Changes in Essential Oil Content and Composition of *Salvia Limbata* C.A. Mey at Different Growth Stages and Altitudes

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Abstract

Background

The present study investigates the effect of growth stage (vegetative, flowering and seed ripening) and altitude (1500, 2000 and 2500 m above sea level) on the content and chemical composition of *S. limbata* essential oil which belongs to *Lamiaceae* family.

Results

According to the oil analysis, 28 components representing 96.5% to 99.7% of the total volatile oil composition were characterized. The main compounds of *S. limbata* oils were α-pinene (14.7-38.7%), β-pinene (12.5-26.2%), allo-aromadendrene (9.2-21.7%), germacrene D (4.2-8.3%), bicyclogermacrene (6.5-14.5 %), and spathulenol (7.5-25.4 %).

Discussion

The obtained results showed that the content and constituents of *S. limbata* essential oil strongly depend on the growth stage and altitude. Our findings revealed that the vegetative stage at 1500 m is the optimal harvest time to obtain the highest content of oil yield. Results of the current study helps to find the optimum situation to gain the highest content of *S. limbata* essential oil but more researches are needed.

1. Introduction

Salvia is the largest and prominent genus of the *Lamiaceae* family, which includes more than 900 medicinal and ornamental species distributed in the world [1]. This genus is found in Central and South America, Western Asia and Eastern Asia [2]. Fifty-eight species of genus *Salvia* are found in Iran which seventeen species are endemic [3,4]. *Salvia limbata* C.A. Mey, a native plant of Iran, is a perennial, herbaceous and aromatic plant (30-60 cm tall) with thick, rounded and bright green leaves. The distribution of this plant within Iran is in Azerbaijan, Lorestan, Shiraz, Kermanshah, Semnan, and Damavand [5]. The genus *Salvia* has always been noticeable in doing research around the world for diverse biological activities and compounds in its essential oil [6,7]. Since the species of genus *Salvia* contain substantial amounts of essential oils, people have been applied for thousands of years in folk medicine to improve health and treat diseases [8,9]. Modern science illustrates that *Salvia* essential oils improve memory and could be effective in treating Alzheimer's in the future [10]. *Salvia* has also been used for treating coughs, colds and wounds and it has been considered as spasmolytic, antiseptic, astringent, and liver protective [11].

Moreover, the phenolic compounds of plants belonging to this genus have shown antiviral, antibacterial, antifungal, antioxidant, antitumor, antidiabetic, anxiolytic, sedative, and anti-inflammatory activities [12,13,,14,15,16,17,18]. Active compounds such as hydrocarbon monoterpenes, hydrogenated monoterpenes, oxygenated monoterpenes, di-terpenes, hydrocarbon sesquiterpenes and oxygenated sesquiterpenes have been obtained from *Salvia* species [19]. The contents of these compounds are influenced by various factors such as planting conditions, harvest time, organ used and growth stage. It seems that ecological factors and genetic are the main significant factors that influence the content of the plant active compounds [20].

To the best of our knowledge, few investigations have been done in the field of autecology and phytochemistry of *S. limbata.* Since the global approach has been perusing the use of medicinal herbs and natural compounds in the pharmaceutical, cosmetic and food industries, there is a strong need to delve more into the issue and do further research to understand how to increase the yield of active ingredients in varied ecological conditions. This could be economically important for the food, cosmetic and pharmaceutical industries. Although phytochemistry of different species of this genus has already been studied in different ecological conditions (mostly in flowering stage), for the first time the effect of different phenological stages and altitudes on the essential oil content and composition of *S. limbata* was investigated in this paper.

2. Materials And Methods

2.1. Plant material

Aerial parts of *S. limbata* at several developmental stages (vegetative, flowering and seed ripening) were harvested in three replicates from its wild habitat at altitudes of 1500, 2000 and 2500 m above sea level from Taleghan rangeland (semi-humid) in Alborz province, Iran (36° 5' 19" N to 36° 19' 19" N and 50° 36' 43" E to 50° 53' 20" E) (Fig. 1). Taleghan is one of *S. limbata*'s main sites with mean relative humidity about 12%, and the average annual temperature of 11.4^{o}C. In this area, there are 150 freezing days and annual precipitation is about 446 mm. Harvested plant materials were dried in the shade and then ground in a grinder (2mm mesh size). A voucher herbarium specimen (MP-300) was lodged at the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

2.2. Isolation of the essential oils

Samples (100 g) of the air-dried aerial parts of *S. limbata* were subjected to hydro distillation using a Clevenger-type apparatus for 4h, according to the method recommended in British Pharmacopoeia. The distilled oils were dried over anhydrous sodium sulfate and stored at 4^{o}C in tightly closed dark vials to be analyzed.

2.3. Chemical composition of the essential oils

For characterization of the volatile oil constituents, samples were subjected to gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-FID analysis was performed by a Shimadzu 15 A gas chromatograph (Dyson Instruments, Newcastle, U.K) equipped with a split/splitless injector (250°C), a DB-5 (60 m length × 0.25 mm internal diameter, 0.25 µm film thickness) capillary column and flame ionization detector (250°C). The helium gas was used as the carrier gas (1 mL/min) and the oven temperature was 60-280°C at a rate of 5°C/min. The injector temperature was 250°C and 1µL of oil sample was injected in the split mode with the split ratio of 100:1.

GC-MS analysis was carried out using a Thermo-Trace GC-MS system (Thermo Electron; San José, CA, USA). This device was equipped with a DB-5 silica column (60 m length × 0.25 mm internal diameter, 0.25 µm film thickness) containing 5%-phenyl 95%-methyl polysiloxane and a mass spectrometer detector (MS). The carrier gas was helium at a flow rate of 1 ml/ min. The column temperature was kept at 60°C for 3 min and then programmed to 250°C at a rate of 5°C/min. The injector and GC/MS interface temperatures were 290°C and 300°C, respectively. The mass spectra were taken at 70 eV in the scan range of m/z 50-550.

2.4. Identification of compounds

The constituents of the essential oils were recognized by calculation of retention indices for all the components, using retention times of C_6-C_{24} n-alkenes series as standards under the same chromatographic conditions. Identification of individual compounds were performed by comparison of their retention indices and mass spectral fragmentation patterns with those reported in the literature, Wiley library (New York, NY, USA) or published mass spectra [8,21]. The relative percentage of essential oil constituents were obtained from the GC-FID peak areas in the chromatogram without the use of correction factors.

2.5. Statistical analysis

The statistical analyses were performed using Graph Pad Prism software (San Diego, CA; version 5.0). Data are presented as mean ± standard deviation (SD) in 5 randomized replicates. One way analysis of variance (ANOVA) and Tukey post-test were used to analyze obtained results. *P*-value < 0.05 was considered as statistically significant difference.

3. Results And Discussion

As shown in Figure 2, comparison of the essential oil yield among different samples revealed that the highest content of essential oil belongs to the harvested aerial parts of *S. limbata* in the vegetative stage at an altitude of 1500 m (0.86% v/w) while no significant difference was observed in the essential oil content among other groups. In a published study authors observed the significant impact of different altitudes and phenological stages on the essential oil yield [22,23]. It was revealed that plant performance is strongly influenced by various factors such as altitude, climate, soil, developmental stages, extraction and analysis methods, genetic factors, abiotic stresses, and slope and modeling techniques can predict these factors in other areas [24,25,26,27, 28]. The results of the current study were compatible with other studies that found the highest content of essential

oil of *Origanum majorana* in the vegetative stage, so they proposed the vegetative stage as the best stage to harvest *Origanum majorana* [29,30, 31,32, 33,34,35,36].Similar results were also found byon the essential oil content of *Nepeta kotschyr* [37]. Moreover, according to a study conducted by the highest yield of essential oil in *Teucrium polium* L. was obtained in the vegetative stage [38]. These results are in agreement with our findings. The accumulation of essential oil in vegetative stage could be due to the fact that plant protection is supplied by phenolic components which are in high amount in this stage [39].

On the contrary, it was reported bythat the highest essential oil in *Satureja mutica* is acquired in the flowering stage[40]. In another study [41] determined that the highest value of essential oil of *Mentha pieperata* in flowering stage, which contradicts our findings. Also, the percentage of essential oils in vegetative stage in *Thymus vulgaris* was the lowest and it rose in flowering stage [42]. One explanation for the increase in essential oil content in the flowering stage is the maintenance of the reproductive stage [43,44] and to attract insects for pollination [35]. Nevertheless, other researches have illustrated that the lowest amount of essential oils in the vegetative stage could be due to the lower activity of some enzymes in synthesizing phenolic compounds in this stage [45]. Since photosynthetic products accumulate in the endosperm during plant growth, it leads to a decrease in the amount of essential oil [46]. It is clear that phenological stages have a great impact on the essential oil metabolism, enzymatic activity and finally essential oil content [45].

As illustrated in Tables 1, 2 and 3, twenty-eight components were identified in the *S. limbata* essential oil by means of GC-FID and GC-MS analysis which represented about 96.5% to 99.7% of the total composition of the obtained essential oil. In the current study, the main identified compounds were α-pinene (14.7-38.7%), β-pinene (12.5-26.2%), allo-aromadendrene (9.2-21.7%), germacrene D (4.2-8.3%), bicyclogermacrene (6.5-14.5 %), and spathulenol (7.5-25.4 %). The molecular structures of the main identified compounds from *S. limbata* essential oil are presented in the Figure 3. Comparing the results of the current study to others, α-pinene (23.7 %), β-pinene (18.7%), sabinene (14.5%), 1, 8-cineole (9.9%) and β-caryophyllene (7.1%) as the major components of *S. limbata* essential oil in the flowering stage [47]. In another research, following GC-MS analysis of the aerial parts of *S. limbata* obtained from Turkey, 42 components were characterized representing 95.6% to 98.1% of the compounds including α-pinene (11.2-24.3%), β-pinene (10.0-20.9%) and sabinene (14.6-17.4%) as the major constituents of the essential oil [48].

Comparing of the monoterpenes and sesquiterpenes contents of the *S. limbata* essential oil at different altitudes and phenological stages in Figure 4, the amount of monoterpenes has decreased from vegetative stage to seed ripening stage; however, the obtained results for sesquiterpenes were reverse. These findings for *Artemisia herba-alba* essential oil were previously observed in another study [49]. Moreover, during the developing plants the amount of sesquiterpenes increased in *Cannabis sativa* L. which are in line with our results[50]. As we found out in our research, the highest amount of monoterpenes was related to the vegetative period at 2000 m, while the highest amount of sesquiterpenes was obtained in seed ripening stage at altitudes of 1500 and 2500 m. [51] 2002 in a research on *Thymus vulgaris* at different growth stages confirmed that the highest content of the monoterpene was related to the vegetative stage.

As shown in Figure 5, the content of α-pinene, β-pinene, alloaromadendrene, germacrene D, bicyclogermacrene, and spathulenol illustrates some changes in different altitudes and developmental stages. The highest percentage of monoterpenes including α-pinene (41.3%) and β-pinene (30.1%) was obtained in the vegetative stage at 2000 m. The contents of α-pinene and β-pinene were decreased to the lowest values in the ripening stage. Moreover, the highest content for alloaromadendrene was measured 20.6% and 20.7% in the ripening stage at 1500 m and 2500 m, respectively without any significant difference between them. However, the lowest quantity was obtained 3.5% for the vegetative stage at 2000 m. The most abundant germacrene D reached in ripening stage at 2500 m (8.3%) and the lowest amount (1.2%) achieved at 2000 m in the vegetative stage. Moreover, high value of bicyclogermacrene was attained in the ripening stage at 1000 m and 2000m (14.5% and 14.3%, respectively) while no significant difference was observed between the mentioned altitudes. On the contrary, the lowest amount was attained in the vegetative stage at 1500 m (4.2%). Furthermore, the highest and the lowest contents of spathulenol (25.4% and 7.2%) were gained in the ripening stage at 2500 m and vegetative stage at 2000 m, respectively. Variation in the percentage of compounds at different stages of phenology and altitudes could be due to the high or low synthesis of compounds by enzymes, which leads to different percentages of compounds in essential oils.

Table 1. The percentage of chemical compositions of *Salvia limbata* essential oil in the vegetative stage at different altitudes

No.	Compounds	RI	1500 m	2000 m	2500 m
1	α-Thujene	922	Tr	Tr	Tr
2	α-Pinene	938	30.4±0.1	41.3±0.2	28.5±0.8
3	Camphene	952	1.2±0.1	1.4±0.2	0.5±0.0
4	Sabinene	975	2.4±0.1	1.9±0.1	4.1±0.1
5	β-Pinene	980	25.4±0.3	30.1±0.1	24.2±0.2
6	Myrcene	985	Tr	Tr	Tr
7	p-Cymene	1025	Tr	Tr	Tr
8	Limonene	1029	0.9±0.1	0.6±0.04	0.4±0.0
9	Z-β-Ocimene	1035	Tr	Tr	Tr
10	Linalool	1085	Tr	Tr	Tr
11	α-Campholenal	1103	Tr	Tr	Tr
12	Trans-Pinocarveol	1125	Tr	0.2±0.0	Tr
13	Trans-Verbenol	1162	Tr	0.3±0.0	0.2±0.01
14	Borneol	1186	Tr	0.2±0.0	Tr
15	Terpine-4-ol	1203	Tr	0.4±0.0	0.2±0.0
16	Myrtenal	1216	Tr	0.4±0.0	0.2±0.0
17	Verbenone	1239	0.3±0.1	0.2±0.0	0.3±0.0
18	Bornyl-acetate	1316	1±0.1	Tr	Tr
19	Eugenol	1340	Tr	0.9±0.1	1.4±0.2
20	β-Caryophyllene	1426	Tr	Tr	0.2±0.0
21	Allo-Aromadendrene	1482	9.5±0.1	3.5±0.2	12.6±0.3
22	γ-Muurolene	1485	Tr	0.6±0.0	Tr
23	Germacrene D	1498	3.7±0.2	1.2±0.1	4.1±0.2
24	Bicyclogermacrene	1505	4.6±0.2	5.5±0.3	8.5±0.9
25	Eugenol-acetate	1521	Tr	0.6±0.0	Tr
26	Spathulenol	1575	12.6±0.1	7.2±0.2	13.8±0.2
27	Caryophyllene oxide	1580	4.3±0.1	Tr	Tr
28	Sclareol	2200	1.9±0.1	0.2±0.0	0.5±0.0
	Monoterpene hydrocarbons		60.3	75.3	57.7
	Oxygenated monoterpenes		1.3	2.6	2.3
	Sesquiterpene hydrocarbons		17.8	10.8	25.4
	Oxygenated sesquiterpenes		18.8	8.0	14.3
	Total		98.2	96.7	99.7

Data are presented as mean±SD; RI indicates retention indices relative to C_6-C_{24} *n*-alkanes; Tr indicates trace (<0.1%)

Table 2. The percentage of chemical compositions of *Salvia limbata* essential oil in the flowering stage at different altitudes

No.	Compounds	RI	1500 m	2000 m	2500 m
1	α-Thujene	922	Tr	Tr	Tr
2	α-Pinene	938	21.2±0.2	29.5±0.4	21.7±0.6
3	Camphene	952	1.3±0.1	1.4±0.2	1.8±0.2
4	Sabinene	975	1.7±0.1	3.3±0.1	1.8±0.1
5	β-Pinene	980	17.3±0.1	26.2±0.2	20.1±0.2
6	Myrcene	985	Tr	Tr	Tr
7	p-Cymene	1025	Tr	Tr	Tr
8	Limonene	1029	1±0.1	1±0.1	0.8±0.1
9	Z-β-Ocimene	1035	Tr	1.2±0.1	Tr
10	Linalool	1085	Tr	Tr	Tr
11	α-Campholenal	1103	Tr	Tr	Tr
12	Trans-Pinocarveol	1125	0.3±0.0	Tr	0.4±0.0
13	Trans-Verbenol	1162	Tr	Tr	0.3±0.0
14	Borneol	1186	Tr	Tr	0.3±0.0
15	Terpine-4-ol	1203	0.6±0.0	0.6±0.0	Tr
16	Myrtenal	1216	0.6±0.0	0.6±0.1	Tr
17	Verbenone	1239	0.2±0.0	0.3±0.0	Tr
18	Bornyl-acetate	1316	Tr	0.2±0.0	Tr
19	Eugenol	1340	1.2±0.1	0.4±0.0	0.5±0.0
20	β-Caryophyllene	1426	Tr	Tr	Tr
21	Allo-Aromadendrene	1482	15.2±0.2	9.2±0.1	14.6±0.2
22	γ-Muurolene	1485	0.5±0.0	3.2±0.1	0.3±0.0
23	Germacrene D	1498	5.7±0.2	4.4±0.1	4.2±0.2
24	Bicyclogermacrene	1505	10.7±0.2	6.5±0.1	13.6±0.2
25	Eugenol-acetate	1521	Tr	Tr	Tr
26	Spathulenol	1575	18.6±0.3	7.5±0.1	15.5±0.3
27	Caryophyllene oxide	1580	1±0.1	2.1±0.2	Tr
28	Sclareol	2200	1.7±0.2	Tr	0.4±0.0
	Monoterpene hydrocarbons		42.5	62.6	46.2
	Oxygenated monoterpenes		2.9	2.1	1.5
	Sesquiterpene hydrocarbons		32.1	23.3	32.7
	Oxygenated sesquiterpenes		21.3	9.6	15.9
	Total		98.8	97.6	96.3

Data are presented as mean±SD; RI indicates retention indices relative to C_6-C_{24} *n*-alkanes; Tr indicates trace (<0.1%).

Table 3. The percentage of chemical compositions of *Salvia limbata* essential oil in the ripening stage at different altitudes

No.	Compounds	RI	1500 m	2000 m	2500 m
1	α-Thujene	922	Tr	Tr	Tr
2	α-Pinene	938	14.9±0.2	19.5±0.2	14.7±0.1
3	Camphene	952	0.9±0.0	1.6±0.2	0.6±0.0
4	Sabinene	975	1.3±0.2	2.2±0.2	1.1±0.1
5	β-Pinene	980	14.2±0.3	17.6±0.3	12.5±0.1
6	Myrcene	985	Tr	Tr	Tr
7	p-Cymene	1025	Tr	Tr	Tr
8	Limonene	1029	0.5±0.0	0.7±0.0	0.4±0.0
9	Z-β-Ocimene	1035	Tr	Tr	Tr
10	Linalool	1085	Tr	Tr	Tr
11	α-Campholenal	1103	Tr	Tr	Tr
12	Trans-Pinocarveol	1125	Tr	Tr	Tr
13	Trans-Verbenol	1162	Tr	0.2±0.0	Tr
14	Borneol	1186	Tr	Tr	Tr
15	Terpine-4-ol	1203	0.3±0.0	0.5±0.0	Tr
16	Myrtenal	1216	0.3±0.0	0.5±0.0	Tr
17	Verbenone	1239	0.2±0.0	0.2±0.0	Tr
18	Bornyl-acetate	1316	Tr	Tr	Tr
19	Eugenol	1340	0.6±0.0	Tr	1.5±0.2
20	β-Caryophyllene	1426	Tr	2.6±0.0	Tr
21	Allo-Aromadendrene	1482	20.6±0.2	16.3±0.2	21.7±0.2
22	γ-Muurolene	1485	0.3±0.0	0.5±0.0	Tr
23	Germacrene D	1498	7.6±0.2	6.3±0.2	8.3±0.3
24	Bicyclogermacrene	1505	14.5±0.0	14.3±0.2	12.4±0.1
25	Eugenol-acetate	1521	Tr	0.9±0.0	Tr
26	Spathulenol	1575	22.4±0.2	12.6±0.1	25.4±0.2
27	Caryophyllene oxide	1580	Tr	Tr	Tr
28	Sclareol	2200	0.7±0.0	Tr	0.4±0.0
	Monoterpene hydrocarbons		31.8	41.6	29.3
	Oxygenated monoterpenes		1.4	1.4	1.5
	Sesquiterpene hydrocarbons		43.0	40.0	42.4
	Oxygenated sesquiterpenes		23.1	13.5	25.8
	Total		99.3	96.5	99.0

Data are presented as mean±SD; RI indicates retention indices relative to C_6-C_{24} *n*-alkanes; Tr indicates trace

4. Conclusion

The data presented in this paper confirmed that the essential oil yield and constituents of *S. limbata* were highly influenced by phenology and altitude. A significant difference in the composition of *S. limbata* essential oil at different growing stages and altitudes was observed. It is noteworthy that the dominant compounds of *S. limbata* essential oil were α-pinene, β-pinene, allo-aromadendrene, germacrene-D, bicyclogermacrene, and spathulenol. Since higher yield of essential oil was obtained in vegetative stage at 1500 m, this stage could be considered as the best stage to harvest plant. Moreover, it was revealed that the highest content of monoterpenes and sesquiterpenes could be obtained at the vegetative stage and ripening stage, respectively. This was the first research on the yield and constituents of *S. limbata* essential oil at different developmental stages and altitudes that could be economically beneficial for the food and pharmaceutical industries as well as other researchers to examine further investigations about this plant.

Declarations

Ethic approval: Not applicable

Consent for publication: Yes

Availability of data and materials: The form of material is in Excel file. The location was in Taleghan rangeland.

Competing interests: No

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Author's contribution: Hossein Azarnivand devised the project, the main conceptual ideas and proof outline. Dr Mohammad Ali Zare Chahouki and Dr Maryam Saffariha worked out almost all of the technical details, and performed the numerical calculations for the suggested experimented Ali Tavili and Dr Samad Nejad Ebrahimi verified the numerical results. Pr. Daniel Potter aided in interpreting the results and worked on the manuscript. Dr. Reza Jahani helped in analysing the data. All authors discussed the results and commented on the manuscript.

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Figures

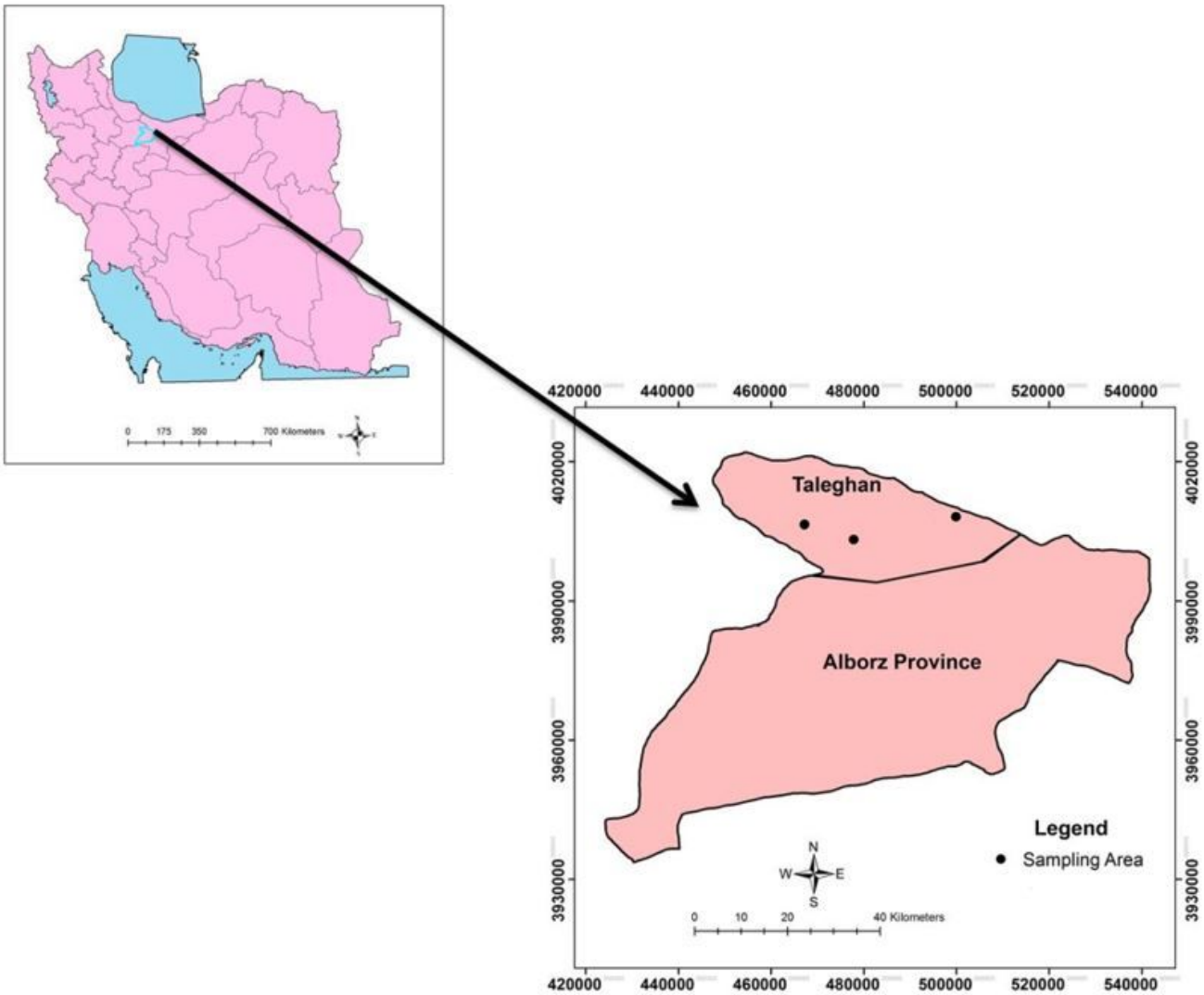
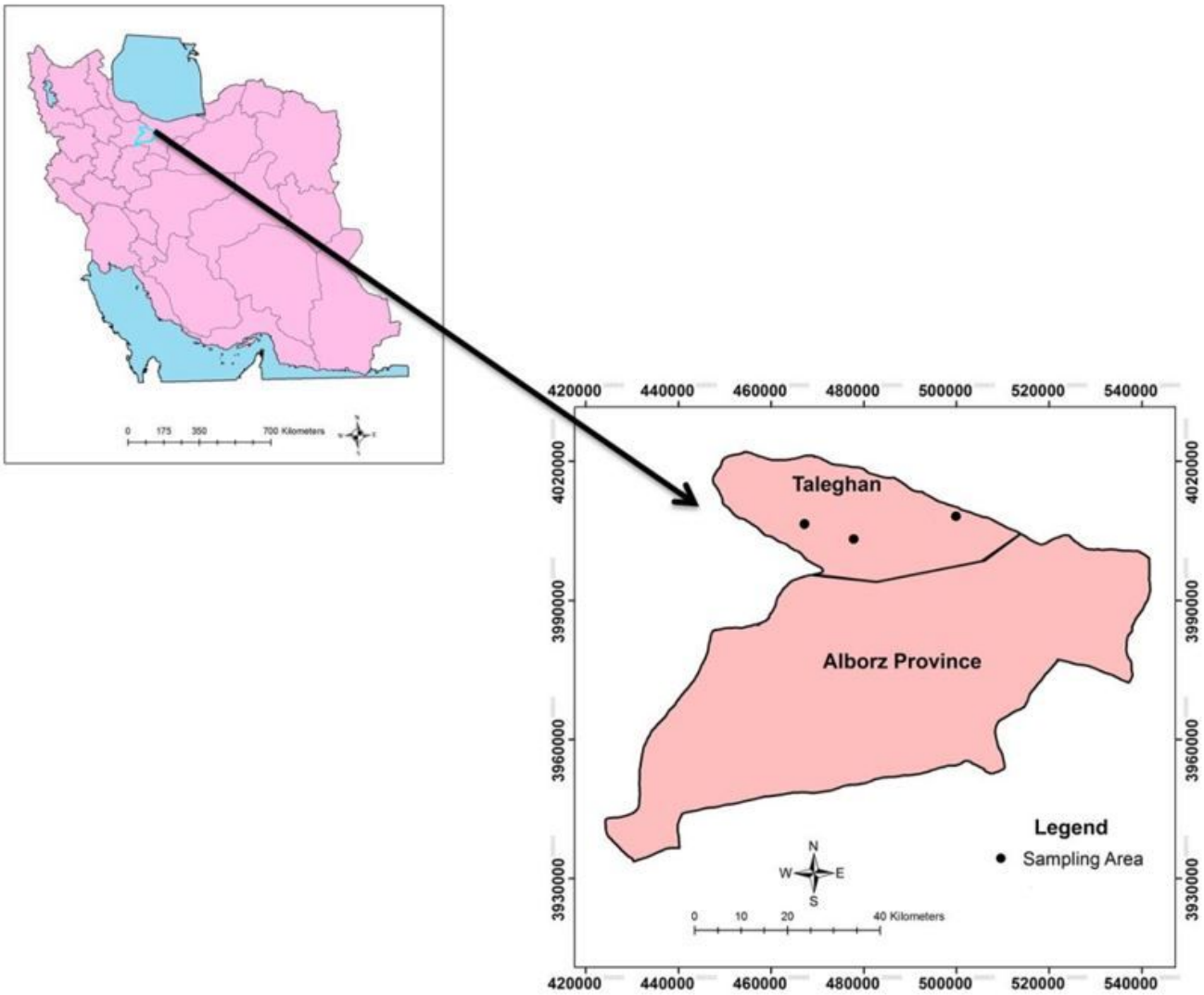


Figure 1

Locations of the study area. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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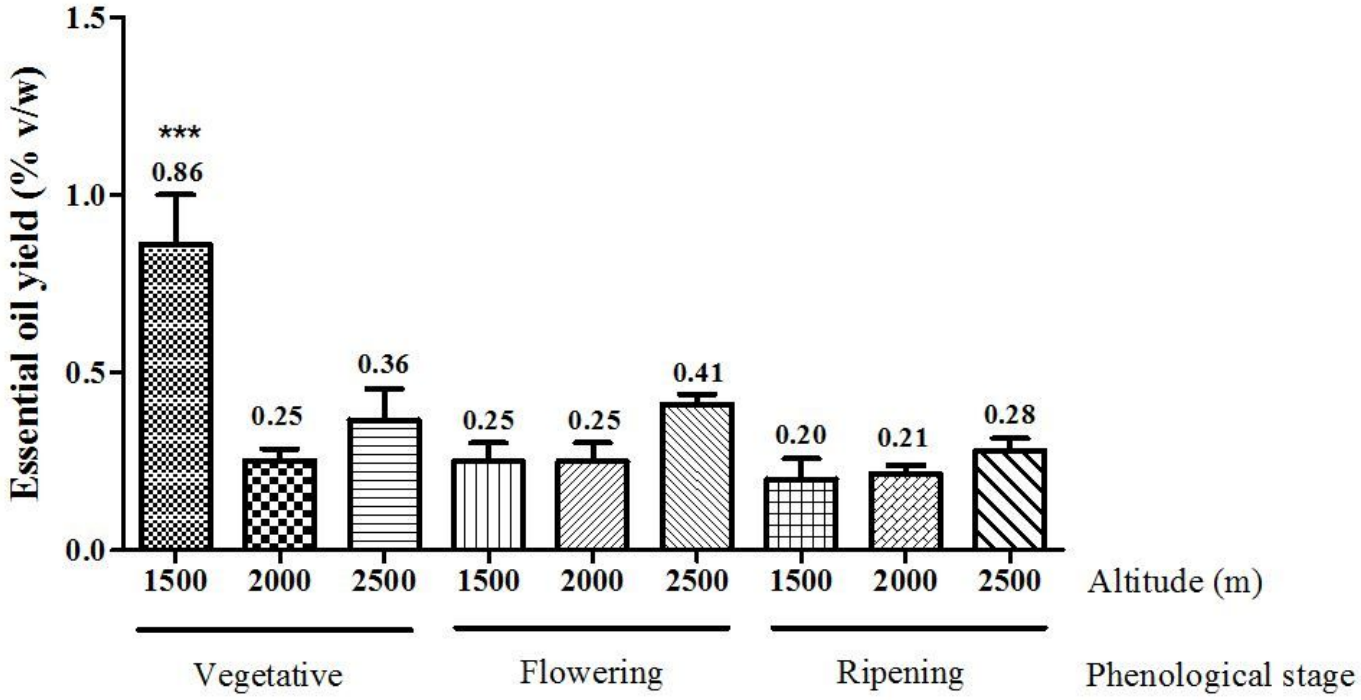
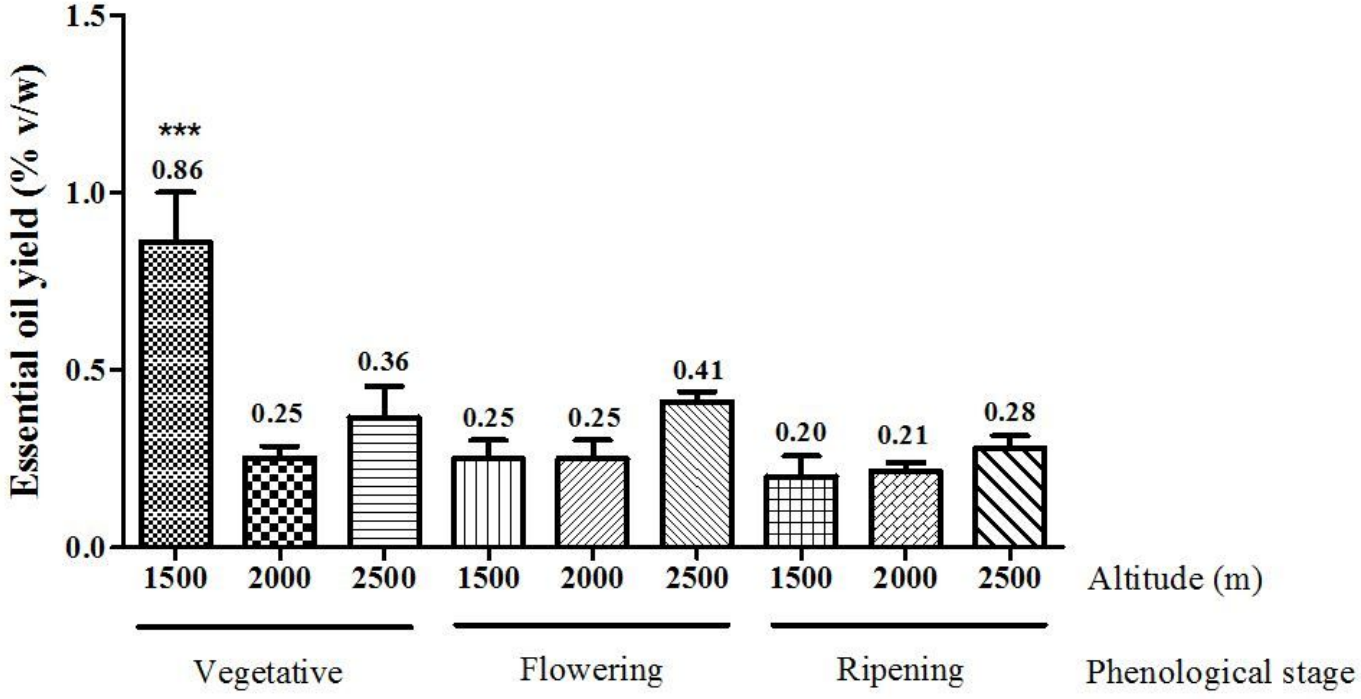


Figure 2

The essential oil content of Salvia limbata at different phenological stages and different altitudes. Data are expressed as mean ± SD (n=5). *** indicates P-value <0.001 compared to other groups



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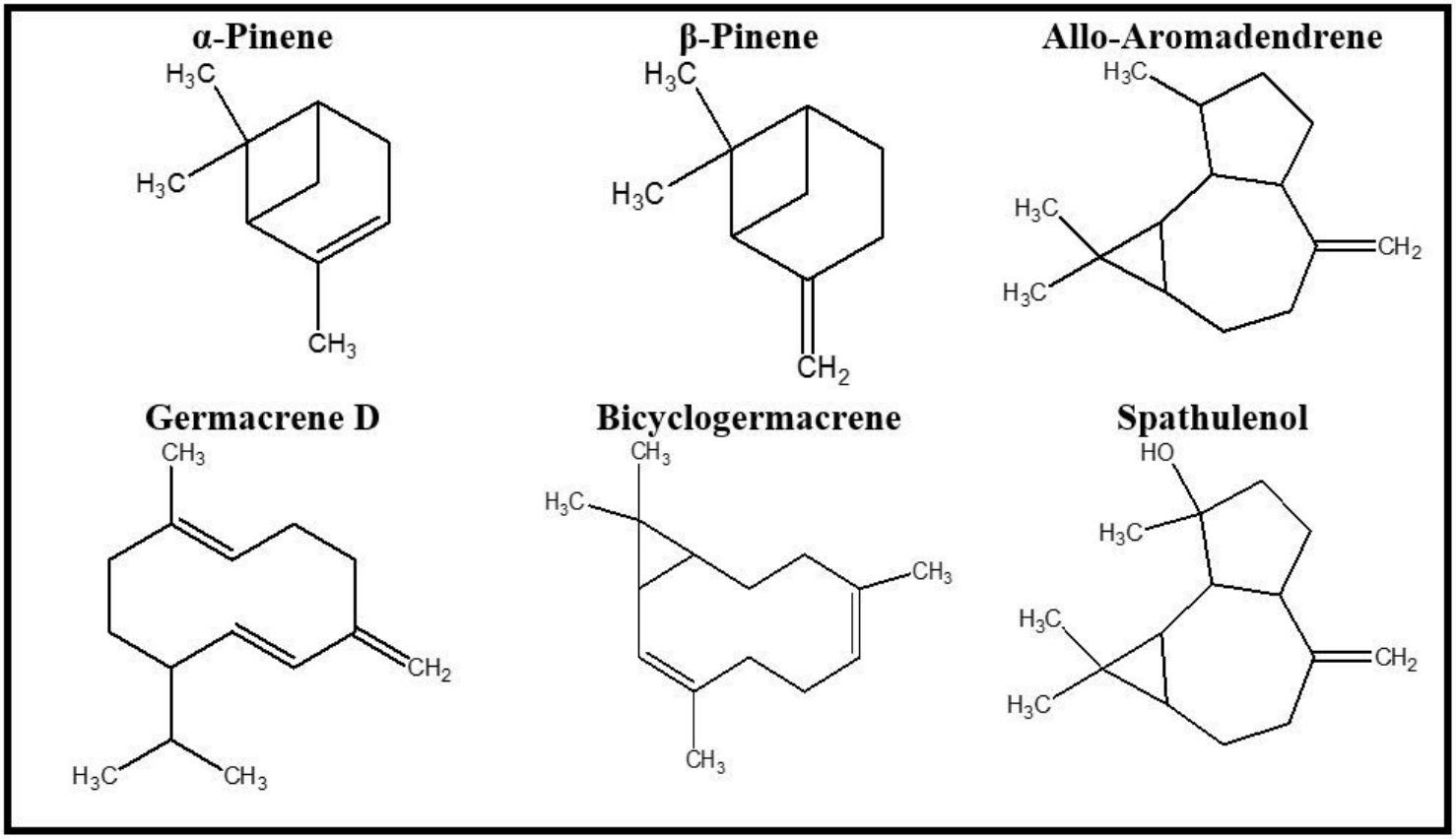
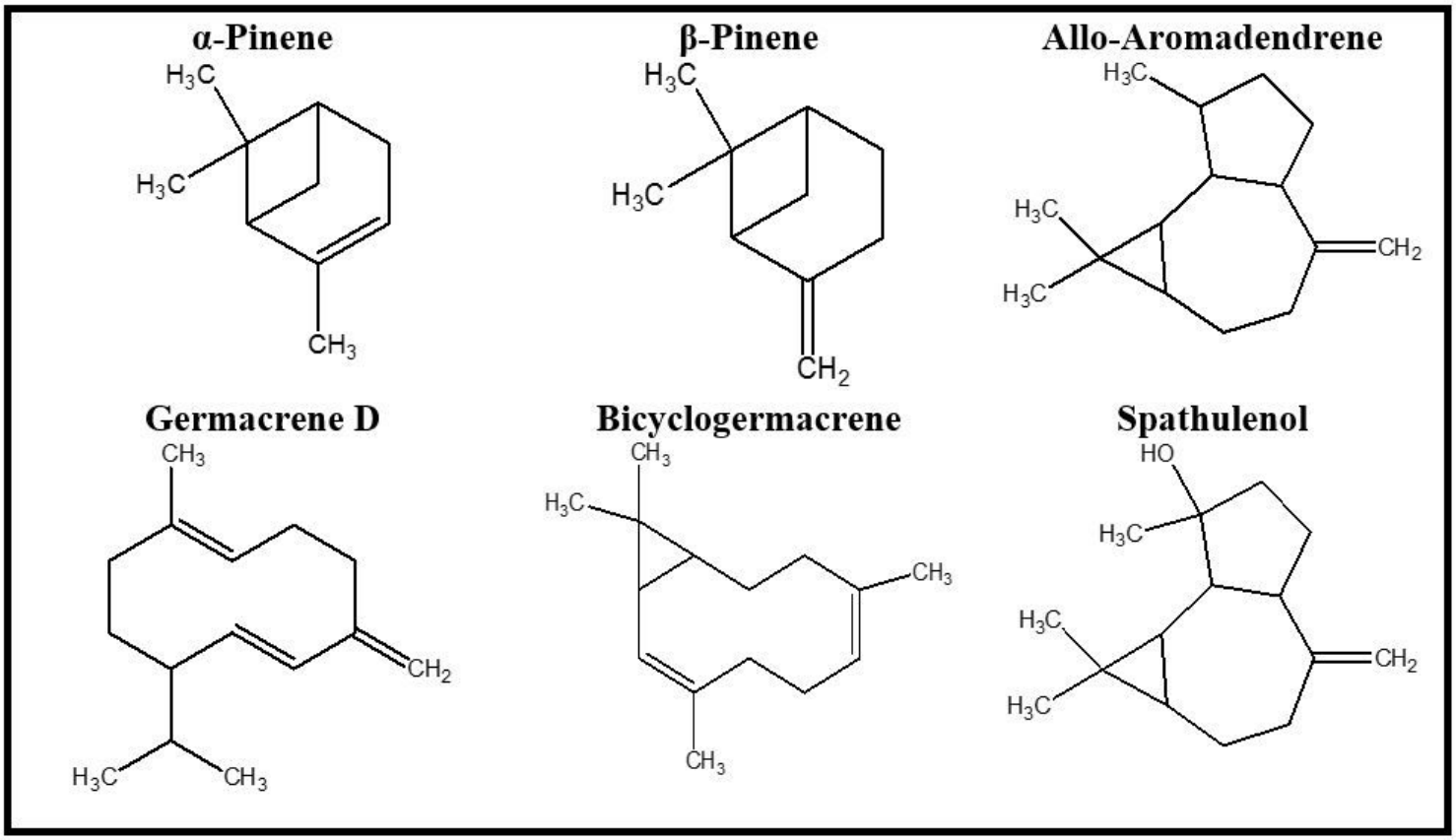


Figure 3

The molecular structures of the main identified compounds from Salvia limbata essential oil



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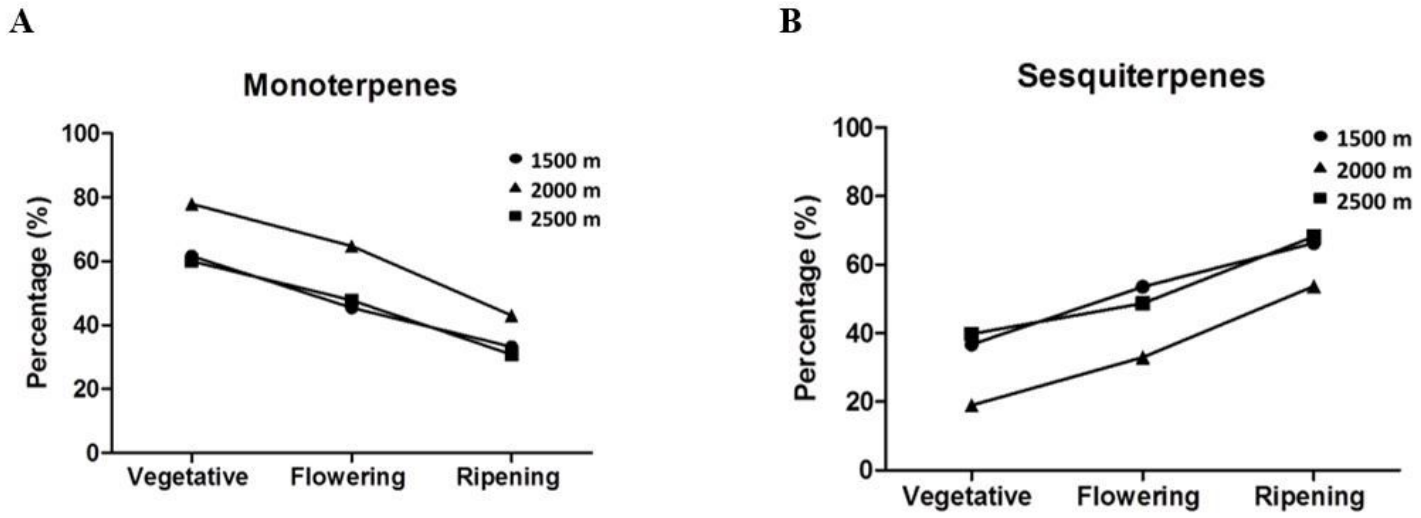
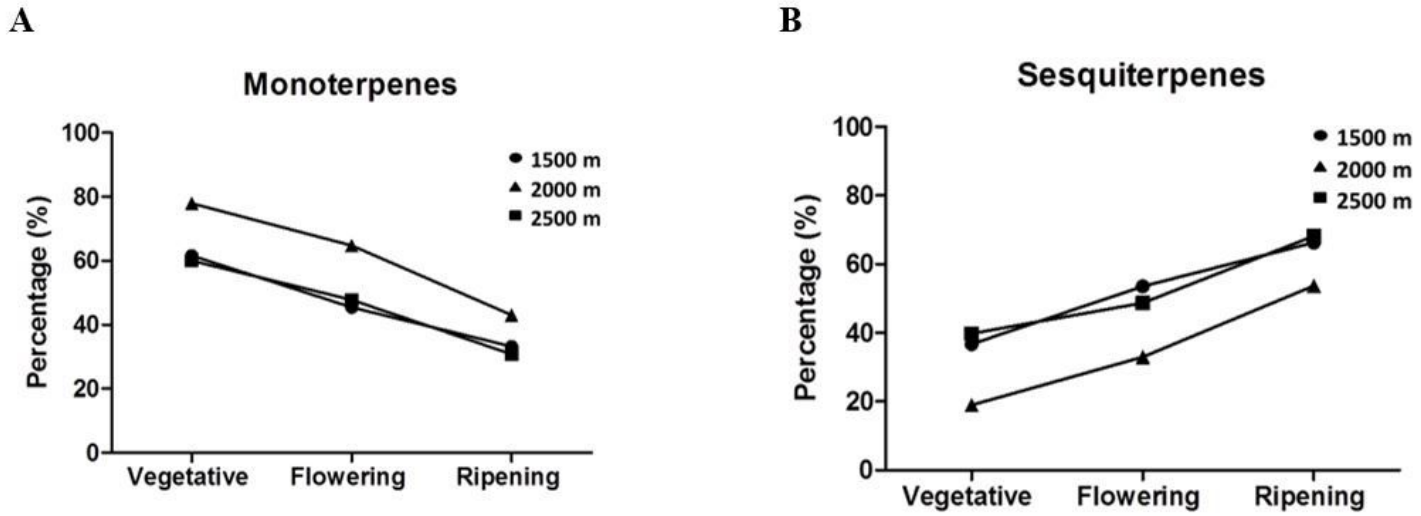


Figure 4

Variation in monoterpenes and sesquiterpenes of Salvia limbata essential oil at different altitudes and phenological stages



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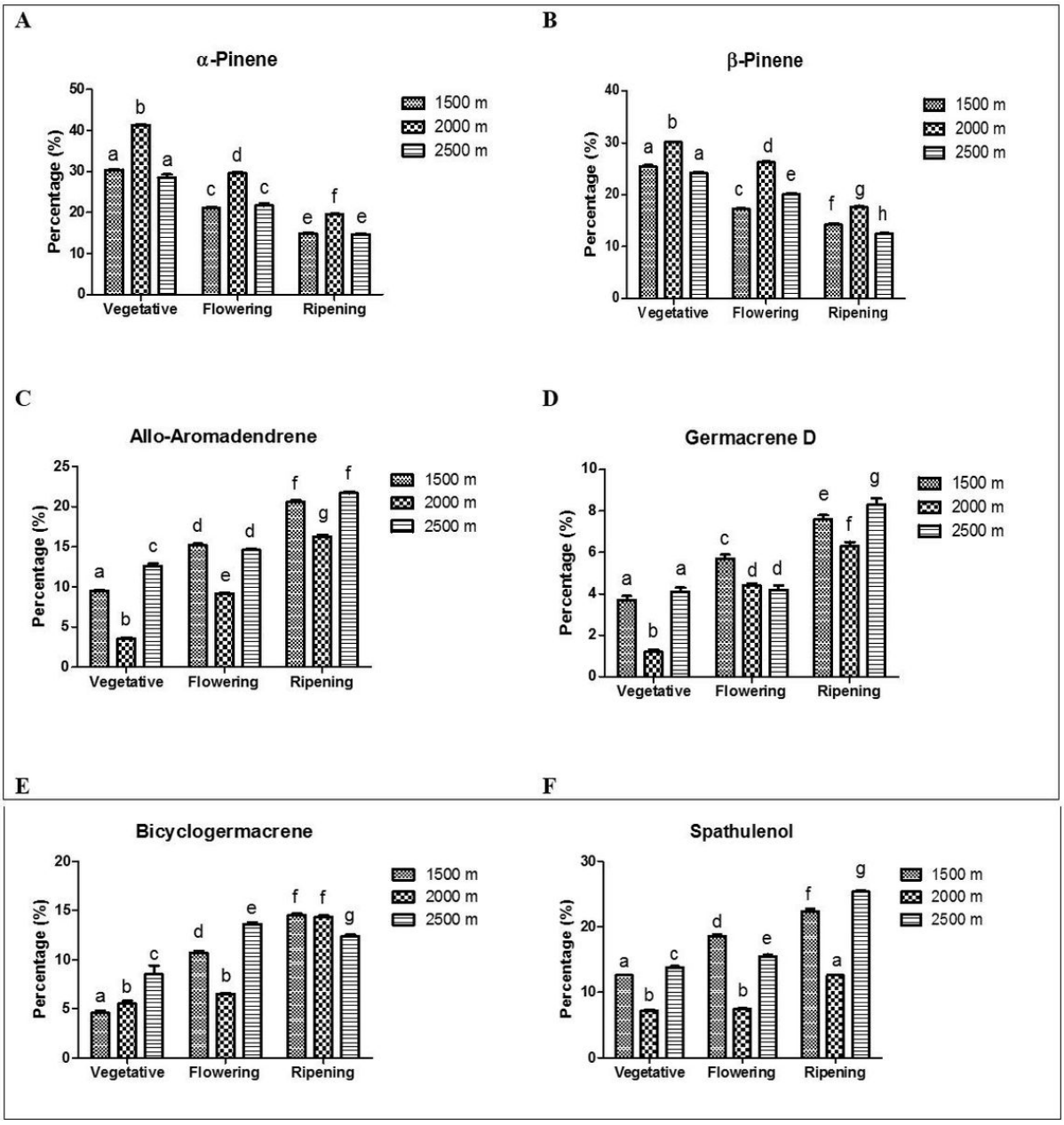
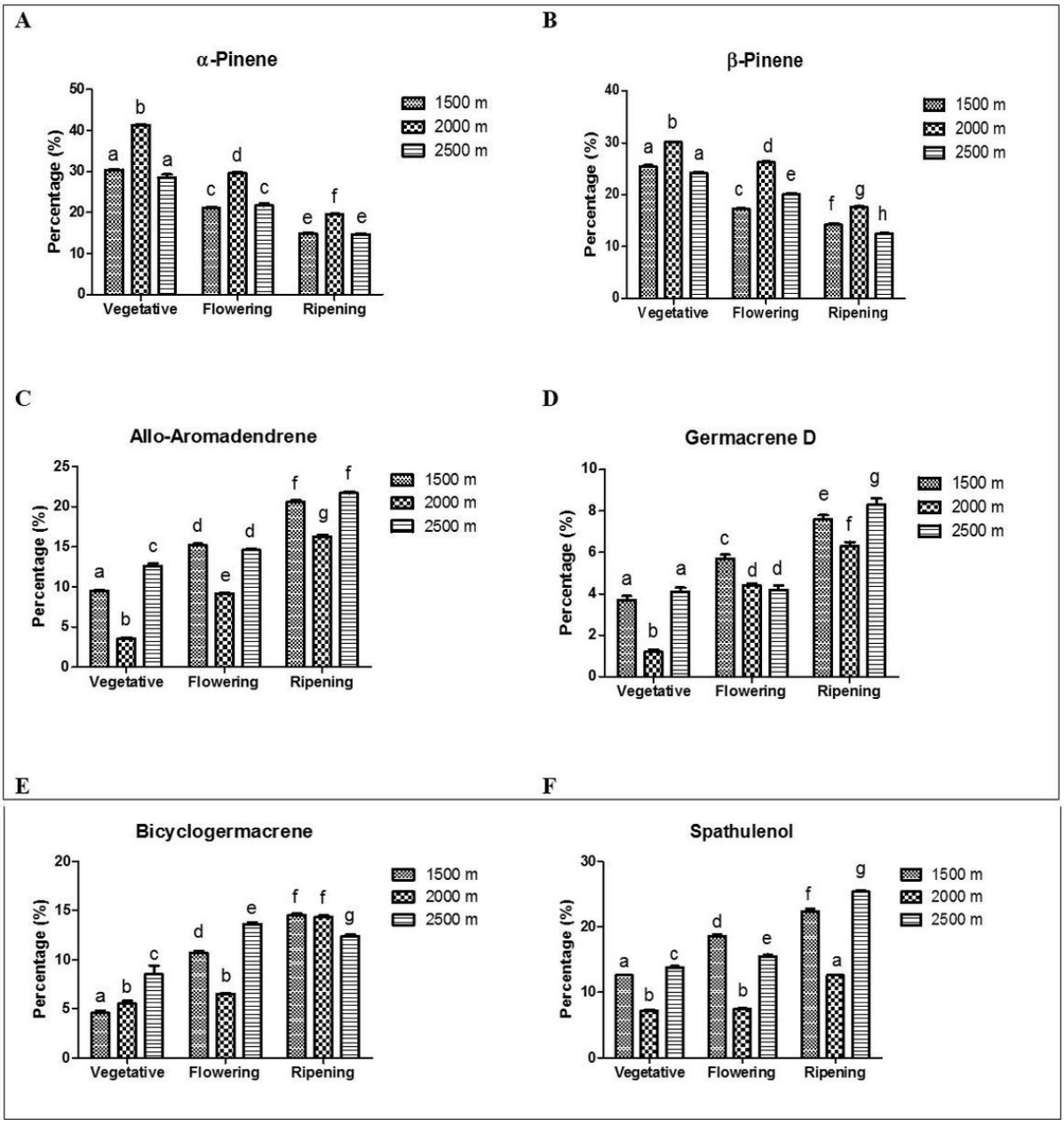


Figure 5

Percentage of α-Pinene (A), β-Pinene (B), allo-Aromadendrene (C), germacrene D (D), bicyclogermacrene (E) and spathulenol (F) at different altitudes and phenological stages. Different letters indicate significant difference among columns ($P < 0.05$). Columns with the same letter are not statistically different.



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