1996), and this reaction is highly induced by more UV radiation at high altitude habitat. Sajjadi & Khatamsaz (2001) investigated the essential oil of *N. heliotropifolia* collected from Hamadan (Iran). Its major components were 1,8-cineole (19.0%), caryophyllene oxide (14.2%) and β-caryophyllene (11.3%). The type and amount of the first main compound was a like between our high altitude population with that was reported by Sajjadi & Khatamsaz (2001), while β-caryophyllene amount, the first main compound, in NHHA population increased more than 1.5 times compared with Hamedan population. Recently, Yarmohammadi *et al.* (2017) compared oil compositions of this species collected from two different localities of Iran (Qazvin and Sefidkhani). They found that phytol (12.8%) and α-copaene (12.0%), were the two major oil compounds in Qazvin population. In Sefidkhani samples, the main components of oil were caryophyllene oxide (14.2%), β-caryophyllene (12.0%), and 1,8-cineole (11.6%). We did not find any similarity in major oil compounds in Qazvin population compared to our studied populations. However, the amounts of 1,8-cineole, major compound of NHLA population, and β-caryophyllene, main compound of NHHA population, were decreased more than 1.5 times in Sefidkhani population. Our findings showed that the amount and kind of major oil parts highly varied under different ecological conditions. Both populations of *N. heliotropifolia* clustered separately in UPGMA tree and also PCO plot. Likewise each of these populations was characterized by special compounds. Low altitude population of *N. heliotropifolia* clustered together with low altitude population of *N. sessilifolia*. Although these populations belong to different species and growth under different ecological conditions, were the most similar in oil compositions. This proved that altitude has strong effects on essential oil compositions. However, Spitaler *et al*. (2006) have suggested that infra-specific investigation on secondary metabolites production performed in plants in different ecological conditions do not inform whether the observed variations show the genetic adaptation to specific environments, or the short-term response to environmental factors.

High altitude population of *N. heliotropifolia* had denser indumentum than low altitude one. Numbers of all glandular trichomes were higher by two times in NHHA population than NHLA population. Solar radiation changes with elevation and plant individuals alter the indumentum density for better adaptation towards habitat conditions. Secretions of glandular trichomes help to reflect solar radiation and protect plant body from intense UV-B radiation. Several investigations (Gianfagna *et al.* 1992; Pérez-Estrada *et al.*, 2000; Horgan *et al.*, 2009) mentioned strong effect of light, altitude, temperature, and nutrients availability on the glandular density in various plant taxa.

The chemical compositions of essential oil and profile varied between lower and higher altitude populations of the same species and they were attendant with difference in trichomes density between these populations. The essential oil in Labiatae species is accumulated and secreted by glandular trichomes, furthermore each type of glandular hair store special compounds. Therefore, each change in trichome type and number alters essential oil amount and its chemical compositions (Askary *et al.,* 2016a, b).

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