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Original Article

Phytochemical Comparison of *Salvia mirzayanii* Rech. & Esfand in Different Ecological Conditions

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ABSTRACT

Salvia mirzayanii Rech. & Esfand is an Iranian endemic plant belonging to the Lamiaceae family which has many pharmacological effects including antioxidant, anti-cholinesterase, antimicrobial, anticancer, anti-inflammatory and enhancing cognition and memory. It is considered as an endemic species of Iran, that is in danger of extinction. The bulks aromatic plants come from wild populations whose essential oils compositions as well as their biological properties are severely affected by the geographical location Therefore, the aim of the present study is to provide more information on the variation of essential oil composition of *S. mirzayanii* collected from four different geographical regions. The aerial parts of four populations of *S. mirzayanii* were collected from different natural habitats of Fars province in southwest of Iran. Chemical composition of *S. mirzayanii* were isolated by hydrodistillation using clevenger type apparatus and analyzed by GC-FID and GC/MS. The major compounds in four populations were α-terpinyl acetate, Eudesm-7(11)-en-4-ol, bicyclogermacrene, **δ**-cadinene and 1,8-Cineole in four populations. Polyphenolics content were identified by HPLC analysis. Predominant phenolic constituents in all extracts were chlorogenic acid, vaniline and rosmarinic acid, *p*-cumaric acid, trans-ferulic acid and quercetin. The DPPH radical inhibition was measured by using a micro-plate reader. The best antioxidant activity was related to ecotype3 with 1281.48 µg/mL and the highest amount of total phenol is related to ecotype1 and ecotype4.

INTRODUCTION

The genus *Salvia* is the largest one among the most important genera of the lamiaceae family consisting of about 900 species, of which 58 are distributed in Iran, 17 of which are endemic [1]. Many *Salvia* species are commonly used in the food, drug, cosmetic and perfumery industries. They are well known among people and widely used as flavorings or fragrances and for the medicinal purposes in the several regions of the world [2,3]. *S. mirzayanii* Rech. L. is an endemic, perennial and bushy aromatic herb which is only distributed in south of Iran [4]. The aerial parts of *S. mirzayanii* are used for diarrhea, stomachache, headache, wound healing and blood sugar by natives of southern parts of Iran [5]. This species grows wild on mountainous in Hormozgan and Fars province in Southern Iran [6]. Previous phytochemical studies on *S. mirzayanii* have led to isolation of volatile oils from aerial parts of these plants which contain a variety of components [7-10], so strong anti-oxidant activity and the immunomodulatory effects have been evaluated in many researches [11-13]. Considering the capability of plant extract and essential oils for substituting synthetic antimicrobial and antioxidant products, the study of potential of *S. mirzayanii* can be useful for industrial application of this plant as a natural product. Therefore, the objective of the present work was to characterize the essential oil composition and polyphenolic content of four *S. Mirzayanii* population.

MATERIALS AND METHODS

Plant Materials

Fresh aerial parts of *S. mirzayanii* were collected from ecotypes that growing in four different regions of Fars province, in Southwest of Iran consist of Larestan (ecotype1), Jahrom (ecotype2), Sarvestan (ecotype3) and Bavanat (ecotype4) with, 668, 1377, 1834 and 1889 meters, altitudes and average temperature, 28, 24, 21 and 19C° respectively (Table 1). Voucher specimen was deposited at the herbarium of medicinal and aromatic plants of Islamic Azad University, Estahban branch (voucher no 165, A, B, C, D). Each sample was labeled and the location was recorded using a Global Positioning System (GPS, Vista Garmin) receiver. Climatic conditions of natural habitat were determined using the nearest meteorology station (Table 1). The harvested plants were dried at room temperature (25 °C) for 10 days. Dry plants were stored in a dark and dry place until essential oil extraction and other experiments.

Essential Oil Extraction

The dried aerial parts of *S. mirzayanii* in each habitat were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus. The essential oil obtained was separated from water and dried over anhydrous sodium sulfate and stored in sealed amber flasks at 4 °C till analysis.

Gas Chromatography (GC)

The essential oil composition was determined by GC and GC-MS analysis. The analysis was performed using a gas chromatograph (Agilent Technologies 7890 GC) equipped with a FID detector, using HP-5MS 5% capillary column (30 m × 0.25 mm, 0.25 μm film thicknesses). The carrier gas was Helium at a flow of 1 ml/min. Initial column temperature was 60 °C and was programmed to increase at 3 °C/min to 280 °C. The injector and detector temperatures were 250 °C and 270 °C, respectively. The samples were injected using the split sampling technique by a ratio of 1:20. The percentage compositions were obtained from electronic integration of peak areas without the use of correction factors.

Gas Chromatography-Mass Spectrometry (GC-MS)

Essential oil was also analyzed by Hewlett- Packard GC-MS (model 6890 series II) operating at 70e V ionization energy. Equipped with a DB-5 capillary column (phenyl methyl siloxane (30 m, 0.25 mm, 0.25 μm film thickness) with He as the carrier gas and a split ratio of 1:50. The retention indices for all the components were determined according to the Van Den Doll method using n-alkanes as standard [14]. The compounds were identified by comparison of retention indices (RRI- AP-5) with those reported in the literature and by comparison of their mass spectra with the Wiley and mass finder 3 libraries or with the published mass spectra [15].

Preparation of Crude Extract

For the total phenolic content test, 7.5 g of air dried and milled aerial parts of the plants were defatted with petroleum ether for 3 h and then extracted twice for 24 h with 200 mL of 90% (v/v) aqueous methanol at room temperature. After filtration through Whatman filter paper (Whatman, Little Chalfont, UK), methanol was evaporated completely using a rotary evaporator. The dry crude extracts were used for total phenolic content assay. For polyphenolic content test using and HPLC analysis, 200 mg of air dried and milled aerial parts were extracted with methanol (≥ 99.9 %), by maceration method, for 6 h. The extracts were then filtered, made up to 10.0 mL in a volumetric flask with methanol, passed through a 0.45 μm filter, and injected into the HPLC system.

Determination of Phenolic Compounds by HPLC

The investigated phenolic compounds on different ecotypes of *S. mirzayanii* was achieved by an Agilent Technologies 1200 HPLC machine, equipped with a Zorbax Eclipse XDB-C18 column (4.6 × 5 μm i.d.; × 150 mm film thickness, RP), and a photodiode array detector (PDA). Elution was monitored at 280 and 230 nm. The column temperature was 30 ºC.

Table 1 Geographical and environmental conditions of *S. mirzayanii* ecotypes growing wild in Southwestern Iran

Region	Altitude (m)	Latitude (UTM)	Longitude (UTM)	Average Temperature (°C)	Average Annual Rainfall
Lar (ecotype 1)	668	27° 42′ N	53° 32′ E	28	180
Jahrom (ecotype 2)	1377	28° 23′ N	54° 02′ E	24	200
Sarvestan (ecotype 3)	1834	29° 12′ N	53° 18′ E	21	250
Bavanat (ecotype 4)	1889	30° 38′ N	53° 42′ E	19	280

The elution was performed by varying the proportion of solvent A (formic acid 1% in deionized water) to solvent B (Methanol (v/v)) as follows: Methanol: formic acid 1% (10:90), at 0 min; Methanol: formic acid 1% (25:75), at 10 min; Methanol: formic acid 1% (60:40), at 20 min and finally, Methanol: formic acid 1% (70:30), at 30 min and Methanol: formic acid 1% (70:30), at 40 min. Prior to injection, samples were filtered through PTFE membrane filter. The injection volume was 20µL and it was done automatically using auto sampler. Identification was based on retention times and overlay curves. All phenolic standards gave linear calibration curves within the concentration range studied (Table 4). The limits of detections (LOD) were calculated from the parameters obtained from calibration curves, using the formula LOD = 3 sa/b, where sa is the standard deviation of the y-intercept of the regression line and b is the slope of the calibration curve [16]. The compounds were injected 5 times to verify the limits of detections (LOD) and limits of quantification (LOQ) of each compound. To validate the reproducibility of the method, the percentage relative standard deviations (%RSD) of peak areas and retention times were calculated.

DPPH Assay

According to our previous work [17], the antioxidant capacity of extracts and the standard antioxidant were assessed on the basis of radical scavenging effect of the stable DPPH free radical. 200 µl of a 40 mg/L solution of DPPH radical in methanol was mixed with 20 µl of 6.25 to 3200 µg/ml extracts andgallic acid respectively, solutions were left at room temperature for30 minutes. The DPPH radical inhibition was measured at 515 nm by using a micro-plate reader model biotek ELx808. The IC_{50}ofeach sample (concentration in µg/ml required to inhibit DPPH radical formation by 50%) was calculated by Matlab software. The antioxidant activity is given by: 100- [(sample Absorbance - blank Absorbance) × 100/ controlAbsorbance].

Determination of Total Phenolic

Total phenolic content (TPC) was determined according to the Folin-Cicocalteu method. Phenols on reaction with an oxidizing agent phosphomolybdate in FCR under alkaline conditions, lead to the formation of a molybdenum blue colored complex, the intensity of which can be measured at 765 nm colorimetrically. For the preparation of calibration curve gallic acid solutions (0.0093,0.0187, 0.0375, 0.075, 0.15 mg/l) were used. 500 µl of each extracts and standards were mixed with 2 ml sodium carbonate solution (7.5%) and 2.5 ml Folin- Cicocalteu's (10%) reagent (FCR). For sixty minutes, the mixture was kept at room temperature. Then, by spectrophotometer (Lambda 950, Perkin-Elmer, USA) the absorbance of the samples was read at 765 nm. For each analysis, the samples were belayed in triplicate. Milligram GA/g dry weight(dw) extract is used to express the TPC values [18].

RESULTS AND DISCUSSION

The yield and composition of essential oils, isolated by hydro-distillation from the aerial parts of *S. mirzayanii* ecotypes are shown in Table 2. The total of 60, 69, 58 and 62 components representing 99.20%, 99.80%, 98.79% and 99.07% of the total were detected at the ecotype1-ecotype4, respectively (Table 2). The highest essential oil yield (1.75%) was obtained in ecotype1 whereas ecotype4 produced the lowest essential oil yield (1.43%). The essential oil yield in ecotype2 and ecotype3 were 1.60 and 1.55% respectively (Table 2). The essential oil of *S. mirzayanii* in the ecotype1- ecotype4 contained oxygenated monoterpenes (42.63, 56.80, 20.71 and 52.0%), oxygenated sesquiterpene (31.50, 19.71, 48.34 and 24.26%), sesquiterpene hydrocarbons (21.46, 16.78, 27.22 and 18.02 %) respectively. The major compounds in ecotype1 were α-terpinyl acetate (24.36%), eudesm-7(11)-en-4-ol (14.63%), bicyclogermacrene (7.80%), δ-cadinene (6.35%),in ecotype2, was detected α-terpinyl acetate (19.74%), linalyl acetate (13.55%), eudesm-7(11)-en-4-ol (9.07%), linalool (7.39%),in ecotype3, contained eudesm-7(11)-en-4-ol (23.56%), α-terpinyl acetate (10.46%), bicyclogermacrene (8.56%), δ-cadinene (7.45%),and in ecotype4 major constituents were α-terpinyl acetate (14.53%), linalyl acetate (12.41%), eudesm-7(11)-en-4-ol), linalool, bicyclogermacrene (7.68%), 1,8-cineole (5.30%), δ-cadinene (5.13%), α-terpineol (4.11%) and germacrene D-4-ol (3.51%). There are some reports on the composition of *S. mirzayanii* (wild type) essential oils. According to these reports, the major components of *S. mirzayanii* essential oils are linalyl acetate, linalool, α-terpinyl acetate and 1,8-cineole [19,20].

Table 2 Essential oil composition of *S. mirzayanii* ecotypes growing wild in Southwestern Iran

No	Components	RI	% ecotype1	% ecotype2	% ecotype3	% ecotype4
1	Tricyclene	919	t*	0.02	0.02	0.01
2	α-Thujene	925	t	0.04	0.06	0.17
3	α-Pinene	932	0.11	0.17	0.15	0.13
4	Camphene	948	t	t	t	t
5	Sabinene	971	0.53	0.65	0.31	0.42
6	β-Pinene	976	0.34	0.48	0.38	0.32
7	Myrcene	987	0.93	1.90	0.35	1.20
8	Unknown	991	0.21	0.23	0.16	0.42
9	α-Phellandrene	1004	t	0.04	t	t
10	α-Terpinene	1015	0.04	0.08	t	0.08
11	p-Cymene	1023	t	0.02	0.06	0.11
12	Limonene	1026	1.09	1.78	0.48	0.81
13	1,8-Cineole	1031	5.92	6.04	4.11	5.30
14	(Z)-β-Ocimene	1033	t	0.34	0	0.17
15	(E)-β-Ocimene	1043	0.21	0.74	0.07	0.62
16	δ-Terpinene	1055	0.06	t	0.05	0.12
17	cis-Sabinene hydrate	1066	0.07	0.16	t	0.28
18	trans-Linalool oxide	1071	0.08	0.22	t	t
19	Terpinolene	1086	0.22	0.41	0.08	0.39
20	Linalool	1099	2.22	7.39	0.58	9.09
21	unknown	1103	t	0.02	t	0.08
22	trans-Pinocarveol	1134	0.03	0.03	t	0.11
23	γ-Terpineol	1166	0.61	0.59	0.30	0.48
24	Terpinen-4-ol	1176	0.15	0.18	0.13	0.31
25	α-Terpineol	1190	2.24	4.09	0.96	4.11
26	n-Decanal	1202	t	0.04	0.06	0.09
27	trans-Carveol	1218	0.11	0.09	0.07	0.11
28	Nerol	1226	0.16	0.41	t	0.58
29	Neral	1239	0.07	0.05	t	t
30	Geraniol	1251	0.23	0.25	0.13	0.29
31	Linalyl acetate	1254	3.73	13.55	1.36	12.41
32	Geranial	1268	0.23	0.27	0.40	0.36
33	n-Decanol	1273	t	0.02	t	0.08
34	δ -Elemene	1334	1.30	1.22	1.63	1.12
35	α-Terpinyl acetate	1349	24.36	19.74	10.46	14.53
36	Neryl acetate	1361	0.26	0.65	0.08	0.74
37	α-Copaene	1373	0.06	0.09	0.07	0.07
38	Geranyl acetate	1380	0.64	1.38	0.36	1.54
39	β-Elemene	1389	1.87	1.77	2.43	1.70
40	Longifolene	1399	0.18	0.12	0.26	0
41	α-Gurjunene	1407	0.85	0.62	1.39	0.95
42	(E)-Caryophyllene	1416	0.92	1.36	1.39	0.65
43	α-Guaiene	1438	1.01	0.37	1.93	0.11
44	α-Humulene	1450	0.07	0.12	0.16	0.09
45	allo-Aromadendrene	1455	0.72	0.22	0.98	t
46	γ -Muurolene	1473	0.30	0.27	0.66	0.43
47	Germacrene D	1478	0.18	0.21	0.25	0.13
48	β-Selinene	1483	0.21	0.36	0.37	0.20
49	δ -Selinene	1488	0.22	0.43	0.30	t
50	Bicyclogermacrene	1494	7.80	4.81	8.56	7.68
51	α-Muurolene	1497	t	0.47	t	t
52	δ -Amorphene	1511	0.56	0.53	0.66	0.58
53	cis-Dihyroagarofuran	1517	3.88	2.63	7.09	2.86
54	δ-Cadinene	1520	6.35	4.84	7.45	5.13
55	α-Cadinene	1534	0.07	0.13	0.16	0.19
56	Elemol	1546	0.37	0.22	0.46	0.26
57	Germacrene D-4-ol	1573	4.22	2.44	5.63	3.51
58	Spathulenol	1576	1.04	0.90	3.04	1.64
59	Caryophyllene oxide	1582	0.14	0.14	0.46	0.20
60	Viridiflorol	1590	0.14	0.11	0.24	0.14

61	Longiborneol	1601	t	0.08	0.17	t
62	Isolongifolan-7-a-ol	1618	0.12	0.06	t	0.09
63	epi-α-Cadinol	1639	2.09	1.21	2.45	1.59
64	α- Muurolol	1645	0.20	t	t	t
66	β-Eudesmol	1648	1.18	0.65	1.38	0.60
67	α-Cadinol	1652	3.29	2.01	3.44	2.24
68	Eudesm-7(11)-en-4-ol	1691	14.63	9.07	23.56	11.13
69	Unknown	1700	0.20	0.19	0.42	t
70	14-hydroxy-d-Cadinene	1807	t	t	0.20	0.11
71	Epi-13-Manoyl oxide	2012	t	t	0.26	0.05
72	Kaurene	2043	t	t	0.17	t
	Monoterpene hydrocarbons		3.52	6.497	2.09	4.67
	Oxygenated monoterpens		42.63	56.8	20.71	52
	Sesquiterpene hydrocarbons		21.46	16.78	27.22	18.02
	Oxygenated sesquiterpenes		31.5	19.71	48.34	24.26
	Diterpens		0.09	0.02	0.43	0.12
	Total		99.20%	99.80%	98.79%	99.07%
	Essential oil yield (%)		1.75	1.60	1.55	1.43

t*: trace

Table 3 Polyphenolic content of *S. mirzayanii* ecotypes grown wild in Southwest Iran determined by HPLC

No	Polyphenolic content (mg/L)	RT [a]	Content in different regions				Linear regression equation [b]	Correlation coefficient
			Ecotype 1	Ecotype 2	Ecotype 3	Ecotype 4		
1	Gallic acid	3.3	ND	ND	ND	ND	Y = 40.507x−33.427	0.999
2	Catechin	8.3	ND	ND	ND	ND	Y = 9.2191x−77.022	0.997
3	Chlorogenic acid	10.5	8.17	19.5	36.18	8.14	Y = 36.796x−682.09	0.999
4	Caffic acid	11.6	ND	ND	ND	ND	Y = 12.586x+42.447	0.999
5	Rutin	12.6	ND	ND	ND	ND	Y = 11.801x−97.719	0.996
6	Vaniline	13.5	13.34	17.50	22.89	13.41	Y = 42.74x+59.464	0.999
7	*p*-Coumaric acid	15.6	ND	8.13	17.49	11.83	Y = 82.241x+287.72	0.997
8	Trans-ferulic acid	16.3	ND	25.32	25.90	18.27	Y = 30.718x−214.48	0.999
9	Sinapic acid	16.5	ND	ND	ND	ND	Y = 0.895x+6.8532	0.998
10	Coumarin	17.4	7.15	ND	ND	ND	Y = 55.203x +186.22	0.999
11	Hesperidin	18.5	ND	ND	ND	ND	Y = 16.849x+40.817	0.997
12	Ellagic acid	19.02	ND	ND	ND	ND	Y=17.803x-185.06	0.992
13	Rosmarinic acid	19.2	60.09	275.45	304.97	183.57	Y = 10.675x−12.921	0.999
14	Quercetin	21.6	14.63	ND	21.45	14.96	Y = 11.801x−97.719	0.996
15	Hesperetin	22.4	ND	ND	ND	ND	Y=30.574x-141.76	0.999
16	Eugenol	23.7	ND	ND	ND	ND	Y=11.32x-147.17	0.994
17	Carvacrol	28.4	ND	ND	ND	ND	Y = 10.675x−12.921	0.999
18	Thymol	28.9	ND	ND	ND	ND	Y = 31.824x−215.65	0.998

Each value in the table was obtained by calculating the average of three experiments. Means with different letters were significantly different at the level of $p < 0.05$.

[a] RT: retention time. ND: not detected. [b]Linear regression equation: Y= area; X= concentration.

Zomorodian *et al*., 2017 showed that the essential oil compounds of *S. mirzayanii* were 1,8-cineole (41.2 ± 1.3%), linalool acetate (11.0 ± 0.5%), and α-terpinyl acetate (6.0 ± 0.4%). Javidnia *et al*. 2002, was reported that δ-cadinene, linalool, α-terpinyl acetate, α-cadinol and spathulenol, as major components of the essential oil of *S. mirzayanii*. However, 1,8-cineole and α-cadinol, which were detected as the main oil components in some report [7,10,21]. Similar our results Valifard *et al* (2014) showed that the leaves of *S. mirzayanii* are rich in α-terpinyl acetate. The comparison of EOs constituents of ecotypes in different altitude and region indicated that the amounts of main compounds were drastically changed with altitude and temperature zone (Table 1). The α-terpinyl acetate as a major compound, showed the maximum concentration at the low altitude (ecotype1:688m) and high average temperature (28 °C) with the amount of 24.36%. This component reduced to 14.53 and 10.46% at the high altitude (ecotype4:1889m and ecotype3:1834m) and low average temperature (19 and 21 °C) respectively. The amount of essential oil decreased with increasing altitude and decreasing temperature from 1.75 to

1.43%, Furthermore, the results of oil analysis indicated that the maximum concentration of Eudesm-7(11)-en-4-ol was observed in ecotype3 and ecotype1(23.56 and 14.63%),whereas the maximum concentration of linalyl acetate was detected in ecotype2 and 4. It is obvious that geographic origin of the plants, climate and soil composition, harvesting time and drying and extraction methods have high impact on both the quantitative and qualitative profile of the essential oil. Differences in essential oil yield and composition in present study can be partly attributed to the differences in environmental conditions in the four geographical location.

Identification and Quantification of Phenolic Compounds by HPLC

HPLC–UV analyses of phenolic compounds extracted of different *S. mirzayanii* population, were carried out by the method validated on the standard phenolic compounds and expressed as mg/l. The amount of 17 investigated polyphenols are listed in Table 3. Three compounds, chlorogenic acid, vaniline and rosmarinic acid were detected in four different ecotype tested, coumarin only detected in ecotype1 (Altitude: 688m and average temperature 28 °C). The *p*-cumaric acid and *trans*-ferulic acid, only detected in ecotypes2,3,4.Also, quercetin was detected in ecotypes1,3,4. The highest amount of chlorogenic acid (36.18 mg/l), vaniline (22.89 mg/l), *p*-cumaric acid (17.49 mg/l), trans-ferulic acid (25.90 mg/l), Rosmarinic acid (304.97 mg/l) and Quercetin (21.45 mg/l) were detected in ecotype3.An earlier review report[22] have shown rosmarinic acid, salvianolic acid B, salvianolic acid A, carnosic acid and caffeic acid were as the major component in *S. mirzayanii*. Similar our study, was reported that phenolic compounds as catechin and rosmarinic acid and common flavonoids as rutin or luteolin were quantified in a methanol extract of the aerial parts of *S. mirzayanii* [23]. A comparison of our results with the previous report suggests differences in polyphenolic content of the plant material, we found seven phenolic compounds as chlorogenic acid, vaniline, coumarin, *p*-cumaric acid, trans-ferulic acid, rosmarinic acid and quercetin, but in previous report, only catechin and rosmarinic acid were detected as phenolic content in *S. mirzayanii* plant extract. Some of this difference in the phenolic compounds of the plants could be attributed to geographical origins, environmental and climatic conditions or harvest time of plants material [24-26]. Figure 1 indicated the antioxidant activitiy of methanolic extract of *S. mirzayanii* ecotypes. According to this results and comparison of means, the lowest IC_{50} (best antioxidant activity) was related to ecotype3with 1281.48 µg/mL. It showed a moderate antioxidant activity in comparison with α-tocopherol (Vit. E) as a standard, and most of it (the least antioxidant effect) is related to ecotype4. According to figure 2, the highest amount of total phenol of *S. mirzayanii* ecotypes is related to ecotype1 and ecotype4, which shows a significant difference compared to ecotype2. Many studies have been carried out on antioxidant activity of many species of the Labiatae family [27,28]. They demonstrated that this family species had a very strong antioxidant capacity. Some of them found that rosemary had the strongest antioxidant effect, but others found this with sage or oregano and basil. Similar our study some authors [29,30] showed poor linear correlation or report total antioxidant activity and phenolic content with no comment, while others [31-33] have demonstrated a linear correlation between the content of total phenolic compounds and their antioxidant capacity.

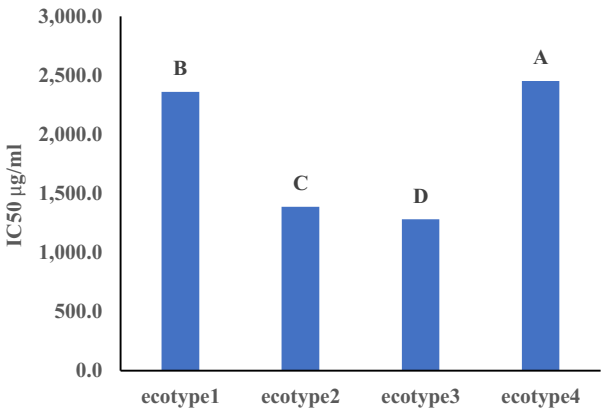

Fig. 1 Antioxidant capacity of *S. mirzayanii* ecotypes

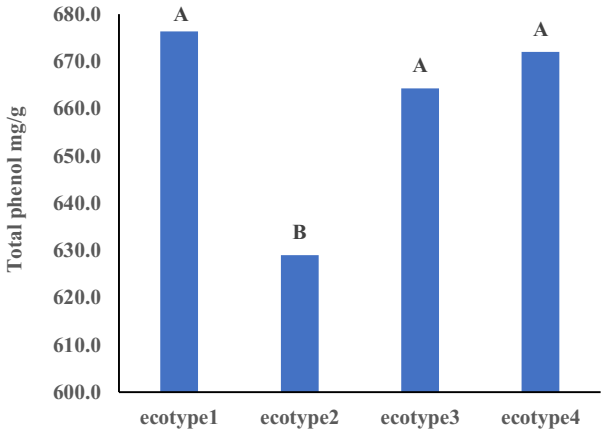

Fig. 2 Total phenolic of *S. mirzayanii* ecotypes

CONCLUSION

The findings of this study showed that the maximum content of EO and the highest concentration of α-terpinyl acetate were obtained by ecotype1 that was obtained at the low altitude and high temperature. On the other hand, even though the plant's growth habitats and genotypes might change its EO content and composition. In this study, ecological conditions significantly affected the phytochemical composition of the *S. mirzayanii.*

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